Scorpion β-toxins affect the activation of voltage-sensitive sodium channels (NaChs). Although these toxins have been instrumental in the study of channel gating and architecture, little is known about their active sites. By using an efficient system for the production of recombinant toxins, we analyzed by point mutagenesis the entire surface of the β-toxin, Bj-xtrIT, an anti-insect selective excitatory toxin from the scorpion Buthotus judaicus. Each toxin mutant was purified and analyzed using toxicity and binding assays, as well as by circular dichroism spectroscopy to discern the differences among mutations that caused structural changes and those that specifically affected bioactivity. This analysis highlighted a functional discontinuous surface of 1405 Å², which was composed of a number of non-polar and three charged amino acids clustered around the main α-helical motif and the C-tail. Among the charged residues, Glu³⁰ is a center of a putative “hot spot” in the toxin-receptor binding-interface and is shielded from bulk solvent by a hydrophobic “gasket” (Tyr²⁶ and Val³⁴). Comparison of the Bj-xtrIT structure with that of other β-toxins that are active on mammals suggests that the hot spot and an adjacent non-polar region are spatially conserved. These results highlight for the first time structural elements that constitute a putative “pharmacophore” involved in the interaction of β-toxins with receptor site-4 on NaChs. Furthermore, the unique structure of the C-terminal region most likely determines the specificity of excitatory toxins for insect NaChs.

Scorpion toxins that modify the gating of voltage-sensitive sodium channels (NaChs) are divided into α and β classes according to their modes of action and binding properties to distinct receptor sites (1, 2). Their high potencies and varying specificities for NaChs subtypes emphasize their importance in the study of channel function and architecture (2). α-Toxins affect channel inactivation upon binding to receptor site-3, whereas β-toxins modulate the activation properties of the channel when interacting with receptor site-4 (3). Among β-toxins, two distinct groups, the excitatory (e.g. AahIT from Androctonus australis hector, Aah) and the depressant (e.g. LqhIT² from Leiurus quinquestriatus hebraeus, Lqh), show specificity for insects (4). Despite different symptoms in blowfly larvae and effects on the sodium current induced by excitatory and depressant toxins, they compete with one another on binding to cockroach NaChs (5). The specificity of excitatory and depressant toxins for insects has prompted baculovirus engineering in an effort to increase their insecticidal efficacy (e.g. 6–9). Interestingly, the β-toxins, Ts1 (from Titus serrulatus) and Lqhβ1 (Fig. 1), which bind with high affinity to both insect and rat brain NaChs, compete with excitatory and depressant toxins upon binding to insect NaChs (10–15). The competitive binding interactions among various β-toxins imply commonality in receptor site-4 (13, 14, 16), which has been shown to be associated with domain-2 of both insect and mammalian NaChs (17–19).

Several excitatory toxins have been identified thus far (e.g. AahIT, LqhIT¹ from Leiurus quinquestriatus quinquestriatus, and Bj-xtrIT from Buthotus judaicus (current name Hottentotta judaica); Fig. 1A), and their modes of action on insects have been characterized. These toxins generate contraction paralysis in fly larvae as a result of nerve repetitive activity (4, 20–23). Under voltage-clamp conditions, excitatory toxins shift insect NaCh activation to more negative membrane potentials (17, 20, 23, 24), which also typifies the effect of scorpion β-toxins on mammalian NaChs (25). Unfortunately, the difficulty of producing active excitatory toxins using a variety of expression systems (8, 26–28) delayed the clarification of their functional surface. Bj-xtrIT, however, has been produced recently in a functional recombinant form using an efficient bacterial expression system (23), which enabled determination of its three-dimensional structure (Fig. 1, B and C; Ref. 29) and unraveling of the importance of the C-tail for activity (23). The core structure of Bj-xtrIT is very similar to those of other “long chain” scorpion toxins active on NaChs (α-helix packed against a three-stranded β-sheet stabilized by three spatially conserved disulfide bonds; Fig. 1), but varies prominently in the spatial arrangement of its forth disulfide bond (Fig. 1; Refs. 29, 30). Moreover, the C-terminal region of Bj-xtrIT (residues 60–76), and most likely that of all excitatory toxins, contains an additional unique α-helix, α₂ (residues 63–69 in Bj-xtrIT; Ref. 29). Bj-xtrIT shares only 44–49% sequence similarity with the other excitatory toxins and contains an additional five-residue

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segment (residues 21–25) preceding the conserved helix, \( \alpha_1 \) (residues 25–34; Fig. 1). Nevertheless, Bj-xtrIT competes with the excitatory toxin, AahIT, on binding to insect NaChs and induces identical modulation of the sodium current in cockroach axons (20, 23), which suggests similarity in the functional surface of these toxins.

Here we provide the first detailed report of the entire functional surface of a scorpion excitatory toxin, Bj-xtrIT, which affects specifically insect NaChs. The functional surface is composed of a region surrounding a putative "hot spot" and the C-tail that most likely provides the specificity for insects. A negatively charged hot spot residue (Glu30 in Bj-xtrIT) adjacent to a nonpolar cluster appears in all \( \beta \)-toxins, suggesting that they constitute the "pharmacophore" of toxins that bind to receptor site-4 on various NaChs.

**MATERIALS AND METHODS**

**Bacterial Strains**—Escherichia coli DH5\( \alpha \) cells were used for plasmid constructions, and the BL21 strain for toxin expression using the pET-11c vector as described (23).

**Toxicity Assays**—Four-day-old blowfly larvae (Sarcophaga gregaria; 150 ± 20-mg body weight) were injected inter-segmentally. A positive result was scored when a characteristic paralysis (immobilization and contraction) was observed up to 5 min after injection. Five concentrations of each toxin were injected to larvae (nine larvae in each group) in
three independent experiments. ED$_{50}$ (effective dose 50%) values were calculated according to the sampling and estimation method of Reed and Muench (31).

**Binding Studies**—Insect synaptosomes were prepared from whole heads of adult cockroaches (Periplaneta americana) according to a method described previously (32). Bj-xtrIT was radioiodinated by iodogen using 5 µg of toxin and 0.5 mCi of carrier-free Na$^{125}$I. Concentration of labeled monomiodotoxin was determined according to the specific activity of 125I (2500–3000 dpm/mmol; Refs. 33, 34). Toxin binding assays were performed as was described in detail previously (11). Membrane-bound toxin was separated from the free radio-ligand by rapid filtration (under vacuum) through glass-fiber filters (GF/C Whatman, Maidstone, UK). Hot saturation assays were performed using increasing concentrations of the radio-labeled toxin, with a constant amount of membranes. Data were analyzed using the iterative program LIGAND (Elsevier Biosoft, Cambridge, UK) using “Hot Saturation” analysis. Equilibrium competition binding experiments were carried out with increasing concentrations of the unlabeled toxin in the presence of a constant low concentration of the radioactive toxin. Competition experiments were analyzed by KaleidoGraph (Synergy Software version 3.08) using a non-linear fit to the Hill equation (34) for determination of the inhibitory concentration 50% (IC$_{50}$). K$_{i}$ values were calculated by the equation K$_{i}$ = IC$_{50}$/(1 + [L*/K$_{a}$]), where L* is the radioactive ligand concentration and K$_{a}$ is its dissociation constant. Each experiment was performed at least three times, and each data point represents the mean of two to three samples (with up to 12% deviation between samples). Data are presented as mean ± S.E. of a number (n) of independent experiments.

**CD Spectroscopy**—CD spectra were recorded at 25 °C using a model 202 circular dichroism spectrophotometer (Aniv Instruments, Lake-wood, NJ). Toxins (140 µm) were dissolved in 5 mM sodium phosphate buffer, pH 7.0, and their spectra (180–260 nm) were measured three times using a 0.1-mm path length quartz cuvette. Blank spectrum of the buffer was run under identical conditions and subtracted from each toxin spectrum.

**Site-directed Mutagenesis and Production of Recombinant Toxic Variants**—Expression of Bj-xtrIT has been described (23). Mutations in the toxin were generated by PCR using complementary oligonucleotide primers (purchased from Sigma) with the expression vectors as DNA templates (23). Toxic mutants were produced similarly to the unmodified toxins. Sequences were verified prior to expression. Quantification of purified recombinant toxins was performed by amino acid analysis using an ABI system 420A/130A synthesizer (Applied Biosystems Inc., Foster City, CA) after hydrolysis by 6 N HCl under vacuum (18 h at 110 °C).

**Three-dimensional Models and CSU Analysis**—Three-dimensional models of Bj-xtrIT were constructed according to the crystal structure (Protein Data Bank accession no. 1BCG) using the Swiss Model Homology Modeling server and the Swiss-PDB Viewer program. Intra-molecular Contacts of Structural Units (CSU) were obtained using the CSU software (35).

**RESULTS**

Bj-xtrIT binds with high affinity (K$_{d}$ = 0.148 ± 0.032 nm, n = 4) to a single site on NaChs in cockroach neuronal membranes (Fig. 2A, inset). This site is also recognized by other anti-insect excitative (e.g., AahIT) and depressant (e.g., LqhIT$_{2}$) toxins (23) and by a β-toxin (Lqhβ1) active on both insects and mammals (11). To elucidate the entire functional surface of Bj-xtrIT, we mutated all solvent-exposed residues (Fig. 1C). Each mutant was produced in a recombinant form and analyzed for toxicity to fly larvae and binding properties on cockroach neuronal membranes. These assays were accompanied by CD spectroscopy to discern between effects resulting from putative structural perturbation and those reflecting putative interaction with the channel receptor. Overall, the changes in toxicity reflected the changes in binding affinity, and the CD spectra of most mutants were indistinguishable from that of the unmodified toxin. Residues whose substitution decreased substantially the toxicity (ED$_{50}$ mutant/ED$_{50}$ wild-type < 10) and binding affinity (K$_{d}$ mutant/K$_{d}$ wild-type > 30) with no alteration of the CD spectrum were considered significant for activity and assigned to the putative functional surface. Residues whose substitution reduced the activity but also altered the CD spectrum (S29A, W44E, Y68A/L) were excluded from the functional surface.

**Mutagenesis of Residues in the N-terminal Region and the Loop Preceding α1**—The N-terminal region (residues 1–15) of Bj-xtrIT and other excitatory toxins is rich in charged residues, several of which are involved in intra-molecular hydrogen bonds (29). Substitution of residues in this region had minor effects on activity, except for Glu$^{25}$ (Fig. 2A, Table I). Mutations E15Q (charge neutralization) and E15D (shorter side-chain) reduced the binding affinity over 100-fold (Fig. 2, A and B, Table I). In contrast, almost all substitutions in the region composed of residues 17–24 (precedes α1 and includes α1) affected toxin activity (Fig. 2A). Mutations V19A, N20A, I22A, P24A, and P24G, but not S17A or A21G (smaller side-chain) significantly decreased the activity (Table I; Fig. 2A). Increase in side-chain size at positions 19 (V19W), 23 (A23V), and 20 (N20Q or N20W), but not 21 (A21V) and 22 (I22L, I22F, or I22W), decreased the activity (Table I). These results suggest that the solvent-exposed region preceding α1 may be part of the interacting surface of Bj-xtrIT with its receptor site.

**Modification of Amino Acid Residues in α1**—Among the ten amino acids encompassing α1 (positions 25–34), substitution of four residues (His$^{25}$, Tyr$^{26}$, Glu$^{30}$, Val$^{34}$) reduced the activity (Fig. 2; Table I). The importance of the hydrophobic moiety and the charge of His$^{25}$ for function were demonstrated by the decreased activity of mutants H25A and H25N (Table I) but not H25R (not shown). Neutralization of the negatively charged side-chain of Glu$^{30}$ (E30Q) reduced the binding affinity 75-fold, suggesting a functional role for this residue. A conserved substitution (E30D) also had an effect (Table I), thus emphasizing the importance of side-chain length at this position. The most prominent effect on activity was obtained upon charge inversion (mutation E30R), which practically abolished the activity (Table I; Fig. 2). These results suggest that Glu$^{30}$ may interact with a positively charged residue of the receptor site.

Because the side-chains of Tyr$^{26}$ and Val$^{34}$ project to the solvent in the same plane as that of Glu$^{30}$ (29), we examined their role in toxin function. Substitution of both residues by Ala had little effect on toxicity (not shown), whereas increase in side-chain size (Y26W or V34W) decreased the binding affinity 950- and 16,300-fold, respectively (Table I). We further examined the importance of the chemical nature of Val$^{34}$ by its substitution with a polar residue of a similar size (V34S). The substantial effect on activity of this mutation together with the above results suggest that a hydrophobic side-chain of limited size is important at this position for toxin function. These results imply that Glu$^{30}$ flanked by Tyr$^{26}$ and Val$^{34}$ may be part of the putative contact surface of the toxin with its receptor site.

**Modifications in Other Loops and the C-terminal Region**—We mutagenized most of the solvent-exposed charged and hydrophobic residues in the loops encompassing amino acids 35–62 (Fig. 1, A and C). Mutations Y35A, Y35W, Y36A, E38I, E53A, D54A, D55A, K56T, K56A/P60S, and K62A had small effects on toxin activity (Fig. 2; Table I). Similarly, mutations D63N, K66A, and K67A at the C-terminal α2 motif (Fig. 1) had minor effects on activity (Fig. 2).

Tyr$^{68}$ is conserved in excitatory toxins (Fig. 1A) and may interact with Ile$^{64}$ (3.2 Å in distance) and with Trp$^{44}$ (4.4 Å in distance; Ref. 29). A Y68W mutation, which maintained the aromatic nature at this position, had no effect on toxin function (not shown), whereas substitutions to Ala or Leu were detrimental to both the structure (alteration of CD) and activity (not shown). Substitutions W44A and W44L (Leu is found in other excitatory toxins; Fig. 1A) had only little effect on activity (Table I), presumably because the hydrophobic interaction with Tyr$^{68}$ was retained. Mutation I64A decreased the binding af-
A. FIG. 2. Mutagenic dissection of Bj-xtrIT. A, effect of substitutions on toxin activity (see Table I for $\Delta \Delta G$ values). Changes in activity were determined by binding to cockroach neuronal membranes ($K_i$) and are presented as the ratio of $K_i$ (mutant over WT, ratio $K_i$ mut/wt). $B$, $K_i$ value of the unmodified recombinant Bj-xtrIT. Inset, Scatchard plot of equilibrium-binding saturation curve obtained with increasing concentrations of $^{125}$I-Bj-xtrIT (5 pm to 1 nm) incubated 60 min at 22°C with cockroach membranes (7 μg protein/ml). $B/F$ designates bound ligand over the free ligand. Nonspecific binding, determined in the presence of 1 μM Bj-xtrIT, was subtracted. Data were analyzed by LIGAND (see "Materials and Methods"). The binding parameters obtained are: $K_d = 149 \pm 32$ pm, $B_{max} = 3.0 \pm 0.8$ pmol/mg (n = 4). A representative experiment is shown.

B. Competitive binding of $^{125}$I-Bj-xtrIT to cockroach neuronal membranes. Membranes (7 μg/ml) were incubated 60 min at 22°C with 50 μM $^{125}$I-Bj-xtrIT and increasing concentrations of the various mutants. Nonspecific binding, determined in the presence of 1 μM Bj-xtrIT (corresponding to 15–20% of total binding), was subtracted. The $K_i$ values (in nm) are: Bj-xtrIT WT, 0.165 ± 0.09 (n = 6); E15Q, 20 ± 1.7 (n = 3); V19A, 5.4 ± 0.6 (n = 3); I22A, 13 ± 2 (n = 3); H25N, 20 ± 1.6 (n = 3); E30Q, 12.3 ± 1.8 (n = 3); V71A, 16 ± 3.5 (n = 3); I74A, 17.4 ± 0.6 (n = 3).
finity 50-fold (Table I), thus endorsing the putative importance of its interaction with Tyr69. On the basis of these results, residues 64 and 68 were not assigned to the functional surface.

Previously, we have shown that deletion of C-terminal residues (Asp70–Ser79) abolished the activity of Bj-xtrIT, whereas deletion of the two terminal residues Pro75 and Ser76 (mutant ΔPro75–Ser76) had no effect (29). To better specify the residues that are important for activity in this region, all C-tail residues were analyzed. A D70A mutation had only a minor effect, whereas V71A, Q72A, I73A, and I74A affected significantly the activity of Bj-xtrIT (Table I; Fig. 2). Substitution of Ile73 to Phe, whereas V71A, Q72A, I73A, and I74A affected significantly the activity of Bj-xtrIT, all C-tail residues that are important for activity in this region, all C-tail residues were analyzed. A D70A mutation had only a minor effect, whereas V71A, Q72A, I73A, and I74A affected significantly the activity of Bj-xtrIT (Table I; Fig. 2). Substitution of Ile73 to Phe, whereas V71A, Q72A, I73A, and I74A had a smaller effect compared with the Ala mutants (Table I). These results suggest that the side-chain length of these residues is important for toxin function. Furthermore, insertion of a negative charge at the C-tail (mutant I73E) was detrimental (6900-fold decrease in binding affinity; Table I). These results suggest that the overall hydrophobic nature of the C-terminal region is important for the activity of Bj-xtrIT.

**DISCUSSION**

Molecular dissection of Bj-xtrIT illuminates a discontinuous functional surface (1405 Å²) composed of amino acid residues of the α-helical motif, its preceding loop, the C-terminal region, and Glu15. Although the loop preceding α1 is unique to Bj-xtrIT and is shorter in other excitatory toxins (Fig. 1), this non-polar cluster seems to be part of the contact surface with the receptor site (Table I; Fig. 3, A and B). In contrast to previous suggestions that positively charged residues have a mandatory role in the activity of scorpion β-toxins (19, 36, 37), the only positively charged residue with a functional role in Bj-xtrIT is His25. Because His25 is not conserved in excitatory toxins or other β-toxins (Fig. 1), the role of the residue at this position in other toxins deserves further investigation. Notably, two negatively charged residues (Glu15 and Glu30) play a critical role in Bj-xtrIT activity. Glu15, whose side-chain projects to the solvent, is conserved in excitatory toxins, and is distant from all other residues involved in function (12 Å from Glu30 and 13 Å from His25; Fig. 3). Glu30 is conserved in the α-helix of all β-toxins, and its substitution to Arg (E30R) had the most detrimental effect on toxin activity.

A recent survey on protein-protein binding interfaces reveals that the free energy of binding is mostly concentrated in hot spots formed by a small number of residues (38). At these hot spots, the high-energy interactions are protected from bulk solvent by surrounding residues that form a shielding “gasket.” Substitution of the gasket residues to Ala has been found in many cases to have little effect on the binding energy, implying that the Ala side-chain and protein backbone are sufficient to occlude solvent from the hot spot. Conversely, substitution by Trp may disrupt the contact surface and hence the shield, leading to a marked decrease in binding affinity (38). The mutagenesis results of Tyr26, Val34, and Glu30 fit well with this scenario and suggest that Glu30 serves as a point of high-energy interaction with the receptor. The reduced binding en-

**TABLE I**

Effects of mutations on the energy of Bj-xtrIT binding to cockroach sodium channels

| Bj-xtrIT mutant | ΔG kcal/mol | Bj-xtrIT mutant | ΔG kcal/mol |
|-----------------|-------------|-----------------|-------------|
| E15Q            | 2.84        | Y26A           | 0.70        |
| E15D            | 3.05        | Y28W           | 4.06        |
| V19A            | 2.06        | E30Q           | 2.55        |
| V19L            | 1.48        | E30D           | 2.24        |
| V19W            | 2.56        | E30R           | 5.53        |
| N20A            | 3.61        | V34S           | 2.73        |
| N20W            | 3.65        | V34W           | 5.74        |
| N20Q            | 4.26        | W44A           | 1.22        |
| A21G            | 1.98        | W44L           | 0.90        |
| A21V            | 1.03        | I64A           | 2.32        |
| I22A            | 2.59        | V71A           | 2.71        |
| I22L            | 1.33        | Q72A           | 4.25        |
| I22F            | 0.86        | I73A           | 4.06        |
| I22W            | 0.05        | I73E           | 5.17        |
| A23G            | 3.56        | I73F           | 2.32        |
| A23V            | 1.79        | I74A           | 2.76        |
| P24G            | 2.93        | I74F           | 1.72        |
| P24A            | 3.24        | I74L           | 1.77        |
| H25A            | 3.91        | I74V           | 2.11        |
| H25N            | 2.86        |                 |             |

**Fig. 3.** Residues involved in Bj-xtrIT function. A, two clusters constitute the functional surface. One cluster appears at α1 and its vicinity, and the other cluster appears at the C-terminal region. The toxin model includes the C-terminal residues 74–76 and is taken from Gurevitz et al. (30). B, residues that comprise the functional surface of Bj-xtrIT. C, Bj-xtrIT at 180° angle on the y axis relative to B. D, superposition of the Cα backbone of Bj-xtrIT (red), Cn2 (blue), and Ts1 (green). The black dashed ellipse indicates the point of divergence of the C-terminal region in the three toxins (30). Note the spatial conservation of Glu with the putative hydrophobic gasket in the three toxins. The residues in A–C are colored as in Fig. 1C. Residues whose substitution did not affect activity are in gray.
energy of E30Q and E30R (\(\Delta G = 2.55\) and 5.53 kcal/mol, respectively) compared with the smaller effect obtained with E30D (\(\Delta G = 2.24\) kcal/mol) indicates that the negatively charged Glu\(^{10}\) most likely interacts electrostatically with a positively charged receptor counterpart. The little effect obtained upon Y26A and V34A substitutions and the prominent effect produced by the Y26W and V34W mutations (Table I) suggest that these residues participate in the gasket that shields the electrostatic interaction of Glu\(^{10}\) with the receptor site. This suggestion is corroborated by the reduced activity obtained upon a V34S substitution, introducing a polar side-chain that presumably disrupts the hydrophobic seal around Glu\(^{10}\) (Fig. 3; Table I; Ref. 38).

Although receptor site-4 of insect and mammalian NaChs has been shown to involve domain 2 (17–19), the composition and topology of this receptor are still elusive. Assuming that the receptor site reflects, at least to some extent, the mirror image of the functional surface of the toxin, it is conceivable that the receptor site-4 of insect and mammalian NaChs may unravel the mechanism by which these toxins modulate NaCh function. These data may be instrumental in the design of novel drugs and insecticides.

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