The Putative Bioactive Surface of Insect-selective Scorpion Excitatory Neurotoxins*

Oren Froy‡, Noam Zilberberg‡, Dalia Gordon‡, Michael Turkov‡, Nicolas Gilles§, Maria Stankiewicz¶, Marcel Pelhate¶, Erwann Loret¶, Deena A. Oren**, Boaz Shaanan***, and Michael Gurevitz‡‡‡

From the ‡Department of Plant Sciences, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, 69978 Tel Aviv, Israel, the §Commissariat à l’Energie Atomique, Département d’Ingénierie et d’Etudes des Protéines, Bat. 152, C. E. Scolay, F-91191 Gif-sur-Yvette Cedex, France, the ¶Laboratoire de Neurophysiologie, Faculté de Médecine, Université d’Angers, F-49045 Angers, France, the §§IBSM-LIDSM-CNRS, UPR 9027, 31 Chemin Joseph Aiguier, BP 71, 13402 Marseille Cedex 20, France, and the ‡‡‡Wolfson Centre for Applied Structural Biology and the ‡‡‡Department of Biological Chemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Giv’at Ram 91904, Jerusalem

Scorpion neurotoxins of the excitatory group show total specificity for insects and serve as invaluable probes for insect sodium channels. However, despite their significance and potential for application in insect-pest control, the structural basis for their bioactivity is still unknown. We isolated, characterized, and expressed an atypically long excitatory toxin, Bj-xtrIT, whose bioactive features resembled those of classical excitatory toxins, despite only 49% sequence identity.

With the objective of clarifying the toxic site of this unique pharmacological group, Bj-xtrIT was employed in a genetic approach using point mutagenesis and biological and structural assays of the mutant products. A primary target for modification was the structurally unique C-terminal region. Sequential deletions of C-terminal residues suggested an inevitable significance of Ile78 and Ile74 for toxicity. Based on the bioactive role of the C-terminal region and a comparison of Bj-xtrIT with a Bj-xtrIT-based model of a classical excitatory toxin, AaHIT, a conserved surface comprising the C terminus is suggested to form the site of recognition with the sodium channel receptor.

Scorpion venom contains a diversity of protein toxins, which modify the properties of neural ion channels. The excitatory toxins are highly specific to insects, have a characteristic structure and mode of action, and define a unique receptor-binding site on the insect sodium channel (1, 2). Unlike all other “long” scorpion toxins with 60–65 amino acid residues and a highly conserved pattern of four disulfide bridges, the excitatory toxins reported thus far are composed of 70 amino acid residues, and one of their disulfide bonds is shifted (3, 4). Excitatory toxins such as AaHIT, LqqIT2, and AmmIT induce spastic paralysis caused by repetitive activity of motor nerves (5–7) resulting from increased sodium current and slowed inactivation (8–10). AaHIT and other excitatory toxins define a high affinity (Kd = 1–3 nM), low capacity (1–2 pmol/mg of protein) binding site in insect neural membrane (1, 11). Their high potency makes them as an attractive model for development of anti-insect-selective biocides. Indeed, AaHIT played a prominent role in early attempts to engineer entomopathogenic baculoviruses (12–17).

It has become apparent that detailed structure-activity analysis of these toxins depends on their efficient overproduction in heterologous systems. However, many attempts to establish a genetic system for high level production of AaHIT in COS cells (18), insect cells (12–14), yeast (19), or bacteria (20) have been very limited, and toxin detection could be achieved only with antibodies or by neurotoxic symptoms. The difficulty in obtaining reasonable amounts of pure protein also hampered three-dimensional structure determination of this toxin group.

Indeed, only one report describing the secondary structure and overall fold of AaHIT has been published (21). Other obstacles limiting a genetic study of excitatory toxins are the small number of toxins identified thus far and their prominent sequence similarity. Only two, highly homologous excitatory toxins (AaHIT and LqqIT1) have been sequenced during the last 3 decades (5, 6), and two additional excitatory toxins have been isolated (AmmIT (7) and BjIT1 (9)).

Recently, we have determined the three-dimensional structure of a novel excitatory toxin, Bj-xtrIT (3), and have shown that, despite the unique architecture of its C-terminal module, the core consisting of an α-helix and a three-stranded antiparallel β-sheet was similar to those found in various pharmacologically distinct scorpion toxins, e.g. AaH II and Cε9v3 (22). Evidently, the difference in bioactivity of the various toxins is dictated by their non-similar exteriors composed of various amino acid side chains, loops, and turns connecting the conserved secondary structure elements and the C termini. Herein we report the purification, cloning, and functional expression of Bj-xtrIT and its utilization in a genetic modification study. By combining site-directed mutagenesis with functional and structural analyses, we delineated specific amino acid residues clustered on a molecular surface forming the putative bioactive domain of scorpion excitatory neurotoxins.

EXPERIMENTAL PROCEDURES

Biological Material

Buthotus judaicus scorpions were collected at the Carmel mountains of Israel, and their venom was collected upon stinging a Parafilm membrane. Sarcophaga falculata blowfly larvae, Spodoptera littoralis noctuid larvae, and Periplaneta americana cockroaches were bred in the

* This work was supported by Grant IS-2486-94C from the United States-Israel Binational Agricultural Research and Development Fund (to M. G. and B. S.); by Grant 891-0122-95 from the Israeli Ministry of Agriculture (to M. G.); by Grant 46697 from the Israel Academy of Sciences and Humanities (to M. G.); and by the Do‘at Consortium, a Magent project administered by the Chief Scientist of the Ministry of Industry and Trade, Israel (to B. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed. Tel.: 972-2-6585241; Fax: 972-2-6585573; E-mail: boazsh@vms.huji.ac.il.

§§ To whom correspondence should be addressed. Tel.: 972-3-6409844; Fax: 972-3-6406100; E-mail: mamgur@ccsg.tau.ac.il.
laboratory. Albino laboratory ICR mice were purchased from the Le-veinstein farm (Yokneam, Israel). Escherichia coli strains DH5α and BL21 were used for plasmid construction and expression, respectively. The translational vector used, PET-11cK, is a PET-11c derivative (23) bearing a gene for kanamycin resistance.

**Purification and N-terminal Sequence of Native Bj-xtrIT**

Crude venom (7.7 mg) from 35 scorpions was lyophilized, dissolved in 3 ml of 10 mM ammonium acetate (pH 6.7), and subjected to three successive chromatographic steps. 1) Cation-exchange chromatography was carried out on a 2.5-ml CMS25-cellulose column (Whatman) equilibrated in 10 mM ammonium acetate (pH 6.7). After elution of 5 ml, a linear gradient of 10–400 mM ammonium acetate (pH 6.7) was applied at room temperature (20 °C) with a flow rate of 0.6 ml/min, and 12 ml fractions were collected. 2) Anion-exchange chromatography was performed on a 0.8-ml DEAE-Sephadex column (Sigma) equilibrated in 10 mM ammonium acetate (pH 8.0). The active fraction obtained in step 1 was chromatographed using a linear gradient of 0.01–1.0 M ammonium acetate (pH 9.0) at a flow rate of 0.5 ml/min, at room temperature. Fractions were collected in 1.5-ml aliquots. 3) Reverse-phase HPLC (1) was carried out on a Vydac C18 column (250 × 10 mm) using 0.1% trifluoroacetic acid as solvent A and 0.1% trifluoroacetic acid and acetonitrile as solvent B. Elution was performed with a linear gradient of solvent B: 10 min with 0–23%, 40 min with 23–35%, 15 min with 35–40%, and a final 10 min with 40–100%. Amino acid sequence analysis was performed using automated Edman degradation using an Applied Biosystems gas-phase sequencer connected to its corresponding phenylthiohydantoin analyzer and data system.

**Cloning of Bj-xtrIT**

Fifteen venom gland segments (telsons) were ground for RNA purification. Isolation of poly(A)+ RNA and cDNA synthesis were performed as described previously (24). A library in E. coli strain DH5α was obtained by cloning 50 ng of cDNA into the Smal polylinker site of pBluescript phagemid (Stratagene). A degenerate oligonucleotide (primer 1), 5'-AAAGAAGAAGAAATCGGATATCCGCTATGAGGGTATTATTTGG-3' and primer 2, 5'-ATTCTTCGAGCCCTTTTTTCTG-3', designed according to the N-terminal amino acid sequence, was PCR-amplified with a second oligonucleotide (primer 3), 5'-ATGTTATATCTCAATAATTTTTGATTTATCTC-3', and the KS HI site (underlined), and the KS HI site (underlined). This primer was employed with primer 4 (5'-AACATATGAA-3') to construct the Bj-xtrIT.

**Construction of Expression Vectors**

Oligonucleotide primers were used to reconstruct via PCR the cDNA termini of Bj-xtrIT 5'8E, Bj-xtrIT 3'8K, and LqhIT (formerly termed LqhIT) (25). For Bj-xtrIT, primers 4 (5'-GGAGTTCCATATGGAGAA-GAACCCTATCCCTGCTG-3') and 5 (5'-CCGAGTACATTTACCAATATATTTATTGTGAAATC-3') were designed to the sequence of the noding strand of the region including the C terminus of Bj-xtrIT and with a terminal BamHI site (underlined), and the KS primer (Stratagene). The 3' primer was designed according to the N-terminal amino acid sequence, and the KS primer was designed according to the sequence of the cloning site of the expression vector pET-11cK.

**Electrophysiological Experiments**

Recordings in current-clamp and voltage-clamp conditions were performed on cockroach isolated giant axons using the double oil-gap single fiber technique (33). Axon isolation and the recording technique were previously described in detail (34). The isolated axon was superfused by physiological saline (buffered to pH 7.2 with 1 mM HEPES) containing 210 mM NaCl, 3.1 mM KCl, 5.2 mM MgCl₂, and 5.4 mM CaCl₂. Potassium currents were blocked in voltage-clamp conditions by superfusion with 0.9 mM 3,4-diaminopyridine (Sigma), and sodium currents with 0.5 mM tetraethylammonium (Sigma). Lyophilized recombinant and native Bj-xtrIT toxins were dissolved in normal saline to a concentration of 1 μM in the presence of 25 mM histidine. Action potentials were recorded in response to 10-s pulses (35). The best fit for LqhIT was obtained using the two-site model (τ = 0.997 by LIGAND).

**Circular Dichroism Spectroscopy**

Toxins were dissolved in 20 mM phosphate buffer (pH 7). Spectra were measured at 20 °C in 0.05-mm path length cells from 260 to 178 nm with a JOBIN-YVON CD spectrophotometer (Model V-6). Calibration was performed with (+)-10-camphorsulfonic acid. A ratio of 2.2 was found between the positive CD band at 290.5 nm and the negative band at 192.5 nm. Data were collected at 0.5-nm intervals with a scan rate of 1 nm/min. CD spectra are reported as Ω per amide. Protein concentration was in the range of 0.5–1 mg/ml as determined on a Beckman amino acid analyzer. Secondary structure content was determined according to the method of Manavalan and Johnson (35).
Modeling

The initial AaHIT model was constructed on the basis of the structural alignment with Bj-xtrIT (3). Using the Insight Homology program (MSI, Cambridge United Kingdom), residues in the Bj-xtrIT model were replaced by homologous residues in AaHIT according to the alignment. As shown in Fig. 1, residues 17 and 26 of Bj-xtrIT are aligned with residues 17 and 21 of AaHIT as a result of an insertion of five residues in Bj-xtrIT. To accommodate in agreement in length of the polypeptide chain around this region, a minor manual adjustment was performed using the program O (37). Further manual adjustments were applied to side chains of several residues in order to alleviate severe steric clash with close neighbors. This initial model was subjected to 2000 steps of energy minimization using the program X-PLOR (38) and the energy parameters described by Engh and Huber (39). During the minimization, protein atoms were allowed to move under the constraints of known protein stereochemistry as applied in X-PLOR. However, since the underlying assumption in modeling AaHIT was its structural resemblance to Bj-xtrIT, the C-α atoms of AaHIT were harmonically restrained (38) to their positions in Bj-xtrIT. The stereochemistry of the final energy-minimized model of AaHIT was examined by the program PROCHECK (40) and found to be in agreement with the stereochemical parameters of proteins in the Protein Data Bank, Brookhaven National Laboratory (Upton, NY).

RESULTS

Purification and Cloning of Bj-xtrIT—Crude venom from 35 B. judaicus scorpions was separated into 19 fractions by ion-exchange chromatography. Fraction IV, which was 19.5% of the crude venom and contained 1.5 mg of protein, produced an excitatory effect on blowfly larvae. Further fractionation of this component by gel-filtration chromatography yielded a major peak constituting 15.6% of the crude venom and containing 1.2 mg of protein. Final purification of the excitatory fraction was achieved by reverse-phase HPLC, yielding three major components, of which the 36-min fraction (31% acetoniiter) contained the toxic activity. The pure toxin (60 µg) constituted −0.8% of the total crude venom protein, and its mass was determined to be 8455 Da by mass spectrometry. The toxicity (ED₅₀) of the purified toxin (Bj-xtrIT) to blowfly larvae was 4.1 ng/100 mg of body weight (Table 1).

The sequence of the 30 N-terminal amino acids of Bj-xtrIT was determined (Fig. 1), enabling synthesis of a degenerate oligonucleotide (primer 1) spanning the first seven amino acid residues. This primer was reacted with an oligo(dT) primer (primer 2) using the cDNA library of B. judaicus (see “Experimental Procedures”), resulting in a PCR product encoding most of Bj-xtrIT cDNA. Primer 3, designed according to the 3’-region of the PCR product, was reacted with the KS primer and the cDNA library as a template to generate the full-length cDNA encoding Bj-xtrIT. Two distinct cDNA sequences encoding toxins that vary at position 38 of the mature polypeptide (Bj-xtrIT.38K and Bj-xtrIT.38E) were identified in a multiple number of experiments. It is noteworthy that, in previous experiments (25), four distinct genes encoding the excitatory toxin of Leiurus quinquestriatus hebraeus have been isolated. These results, together with the report of Bougis et al. (18), indicate polymorphism among the genes encoding excitatory toxins (Fig. 1).

Expression of Bj-xtrIT and Lqh-xtrIT Toxins—An efficient bacterial expression system, employed previously to express an α-toxin (26) and a depressant toxin (27), was utilized in an attempt to produce an active recombinant excitatory toxin. Lqh-xtrIT-a cDNA (Fig. 1), encoding the excitatory toxin of L. quinquestriatus hebraeus (25), and Bj-xtrIT-a and Bj-xtrIT-b cDNAs (Fig. 1), encoding the aforementioned variants of B. judaicus excitatory toxin, were engineered into a pET-11cK expression vector. Following isopropyl-β-D-thiogalactopyranoside induction, the toxins, accumulating within E. coli inclusion bodies, were subjected to a denaturation/renaturation procedure (26, 27). Folding conditions to obtain a functional toxin conformation were achieved by dissolving the denatured protein under conditions of various ammonium acetate concentrations (0–0.25 M), temperatures (4–37 °C), pH values (5.0–9.0), β-mercaptoethanol concentrations (0–0.5 M), and incubation periods (0–96 h). Both Bj-xtrIT variants eluted under similar conditions from the C₁₈ column, resulting in a major active peak. However, negligible activity (as determined by the toxicity to blowfly larvae and the ability to displace the depressant

### TABLE I

Bioactivity of Bj-xtrIT variants and mutants

| Toxin variant | ED₅₀ ng/100 mg body weight |
|---------------|---------------------------|
| Bj-xtrIT.a    | 4.1                       |
| Bj-xtrIT.b    | 8.6                       |
| Bj-xtrIT.38K  | 8.6                       |
| Bj-xtrIT.38E  | 39.5                      |
| B. judaicus   |                            |

*ND, not detectable.

**Fig. 1. Comparison among scorpion excitatory neurotoxins.** The Bj-xtrIT sequences have been scanned against the data base, and all sequences with significant relatedness are included. Alignment is with the sequence of Bj-xtrIT-a (Bj-xtrIT.38K, EMBL accession number AJ012312). The AaHIT variants (EMBL accession numbers as follows: AaHIT-a, M27705; AaHIT-b, M27706; AaHIT-c, M27707; and AaHIT-d, X58376) are from Androctonus australis Hector (18, 21, 36). LqhIT₁ is from Leiurus quinquestriatus quinquestriatus (6). Lqh-xtrIT (formerly termed LqhIT₁) variants are from L. quinquestriatus hebraeus (25). Identical residues are designated by dots. Dashes indicate gaps in the aligned sequences.
Scorpion Excitatory Neurotoxins

Bioactivity of Bj-xtrIT Natural Variants—The biological activity of the native (Bj-xtrIT-n) and recombinant (Bj-xtrIT.38E and Bj-xtrIT.38K) toxins was assessed by toxicity, binding, and electrophysiological assays. Injection of blowfly larvae provided ED\textsubscript{50} values of 4.1, 8.6, and 8.6 ng/100 mg of body weight (Table I) for the three toxins, respectively. Injection of Bj-xtrIT.38K into S. littoralis lepidopteran larvae generated paralytic symptoms, with a PU\textsubscript{50} value of 1.4 μg/100 mg of larva. Binding studies were performed using AaHIT and the depressant toxin LqhIT\textsubscript{2}; a high affinity site, shown to be located on insect sodium channels, has been described. Both Bj-xtrIT and AaHIT from a low affinity site with high capacity. Thus, Bj-xtrIT and AaHIT bind to the same receptor site on the cockroach neural membranes.

In current-clamp experiments, Bj-xtrIT depolarized the resting membrane potential and generated spontaneous action potentials. Application of the toxin (1 μM) caused a 8–15-mV depolarization in <6 min (Fig. 3A); this effect could be blocked by 0.5 μM tetrodotoxin (data not shown). Depolarization was accompanied by persistent repetitive firing of action potentials (starting at ~52 mV and observed 5–15 min after toxin application) even in the absence of electrical stimulation (Fig. 3B). Artificial repolarization, by passing a constant hyperpolarizing current, did not prevent a later depolarization accompanied by repetitive activity (Fig. 3C). The repetitive activity induced by the toxin was typified by action potentials with slightly decreased amplitude (70–80 mV rather than 95–100 mV in control conditions). The frequency during the action potential bursts varied between 80 and 200 Hz, and no “plateau potential” was recorded. The native (Bj-xtrIT-n) and recombinant (Bj-xtrIT.38E) toxins were equally potent on the axonal preparation.

In voltage-clamp experiments, Bj-xtrIT changed neither the amplitude nor the kinetics of the K+ current recorded in the presence of 0.5 μM tetrodotoxin (data not shown). During the first 5–8 min after toxin application, Na+ currents recorded at voltage pulses of −20 or −10 mV (from a holding potential of −60 mV) increased slightly, and an inward component developed. At −50 or −40 mV, inward Na+ currents could be measured, whereas no such currents existed under control conditions. The maximum inward Na+ current was obtained at −30 mV (Fig. 3, D panel b and F). Furthermore, a small inward constant current developed progressively up to 80–100 nA at holding potential during the first 5–15 min (Fig. 3F, panel b). These data imply that Bj-xtrIT-n as well as Bj-xtrIT.38E open Na+ channels at very negative potential values. As illustrated in Fig. 3D (panels a and b), it was possible to calculate the relative sodium conductance (g\textsubscript{Na+} = I\textsubscript{Na+}/(E\textsubscript{Na} − E\textsubscript{Na})) under control conditions and in the presence of toxin (Fig. 3E). Both the native and recombinant toxins caused a shift in the Na+ activation voltage curve to more negative values by 12–15 mV, and the overall sodium conductance of the membrane surface increased, especially between −60 and −20 mV. A decrease in this conductance could be observed 15–20 min after toxin application, when the holding current reached −80 nA. At this stage, it became evident that the axoplasmic concentration of sodium ions had increased, resulting in a decrease in the equilibrium potential for Na+ ions (E\textsubscript{Na}). After the peak Na+
current, a maintained inward current (without inactivation) developed at $-30\, mV$ to a higher extent than at positive potential values (Fig. 3D). This finding indicates that the inactivation process slows down less drastically than with α-type scorpion toxins, which favor plateau potentials. In summary, Bj-xtrIT causes repetitive activity of short action potentials, which explains the contractive response manifested by excitatory toxins.

**Modification of the C Terminus of Bj-xtrIT—Comparison of the three-dimensional structure of recombinant Bj-xtrIT (3) with the known structures of other toxins (22) revealed a striking similarity of a large portion of the molecule including the α-helix (residues 24–34) (Fig. 1), the β-sheet, and the configuration of three of the four disulfide bonds. However, an additional α-helix (residues 63–69) (Fig. 1) and a long C-terminal tail are unique to Bj-xtrIT (3) and therefore have become primary targets for modification to determine their involvement in the characteristic neuropharmacology of scorpion excitatory toxins. We used the *E. coli* expression and *in vitro* reconstitution system to produce and analyze a Bj-xtrIT.38E mutant (Bj-xtrIT.DC-ter) in which the entire C-terminal tail following Cys69, namely, Asp70-Val71-Gln72-Ile73-Ile74-Pro75-Ser76, was replaced with a single Gly residue (primer 7; see “Experimental Procedures”). This deletion did not affect the folding capacity of the mutant toxin as was suggested by the HPLC profile of the product obtained under similar folding conditions used for renaturation of Bj-xtrIT.38E. The HPLC-purified mutant polypeptide was analyzed for its bioactivity and secondary structure as detailed in Table II.

**Table II: Comparison of secondary structure content among scorpion neurotoxins**

| Toxin          | Measurement | H | A | P | T | O |
|----------------|-------------|---|---|---|---|---|
| CsE v3         | CD          | 15| 32| 4 | 23| 28|
| CsE v3         | x-ray       | 14| 29| 0 | 24| 40|
| AaH II         | CD          | 21| 31| 0 | 23| 29|
| AaHIT          | 2-D NMR     | 27| 7 | 4 | 28| 33|
| Bj-xtrIT.38E  | CD          | 34| 29| 0 | 16| 21|
| Bj-xtrIT.38K  | CD          | 11| 15| 15| 25| 33|
| Bj-xtrIT.DC-ter| CD          | 20| 18| 3 | 23| 36|

- H, α-helix; A, antiparallel β-sheet; P, parallel β-sheet; T, β-turn; O, other structures; x-ray, structure from x-ray data (CsE v3 (42) and AaH II (43)); 2-D NMR, structure from two-dimensional NMR (21).

Fig. 3. Effect of Bj-xtrIT on electrical activity and sodium currents of a cockroach isolated axon. A: two superimposed recordings of resting potentials and evoked action potentials under control conditions. A 0.5-ms current pulse of 10 nA evoked only one action potential of 95 mV in amplitude and 0.5 ms in duration (~20 mV above the resting potential). Six minutes after Bj-xtrIT application, the resting potential decreased by 6 mV; the action potential reached its threshold more rapidly; and a second spontaneous action potential was measured 6.5 ms later, giving an instantaneous frequency of 154 Hz. B and C: spontaneous activity of the action potential after 7 and 10 min, respectively, after Bj-xtrIT application. Action potential frequencies higher than 200 Hz were obtained, and no “plateau potentials” could be measured. After a 10-mV artificial hyperpolarization generated by a constant current, a slow spontaneous depolarization reached the threshold for a sustained repetitive firing of short action potential (C). D: families of Na+ currents recorded in the presence of 0.5 mM 3,4-diaminopyridine during 5-ms voltage pulses. Panel a, control; panel b, after 6 min in the presence of Bj-xtrIT. Note that after Bj-xtrIT application, Na+ currents developed at more negative potential values than in the control. The horizontal lines indicate the zero current level; E: voltage dependence of the sodium conductance (expressed as gNa/ gNa.max.control) calculated from current families as illustrated in D. Note the shift of the activation curve by 12.5 mV toward more negative potentials after addition of Bj-xtrIT. F: panel a, Na+ current during a 300-ms voltage pulse to $E_m = -30\, mV$ showing a sustained current, which did not inactivate during the pulse, and the existence of an inward holding current before and after the pulse (dotted line indicates zero current level); panel b, superimposed recordings before and after application of 0.5 μM tetrodotoxin, which suppressed the Na+ peak and maintained the holding current.
structure content (Table II). No toxicity could be detected upon injection into blowfly larvae of quantities up to 16 μg/100 mg of larva (Table I). In binding assays, Bj-xtrIT.C-ter displaced LqhIT2 from the high affinity binding site shared by depressant and excitatory toxins (31) at concentrations above 400 nM compared with 1.4–3.0 nM unmodified toxin (Fig. 2). The CD spectrum of the tailless mutant resembled the spectrum of Bj-xtrIT.38K (Fig. 4), and its secondary structure content was practically similar to those of the two unmodified variants, Bj-xtrIT.38K and Bj-xtrIT.38E (Table II), suggesting similarity in overall structure. To elucidate specific residues at the C terminus that could participate in bioactivity, the C terminus was analyzed by sequential deletions. Deletion of the ultimate Ser alone or together with the penultimate Pro revealed mutant toxins with ~5-fold decreased activity. Any truncation beyond the last two residues resulted in an inactive mutant toxin (Table I).

**DISCUSSION**

In this study, we provide insight into the putative bioactive surface of scorpion excitatory neurotoxins displaying anti-insect selectivity. The atypical features of Bj-xtrIT, a unique representative of this pharmacological group from the Israeli black scorpion (*B. judaicus*), enabled high yield expression, proper in vitro folding, and crystallization (3). With these prerequisites, a genetic modification approach has become available for the elucidation of the bioactive site of these important effectors of insect sodium channels.

**Uniqueness of Bj-xtrIT**—Bj-xtrIT is the longest scorpion neurotoxin affecting sodium channels that has been described thus far (76 residues; molecular mass of 8455 Da). The added length of Bj-xtrIT is manifested by an additional pentapeptide (residues 21–25) preceding the α-helical motif (residues 24–34) in the toxin core (3) and in one additional C-terminal residue (Fig. 1). Its deduced amino acid sequence differs substantially from all highly conserved sequences of known excitatory toxins (49% similarity) (Fig. 1). Whereas all excitatory toxins contain two prolines and four glycines, Bj-xtrIT is relatively rich in these amino acids (five and seven, respectively), which may suggest higher conformational flexibility. Despite the differences, Bj-xtrIT displays, with high fidelity, the characteristic features of excitatory toxins, e.g., poisoning symptoms, displacement from the receptor-binding site (Fig. 2), sodium channel modification (Fig. 3), CD spectrum (Fig. 4), and immunoreactivity with antibodies raised against Lqh-xtrIT (data not shown). Although it has been shown in quantitative radio immunonassays performed with scorpion α-toxins that cross-reactivity demanded at least 75% sequence homology (44), Bj-xtrIT and the other excitatory toxins, varying substantially in sequence, seem to share rather conserved immunogenic epitopes. The molecular mass, ED50 values for blowfly larvae, amino acid composition, and overall length of Bj-xtrIT differ substantially from those determined for a *B. judaicus* excitatory toxin, BjIT1, detected and partly characterized by Lester et al. (9); yet it cannot be ruled out that BjIT1 and Bj-xtrIT are identical.

The uniqueness of Bj-xtrIT, compared with other excitatory toxins, seems to be of great importance and provides the following advantages: 1) amenability to genetic dissection and structural analysis due to the easy production and ability to fold in vitro; 2) distinction between conserved versus variable regions, which enables a mutagenic approach to determine functionally important residues; and 3) an applied potential arising from its prominent anti-lepidopteran toxicity (PU50 = 1.4 μg/100 mg of body weight) compared with the effect (PU50 = 2.5 μg/100 mg of body weight) induced by the classical excitatory toxin AaHIT (45). It is possible that the metabolic fate of Bj-xtrIT in the hemolymph differs from that of AaHIT with respect to accessibility barriers and to degradation processes. These features place Bj-xtrIT as a preferred candidate among scorpion toxins for improving the insecticidal efficacy of baculoviruses.

Further evidence for the structural uniqueness of Bj-xtrIT is provided by its three-dimensional features (3). Unlike other known long toxin structures (Ref. 22 and reviewed in Ref. 2), Bj-xtrIT consists of two structural entities: a major entity (residues 1–59) encompassing the α/β-core found in all other toxins and a minor entity (residues 60–76) comprising the additional α-helix and the seven-residue C-terminal tail. The pronounced ability of mutant Bj-xtrIT.C-ter to fold into a major isoform with secondary structure content (Fig. 4 and Table II), similar to that of the unmodified toxin variants, suggests that the C-terminal tail is devoid of typical secondary structure and has no effect on the folding capability of the entire molecule. Although the additional α-helix observed in Bj-xtrIT (residues 63–69) (3) is contiguous to the bioactive C-terminal tail, its actual role remains to be determined.

Interestingly, the α-helix in the conserved α/β-core (residues 24–34) is preceded by an additional four-residue helical motif (residues 19–22), which is part of the five-residue insertion unique to Bj-xtrIT (Fig. 1). The existence of additional helical elements explains the relatively high content of α-helical secondary structure observed in the singular value decomposition analysis of excitatory toxins (Table II).

**Putative Toxic Surface of Scorpion Excitatory Toxins**—The ability of Bj-xtrIT to displace AaHIT from its receptor-binding site, despite their moderate sequence homology, implies a common bioactive surface. The effect of the modifications introduced at the C-terminal tail of Bj-xtrIT on the bioactivity strongly suggests that the putative active surface is located in the vicinity of the C-terminal region. Comparison of the three-dimensional structure of Bj-xtrIT with the AaHIT three-dimensional model, with particular emphasis on the C-terminal module, could therefore highlight conserved and hence functionally significant residues in this region. Taking the conserved bioactive Ile73 and Ile74 as two common reference points, another 11 residues comprising Lys1, Lys2, Asn28, Thr32, Lys33, Tyr36,
FIG. 5. Putative interaction surface of scorpion excitatory toxins. A, a ribbon diagram of Bj-xtrIT structure (3) with the side chain residues indicated in B. B and C, the putative bioactive surfaces of Bj-xtrIT and AaHIT, respectively, highlighted by the ellipsoid. The model of AaHIT (C) was constructed according to the solved structure (3) of Bj-xtrIT (B). Colors are according to the side chain character: blue, positive; red, negative; magenta, aromatic; green, hydrophobic; yellow, polar; white, glycine. Note the position of the essential residue, the last residue observed (36). Acetylation of residues that belong to the putative bioactive surface of some residues belonging to this molecular surface has also been provided by chemical modifications introduced to AaHIT (36). Acetylation of residues that belong to the putative bioactive surface, e.g., Lys\(^{39}\) and Lys\(^{51}\) (Lys\(^{39}\) and Lys\(^{56}\) in the Bj-xtrIT sequence), substantially decreased the activity without perturbation of the CD spectrum of the modified polypeptides. Conversely, acetylation of Lys\(^{34}\), carbothoxylation of the imidazolyl ring of His\(^{39}\), and modification of Arg\(^{50}\) by 1,2-cyclohexanedione had no effect on the toxicity of AaHIT (36). These positions in Bj-xtrIT are occupied by Ser\(^{39}\), Tyr\(^{35}\), and Thr\(^{65}\), respectively, and in addition to the lack of conservation, they are either buried or remote from the putative bioactive surface.

On the basis of the structure and the putative active surface, it is possible to interpret the similar activity of Bj-xtrIT.38K and Bj-xtrIT.38E variants. Although the last three residues of the C-terminal tail could not be observed in the crystal structure (3), it is likely that they cover residue 38 and thus reduce the effect of its charge on the bioactive surface (Fig. 5). Other residues that are conserved in Bj-xtrIT and AaHIT are located farther away from the C terminus and are less exposed to the solvent. As suggested by the crystal structure of Bj-xtrIT, it is clear that Asp\(^{70}\) is somewhat buried in the structure, and its conservation might be due to its structural role in stabilizing the C-terminal module through hydrogen bonds with Asn\(^{3}\) and Tyr\(^{48}\) and a salt bridge with Lys\(^{66}\) (3). The residues in the conserved surface lie, as was proposed by Fontecilla-Camps (22), on loops outside the αβ-core, whereas the conserved residues not included in the bioactive surface may play a structural role.

The putative toxic surface of Bj-xtrIT (and, most likely, of all other excitatory toxins) is the first structural element described thus far that is able to recognize exclusively sodium channels of insects. As such, it becomes an important tool for the molecular characterization of these channels and may be of practical value in the design of novel insect-pest control agents.

Acknowledgment—We thank Prof. M. Adams (University of California, Riverside, CA) for help and critical comments.

REFERENCES

1. Zlotkin, E. (1987) *Endeavour* **11**, 168–174
2. Gordon, D., Savarin, P., Gurevitz, M. & Zinn-Justin, S. (1998) *J. Toxicol. Toxin Rev.* **17**, 131–158
3. Oren, D., Froy, O., Amit, E., Kleinberger-Doron, N., Gurevitz, M. & Shaanan, B. (1998) *Structure* **6**, 1185–1192
4. Darbon, H., Zlotkin, E., Kopeyan, C., Van Rietelschoten, J. & Rochat, H. (1982) *Int. J. Pept. Protein Res.* **20**, 320–330
5. Zlotkin, E., Rochat, H., Kopeyan, C., Miranda, F. & Lissitzky, S. (1971) *Biochimie* **53**, 1073–1078
6. Kopeyan, C., Mansuelle, P., Sampieri, F., Brando, T., Bahrani, E. M., Rochat, H. & Granier, C. (1990) *FEBS Lett.* **261**, 423–426
7. Zlotkin, E., Teitelbaum, Z., Rochat, H. & Miranda, F. (1979) *Insect Biochem.* **9**, 347–354
8. Pelhate, M. & Zlotkin, E. (1982) *J. Exp. Biol.* **97**, 67–77
9. Lester, D., Lazarovici, P., Pelhate, M. & Zlotkin, E. (1982) *Biochim. Biophys. Acta* **701**, 370–381
10. Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M. F. & Rochat, H. (1985) *Arch. Biochem. Biophys.* **240**, 877–887
11. Gordon, D., Jover, E., Couraud, F. & Zlotkin, E. (1984) *Biochim. Biophys. Acta* **778**, 349–358
12. Maeda, S., Voit, S. L., Hanzlik, T. N., Harper, S. A., Majuma, K., Maddox, D. W., Hammock, B. D. & Fowler, E. (1991) *Virology* **164**, 777–780
13. Stewart, L. M. D., Hirst, M., Ferber, M. L., Merryweather, A. T., Cayley, P. J. & Possee, R. D. (1991) *Nature* **352**, 85–88
14. McCutchen, B. F., Choudary, P. V., Cresshaw, R., Maddox, D. & Kamita, S. G., Palchak, N., Volvah, S., Fowler, E., Hammock, B. D. S. & Maeda, S. (1991) *Bio/Technology* **9**, 848–852
15. Cory, J. S., Hirst, M. L., Williams, T., Hails, R. S., Goulson, D., Green, B. M., Carty, T. M., Possee, R. D., Cayley, P. J. & Bishop, D. H. (1994) *Nature* **370**, 138–140
16. Jarvis, D. L., Reilly, L. M., Hoover, K., Schultz, C., Hammock, B. D. & Guarino, L. A. (1996) *Biol. Control* **7**, 228–235
17. Gerishburg, E., Stockholm, D., Froy, O., Rashi, S., Gurevitz, M. & Chejanovsky, N. (1998) *FEBS Lett.* **422**, 132–136
18. Bougis, P. E., Rochat, H. & Smith, L. A. (1989) *J. Biol. Chem.* **264**, 19259–19265
