Structure and Function of *Cerebratulus lacteus* Neurotoxin B-IV: Tryptophan-30 Is Critical for Function while Lysines-18, -19, -29, and -33 Are Not Required[†]

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ABSTRACT: The *Cerebratulus lacteus* B-toxins are a family of polypeptide neurotoxins known to bind to crustacean voltage-sensitive sodium channels. We have previously shown that in the most abundant homolog, toxin B-IV, Arg-17 in the N-terminal helix and a positive charge at position 25 in the loop region are essential for function. In this report, we target a tryptophan residue at position 30, as well as lysine residues found in both the N-terminal helix and loop regions by polymerase chain reaction mutagenesis, to determine their contributions to toxin activity. Substitution of Trp-30 with a serine causes a more than 40-fold reduction in specific toxicity, whereas replacement by tyrosine and phenylalanine is well tolerated. The secondary structures of both these muteins are identical to that of the wild-type toxin as determined by circular dichroism spectroscopy. Thermal denaturation experiments also show that their conformational stabilities are intact. These results demonstrate that an aromatic residue at this position is required for toxin function. Charge neutralizing substitutions of Lys-18 and Lys-19 located in the N-terminal helix have very little effect on toxicity, suggesting the nonessentiality of these residues. Similar results are also obtained for the charge neutralizing muteins for Lys-29 and Lys-33 in the loop region. Interestingly, reduction experiments demonstrate that both K29N and W30S are more sensitive to reducing agent than wild-type B-IV, raising the possibility that the loop sequence may modulate toxin stability.

Many polypeptide neurotoxins modulate the kinetics of voltage-sensitive sodium channels in diverse species, frequently displaying a high degree of selectivity for vertebrate (*1*, *2*), insect (*3*, *4*), or crustacean (*5*, *6*) channels. These toxins may therefore serve as important tools for studying structural features unique to channels of different phyla. *Cerebratulus lacteus* is an intratidal zone dweller of the northeastern coast of the United States, and belongs to the group commonly known as ribbon worms. The organism, which preys on molluscs, is known to elaborate a family of polypeptide neurotoxins which bind with high affinity to voltage-sensitive sodium channels in crustacean nerves. The most abundant member of this family, toxin B-IV, induces essentially instantaneous paralysis of crayfish at 20−30 ng/g of body weight, while having little or no effect on mammals (*7*). Electrophysiologically, B-IV delays inactivation of neuronal sodium channels, in common with α-scorpion and sea anemone toxins known to interact with Catterall's site III. However, B-IV displays neither sequence homology nor a disulfide bridging pattern similar to those found in these well-characterized polypeptide toxins. Secondary structure analyses by circular dichroism, laser Raman spectra, and two-dimensional NMR reveal that B-IV is composed of 55% α-helix and is folded into an antiparallel two-helical bundle, whereas scorpion and anemone toxins exist mostly in β-structures and have very little helix. While the unique structural motif of B-IV is not found in any previously described neurotoxins, similar two-helical hairpin conformations have been seen in several polypeptides including the plant seed protein crambin (*8*, *9*) and the *E. coli* heat-stable enterotoxin STb (*10*). These results indicate that B-IV represents a novel class of sodium channel toxin. Because of its unique primary, secondary, and folded structure, as well as crustacean-selectivity, B-IV can be used as a model polypeptide toxin for probing the binding site on crustacean sodium channels, and could ultimately provide valuable information for development of new drugs such as highly specific insecticides.

Our laboratory has cloned a synthetic gene for B-IV and developed a bacterial expression system (*11*) for producing recombinant toxin. In order to understand the molecular basis for its activity, we have pursued structural and functional characterization of this toxin by site-directed mutagenesis. An initial working model for B-IV has been generated based on biochemical (*12*) and two-dimensional NMR data (*13*), in which major N-terminal (residues 13−26) and C-terminal (residues 33−49) helices are linked by a type-I reverse turn involving Residues 28−31. The very recently reported solution structure of B-IV reveals that these two helices actually encompass residues 11−23 and 34−49 and are joined by a multiturn loop from residues 24 to 33 (*14*). This model has provided an important framework in which to interpret our previous mutagenesis experiments. We have shown that Arg-17 as such and a positive charge at position 25 are essential for toxin activity, whereas within the N-terminal helix the two carboxylate groups, Glu-13 and Asp-21, are not required (*15*). The less ordered N-terminus (residues 1−10) and C-terminus (residues 50−55) are

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relatively less important for activity, based on limited analysis of these regions (*15*, *16*).

In toxin B-IV, one of the two tryptophan residues, Trp-30, is located within the loop region linking the two helices. Previous chemical modification demonstrated that while alkylation of Trp-5 has very little apparent effect on toxin function, modification of both Trp-5 and Trp-30 results in almost complete ablation of activity (*17*). In order to assess the functional contribution of Trp-30 in the absence of changes at position 5, we have mutagenized Trp-30 to tyrosine, phenylalanine, or serine. This approach also allows us to determine the importance of the aromatic group at position 30. Finally, we have explored the roles of the flanking cationic residues Lys-29 and Lys-33 in the loop region, as well as Lys-18, Lys-19, and Leu-22 within the N-terminal helix. Our results indicate that an aromatic residue at position 30 is essential for toxin B-IV activity, whereas neither Lys-29 nor Lys-33 within the turn region is important for function.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents. All restriction enzymes, T4 ligase, and *Taq* DNA polymerase were purchased from GIBCO Life Technologies, Inc., and RNase, DNase, and protease inhibitors were from Sigma. Isopropyl 1-thio-β-D-galactopyranoside, lysozyme, and Sequenase kits were purchased from Amersham. Primers for polymerase chain reaction (PCR)[1] mutagenesis were synthesized by the DNA core facility in the Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine. Bovine factor Xa was from Pierce and small crayfish *Procambarus clarkii* from Carolina Biologicals. All other chemicals were of the highest quality available and purchased from standard suppliers.

Plasmids and Bacterial Strains. The expression vector pMH-8, which encodes the synthetic gene for B-IV fused to T7 gene-9 of bacteriophage, was used to express high levels of wild-type toxin and each mutein (*11*). *Escherichia coli* strains XL-1 blue, JM109, and DH10α were used as hosts for plasmid constructions. The strain BL21(DE3), which contains the RNA polymerase gene of T7 bacteriophage under control of the *lac* UV5 promoter, was used as the expression host (*18*).

Mutein Construction, Expression, and Purification. A two-step overlap extension PCR method (*19*) was used in this study. Briefly, pMH-8 containing the wild-type B-IV gene was used to construct all mutants. In the first step, complementary oligonucleotide primers bearing the desired mutations and two other primers flanking the template gene were used to generate two PCR fragments with overlapping ends. In a second step, annealing of these oligonucleotides allows extension to yield the final products. PCR reactions were carried out for 30 cycles following a 4 min "hot start" at 94 °C according to protocols provided by Perkin-Elmer. Melting temperatures were adjusted according to the T_m values for each pair of primers used. The final products were cleaved with appropriate restriction enzymes and cloned back into pMH-8. Sequences of all the muteins were confirmed prior to protein expression by the dideoxy method using Sequenase 2.0.

[1] Abbreviations: PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography.

Muteins were expressed and purified as described previously (*16*). In brief, plasmids containing the desired mutation were transformed into *E. coli* strain BL21(DE3). Cell cultures were induced at 37 °C for 2 h with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside after reaching mid-log phase. Clarified cell lysates were treated with streptomycin sulfate to eliminate nucleic acid and subsequently concentrated by ammonium sulfate precipitation. Fusion proteins were further purified by anion exchange chromatography on DE-52 columns. Following reoxidation of disulfide bonds, the recombinant B-IV was cleaved from the fusion protein with protease factor Xa for 15 h at room temperature and, in the final step, purified by reverse-phase HPLC[1] on a C_4 column.

Analytical Methods. Amino acid compositions were determined using a Waters Pico-Tag system. Hydrolysates were prepared *in vacuo* in the presence of 6 N HCl at 110 °C for 22 h. The dried hydrolysates were derivatized with phenyl isothiocyanate and quantified by HPLC according to the Pico-Tag manual.

Secondary structures of wild-type toxin and muteins were determined by circular dichroism spectropolarimetry using a Jasco J-710 instrument calibrated with d_{10}-camphorsulfonic acid. Each spectrum represents the accumulation of four scans of the same sample buffered with 5 mM sodium phosphate at pH 6.8. In thermal stability experiments, muteins were slowly heated from room temperature to 80 °C at a rate of approximately 1 °C/min in a water-jacketed cuvette, and their spectra were measured at 5 °C intervals. Prior to recording, samples were allowed to equilibrate at each temperature for 2 min. Subsequently, the muteins were renatured by gradually returning the temperature to 23 °C. Secondary structure estimations were derived from the spectral data by a least-squares fit to a composite structure based on the following proteins of known secondary structures: myoglobin, lysozyme, ribonuclease A, papain, cytochrome *c*, hemoglobin, α-chymotrypsin, trypsin, and horse liver alcohol dehydrogenase. Mutein stabilities were also assessed by measuring the time course of loss in helicity in the presence of 1mM DTT at 37 °C under nondenaturing conditions.

Functional Characterization of B-IV Muteins. To estimate the biological activities of each mutein, quantal bioassays using small (8−15 g) specimens of the crayfish *Procambarus clarkii* (*7*) were performed, and the data were normalized to those of the wild-type B-IV obtained on the same day. Toxins dissolved in van Harreveld's saline (*20*) are injected under the carapace at the junction of the cephalothorax and tail. Ten minutes later, the ability of treated animals to right within 2 min of being placed on their backs is determined. Animals incapable of righting in two separate trials prior to injection are eliminated from all test groups. Each point of the dose−response curves represents testing a total of 20−30 animals in at least 2 separate experiments. Data are fitted to a first-order regression using Sigmaplot 3.0, and PD_{50} values, defined as that concentration yielding paralysis of 50% of the test animals, are calculated from the resulting curves.

RESULTS

In order to ascertain the contribution of Trp-30 to the activity of toxin B-IV and to determine the role in toxin structure and/or function of the surrounding residues, we have

Table 1: Amino Acid Compositions (Residues per Mole) of Wild-Type and Mutant Forms of *C. lacteus* Toxin B-IV[a]

amino acid	wild-type	K18Q	K18,19Q	L22D	K29N	K33N	W30Y[b]	W30S[b]	W30F[b]
Asx	5.2 (5)	5.0 (5)	5.0 (5)	**5.9 (6)**	**6.0 (6)**	**6.5 (6)**	5.4 (5)	4.9 (5)	5.2 (5)
Glx	4.3 (4)	**4.9 (5)**	**6.2 (6)**	3.8 (4)	4.0 (4)	4.2 (4)	4.3 (4)	4.0 (4)	4.0 (4)
Ser	1.2 (1)	1.1 (1)	1.0 (1)	0.9 (1)	0.8 (1)	0.7 (1)	1.2 (1)	**1.7 (2)**	1.0 (1)
Tyr	1.8 (2)	1.6 (2)	1.8 (2)	1.6 (2)	2.2 (2)	1.5 (2)	**2.5 (3)**	1.5 (2)	1.6 (2)
Leu	1.1 (1)	0.9 (1)	0.6 (1)	**0.2 (0)**	1.2 (1)	1.2 (1)	1.1 (1)	0.9 (1)	1.0 (1)
Phe	0.1 (0)	0.2 (0)	0.1 (0)	0.1 (0)	0.2 (0)	0.3 (0)	0.2 (0)	0.1 (0)	**0.8 (1)**
Lys	9.4 (10)	**9.3 (9)**	**7.5 (8)**	9.4 (10)	**9.1 (9)**	**8.7 (9)**	10.3 (10)	9.5 (10)	9.4 (10)
yield[c]	5.0	3.3	4.3	2.4	4.1	4.4	4.5	4.6	4.3

[a] Only those residues whose compositions are predicted to be altered by mutagenesis are shown. The compositions for all other residues are identical to those of the wild-type toxin. [b] The loss of one tryptophan in each of these muteins is determined spectrophotometrically. [c] Reported yields represent mG HPLC-purified toxins obtained after purification and factor Xa-catalyzed hydrolysis of the fusion protein from 2 L of induced *E. coli*, and are not corrected for losses during any of the purification steps.

generated and characterized a variety of muteins in the loop region ^{24}Ile-Arg-Cys-Gln-Gly-Lys-Trp-Ala-Gly-Lys^{33}. Replacing Trp-30 with tyrosine or phenylalanine is designed to conserve the aromatic group at this site, whereas the serine replacement is designed primarily as a side-chain truncation. Additionally, if donation of a hydrogen bond by the indole nitrogen of Trp-30 were important in binding, replacement by serine might allow substantial affinity to be retained. To analyze the role of the positively charged residues, Lys-29 and Lys-33 were replaced in separate experiments by the straight chain polar amino acid asparagine. In addition, we have extended our previous characterization of functionally essential features lying within the N-terminal helix. The roles of Lys-18 and Lys-19 were evaluated by characterizing muteins K18Q and K18,19Q. Finally, in order to complete our definition of the binding epitope located within the N-terminal helix of B-IV, we also analyzed replacement of Leu-22. Two-step overlap extension PCR mutagenesis was used to generate all muteins (*19*) which were then cloned into plasmid pMH-8. The mutated plasmids were then used to transform *E. coli* strain BL21(DE3) for production of the gene-9/B-IV fusion proteins as described previously (*11*). The amounts of fusion proteins produced, as well as the final yield of each mutein following protease factor Xa digestion and HPLC purification (Table 1), are very similar to those obtained for the wild-type toxin. The amino acid compositions of each mutein were determined, and the residues for which mutations were generated are shown in Table 1. With the exception of lysine values, which appear to be underestimated in most cases, all are consistent with the designed mutations and the absence of other changes. That the compositions lack the residues known to be absent from wild-type B-IV (methionine, valine, and phenylalanine) underscores the purity of the toxin samples used for subsequent characterization.

Functional Characterization of Muteins. (A) Loop Region. High-resolution NMR studies define the loop region as including residues from 24 to 33. Within this region, we have previously demonstrated the importance of Arg-25 (*15*). Therefore, in the present study, the major targets are Lys-29, Trp-30, and Lys-33. As shown previously, toxin B-IV is inactivated when both Trp-5 and Trp-30 are alkylated with HNB-Br, although modification of Trp-5 has little or no effect on activity (*17*). While these findings suggest a possible functional role for Trp-30, direct data showing that modification of this residue *by itself* abolishes activity are lacking. Site-directed mutagenesis allows a direct test of this question, as well as permitting additional insight into the mechanism by which activity is lost. In order to determine whether a tryptophan is important, rather than simply aromaticity, we have in the present study generated the muteins W30Y, W30F, and W30S, and compared their activities to that of wild-type B-IV by quantal bioassays as described under Experimental Procedures. Dose−response curves for these muteins are compared to those of wild-type toxin in Figure 1, and their PD_{50} values are contrasted in Figure 2. Although not all of the dose−response curves shown in Figure 1 have identical slopes, it should be noted that PD_{50} values are not slope-dependent. The loss of activity for W30Y is relatively small (PD_{50} = 1.4 μg/mL compared to the wild-type PD_{50} of 0.33 μg/mL), while W30F is even more active, with a PD_{50} of 0.39 μg/mL. However, mutein W30S displays a PD_{50} of 13.4 μg/mL, corresponding to a 41-fold decrease in toxicity. These results highlight the importance of an aromatic residue at position 30 with tryptophan required for optimal activity.

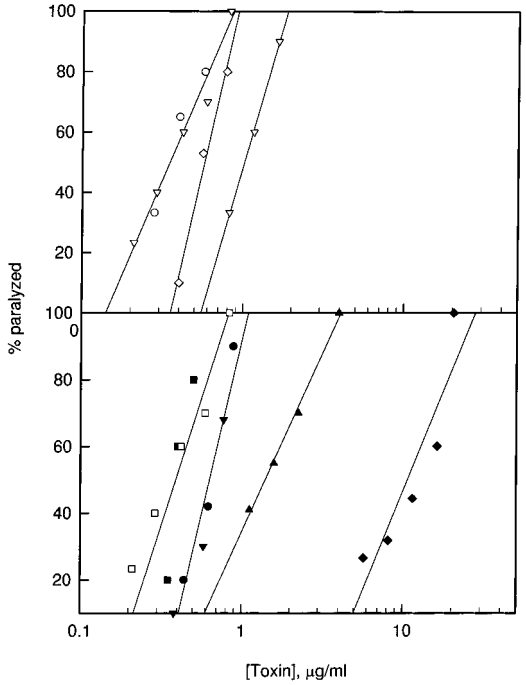

FIGURE 1: Biological activity of the N-terminal helix and loop region B-IV muteins. (Upper panel) Toxicity of wild-type (▽) B-IV is compared to that of the muteins L22D (○), K18Q (◇), and K18,19Q (▽) in the N-terminal helix. (Lower panel) Comparison of the activities of wild-type (□) B-IV to that of the muteins K29N (●), K33N (▼), W30Y (▲), W30F (■), and W30S (◆) in the loop region. Each point represents assay of a total of 20−30 animals performed in at least 2 separate experiments.

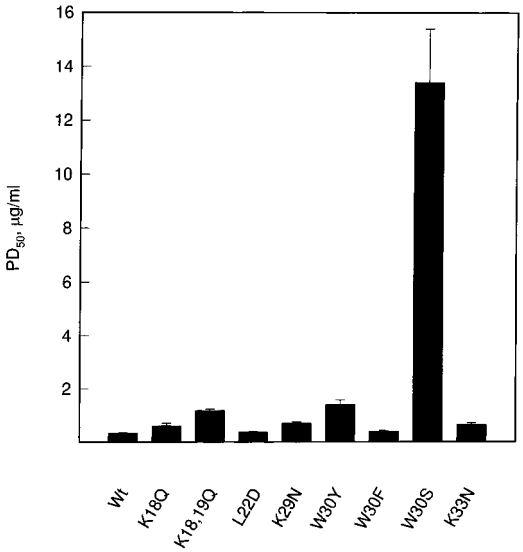

FIGURE 2: Comparison of PD_{50} for all muteins. The PD_{50} values for K18Q (0.60 ± 0.09), K18,19Q (1.17 ± 0.06), L22D (0.36 ± 0.02), K29N (0.70 ± 0.05), K33N (0.64 ± 0.07), W30F (0.39 ± 0.03), W30Y (1.40 ± 0.17), and W30S (13.4 ± 2.6), calculated from the data depicted in Figure 1, are compared to that of wild-type B-IV (0.33 ± 0.02).

The importance of cationic residues in the activity of polypeptide neurotoxins is well established (*21*−*24*), and we have previously demonstrated by chemical modification that when 2−3 lysines (of the 10 in B-IV) are modified with pyridoxal phosphate at pH 6.5,[2] the specific toxicity is reduced by 50-fold. However, C-terminal truncation analysis indicates that neither Lys-53 nor Lys-54 contributes to this loss of activity (*15*). Since we know that important binding determinants exist in both the N-terminal helix and loop region, we examined the roles of lysines in these two regions. In an effort to identify which of the remaining eight lysines is essential, we have mutagenized Lys-29 and Lys-33 to asparagine, and Lys-18 and -19 to glutamine (cf. below) in the present study. As depicted in Figure 1, both muteins K29N and K33N have very similar toxicities as compared to that of wild-type B-IV, indicating that the positive charges at these loop sites are nonessential for function.

(B) N-Terminal Helix. We have previously found that Arg-17 within the N-terminal helix of toxin B-IV (residues 11−23), as well as Arg-25 in the loop region, is essential for activity (*15*), whereas Glu-13 and Asp-21 located in the N-terminal helix are not. The two cationic sites thus delimit an epitope for toxin binding to sodium channels. To determine whether additional nearby charged groups also contribute to this functional epitope, we tested the activity of mutein K18Q. As shown in the upper panel of Figure 1, its activity is identical to that of the wild-type toxin. In a parallel experiment, the activity of the double mutein K18,19Q is observed to decrease to a small extent, with a PD_{50} of 1.17 μg/mL. Based on these results, we conclude that neither of the two lysine residues in the N-terminal helix is essential.

Comparison of specific toxicities between B-toxins reveals that B-IV is 15-fold less active than its close homolog B-II (*7*). Previous analyses of their N-terminal regions show that the replacement of Ala-3 and -8 in B-IV by serine in B-II makes only a small contribution to this difference (*16*). Within the N-terminal helix and the loop region, 20 of the 23 residues are identical in the 2 toxins, with the exceptions being Lys-19, Leu-22, and Arg-25 in B-IV. Because neither of the B-IV to B-II replacements K19Q (this work) or R25K (*15*) significantly affects activity, we sought to determine the importance of the third site in this region, Leu-22, in discriminating between the two toxins. We therefore constructed and characterized the mutein L22D, in which this residue is replaced by its B-II counterpart. The mutein shows no difference in toxicity compared to that of wild-type B-IV. We therefore conclude that Leu-22 plays no role in defining potency in these *Cerebratulus* toxins.

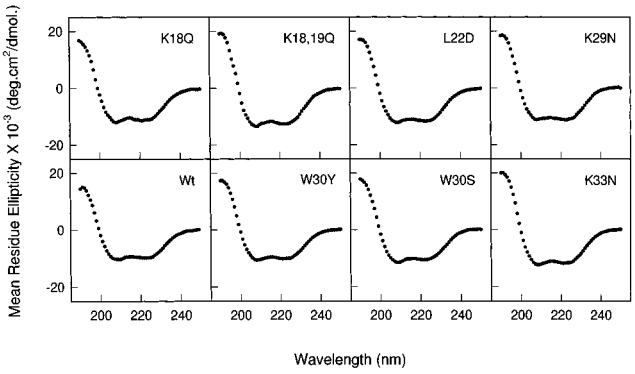

FIGURE 3: Circular dichroic spectra of the wild-type and mutant B-IVs. The CD spectra of all muteins in this study (as indicated in each panel) are compared to that of the wild-type B-IV. Spectra were recorded and their secondary structures estimated as described under Experimental Procedures.

Structural Characterization of Muteins. Changes in secondary structures of these muteins were monitored by far-UV circular dichroism spectropolarimetry over the range of 190−250 nm. As shown in Figure 3, the spectra for all muteins in this study are indistinguishable from that of the wild-type toxin. Their α-helix contents as estimated by the JASCO secondary structure determination program are identical. Together with the fact that the yields of all muteins and their HPLC profiles are very similar to those of the wild-type toxin, we conclude that their overall folding patterns are unperturbed.

W30S shows the most dramatic decrease in toxicity among all muteins in the present study. It is important to understand whether its aromatic ring interacts directly with the sodium channel, or whether this residue plays a structural role. To evaluate a potential structural contribution of Trp-30, we have probed the structural stability of this and other muteins in the loop region. Loss of dichroic signal was recorded as a function of temperature, and the residual α-helical contents after renaturation of each toxin from 80 °C to room temperature were calculated. As shown in Table 2, more than 90% of the helical content is recovered upon renaturation for all muteins including W30S. These results indicate that the overall stabilities of these muteins are largely unperturbed.

Disulfide bonds in many polypeptides stabilize their folded native structures and in the case of B-IV, as demonstrated by inactivation of toxin upon reduction, are crucial for its function (*12*). We therefore examined the integrity of disulfide bonds in these muteins, as well as their susceptibility to reduction. Because none of the toxins react with DTNB at room temperature in the presence of 1% SDS (data

[2] D. L. Lieberman and K. M. Blumenthal, unpublished data.

Table 2: Structural Stabilities of Wild-Type and Mutant Forms of Toxin B-IV

toxin form	% recovery (α-helix)[a]	k_{red} (s^{-1})[b]
wt	97	0.91 ± 0.17
K29N	98	3.33 ± 0.34
K33N	97	1.15 ± 0.27
W30Y	95	1.04 ± 0.16
W30S	97	3.60 ± 0.93

[a] Defined as the helical contents recovered after thermal denaturation and subsequent return to room temperature. [b] k_{red} is the rate constant for reduction of helical content in the presence of 1 mM DTT at 37 °C.

not shown), we conclude that all four disulfide bonds are intact in all toxins. It is known that mild reduction of B-IV attenuates the dichroic signal at 222 nm, reflecting a loss of helix content. To measure the reduction rates, wild-type and mutant forms of B-IV were incubated at 37 °C in the presence of 1 mM dithiothreitol at pH 6.8, and the rate of loss of α-helix was measured as a function of time. As shown in Table 2, the rate constants for reduction of helical contents in W30Y and K33N are similar to the value obtained for wild-type toxin. However, with both K29N and W30S, helicity was lost 3−4 times more rapidly, demonstrating that while the overall folding of these muteins is generally intact, truncations of side chains at either position 29 or position 30 destabilize the toxin.

DISCUSSION

Neurotoxin B-IV represents a novel class of sodium channel toxin owing to its unique primary sequence, folding pattern, and channel selectivity. For the past few years, we have been interested in analyzing the underlying structure−function relationships of this toxin, having successfully cloned the synthetic gene and produced recombinant protein in a bacterial expression system (*11*). Structural analysis of B-IV by two-dimensional NMR reveals that it consists mainly of two antiparallel α-helices, incorporating residues 11−23 and 34−49, linked by two interlocking inverse γ-turns and two interlocking β-turns between residues 24 and 33 (*14*). The remainder of the molecule is less ordered. Residues of both the N-terminus (residues 1−10) and C-terminus (residues 50−55) have been probed previously by chemical modification and/or mutagenesis studies. Replacement of Ala-3 and -8 with serine results in a 2−3-fold increase in specific toxicity. The fact that the secondary structure of this mutated toxin is unchanged suggests that the presence of hydrogen bond donors at these positions is responsible for the enhanced activity (*16*). We have recently demonstrated much larger effects on activity upon mutagenesis of charged residues in the N-terminal helix and the loop region. When either Arg-17 or Arg-25 is neutralized by replacement with glutamine, the loss of activity is minimally 700-fold, and all Arg-17 muteins tested are totally devoid of toxicity. Thus, electrostatic interactions involving Arg-17 and Arg-25 are absolutely essential for toxicity (*15*).

In order to more completely define this structure−function map, we have in the present study investigated the importance of amino acid residues in the loop region linking the two helices. Toxin derivatives in which both Trp-5 and -30 are alkylated are approximately 20-fold less active than the wild-type B-IV. Both NMR data and its chemical reactivity indicate that Trp-30 is accessible to solution. In the present study, replacement of this residue by alternative aromatic ones is well tolerated, with decreases in activity ranging from 20% for W30F to less than a 5-fold decrease for W30Y. However, when the aromatic side chain is truncated to serine, the toxicity is dramatically reduced by greater than 40-fold. These data strongly suggest the essential contribution of Trp-30 is its ability to provide an aromatic function at this position, rather than to donate a hydrogen bond via its indole nitrogen. Because the structures of all of these muteins are largely unperturbed, these results suggest that the indole ring of Trp-30 may make important contact with the sodium channel.

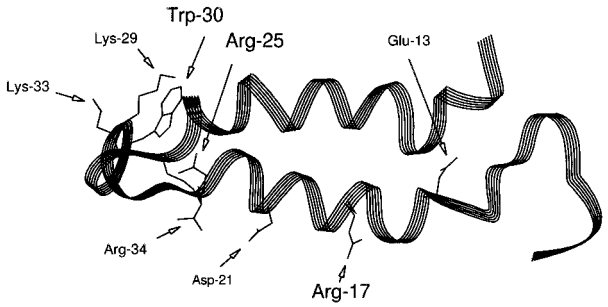

FIGURE 4: Backbone structure of neurotoxin B-IV is represented in the ribbon format, with the side chains of selected targeted residues indicated as lines. Residues annotated in the larger font (Arg-17, Arg-25, and Trp-30) have been shown to be essential in either this study or the study of Wen and Blumenthal (*15*). The structure depicted is based on the coordinates described in Barnham et al. (*14*) and deposited in the Protein Database (pdb1vib).

Although this does not affect the calculated PD_{50} values, it is apparent that not all of the toxin derivatives characterized display dose−response curves having identical slopes (Figure 1). A great many alternative mechanisms could explain this behavior, all of which would exert their effects primarily at low toxin concentrations. For example, mutant toxins might display slower on-rates or more rapid dissociation, either of which could yield an abnormally lower percentage of paralyzed animals. Such effects might reasonably be expected to be exacerbated at low toxin concentrations, resulting in a change in slope. It is also possible that certain mutants might be cleared from the animal more rapidly than others, or than wild-type. While our data do not allow us to distinguish among these possibilities, we wish to reemphasize that the nonparallel slopes observed have no effect on PD_{50} values, which represent the basis for our comparison of activities.

Chemical modification with pyridoxal 5′-phosphate also implicates lysine residues in toxin function.[2] We have been investigating the role of individual lysines by either deleting the residue or eliminating its charge and have recently demonstrated that truncation of the toxin by successive removal of Lys-53 and-54 has little effect on toxicity (*15*). Our present demonstration that Lys-18 and -19 can be neutralized without affecting toxicity, in conjunction with our previous results, shows that within the N-terminal helix only Arg-17 provides a crucial binding determinant. Figure 4 shows a backbone model of neurotoxin B-IV, based on the recent data of Barnham et al. (*14*). The positions of the side chains of all of the residues we have found to be essential in this and our earlier study are also indicated. It is apparent that while loop residues Arg-25 and Trp-30 are oriented toward the same face of the molecule, the third essential residue, Arg-17, points in a different direction.

Moreover, it is now clear that none of the lysines found in either the N-terminal helix or the connecting loop are essential. We therefore speculate that one or more of the four remaining lysines, three of which are in the C-terminal helix, are important for toxin function. The recently published high-resolution structure of this toxin will greatly facilitate identification of such residues.

The role of turns in determining the structure and stability of proteins is an area of active investigation. Brunet *et al.* (*25*) and Predki and Regan (*26*) have proposed that turns play a mostly passive role, simply connecting other elements of protein secondary structure. Alternatively, Zhou and DeGrado have shown that in the B1 domain of streptococcal protein G the identity of amino acids within a turn can exert significant influence on both the stability and folding of the protein (*27*). To investigate whether the residues in the loop region play a structural role in B-IV, we have examined the secondary structures and thermal stabilities of muteins in this region. Similar protein yields, HPLC elution profiles, and identical α-helical contents lead to the conclusion that sequences of the loop are not important for the overall folding of the toxin. Although the presence of four disulfide bonds in these toxins could well mask any potential destabilization, the fact that all muteins refold indistinguishably after thermal denaturation is also consistent with this conclusion. In contrast, our reduction experiments indicate that K29N and W30S are 3−4 times more sensitive than wild-type toxin, raising the possibility that the loop sequence modulates B-IV stability. Surprisingly, Chou−Fasman analysis predicts both substitutions to strengthen the 28−31 β-turn (*28*). That we observe the opposite suggests specific stabilizing interactions between Lys-29 and Trp-30 may exist. These could take the form of the cation−π interactions well documented in other systems (*29*, *30*). Alternatively, hydrophobic interactions between the methylene groups of Lys-29 and the indole group of Trp-30 might also contribute. Finally, NMR analyses reveal that the aromatic protons of Trp-30 have long-range NOE connectivities with several neighboring residues including Cys-37 (*13*). Such interactions would necessarily be abolished in W30S, leading us to speculate that the elevated rate of reduction seen in this mutein results from the destabilization of the Cys-26/Cys-37 disulfide bond.

Fourteen nonconserved amino acids potentially contribute to the 15-fold difference in potency between *Cerebratulus* B-toxins II and IV. While we have demonstrated that Arg-17, Arg-25, and Trp-30 are essential for function in B-IV, no analyses have identified sites responsible for the difference in toxicity between these two homologs. In this and previous studies, the B-II to B-IV substitutions of serine for Ala-3 and -8, glutamine for Lys-19, aspartic acid for Leu-22, and lysine for Arg-25 individually cause only very minor changes in toxicity. Thus, these nonconserved sites play little or no role in defining the differential activities of the two toxins. Therefore, the seven sites within the C-terminal helix at which B-II and B-IV differ must account for the difference in potency. The role of the C-terminal helix in toxin activity remains to be analyzed.

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