Role of Electrostatic Interactions in Defining the Potency of Neurotoxin B-IV from *Cerebratulus lacteus*

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Paul H. Wen and Kenneth M. Blumenthal‡

From the Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0524

Chemical modification implicates arginine residues of the *Cerebratulus lacteus* neurotoxin B-IV in biological activity. In the present study, we used site-directed mutagenesis to assess the functional contributions of each of these residues. Panels of mutants at each site have been constructed by polymerase chain reaction and recombinant proteins expressed and purified to homogeneity using an *Escherichia coli* expression system developed in this laboratory. All substitutions for Arg-17 (Gln, Ala, or Lys) yield proteins having undetectable levels of activity, while charge neutralizing replacement of Arg-25 (R25Q) causes a 400-fold reduction in specific toxicity. However, the R25K mutein is almost as active as natural toxin. Circular dichroism spectroscopy indicates that there are no major conformational changes in any of these muteins. These results therefore demonstrate the requirement for a guanidinium group at position 17, and a positive charge at position 25. NMR analyses (Hansen, P. E., Kem, W. R., Bieber, A. L., and Norton, R. S. (1992) *Eur. J. Biochem.* 210, 231–240) reveal neurotoxin B-IV to contain two antiparallel a-helices, which together include 57% of the sequence. Both Arg-17 and Arg-25 lie on the same face of the N-terminal helix (residues 13–26), as do the carboxyl groups of Gln-18 and Asp-21. However, charge neutralizing mutations of the latter two sites have no effects on biological activity. Arg-34, situated near the N terminus of helix 2 (residues 33–49) is also important for activity, as its replacement by Gln or Ala diminishes activity by 20- and 80-fold, respectively. However, unlike Arg-17 and Arg-25, thermal denaturation experiments suggest that R34Q may be structurally destabilized relative to wild-type B-IV.

Voltage-dependent ion channels are integral plasma membrane proteins responsible for the generation of action potentials in electrically excitable cells (1, 2). Several groups of neurotoxins that bind specifically to these channels have been used as probes both for identification and functional mapping of their target macromolecules (3–5), which include voltage-sensitive sodium channels. These toxins interact with sodium channels at a variety of receptor sites, one of which (site III: Refs. 6 and 7) serves as the acceptor for many polypeptide neurotoxins. This site presents a novel class of neurotoxin, as it displays no sequence homology with those identified previously. In addition, while scorpion and sea anemone toxins exist in mostly b-sheet conformations, the secondary structure of B-IV consists largely of a-helices (15, 19, 20). Recent two-dimensional NMR data show that B-IV is composed of two antiparallel a-helices, incorporating residues 13–26 and 33–49 (21). This two-helix bundle folding pattern is not present in any previously characterized polypeptide neurotoxins.

We have begun to investigate important structural elements and to characterize the essential residues in this structurally unique, crustacean-selective neurotoxin. Blumenthal and Kem showed that nitration of Tyr-9 or alkylation of Trp-30 both result in ablation of biological activity (25, 26). While other chemical modification analyses implicate lysine, arginine, and exemplified by the insect-selective and crustacean-selective toxins derived from scorpion venoms (8–11), and mollusk-selective toxins from Conus snail (12, 13), discriminate efficiently among the sodium channels of various phyla, implying that sequence-specific structural differences among the various toxins define both their function and selectivity. As such, these neurotoxins may have important applications in the design of new drugs, such as highly specific insecticides.

The heteronemertine *Cerebratulus lacteus* produces a family of four structurally homologous, crustacean-selective polypeptide neurotoxins designated B-I to B-IV, that induce repetitive, repetitive action potentials in crab and lobster walking leg nerves (14). Neurotoxin B-IV, the most abundant of the B-toxins, is selectively toxic to crustaceans, inducing paralysis at mean concentrations of about 20 ng/g of body weight (14, 15). This toxin prolongs the repolarization phase of the action potential in crustacean nerves, similar to the actions of the other polypeptide neurotoxins mentioned above (14). Electrophysiological experiments have demonstrated that B-IV causes a small depolarization of the resting potential in lobster and crayfish walking leg nerve, and that this effect can be blocked by tetrodotoxin, a known sodium channel blocker, or by replacing external Na+ ions with the impermeant choline. Biochemical analyses show specific high affinity binding of [125I]labeled toxin B-IV to a single class of receptor sites on lobster axon membrane vesicles. While binding competition between B-IV and sea anemone or scorpion toxins has not been observed, the capacity of the binding site for *Cerebratulus* neurotoxin is very similar to that of saxitoxin in the same tissue (16). Furthermore, photoaffinity labeling shows that B-IV binds to a 40-kDa protein on lobster nerve membrane, which is similar in size to the b-subunit of mammalian nerve and muscle sodium channels (17). The data above demonstrate that the binding site for B-IV is distinct from sodium channel site III, which is targeted by both a-scorpion and sea anemone toxins (16, 18).

Despite their being generally similar in terms of size and overall basicity, the 55-residue, 6-kDa neurotoxin B-IV represents a novel class of neurotoxin, as it displays no sequence homology with those identified previously. In addition, while scorpion and sea anemone toxins exist in mostly b-sheet conformations, the secondary structure of B-IV consists largely of a-helices (15, 19, 20). Recent two-dimensional NMR data show that B-IV is composed of two antiparallel a-helices, incorporating residues 13–26 and 33–49 (21). This two-helix bundle folding pattern is not present in any previously characterized polypeptide neurotoxins.

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Arginine Residues in Activity of Neurotoxin B-IV

Mutant codons are underlined within the primer sequences. Residues given in parenthese represent wobble positions.

| Mutation | Strand | Primer |
|----------|--------|--------|
| Y9F      | Sense  | 5′-GTGCGATTTCCGGGC-3′ |
| E13Q     | Antisense | 3′-GACCGTGTTTCAAGTCGTTC-5′ |
| E13A/G   | Antisense | 3′-GACCGTGTTTCAAGTCGTTC-5′ |
| D21N     | Sense  | 5′-CACTGCCAGAAAAATACCATTTG-3′ |
| D21A/P   | Sense  | 5′-CACTGCCAGAAAAATACCATTTG-3′ |
| R17Q/A/K | Sense  | 5′-ACAATGCGCAGTACCAAGTAA-3′ |
| R52Q/A/K | Sense  | 5′-TTTGCAGCCGCTAGTTTTTAT-5′ |
| R53Q/A/K | Sense  | 5′-TTTGCAGCCGCTAGTTTTTAT-5′ |
| E55X/K54X/K55X | Sense | 5′-AAATG(CT)AA(CT)AAGTAAATAAGA-3′ |

1 D. L. Lieberman and K. M. Blumenthal, unpublished data.
2 The abbreviations used are: PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; DTNB, 5,5′-dithio-

carboxylate groups in toxin function, identification of uniquely essential residues within a chemically equivalent group has only become possible with our development of a bacterial expression system for B-IV (15). With the availability of molecular biological techniques, we have begun targeting individual amino acids of this neurotoxin by site-directed mutagenesis of the synthetic B-IV gene. Initial studies show that the double substitution A35S/A85S enhances toxin activity 2-fold (27), and have been interpreted as indicating a role for hydrogen-bonding potential in the N-terminal region in activity.

Both chemical and mutational alterations, carried out in this laboratory and elsewhere, have revealed that cationic residues in sea anemone neurotoxins (28, 29) and a-scorpion toxins (30, 31) are important determinants of their activity. Because of this well known importance of cationic residues to function in polypeptide neurotoxins, and the results obtained upon modification of natural B-IV with 1,2-cyclohexanediol,1 we have explored the functional consequences of replacing each of the three arginine residues (Arg-17, Arg-25, and Arg-34) with lysine, glutamine, or alanine. We also examined the roles of Glu-13, Asp-21 by characterizing charge-neutralized muteins at these sites. Finally, we characterized mutant toxins that were serially truncated at the C terminus in order to assess the role of a cationic cluster in this region. Our results indicate that within the N-terminal helix, an arginine residue at position 17, and a positive charge at position 25 are essential for function, whereas the third arginine, Arg-34, contributes as well to intramolecular stabilization of the two-helix bundle. In contrast, the carboxylate groups of Glu-13, Asp-21 and Glu-55, and ε-amino groups of Lys-53 and Lys-54 are nonessential for both structure and function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes, T4 ligase, and Taq DNA polymerase were obtained from Life Technologies, Inc., and bovine Factor Xa was from either Boehringer Mannheim or Pierce. Isopropyl-1-thio-β-D-galactopyranoside was purchased from United States Biochemical Corp. Primers for PCR2 mutagenesis were synthesized by the DNA core facility in the Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine. Sequenase kits were purchased from United States Biochemical Corp., and the crayfish Procambarus clarkii from North Carolina Biological. All other chemicals and reagents were obtained from standard suppliers and were of the highest grades available.

**Bacterial Strains and Plasmids—**Escherichia coli strains XL-1, JM 109, or HB101 were used as hosts for plasmid constructions. The expression plasmid pMH8, containing the wild-type B-IV gene, was used as the template in all PCR reactions (15). Complementary oligonucleotide primers (Table I) bearing the desired wobble mutation and two other primers flanking the template gene were used to generate two PCR fragments having overlapping ends. In a second PCR step, annealing of these oligonucleotides allows extension by Taq polymerase to yield recombinant products containing the desired mutation. All PCR reactions were carried out for 30 cycles following incubation for an initial 3 min at 94 °C to completely denature the template. Melting temperatures were adjusted according to the Tm values for each pair of primers used. The final products were cloned into the polylinker region of plasmid pSR9 after cleavage with appropriate restriction enzymes, and the sequences of plasmids carrying the desired mutations then confirmed by the dyeoxy method using Sequenase 2.0 (United States Biochemical Corp.).

Standard procedures developed in this laboratory were utilized to express and purify all proteins (15, 27). Briefly, plasmid containing the desired mutation was transformed into E. coli strain BL21(DE3), and gene θ-B-IV (mutein) fusion protein synthesis induced in mid-to-late log phase (A600 = 0.85) by addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Following purification by anion exchange chromatography on DE-52, and reoxidation of disulfide bonds, B-IV muteins were liberated using restriction protease Factor Xa (E:S = 1:4.000–8.000). Final purification of each mutein was accomplished by reverse-phase HPLC3 on a C4 column.

**Analytical Methods—**Amino acid compositions were determined using a Waters Pico-Tag system. Samples containing 0.2–2 nmol of protein were hydrolyzed in the presence of 6 N HCl at 110 °C for 22 h. Hydrolysates were then dried, derivatized with phenylisothiocyanate, and quantified according to the Pico-Tag manual. Secondary structures of all muteins were determined by circular dichroism spectropolarimetry in a Jasco J-710 instrument calibrated with d16-camphorsulfonic acid. For assessment of conformational stability, muteins in 6 mM phosphate buffer were gradually heated from 23°C to 80°C in a water-jacketed cuvette, and their spectra measured at 10-degree intervals. Samples were equilibrated for 10 min at each temperature prior to recording their spectra, and were then renatured by gradually returning the temperature to 23°C. The resulting circular dichroic spectra were deconvoluted using the convex constraint algorithm described by Perrin et al. (34). Changes in secondary structures for all muteins as well as wild-type toxin were then calculated. The sensitivity of the ε-helical content of each mutein to reduction was also assessed by measuring the time course of loss in helicity upon incubating samples in 1 mM dithiothreitol at 37°C.

**Functional Characterization of B-IV Muteins—**The biological activity of each mutein was measured in the crayfish P. clarkii by quantal bioassay as described previously (14) and normalized to that of the wild-type toxin analyzed on the same day. Each point of the dose-response analysis represents assay of a total of 15–40 animals in at least two separate experiments. PD50 values, defined as the toxin concentration at which 50% of the crayfish are paralyzed, were calculated for each mutein using Macintosh’s KaleidaGraph software.

**Molecular Modeling of Toxin B-IV—**An initial model structure for...
neurotoxin B-IV has been generated using the molecular modeling software Insight/Discover (Biosym Technologies, San Diego, CA). Based on two-dimensional NMR data (21), two α-helices were created encompassing residues 13–26 and 33–49; subsequently, a type-II β-turn involving residues 28–31 was generated yielding a hairpin shaped structure. This unconstrained model was then energy-minimized (500 steps of steepest descent minimization) and subjected to constrained molecular dynamics in order to achieve proper juxtapositioning of the cysteine pairs known to participate in the four disulfide bonds of B-IV (Cys-12/Cys-48, Cys-16/Cys-52, Cys-23/Cys-41, and Cys-26/Cys-37). Finally, 100 iterations of steepest descents were performed to allow unfavorable geometric strain in the molecule to relax.

**RESULTS**

In order to analyze the role of the three arginines and two side chain carboxylate groups in toxin B-IV, we have generated and characterized multiple muteins at each site, designed to conserve charge, conserve polarity, or truncate the targeted side chain. In the example of arginine residues, replacement by lysine allows us to ascertain whether an arginine specifically, or merely a positive charge, is required at a given position. Conversion to a straight chain polar amino acid, e.g. Arg → Gln, assesses the importance of the positive charge, while the consequences of side chain truncation are estimated by replacement with alanine. The same principles were also applied for the two carboxyl groups in the present study, Glu-13 and Asp-21. For Asp-21, we also examined the role of the N-terminal helix (Glu-13 to Gly-26) in the toxin as a structural determinant of activity by replacing this residue with proline, a strong helix breaker (35). Most muteins were produced using a two-step overlap extension PCR protocol, which in our hands allows retrieval of mutated DNA sequences with high efficiencies. Plasmids containing the desired mutations were then used to program expression of fusion proteins in E. coli strain BL21(DE3). The amounts of the fusion proteins obtained in all cases were between 75 and 100 mg/liter of cell culture. Final yields of all muteins after reoxidation and digestion with protease Factor Xa and HPLC purification were between 5 and 15 mg/400 mg of fusion protein, very similar to those obtained for the wild-type toxin (15). The reverse-phase HPLC profiles for all muteins show a single homogeneous peak having a retention time very similar to that of the wild-type protein. Amino acid compositions (Table II) are in all cases consistent with the designed mutations.

**Functional Characterization of Muteins**

**Role of Arginine Residues**—Our major targets in this study are the three arginines found at positions 17, 25, and 34. Previous chemical modification of B-IV by 1,2-cyclohexanediol-1,2-epoxide caused inactivation of the toxin without measurably altering its secondary structure. However, it was not possible to target any single, uniquely reactive arginine using this reagent. In contrast, mutation of each arginine was readily accomplished by overlap extension wobble PCR, allowing substitution of Lys, Gln, or Ala at each site probed. The activities of the resulting purified muteins were then compared with that of wild-type B-IV by quantal bioassay as described under “Experimental Procedures.” Dose-response analyses for each arginine mutein are depicted in Fig. 1, and their PD₅₀ values compared in Fig. 2. None of the Arg-17 muteins displays detectable activity, even at concentrations 500–1,000 times greater than the PD₅₀ of wild-type B-IV. These data are consistent with the essentiality of Arg-17 for toxicity. In contrast, the Arg-34 muteins display more modest reductions in activity, with the PD₅₀ values for R34Q and R34A being between 20- and 70-fold higher than for wild-type protein. Interestingly, R34K is only 8-fold less active, indicating that this mutein, which retains a positive charge at position 34, is similar in activity to the wild-type toxin.

Arg-25 represents an intermediate situation. Replacement of this arginine by glutamine causes approximately a 400-fold reduction in activity. However, conversion to a lysine at this position does not affect the activity of B-IV greatly, as the PD₅₀ for R25K is increased only 2-fold relative to the wild-type toxin. Thus the presence of a cationic residue at position 25 is essential for normal B-IV function.

**Role of Lysines 53 and 54**—The C-terminal region of toxin B-IV is highly cationic, with four of the last seven residues being lysine. We have begun characterization of these lysine residues by individually converting the codons for Lys-53, Lys-54, and Glu-55 to translational terminators. Following expression and purification, the amino acid compositions of these proteins (Table II) are consistent with predicted values, and their secondary structures indistinguishable from wild-type B-IV. Activities of the resulting chain termination muteins were characterized as described above. As depicted in Fig. 2, none of the C-terminal residues are essential for activity, as in no case does truncation result in more than a 2-fold increase in PD₅₀. Since the yields of each termination mutein are very similar to wild-type protein, it is also unlikely that any of these sites affect the folding pathway or disulfide bond formation to a significant degree, despite their proximity to Cys-52.

**Role of Carboxyl Groups**—As noted above, four carboxyl...
groups are present in B-IV, and deletion of the C-terminal Glu-55 has no significant effect on activity. In order to assess the functional contribution of Glu-13 and Asp-21, each site has been replaced with both Ala and the corresponding amide. In neither case does ablation of the negative charge or truncation of the side chain affect the activities of these toxins, as demonstrated in Figs. 1 and 2. Thus, neither Glu-13 nor Asp-21 are essential residues. The secondary structures of the E13Q/A and D21N/A muteins are also indistinguishable from that of wild-type B-IV. In contrast, substitution of a proline for Asp-21 results in a 75% reduction in helical structure as measured by CD. Somewhat surprisingly, the activity of this mutant toxin is decreased only 10-fold. A possible explanation for this seemingly anomalous result will be presented under “Discussion.”

Tyrosine 9—Of the two tyrosines in toxin B-IV, Tyr-9 has been identified as an essential residue for biological activity based on the loss of more than 99% of specific toxicity upon its nitration with tetranitromethane (25). Nitration has been shown to decrease the $pK_a$ of the phenolic hydroxyl group to approximately 6.6, indicating that this group would be largely anionic under conditions of the bioassay. To assess whether the loss of activity seen upon nitration is related to steric effects occurring upon introduction of a nitro group rather than perturbation of the ionic properties of the phenolate side chain upon modification, we constructed and expressed the mutein Y9F. Measurement of its biological activity revealed that Y9F is only about 5-fold less active than wild-type B-IV. Thus, it is likely that the abolition of activity observed upon nitration of Tyr-9 is a steric effect resulting from the presence of the nitro substituent, rather than from changes in the phenolate $pK_a$.

**Structural Characterizations of Muteins**

Prior to characterizing the biological activities of these muteins, their far UV circular dichroism spectra were measured over the range 190–250 nm in order to determine whether any mutationally induced alterations in secondary structure exist. The results shown in Fig. 3 indicate that the $\alpha$-helical contents of all muteins except D21P are essentially identical to that of wild-type B-IV. In contrast, for mutein D21P, 75% of the total helical content found in the wild-type toxin was lost. Given the known helix-breaking properties of proline, this result was not unexpected.

The losses of activity seen upon replacement of Arg-17, -25, or -34 could point to a direct involvement of the guanidinium group in binding to the channel. Alternatively, the mutated residue could stabilize the three-dimensional structure of toxin B-IV or be an important folding determinant. To distinguish

![Fig. 1. Biological activity of carboxylate and arginine muteins. Upper panel, toxicity, expressed as paralytic activity, of wild-type (+) B-IV compared with that of the carboxyl group muteins E13Q (O)/D21A (†), E13A (square)/D21N (△), and D21P (○). Solid lines represent fits of the data to linear functions for these sets of muteins. The differences in PD50 within each set are statistically insignificant. Lower panel compares the activities of wild-type (+) B-IV with those of the arginine muteins R25Q (M), R25K (★), R34Q (†), R34A (△), and R34K (○). Each point represents assay of a total of 15–40 animals in at least two separate experiments.](image1)

![Fig. 2. Comparison of PD50 for all muteins. Muteins are categorized into six groups and their PD50 values (mean values of two or more separate experiments ± S.D.) compared with that of wild-type B-IV. No activity could be detected for any of the Arg-17 muteins, which are therefore not included in the figure.](image2)

![Fig. 3. Circular dichroic spectra of arginine and carboxylate muteins. The CD spectra of R17Q (□), R17A (○), and R17K (△) in panel A, R25Q (○) and R25K (△) in panel B, R34Q (○), R34A (◆), and R34K (△) in panel C, as well as E13Q (○), D21N (□), and D21P (△ with thin solid line) in panel D are compared with that of wild-type B-IV shown by thick solid line in each panel. Spectra were recorded as described under “Experimental Procedures” and deconvoluted using the convex constraint algorithm described by Perczel et al. (34).](image3)
Arginine Residues in Activity of Neurotoxin B-IV

TABLE III

| Structural Stabilities of wild-type and mutant B-IV |
|---------------------------------------------------|
| Toxin form | Recovery (α-helix) | k_red \(^a\) |
|------------|-------------------|-------------|
| Wild-type  | 96              | 0.37 ± 0.02 |
| R17Q       | 96              | 0.20 ± 0.14 |
| R17A       | 94              | 0.40 ± 0.09 |
| R17K       | 99              | 2.70 ± 0.35 |
| R25Q       | 93              | 0.57 ± 0.16 |
| R25K       | 99              | 0.20 ± 0.01 |
| R34Q       | 77              | 0.42 ± 0.16 |
| R34A       | 99              | 0.61 ± 0.10 |
| R34K       | 96              | 0.65 ± 0.05 |

\(^a\) Defined as the percent of α-helix 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 dithiothreitol at 37 °C.

among these possibilities, the structural stabilities of all muteins were assessed by thermal denaturation experiments. Circular dichroism spectra were recorded as a function of temperature, and secondary structure compositions then calculated by deconvolution using the convex constraint algorithm (34). The calculated residual α-helical contents for each mutein are shown in Table III. At temperatures up to 80 °C, all muteins denatured to similar extents (50–65%; data not shown), similar to the behavior observed for the wild-type toxin. Upon returning the temperature to 23 °C, more than 90% of the helical structure for the wild-type toxin is recovered. This behavior is also observed for all the muteins except R34Q, which regains only 77% of its original helicity upon renaturation. This result raises the possibility that the Arg-34 muteins may be slightly destabilized as compared with both wild-type B-IV and the other arginine muteins.

The disulfide bonds of B-IV and other polypeptide neurotoxins have been shown to be crucial for toxicity (36). In order to measure the integrity of their four disulfides, we treated the arginine muteins with DTNB, a sulphydryl group modifier. Because neither the arginine muteins, nor wild-type B-IV are reactive with DTNB, we conclude that all four disulfide bonds are intact (data not shown). Disulfide bonds function to stabilize folded proteins, and it is known that many small, disulfided polypeptides are readily denatured upon reduction. Thus, the stability of our muteins might also be estimated by observing the effect of mild reduction upon secondary structure. Samples of wild-type and mutated B-IV were therefore incubated at 37 °C in the presence of 1 mM dithiothreitol, and their helical contents measured as a function of time. The rate constants for reduction of each polypeptide are presented in Table III. The initial rates of reduction are very similar for all muteins except R17K, in which helicity is lost approximately 7 times more rapidly.

In order to determine the number of disulfide bonds remaining after reduction, the reduced polypeptides were purified by reverse-phase HPLC and reacted again with DTNB in the presence of 0.1–0.5% SDS. Under these conditions the number of free sulphydryl groups per molecule is very similar for all muteins (data not shown). In each case one of the four disulfide bonds is reduced after 30 min. Taken together, these analyses confirm that the loss of activity seen in these muteins is not due to the lack of intact disulfide bonds, and that their global conformations are generally comparable with those of the wild-type toxin. The slightly diminished structural stabilities seen in R34Q and R17K may be due to local perturbations.

DISCUSSION

Recent two-dimensional NMR studies (21) indicate that C. lacteus neurotoxin B-IV is composed of two antiparallel α-helices, which together contain 57% of the sequence. This motif is absent in all other polypeptide neurotoxins characterized to date. We have therefore employed site-directed mutagenesis to identify critical elements involved in the function of this novel class of sodium channel toxins. Previous chemical modification studies suggested the importance of cationic residues, as well as carboxylate groups, to toxicity. In the present study, we demonstrate that a subset of arginine residues, located within the N-terminal helix, is essential for the activity of neurotoxin B-IV and most likely directly involved in binding. In contrast, residues in the C-terminal half of the molecule contribute less significantly. Protein yields, SDS-polyacrylamide gel electrophoresis mobilities, and HPLC elution profiles for all muteins are very similar, indicating that none of the substitutions interfere with the abilities of these polypeptides to fold. Functional analysis of the mutated toxins reveals that each of the three arginines contributes differentially to neurotoxicity. Because replacement of Arg-17 by either lysine, glutamine, or alanine reduces toxin activity to undetectable levels without discernible effect on overall structure, there is little doubt that this residue is essential \textit{per se}. Despite the fact that the detailed structure of the toxin-channel complex remains unknown, these data strongly suggest that Arg-17 is an important binding determinant. Similarly, the activity of mutein R25Q is diminished by around 400-fold. However, because its replacement by lysine causes only minimal changes in activity, we conclude that a positive charge at position 25, rather than a guanidinium function, is essential. These results strongly suggest that both Arg-17 and Arg-25 are involved in defining the functional binding epitope of neurotoxin B-IV. Finally, replacement of Arg-34 causes the smallest changes in toxicity, in that only 20- (R34Q) and 70-fold (R34A) reduced activity is observed. The corresponding lysine substitution (R34K) causes only an 8-fold decrease in toxicity, again indicating the importance of a positive charge at this position. However, as discussed below, this arginine residue may also serve as a structural determinant in toxin B-IV.

To demonstrate that the loss of activity observed upon neutralization of Arg-17 or Arg-25 is not due to concomitant alterations in toxin conformation, secondary structures and thermal stabilities were probed by circular dichroism. With the sole exception of D21P, whose helix content is substantially reduced, the secondary structures of all the muteins characterized contain 55% α-helix, identical to that of the wild-type toxin. The loss in helicity in D21P is not unexpected, based on proline’s being a strong helix breaker. Unexpectedly, this mutein is only 10-fold less active. While this result seems inconsistent with our hypothesis that helical structure is important in maintaining normal biological activity, it must be remembered that the presently available structural data do not enable us to determine the actual folding of the bound form of the toxin. The possibility that interaction with the channel stabilizes the N-terminal helix and that channel-bound D21P thus has a higher helical content than observed here in solution cannot be excluded.

From the information available (20, 21), toxin B-IV can be roughly divided into three major structural features: an antiparallel two-helix bundle encompassing residues 13–26 and 33–49, a less ordered and poorly defined N terminus including residues 1–12, and a “helix-like” five-residue C-terminal region. Based on this information, we generated an initial model for the toxin intended only to depict these elements (Fig. 4). In agreement with the known periodicity of an α-helix, Arg-17 and Arg-25 are both situated in the same spatial plane. Because Glu-13 and Asp-21 also lie on this face, electrostatic interactions among the carboxylate and guanidinium groups might be
expected to stabilize the N-terminal helix. Loss of stabilization would then be reflected in altered thermal denaturation profiles for some of the muteins. However, the patterns of melting and refolding of all muteins except R34Q are indistinguishable from that of the wild-type toxin, arguing that neither Arg-17 nor Arg-25 alone helps to direct the folding of toxin B-IV. This finding reinforces our conclusion that both Arg-17 and Arg-25 contribute directly to the B-IV binding epitope.

As alluded to above, the role played by Arg-34 is less clear. While the secondary structure of mutein R34Q is identical to that of the wild-type toxin, its ability to refold after thermal denaturation is modestly impaired. This impairment raises the possibility that Arg-34, which is located at the beginning of the C-terminal helix, plays a significant role in toxin folding. We believe that removal of intramolecular hydrogen bonding between Arg-34 and neighboring residues, rather than its interaction with the helix dipole, is responsible for the diminished stability of R34Q.

Secondary structural elements in small polypeptides are frequently stabilized by disulfide bonds, and it has been shown previously that the integrity of its four disulfide bonds is crucial for toxin B-IV activity (36). As a further check of conformational stability, we examined the effect of reducing agent upon α-helical content. Of all the muteins examined, only R17K shows a rate of reduction significantly greater than that of the wild-type toxin, leading us to speculate that a local conformational perturbation might occur upon substitution of this arginine with a lysine and that this perturbation could then diminish the stability of the adjacent Cys-16/Cys-52 disulfide bond. In order to test this possibility, we used DTNB as a probe for free sulfhydryl groups. Our results show that under denaturing conditions, all muteins, including R17K, are fully oxidized. Moreover, when reduced under the conditions described under “Results,” both wild-type toxin and the muteins yield very similar results; approximately one cystine is reduced in all cases. These results indicate that the global conformations of the arginine muteins are intact and therefore do not contribute to the changes in specific activities.

We have also determined that the four carboxyl groups in this toxin, as well as the C-terminal cationic region, are non-essential. Neither deletion of the C-terminal glutamic acid nor further truncation of the polypeptide by successive removal of the two lysines at positions 53 and 54 causes substantial losses of activity, indicating the dispensability of these C-terminal charged residues for B-IV-channel interaction. Similarly, neutralization of either Glu-13 (E13Q/A) or Asp-21 (D21N/A) has no substantial effect on bioactivity.

Previous analysis of functionally important regions in toxin B-IV focused on the N-terminal decapeptide. The Chou-Fasman and other algorithms strongly predict that this region exists as an α-helix ($P_\alpha = 1.12$) (22), although we now know from NMR (21) that the first helix actually starts at Glu-13. Replacement of helix-favoring residues within residues 1–10 by helix breakers (e.g. A3S/A3S) (a) has no effect on total helix content of the protein and (b) results in a 2–3-fold increase in specific toxicity (27). These results indicate a positive correlation between N-terminal hydrogen bonding capacity and biological activity. Also found in this region is Tyr-9, previously defined as essential to activity based on the abolition of toxicity caused by its nitration (25). Our finding that the toxicity of mutein Y9F is only 5-fold less than that of wild-type B-IV strongly suggests a steric basis for the inactivation seen in the nitration experiment. We conclude that electrostatic interactions made by Arg-17 and Arg-25 are of primary importance in binding, while hydrogen bonding is not.

While no polypeptide neurotoxins structurally related to B-IV are known, similar antiparallel two-helix motifs are present in the plant seed protein crambin, a 46-amino acid polypeptide containing 45% α-helix and three disulfide bonds (23), and in the E. coli heat-stable enterotoxin STb, a 48-amino acid protein containing 70% α-helix and two disulfides (24). There is essentially no sequence homology among these proteins. Like B-IV, recent site-directed mutagenesis studies on STb demonstrate that positively charged residues located in its N-terminal helix are important for toxic activity, echoing our results that cationic residues within the corresponding helical structures of B-IV are essential functional elements. More interestingly, crambin, a homologue to the thionin family of membrane-active plant toxins, has been reported to display a local anesthetic activity in lobster walking leg axon (37). It is thus tempting to speculate that both B-IV and crambin have related actions toward crustacean nerve targets. A more complete understanding of B-IV function, particularly its specificity, should be gained once the three-dimensional structure of this toxin becomes available.

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