Functional Expression and Genetic Alteration of an Alpha Scorpion Neurotoxin†

ARTICLE *in* BIOCHEMISTRY · AUGUST 1996

Impact Factor: 3.02 · DOI: 10.1021/bi9528309

Functional Expression and Genetic Alteration of an Alpha Scorpion Neurotoxin†

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Received November 29, 1995; Revised Manuscript Received May 15, 1996⊗

ABSTRACT: The alpha neurotoxin LqhαIT is toxic to both insects and mammals but exhibits a bioactivity ratio favoring insects (insect/mammal ∼2). With the objective of increasing this ratio by genetic manipulation of the amino acid sequence, a cDNA clone encoding LqhαIT was used to produce recombinant variants of the toxin in a high efficiency bacterial expression system. The unmodified recombinant toxin, isolated from inclusion bodies and renatured *in vitro*, exhibited chemical and biological properties indistinguishable from those of the authentic native toxin. Alteration of the toxin by site-directed mutagenesis led to a substantial reduction in anti-mammalian toxicity (mouse LD_{50} reduced 6.4-fold) but only a slight reduction (×1.5) in the insect ED_{50} value for paralysis. The reduction in anti-mammalian toxicity was correlated with a ∼2-fold reduction of its potency for slowing of sodium channel inactivation in mammalian neurons, while no change in mutant toxin binding affinity to insect neuronal receptors was registered. These results demonstrate for the first time expression of a recombinant sodium channel neurotoxin in *Escherichia coli* and the use of site-directed mutagenesis to improve phylogenetic selectivity. This recombinant approach provides a promising strategy for optimizing the selective toxicity of peptide neurotoxins.

Alpha scorpion toxins from the subfamily Buthinae are structurally related polypeptides which prolong sodium channel inactivation (Zlotkin et al., 1978; Rochat et al., 1979). These toxins bind to site 3 on mammalian sodium channels in a voltage-dependent manner, and lipid-soluble toxins such as veratridine allosterically enhance this binding (Catterall, 1980, 1986). As specific modifiers of the channel inactivation process, alpha toxins serve as pharmacological tools for analyses of channel kinetics and gating properties (Catterall, 1980, 1986). The insecticidal properties of some of these toxins also make them useful in analyzing structural determinants of phylogenetic selectivity.

LqhαIT,[1] a toxin from the *Leiurus quinquestriatus hebraeus* venom, shows high sequence similarity to previously described α-scorpion toxins and functional similarity in slowing the inactivation process of insect sodium channels (Eitan et al., 1990). Furthermore, the binding of LqhαIT is competitively inhibited by the sea anemone toxin ATXII and enhanced by veratridine (Gordon & Zlotkin, 1993). Radiolabeled LqhαIT binds to a single class of high affinity (K_d = 1.06 ± 0.15 nM) sites in insect neuronal membranes but shows no detectable binding to rat brain membranes (Gordon & Zlotkin, 1993). Nevertheless, LqhαIT is toxic to mice only at relatively high doses, perhaps due to modification of muscle sodium channels (Eitan et al., 1990). LqhαIT thus appears to recognize a conserved alpha-toxin binding site on both insect and mammalian sodium channels, while at the same time having an apparent higher affinity for the insect channel. This toxin thus serves as a suitable model for probing the structural basis of selective toxicity for a peptide neurotoxin. Much information has been obtained from chemical modifications introduced at specific residues of the alpha toxins AaHII and AaHIII from the scorpion *Androctonus australis* Hector, Lqq5 from *Leiurus quinquestriatus quinquestriatus*, and BomIII from *Buthus occitanus mardochei* (Darbon et al., 1983; Kharrat et al., 1989; El Ayab et al., 1986a). Moreover, the interaction of AaHII with its receptor site was studied by using site-directed antibodies (El Ayeb et al., 1986b). However, up to now, no genetic study has been conducted with scorpion neurotoxins that affect sodium channels, mainly due to the lack of a reliable quantitative expression system.

Here we describe the isolation of LqhαIT cDNA, the efficient expression of the recombinant toxin in *Escherichia coli*, and its reconstitution *in vitro* into a fully functional toxin. Site-directed mutagenesis of the toxin produced a selective decrease in its anti-mammalian toxicity, thus improving its phylogenetic selectivity in favor of insects.

† This work was supported in part by an internal grant of the Research Authority at Tel-Aviv University and in part by a Binational Agricultural Research and Development (BARD) Grant No. IS-1982-91 to M.G., E.Z., and M.E.A.

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⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1996.

[1] Abbreviations: AaHII, *Androctonus australis* Hector alpha mammal toxin II; LqhαIT, *Leiurus quinquestriatus hebraeus* alpha insect toxin; Lqq4, *Leiurus quinquestriatus quinquestriatus* alpha mammal toxin 4; RP-HPLC, reversed-phase high pressure liquid chromatography; SDS−PAGE, sodium lauryl sulfate−polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside; TFA, trifluoroacetic acid.

S0006-2960(95)02830-3 CCC: $12.00

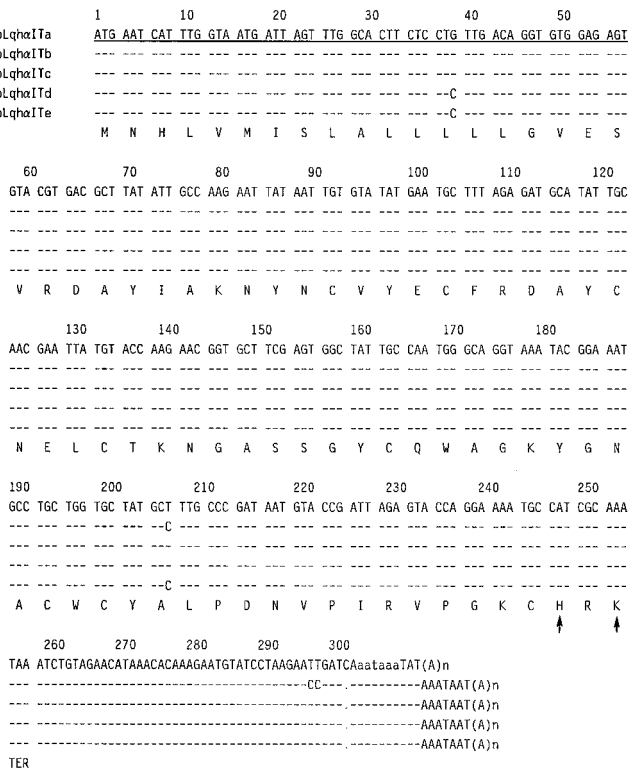

FIGURE 1: Nucleotide sequence of five LqhαIT-cDNAs derived from *L. q. hebraeus.* The putative leader sequence is underlined. The sequence coding for the mature polypeptide starts at position 58. Identity between sequences is indicated by dashes. The His and Lys residues not found at the C-terminus of the toxin purified from the scorpion venom (Eitan et al., 1990) are designated by arrows, and a potential polyadenylation site is indicated by lowercase letters. Dots indicate absence of a nucleotide.

EXPERIMENTAL PROCEDURES

Materials, Strains, and Animals. All chemicals and reagents were from Sigma (USA) or Merck (Germany). Enzymes were obtained from New England BioLabs (Germany) and United States Biochemicals (USA). *E. coli* DH5α strain was used for plasmid constructions and propagation. *E. coli* strain BL21 lysogen with DE3 λ phage derivative, bearing the T7 RNA polymerase gene whose expression is driven by the *lac* promoter, was used for expression. This strain was transformed with a pLysS plasmid bearing a T7 lyzosyme gene and a gene conferring chloramphenicol resistance (Rosenberg et al., 1987). The translational vector pET-11c (Rosenberg et al., 1987), in which we inactivated the *bla* gene by the insertion of a gene encoding NPTII (pET-11cK), was used for expression. *Sarcophaga falculata* blowfly larvae were bred in the laboratory. Albino laboratory mice (strain sabra) were purchased from the laboratory animal farm of the Hadassah Medical School, Jerusalem, and ICR mice from the Levenstein farm, Yokneam.

Cloning and Construction of LqhαIT-cDNA in an Expression Vector. Isolation of cDNA clones coding for LqhαIT followed a modified procedure of inverse-PCR (Zilberberg & Gurevitz, 1993). The PCR product was used as a probe to pull out of a cDNA library (Zilberberg et al., 1992) five different clones (Figure 1). Clone pLqhαITa was further used for expression. Two synthetic oligonucleotide primers (Figure 2) were designed for reconstruction of the 5′ and 3′ termini of the LqhαIT-cDNA. Primer 1, a 29mer, was designed to (i) remove the leader sequence, (ii) create an additional codon for methionine at position −1 of the mature

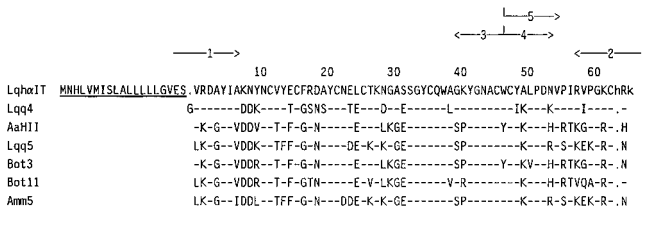

FIGURE 2: Amino acid sequences of several alpha scorpion neurotoxins and oligonucleotide primers used for cloning and for mutagenesis. Numbering follows the sequence of the mature LqhαIT. Residues homologous to those in LqhαIT are designated by dashes. Dots indicate gaps in the aligned sequences. Primers (indicated by arrows) 1 and 2 were used to engineer via PCR both termini of the cDNA encoding LqhαIT presented in its unprocessed form. The deduced signal peptide is underlined, and the additional residues at the C-terminus removed through processing are indicated by lowercase letters. An ATG codon that precedes Val1 and an *Nde*I restriction site were designed in primer 1. In primer 2, representing the sequence of the noncoding DNA strand of LqhαIT sequence prior to maturation, the codons for His64 and Lys66 were deleted, and a *Bam*HI restriction site was designed. The region between amino acid residues 40 and 54 was used to design back to back primers 3, 4, and 5 for modification of Tyr49, Ala50, and Asn54 via inverse-PCR (see Figure 3).

polypeptide sequence, and (iii) provide an *Nde*I restriction site (underlined in Figure 2) immediately juxtaposed to the ATG start codon. Primer 2, representing the noncoding DNA strand, was designed as a 32-mer in which the codons for His-62 and Lys-64 of the deduced amino acid sequence of LqhαIT-cDNA were omitted. These residues are lacking from the sequence of the purified native toxin (Eitan et al., 1990). Whereas the terminal Lys residue is probably removed posttranslationally, the additional codon for His-62 found in the cDNA could be attributed to genetic polymorphism of this gene. Primer 2 was provided with a *Bam*HI restriction site (underlined in Figure 2) immediately adjacent to the 3′-end of the coding sequence. Both primers were PCR-reacted with the LqhαIT-cDNA, and the product, digested with *Nde*I and *Bam*HI, was cloned into the corresponding *Nde*I and *Bam*HI restriction sites flanking gene 10 in the pET-11cK modified vector (Rosenberg et al., 1987). The resulting expression vector, pLqhαIT-X, was used for transformation of *E. coli* cells.

Site-Directed Mutagenesis. Site-directed modifications, introduced into pLqhαITa, were carried out with back to back oligonucleotide primers via PCR (Figure 3). A *Pvu*II fragment, derived from plasmid pLqhαITa containing the unmodified LqhαIT cDNA within the *Sma*I site of the polylinker in pBluescript, was circularized with T4 DNA ligase. The circular fragment, used as template DNA, was PCR reacted in presence of either primers, 3 with 4 or 3 with 5 (Figure 2). In each reaction (30 cycles) 2.5 units of Taq polymerase (United States Biochemicals, USA) was applied to 50 pmol of oligonucleotide primers (synthesized by Biotechnology General, Rehovot, Israel) and 10 ng of cDNA as a template. The amplified linear DNA product was eluted from an agarose gel, treated with T4 DNA polymerase (Boehringer Mannheim, Germany) displaying exonucleolytic activity on 3′ overhanging residues, and further phosphorylated with T4 DNA polynucleotide kinase (New England BioLabs, Germany) prior to ligation. The recircularized DNA was used as a template for PCR in presence of primers 1 and 2 (Figure 2), and the product,

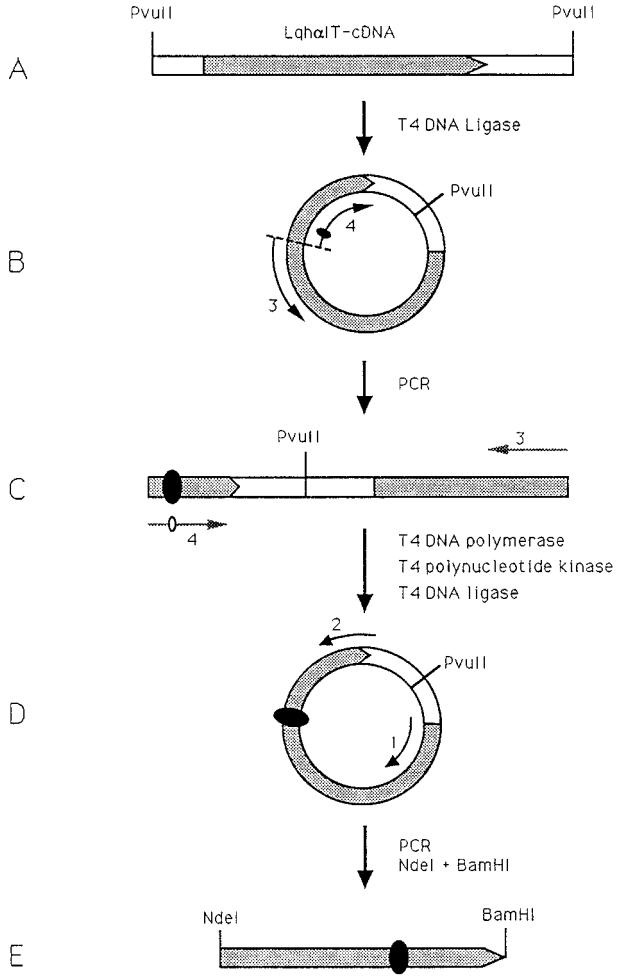

FIGURE 3: Inverse-PCR for site-directed modification of LqhαIT-cDNA. Steps of mutagenesis, described for primers 3 and 4 (the mutation in primer 4 is designated by a black ellipse) as an example, were as follows: A, a 0.8 kb *Pvu*II fragment including the cDNA encoding LqhαIT (dotted) was isolated from the pBluescript vector; B, the linear DNA was circularized by T4 DNA ligase following inverse-PCR in presence of primers 3 and 4 (note position of primers and their "back to back" orientation); C, a mutagenized amplified product was obtained; D, the linear product was circularized again by T4 DNA ligase after "filling-in" and 5′-phosphorylation and used as a template in a second cycle of PCR in the presence of primers 1 and 2; E, the linear amplified DNA was digested with *Nde*I and *Bam*HI prior to cloning into the corresponding restriction sites in vector pET-11cK.

digested with *Nde*I and *Bam*HI, was cloned into the corresponding restriction sites in plasmid pET-11cK. Sequenase II (United States Biochemicals, USA) and primers 1 and 2 (Figure 2) were used for DNA sequencing (Sanger et al., 1977).

SDS−PAGE and Immunoblotting. Protein samples were prepared and separated by electrophoresis on an SDS containing 10% (w/v) polyacrylamide 0.3% (w/v) bisacrylamide gel following an established procedure (Schagger & von Jagow, 1987). Gels were run at 75 V for 2 h. Protein was visualized using Coomassie stain or transferred onto nitrocellulose membrane (Hybond-C, Amersham; 1 h at 170 V with cooling) (Szewczyk & Kozloff, 1985). The relative molecular weight of the recombinant protein was estimated by comparison with molecular weight standards (Sigma, USA). Immunoblots were incubated for 1 h in blocking solution [0.02% (w/v) NaN_3 and 5% (v/v) nonfat dry milk in TBS buffer (12.5 mM Tris, pH 7.4; 50 mM NaCl)] and then incubated overnight with the anti-LqhαIT serum (1:1000 dilution) at room temperature. After three successive washes with 0.1% (v/v) Tween-20 in TBS buffer, the blots were incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma, USA) (1:4000 dilution in TBS buffer). After washes as described above, the detection reaction was performed in 100 mM Tris, pH 9.5, 100 mM NaCl, and 2 mM $MgCl_2$ using BCIP (5-bromo-4-chloro-3-indolyl phosphate disodium salt) and NBT (nitroblue tetrazolium) as substrates (Sigma, USA).

Electron Microscopy. Cells grown in the presence of 0.4 mM IPTG were harvested at stationary growth phase. Aliquots (20 mL) were centrifuged and immediately fixed for 18 h in a solution containing 4% (v/v) glutaraldehyde, 0.5% (v/v) paraformaldehyde, 0.2 M sodium cacodylate, and 5 mM $CaCl_2$. Cells were postfixed in 1% (w/v) OsO_4 for 1 h and then pelleted, washed twice in 0.2 M sodium cacodylate buffer, and left overnight at 4 °C. The pellets were dehydrated through an ethanol series and finally embedded in Polybed 812 resin Kit (Polysciences, USA). Ultrathin sections were stained with uranyl acetate and lead citrate and observed on a JEOL 100B electron microscope.

Purification of Recombinant LqhαIT from E. coli. Recombinant *E. coli* cells were grown in Luria broth medium containing 40 μg/mL kanamycin and 30 μg/mL chloramphenicol. IPTG was added at 0.2 A_{600} to a final concentration of 0.4 mM and growth continued for 16 h. Cells from 1 L of culture were harvested by centrifugation, resuspended in 50 mL of water, and lysed by freezing and thawing. The lysate was sonicated (four bursts of 1 min each on ice), and the insoluble pellet obtained by centrifugation (13000*g* for 15 min) was washed three successive times with washing buffer [25% (w/v) sucrose, 5 mM EDTA, 1% (v/v) Triton X-100 in PBS]. The washed pellet was suspended in a denaturing solution [6 M guanidinium hydrochloride, 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, pH 8.0, and 0.5 M β-mercaptoethanol for 2 h at room temperature (Jaenicke & Rudolph, 1990)]. Most of the material was solubilized and further desalted by precipitation with 6 vol of cold acidic acetone (39:1 acetone and 1 M HCl, respectively) for 2 h at −20 °C. The pellet obtained after centrifugation (5000*g* for 5 min) was washed twice with 80% acidic acetone and air dried for 5 min, and protein renaturation was initiated by solubilization in 20 mL water before increasing the volume to 1 L with 0.2 M ammonium acetate, pH 8.0. After 48 h of incubation at 30 °C, insoluble material was removed by filtration through a Whatman 1 mm paper, and the protein was precipitated with 80% saturated ammonium sulfate for 2 h on ice. The precipitate was collected by filtration through a 50 mm in diameter GF/C Whatman filter and solubilized in 5 mL of water. Final purification was carried out with a Vydac C18 RP-HPLC column (4.6 × 250 mm, 5 μm particle size; LKB, Sweden) using 0.1% (v/v) trifluoroacetic acid (TFA) in water as buffer A, and 0.1% (v/v) TFA in acetonitrile/2-propanol (1:1) as buffer B. The column was equilibrated in 5% buffer B and eluted with a linear gradient of 5−20% buffer B over the first 15 min followed by a 60 min linear gradient to 50% buffer B. Flow rate was 0.5 mL/min. Quantification of the purified recombinant LqhαIT was performed by amino acid analysis.

Blowfly Larvae and Mice Bioassays. Four-day-old blowfly larvae (*Sarcophaga falculata*; 100 ± 20 mg body weight) were injected and scored as positive when a characteristic paralysis (immobilization and contractions) was observed 5 min postinjection. Nine larvae were injected with five

concentrations of each toxin in three independent experiments. To determine toxicity to mammals, groups of five female mice (20 ± 3 g) were injected subcutaneously with each toxin concentration in three independent experiments. ED_{50} and LD_{50} values, for larvae and for mice, respectively, were calculated according to the sampling and estimation method of Reed and Muench (1938).

Electrophysiology. Voltage- and current-clamp experiments on cockroach giant axons (*Periplaneta americana*) were performed using the double oil gap, single fiber technique (Pichon & Boistel, 1967). Insect physiological saline was composed of 0.2 M NaCl, 3.1 mM KCl, 5.4 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM $NaHCO_3$, and 0.1 mM NaH_2PO_4, (pH 7.2). 3,4-Diaminopyridine was used to selectively block the potassium current (Pelhate & Pichon, 1974). Conventional whole-cell patch clamp techniques (Hamill et al., 1981) were used to record sodium currents in acutely dissociated dorsal root ganglion (DRG) neurons from 7−12 day-old rats (Mintz et al., 1992). Pipettes (0.5−3 MΩ resistance) were manufactured from Boralex glass (Dynalab, Rochester, NY), coated with sylgard and fire polished, and then filled with an internal solution containing 75 mM CsF, 75 mM CsCl, 2.5 mM $MgCl_2$, 5 mM HEPES, and 2.5 mM EGTA, pH adjusted to 7.4 with CsOH (Ogata & Tatebayashi, 1993). Whole-cell recordings were established with cells bathed in Tyrode's solution (150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH adjusted to 7.4 with NaOH). Once a gigohm seal was achieved, the cell was lifted from the bottom and placed in front of a linear array of microcapillary tubes which delivered either recording solution (140 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 15 mM tetraethylammonium chloride, 1 mM 4-aminopyridine, 1 mg/mL cytochrome *c*, pH adjusted to 7.4 with 1 M NaOH) or recording solution plus toxin. Currents were recorded using an Axopatch 200A patch clamp amplifier (Axon Instruments, Burlingame, CA), low pass Bessel filtered at 2 kHz and then digitized at 50 μs intervals and stored using the Basic-Fastlab data acquisition system (INDEC Systems, Sunnyvale, CA). Sodium channel currents were corrected for leak and capacitive currents by substracting a scaled current elicited by a 10 mV hyperpolarization from the holding potential (80 mV). Currents were allowed to stabilize before data was collected. Data were discarded if voltage errors due to series resistance remaining after partial compensation were greater than 5 mV. All recordings were made at room temperature (22−24 °C).

Competition Binding Experiments. Native and recombinant LqhαIT were radioiodinated by Iodogen (Pierce Chem. Co. Rockland, USA) according to the method described by Gordon and Zlotkin (1993). Locust (*Locusta migratoria)* synaptosomal membrane vesicles (mvP2L, prepared by an osmotic shock of synaptosomes) were prepared from the central nervous system (CNS) of adult locusts according to established methods (Gordon et al., 1992). The binding assays were performed in the form of equilibrium saturation assays using increasing concentrations of the unlabeled toxin in the presence of a constant low concentration of the labeled toxin (0.1−0.2 nM), as described (Gordon & Zlotkin, 1993; Gordon et al., 1984). Analysis of binding assays was carried out by using the iterative computer program LIGAND (P. J. Munson and D. Rodbard, modified by G. A. McPherson, 1985).

RESULTS

Polymorphism among cDNA Clones Encoding LqhαIT. Five different cDNA clones, pLqhαITa, pLqhαITb, pLqhαITc, pLqhαITd, and pLqhαITe (Figure 1), were isolated from the cDNA library constructed from the scorpion poly(A)$^+$ RNA (Figure 1). These clones vary slightly in their nucleotide sequence, but their deduced amino acid sequence for the coding region is identical. Whether this polymorphism is due to the expression of several genes or whether they represent natural variation among the yellow scorpion population is still unknown. Clone pLqhαITa was used for DNA manipulations and expression.

Expression of a Recombinant LqhαIT in E. coli. Considering the reported difficulties in expressing eukaryotic small polypeptides in the reductive cytosol of *E. coli* (Howell & Blumenthal, 1989; Fiordalisi et al., 1991), our strategy was to overproduce a large amount of insoluble recombinant toxin by utilizing a strong inducible T7 promoter. The constructed recombinant vector, pLqhαIT-X, was used to transform competent *E. coli* DH5α cells. Kanamycin resistant colonies were obtained, their plasmid was purified, and the sequence of the modified cDNA was confirmed by DNA sequencing. Purified pLqhαIT-X vector was used for the transformation of *E. coli* strain BL21 (DE3) (Rosenberg et al., 1987). Kanamycin and chloramphenicol resistant colonies were selected and grown to $A_{600} = 0.2$ before the addition of the inducer IPTG.

Identification, Renaturation, and Purification of the Recombinant Toxin. In order to identify the recombinant toxin, the *E. coli* extract was electrophoresed in a 10% SDS−PAGE. A gradually increasing band of ∼7.5 kDa appeared in lanes containing extracts from induced cells (Figure 4, lanes B−E). This product was missing in the noninduced cells (Figure 4, lane A). Fractionation of the extract into a 16000*g* supernatant and pellet indicated that the recombinant polypeptide resided in the nonsoluble fraction (Figure 4, lanes F and G). This result was supported by transmission electron micrographs illustrating recombinant *E. coli* cells that contain inclusion bodies (Figure 4, left), and by the immunochemical recognition of the toxin by anti-LqhαIT serum (Figure 4, lanes J−L). The bands larger than 7 kDa, appearing in both the Coomassie stained (lane G) and immunoblot (lane L) pellet fractions, could result from different size aggregates of the polypeptide monomer. The active renatured recombinant toxin was further purified by reversed-phase HPLC and analyzed by mass spectrometry and amino acid analysis, which indicated the presence of methionine at the N-terminus (data not shown). Despite the additional Met residue at the N-terminus of the recombinant toxin, its HPLC elution profile was identical to that of the native toxin. It is noteworthy that the amount of soluble, incorrectly folded protein obtained in our experiments under optimal renaturation conditions was minor and in several instances was undetectable as shown in the HPLC profile. The pellet fraction (50 mg derived from 1 L of *E. coli* recombinant culture grown to $A_{600} = 2$) was solubilized by guanidinium hydrochloride and completely denatured and reduced (see Experimental Procedures). Determination of optimal conditions for renaturation of a functional protein was carried out at various ionic strengths (between 0 and 0.25 M ammonium acetate), temperatures (between 4 and 37 °C), pH (between 5 and 9), protein concentrations (between 10 and 200 μg/mL), and time durations (0−72 h). The largest yield of biologically active

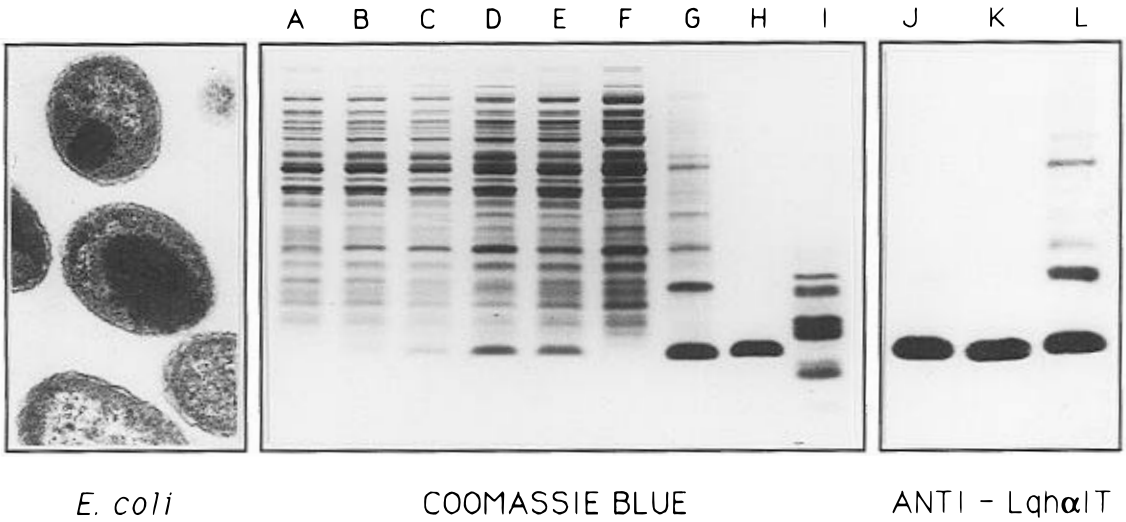

FIGURE 4: Ultrastructural, electrophoretic, and immunochemical identification of the recombinant LqhαIT. Transmission electron micrograph of an LqhαIT granule in IPTG-induced recombinant *E. coli* is shown on the left hand side (magnification ×24000). On the right hand side, lanes A−I illustrate a Coomassie blue stained SDS−PAGE representing the following: A, extract of noninduced recombinant *E. coli* containing the expression vector; B−E, extracts of IPTG-induced *E. coli* containing the vector after 1, 2, 5, and 24 h of induction, respectively; F and G, supernatant and nonsoluble (16000*g*) fractions, respectively, of cells grown for 24 h in the presence of IPTG; H, HPLC-purified recombinant LqhαIT; I, molecular weight markers (6.21, 8.16, 10.6, 14.4, and 16.95 kDa, Sigma, USA). Lanes J−L illustrate a nitrocellulose blot probed with rabbit polyclonal antibodies raised against LqhαIT: J, 1 μg of HPLC-purified recombinant toxin; K, 1 μg of native purified LqhαIT; L, nonsoluble fraction as in lane G.

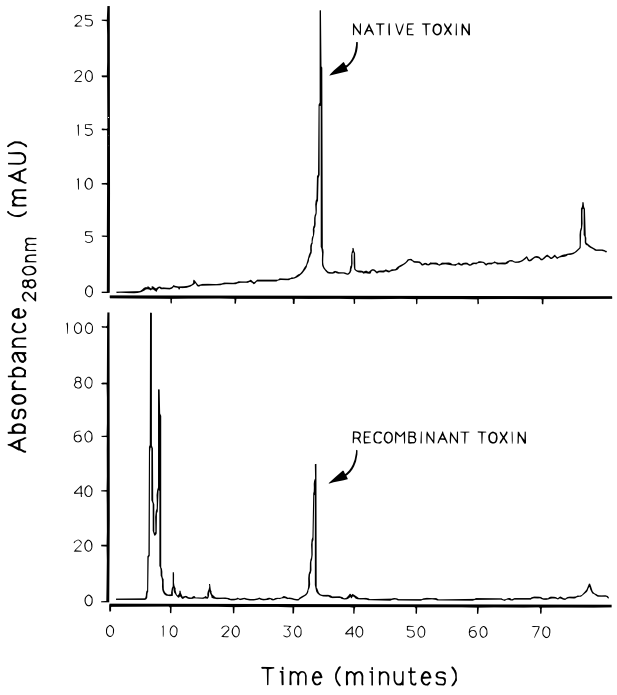

FIGURE 5: Purification of the recombinant LqhαIT by HPLC. Ten micrograms of the native toxin (A) and a sample of the renatured recombinant polypeptide (B) were purified on a Vydac RP-C18 column (4.6 × 250 mm). Buffer A, 0.1% (v/v) TFA. Buffer B, 0.1% (v/v) TFA in acetonitrile/2-propanol (1:1). Gradient: 0 min 5% B; 15 min 20% B; 75 min 50% B. Flow rate: 0.5 mL/min. The toxic fractions are indicated by arrows.

toxin (5−10 mg/L of *E. coli* culture) capable of provoking the typical delayed and sustained spastic contraction paralysis of blowfly larvae (Eitan et al., 1990) was obtained with 50 μg/mL protein incubated for 48 h in the presence of 0.2 M ammonium acetate pH 8.0 at 30 °C. The active renatured recombinant toxin was further purified by reversed-phase HPLC. The recombinant and native LqhαIT toxins had identical HPLC elution profiles (Figure 5).

Functional Characterization of the Renatured Recombinant Toxin. The biological activity of the recombinant toxin

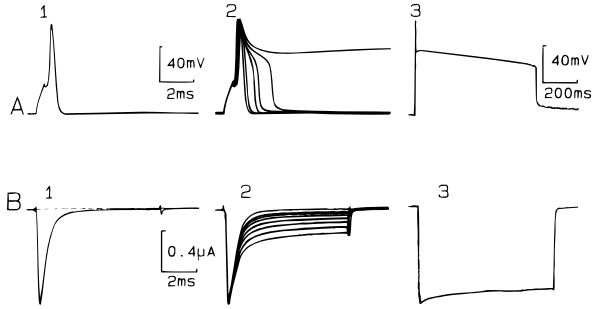

FIGURE 6: Effect of the recombinant LqhαIT on action potentials and sodium currents of an isolated cockroach axon. A, Current clamp experiment: (1) control, action potential evoked by a short (0.5 ms) depolarizing current pulse of 10 nA; (2) series of superimposed recordings of action potentials at intervals of 10 s following the application of 10^{-8} M of the recombinant toxin and resulting in (3) a prolonged action potential recorded 4 min after toxin application. B, Voltage clamp experiment: Effect of the recombinant toxin on sodium currents associated with a step depolarization from a holding potential of −60 to −10 mV of the isolated cockroach axon in the presence of 5×10^{-4} M 3,4-diaminopyridine. (1) Control, sodium current completely inactivated in 4−5 ms. (2) Superimposed records of sodium currents recorded every 10 s during the first 90 s of toxin (10^{-8} M) action. In the presence of toxin, the sodium inactivation is progressively slowed. (3) Record of the maintained sodium current recorded 10 min after toxin application.

was assessed by several criteria: (1) Paralysis of blowfly larvae. The paralytic unit calculated per 100 mg body weight of the *Sarcophaga* fly larvae was 14 ng for the native toxin fraction as compared to 12.8 ng for the recombinant toxin fraction. (2) Electrophysiological properties. The effect of recombinant LqhαIT on the sodium conductance in an insect axonal preparation (Figure 6) was found to be qualitatively and quantitatively identical to that obtained with the native LqhαIT (Eitan et al., 1990). Application of toxin prolonged the evoked action potential (Figure 6A), due to an evident inhibition of sodium current inactivation (Figure 6B). Neither native nor recombinant toxin altered the action potential amplitude, resting membrane potential, or potassium conductance (data not shown). (3) Competitive binding. The

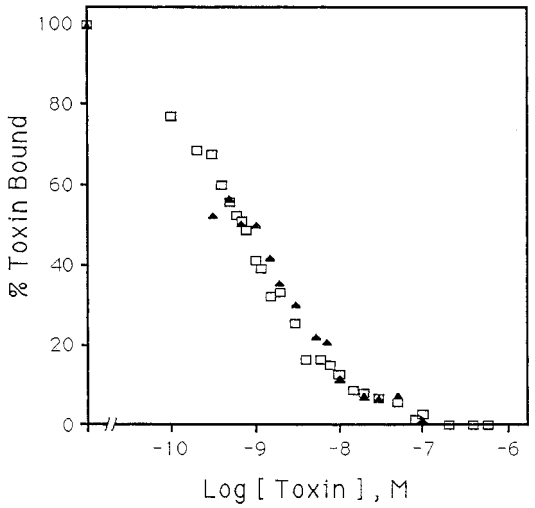

FIGURE 7: Competitive inhibition of native ^{125}I-labeled LqhαIT binding by the native and recombinant LqhαIT toxins. Locust neuronal membranes (25 μg of membrane protein) were incubated for 60 min at 22 °C in the presence of 0.15 nM ^{125}I-labeled LqhαIT and increasing concentrations of native (squares) and recombinant (triangles) toxins. Nonspecific binding of ^{125}I-labeled LqhαIT, determined in the presence of 1 μM LqhαIT (corresponding to 15−25% of total binding), was subtracted. The binding was determined as described [see Experimental Procedures and Gordon and Zlotkin (1993)] and analyzed by the LIGAND computer program.

ability of the recombinant toxin to displace the authentic ^{125}I-labeled LqhαIT from its binding site on locust neuronal membranes was compared to that of the native LqhαIT toxin. As shown in Figure 7, both the native and the recombinant LqhαIT polypeptides were equally capable of competing with the radiolabeled authentic toxin, providing an identical IC_{50} value of 0.7 ± 0.2 nM. The functional, biochemical and immunochemical equivalencies of the recombinant and native LqhαIT toxins firmly argue for an overall conformational identity of both polypeptides.

Site-Directed Modifications. Comparative sequence analysis of LqhαIT with other alpha toxins reveals various degrees of similarity with most differences located at the N-terminal region (Dufton & Rochat, 1984; see Figure 2). The alpha toxin with highest sequence similarity to LqhαIT is Lqq4 (74%). This similarity is particularly prominent at the C-terminal half of both toxins beginning at residue 33. Except for the differences at positions 49, 50, and 54 (LqhαIT sequence), only two more conservative alterations at positions 39 and 59 are detected. This observation was exploited to assess whether the unique amino acid residues, Tyr49, Ala50, and Asn54, are involved in determining the marked toxicity of LqhαIT to insects. First, Tyr49 and Ala50 were replaced by Ile and Lys, respectively (primers 3 with 4; resulting in expression vector pLqhαIT-IK). Then, Asn54 was replaced by Lys in addition to the former modifications (primers 3 with 5; resulting in expression vector pLqhαIT-IKK) (see Table 1). The latter modification generated a carboxy-terminal half practically resembling its counterpart region in Lqq4 alpha anti-mammalian toxin. Both modified clones were used to transform *E. coli* BL21 cells as previously described. The insoluble proteins obtained were totally denatured and then renatured as described in Experimental Procedures. The soluble modified toxins were purified by HPLC and their activities analyzed.

As shown in Table 1, both modified polypeptides showed significantly less anti-mammalian toxicity relative to the unmodified toxin. When tested on ICR mice, the LD_{50} value

Table 1: Biological Activities of the Recombinant Toxins[a]

toxin	LD_{50} (ng/1.0 g of body weight)	ED_{50} (ng/100 mg of body weight)	IC_{50} (nM)
unmodified LqhαIT	250 ± 10	12.8 ± 1.8	0.38 ± 0.09
Y49I + A50K	747 ± 76	18.5 ± 1.9	0.45 ± 0.20
Y49I + A5K + N54K	1605 ± 43	19.1 ± 1.9	0.41 ± 0.18
Lqq4	70	950.0 ± 145	15 ± 4.6

[a] LD_{50} values for the recombinant toxins were determined on ICR mice (20 ± 3 g). ED_{50} was determined for blowfly larvae (100 ± 20 mg). IC_{50} for recombinant toxins was determined on locust synaptosomal membranes. The results are provided with calculated standard deviation values. A, alanine; I, isoleucine; K, lysine; N, asparagine; Y, tyrosine. Y49I + A50K and Y49I + A50K + N54K are the modified toxins obtained with vectors pLqhαIT-IK and pLqhαIT-IKK, respectively. The values of LD_{50} and IC_{50} for Lqq4, an anti-mammalian toxin from the venom of *L. q. quinquestriatus*, are from Kopeyan et al. (1985); the ED_{50} value for Lqq4 is from Gordon et al. (1996).

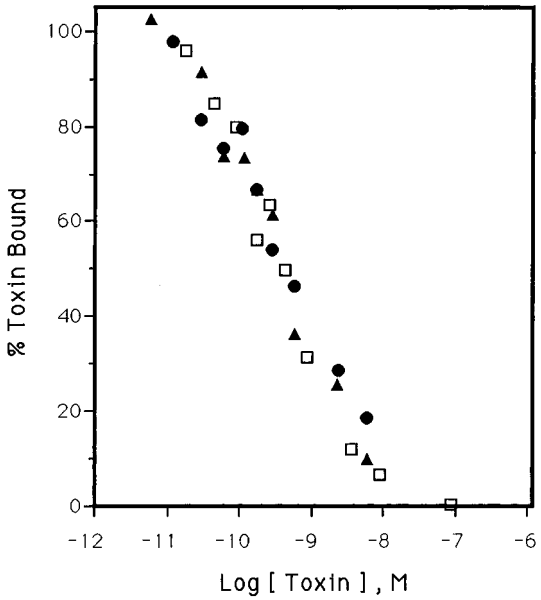

FIGURE 8: Competitive inhibition of recombinant ^{125}I-labeled LqhαIT binding by the unmodified and modified recombinant LqhαIT toxins. Conditions were identical with those described in the legend of Figure 7. Squares indicate the unmodified recombinant toxin; black circles indicate the modified toxin at positions 49 and 50; triangles designate the modified toxin at positions 49, 50, and 54.

for the double mutant increased by 3-fold and the triple mutant by 6.4-fold as compared to the unmodified toxin. On the other hand, the two mutant toxins showed only a slight but significant decrease in potency for insect paralysis—18.5 and 19.1 ng per 100 mg body weight of blowfly larvae, respectively—as compared with 12.8 ng for the unmodified toxin. Moreover, the calculated IC_{50} obtained in radioligand binding assays on locust CNS membranes remained unchanged (IC_{50} = 0.38 ± 0.09 nM) as compared to that obtained with the unmodified toxin (Figure 8).

To determine if reduced anti-mammalian toxicity of the triple mutant toxin could be associated with decreased potency of the toxin at the level of the sodium channel, we examined sodium currents in rat dorsal root ganglion neurons using the whole-cell patch clamp technique. Exposure to 200 nM recombinant unmodified LqhαIT led to a marked slowing of sodium channel inactivation (Figure 9A−C). This effect was fully reversible upon switching to toxin-free recording solution. Subsequent exposure of the same cell to 200 nM of the triple mutant toxin (Tyr49Ile, Ala50Lys, and Asn54Lys) again led to slowing of sodium channel

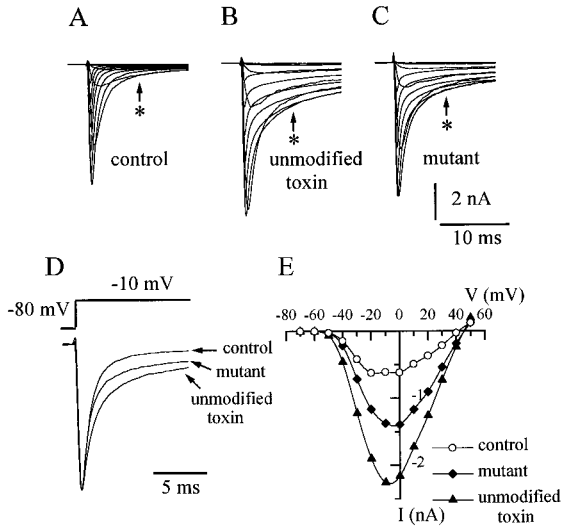

FIGURE 9: Comparison of recombinant unmodified and mutant LqhαIT effects on sodium channel inactivation in rat dorsal root ganglion (DRG) neurons. Currents were recorded using the whole-cell recording technique as described in Experimental Procedures. (A−C) Current−voltage relationship of sodium channels examined by stepping a neuron to test potentials in 10 mV increments from a holding potential of −80 mV (shown in D). Superimposing the family of evoked inward currents shows that when the cell was under control conditions (A) the sodium currents decayed rapidly. However, exposure to 200 nM unmodified LqhαIT (B) caused a clear slowing of the decay of current and an increase in the peak amplitude of current. This effect was completely reversed by washing. Subsequent exposure of the same cell to 200 nM mutant LqhαIT (C) also slowed the decay of sodium current and increased the current amplitude. However, the mutant toxin was less effective than the unmodified toxin. (D) Normalized inward sodium currents evoked in a cell by stepping to a test potential of −10 mV are superimposed (V_H = −80 mV). The mutant toxin is significantly less effective at slowing the decay of sodium current than the unmodified toxin. (E) Current−voltage relationship for currents evoked by stepping a neuron to test potentials in 10 mV increments from a holding potential of −80 mV (A−C). The amplitudes of currents measured 8 ms after the beginning of voltage steps (arrow marked with asterisk in A−C) were plotted as a function of the test potential. The current−voltage relationship in the presence and absence of toxin shows that the mutant toxin is approximately 2-fold less effective on sodium currents than the unmodified toxin (see text).

inactivation, but to a significantly lesser extent. The normalized currents are shown in Figure 9D. To quantify the potency difference between the unmodified and triple mutant recombinant toxins, amplitudes of evoked sodium currents (measured 8 ms after the start of each voltage step; see asterisk arrow in Figures 9A−C) were plotted as a function of the test potential to yield the current−voltage relationships shown in Figure 9E. The unmodified recombinant toxin increased the current 274% over control (at the peak of the *I/V* curve), while the triple mutant was about 2-fold less potent, causing 127% increase (Figure 9E). In six experiments, the unmodified recombinant toxin increased current in an average of 228% compared to 119% for the triple mutant LqhαIT. In the same six neurons, the unmodified toxin caused approximately a 19% increase in the peak amplitude of inward sodium currents evoked by voltage steps to −10 mV (Figure 9A−C), while the modified toxin generated only a 8% increase in peak current amplitude. These data indicate that the mutant forms of LqhαIT show reduced potency as compared to the unmodified recombinant toxin in slowing the rate of sodium current inactivation.

DISCUSSION

In this study we have shown the successful high efficiency expression of an alpha scorpion toxin in a bacterial expression system and, through site-directed mutagenesis, increased its relative phylogenetic selectivity for insects over mammals. Although several toxins previously have been expressed in eukaryotic systems (Bougis et al., 1989; Dee et al., 1990; Stewart et al., 1991; Tomalski & Miller, 1991; Maeda et al., 1991), their expression in *E. coli*, a preferred system for recombinant DNA manipulations, was not achieved, possibly due to the reducing environment within the cytosol, the lack in recognition of regulatory sequences, and/or the rapid degradation of newly synthesized polypeptides (Howell & Blumenthal, 1989; Fiordalisi et al., 1991). These difficulties were overcome in this study by enforcing the accumulation of LqhαIT in *E. coli* within inclusion bodies and then exploiting the competence of small denatured polypeptides to refold into their native conformation *in vitro* (Marston, 1986; Sabatier et al., 1987), thus yielding large amounts (up to 5 mg/L of *E. coli* culture) of functional recombinant protein. It is noteworthy that the production of recombinant polypeptides in heterologous expression systems is sometimes limited due to instability of the products caused by destabilizing amino terminal residues such as Lys, Arg, Phe, Leu, and Asp (Bachmair et al., 1986). It is therefore quite plausible that the additional methionyl residue preceding Val at the N-terminus of the recombinant toxin contributes to its stability throughout the expression and refolding experiments.

The functional recombinant toxin purified after renaturation was subjected to a thorough analysis and comparison with the native toxin according to biochemical, immunochemical, and biological criteria. Biochemically, three parameters were analyzed: (1) migration on SDS−PAGE (Figure 4); (2) HPLC elution profile (Figure 5); and (3) displacement of the native toxin from its receptor site on a locust synaptosomal preparation (Figure 7). Immunochemically, the recognition of the recombinant LqhαIT by anti-LqhαIT serum on a Western blot (Figure 4) and by radioimmunoprecipitation (data not shown) was demonstrated. Biological parameters examined were (1) paralysis of blowfly larvae and (2) modification of sodium currents in isolated cockroach axons (Figure 6). These parameters were found to be practically identical for both the recombinant toxin and the native authentic toxin. Since toxin activity is most likely related to the native conformation, toxicity determination is one of the best tests for monitoring reoxidation−renaturation phenomena (Sabatier et al., 1987). Furthermore, the unchanged binding affinity to the insect neuronal preparation, as compared to the native toxin, implies that there were most likely no mixed nonactive isoforms with the recombinant toxin purified by HPLC. The estimated amount of active recombinant toxin obtained by renaturation is approximately 20% of the overall amount of recombinant LqhαIT produced in *E. coli.* Together with the fact that correct folding involves the formation of specific four disulfide bridges, the high percentage of active toxin obtained implies that refolding of the denatured protein was in favor of its active form. Due to the fact that several milligrams of active LqhαIT were reconstituted from one liter of *E. coli* culture, and considering the functional identity between the recombinant and the native toxins, this efficient system is highly amenable for a molecular genetic study. The genetic

approach employed in this study was based on comparison between the two highly homologous, yet functionally distinct alpha toxins, LqhαIT and Lqq4 (Kopeyan et al., 1985). Despite their high sequence similarity (74%), LqhαIT is highly toxic to insects, is practically nontoxic to mice strain sabra (Eitan et al., 1990), and has an LD_{50} value of 250 ng/g of body weight when injected to ICR mice. Conversely, Lqq4 is toxic to both strains of mice (LD_{50} to ICR mice of 70−80 ng/g) but is only weakly toxic to insects (see Table 1). It was previously postulated that the C-termini of insecticidal scorpion toxins comprise a structural motif near the "conserved hydrophobic surface" contributing to their specificity (Fontecilla-Camps, 1989; Darbon et al., 1991). We therefore modified the C-terminal half of LqhαIT to resemble the equivalent region of the alpha anti-mammalian toxin, Lqq4.

Utilizing the *E. coli* expression system and functional reconstitution procedure described in this study, appropriate toxin mutants were generated. Modification of the toxin at three sites, Tyr49Ile, Ala50Lys, and Asn54Lys, resulted in a marked decrease in anti-mammalian toxicity (6.4-fold) but little change in its biological activity against insects. The decreased anti-mammalian toxicity was correlated with a ∼2-fold reduction in potency against mammalian sodium channels. This decreased potency of the mutant toxin might be a contributing factor in the overall decrease in toxicity, but firm conclusions along this line will depend on a direct comparison between mammalian and insect sodium channels. Furthermore, it should be emphasized that a variety of factors, including metabolism, transport, and pharmacokinetics, may contribute to the LD_{50} value. For example, it is possible that the substitution of lysine residues at positions 50 (Ala50Lys) and 54 (Asn54Lys) leads to increased proteolysis *in vivo*, thus providing a second mechanism underlying decreased mammalian toxicity. The relative contributions of these respective factors will be addressed in future studies.

In summary, we have demonstrated the high efficiency production of LqhαIT by bacterial expression and shown that the relative activity of LqhαIT against insects vs mammals can be independently modified by site-directed mutagenesis. In particular, our findings show that alteration of the C-terminal domain of the toxin reduces anti-mammalian toxicity without significantly affecting insecticidal potency. Our approach demonstrates the feasibility of improving the phylogenetic selectivity of a polypeptide neurotoxin by structural modification. Since alteration of the C-terminal domain of LqhαIT produces no apparent change in biological activity against insects, future experiments will focus on modification of the N-terminal half of the molecule with the objective of producing further increase in selective toxicity of this polypeptide.

ACKNOWLEDGMENT

We thank Dr. E. Loret for useful discussions, Dr. M. Fainzilber for help and advice with the toxin purification, and Prof. I. Hammel for helping with the electron microscopy and the figures.

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BI9528309