Importance of the Conserved Aromatic Residues in the Scorpion α-Like Toxin BmK M1

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About one-third of the amino acid residues conserved in all scorpion long chain Na⁺ channel toxins are aromatic residues, some of which constitute the so-called “conserved hydrophobic surface.” At present, in-depth structure-function studies of these aromatic residues using site-directed mutagenesis are still rare. In this study, an effective yeast expression system was used to study the role of seven conserved aromatic residues (Tyr5, Tyr14, Tyr21, Tyr35, Trp38, Tyr42, and Trp47) from the scorpion toxin BmK M1. Using site-directed mutagenesis, all of these aromatic residues were individually substituted with Gly in association with a more conservative substitution of Phe for Tyr5, Tyr14, Tyr35, or Trp47. The mutants, which were expressed in Saccharomyces cerevisiae S-78 cells, were then subjected to a bioassay in mice, electrophysiological characterization on cloned Na⁺ channels (Na⁺,1.5), and CD analysis. Our results show an eye-catching correlation between the LD₅₀ values in mice and the EC₅₀ values on Na⁺,1.5 channels in oocytes, indicating large mutant-dependent differences that emphasize important specific roles for the conserved aromatic residues in BmK M1. The aromatic side chains of the Tyr5, Tyr21, and Trp47 cluster protruding from the three-stranded β-sheet seem to be essential for the structure and function of the toxin. Trp38 and Tyr42 (located in the β₂-sheet and in the loop between the β₂- and β₃-sheets, respectively) are most likely involved in the pharmacological function of the toxin.

Scorpion neurotoxins targeting voltage-gated sodium channels are single chain polypeptides composed of 60–70 amino acids cross-linked by four disulfide bridges. They have been divided into two major classes, α- and β-toxins. Scorpion α-toxins, the most extensively studied group, can prolong the action potential by slowing the inactivation of Na⁺ currents with no direct effect on activation (1–3).

According to their different pharmacological properties, the α-toxins can be further divided into three subgroups, classical α-, α-like, and insect α-toxins (4, 5). The classical α-toxins (e.g., AaH II and Lqh II) are highly toxic to mammals, whereas the insect α-toxins (e.g., Lqh α insect toxin) are highly toxic to insects. The more recently characterized α-like toxins (e.g., Lqh III and BmK M1) act on both mammals and insects, but are unique in their inability to bind to rat synaptosomes despite a high toxicity by intravenous injection. Although three-dimensional structures for the classical α-toxins (6, 7), α-like toxins (8, 9), and insect α-toxins (10) have been elucidated, in-depth structure-function studies of these long chain toxins using site-directed mutagenesis are still rare, mainly because of folding problems; and the focus has often been on the charged residues in the toxins (11, 12). Here, we report the importance of the conserved aromatic residues in α-toxins identified by mutagenesis analysis using the α-like toxin BmK M1 as template.

BmK M1 is a toxin from the venom of the scorpion Buthus martensii Karsch, which resides in eastern Asia, and is composed of 64 amino acids cross-linked by four disulfide bridges (3, 7). BmK M1 has been the subject of different studies: its three-dimensional structure was determined by x-ray crystallography at 1.7Å resolution (8); the pharmacological properties of Na⁺ channels have recently been investigated (13); and gene cloning and expression of wild-type BmK M1 have also been carried out (14, 15).

Alignment of the amino acid sequences of several α-toxins shows that seven aromatic residues, including Tyr5, Tyr14, Tyr21, Tyr35, Tyr42, Trp38/Trp39, and Trp47/Tyr47, are notably conserved (Fig. 1A) (5). In a previously performed structural analysis, a conserved hydrophobic surface (CHS)1 was identified (7). The CHS is assumed to be part of the functional site of scorpion toxins targeting sodium channels (16, 17). Tyr5, Tyr35, and Trp47 are located on the so-called Face A surface of the toxin (CHS). Tyr14 and Tyr21 are situated on the surface opposite to Face A, called Face B. Trp38 and Tyr42 are located in the β₂-sheet and in the loop between the β₂- and β₃-sheets, respectively (Fig. 1B).

Seven of the 15 residues conserved in scorpion toxins (5) are aromatic residues and have been studied in this work. Based on an efficient yeast expression system (15), the importance of the above-mentioned aromatic residues in the scorpion toxin BmK M1 was analyzed by site-directed mutagenesis. The results from mutagenesis and expression, characterization, bioassays, and electrophysiological analysis of the mutants are reported here. Based on these findings, the important role of these conserved aromatic residues is discussed.

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1 The abbreviations used are: CHS, conserved hydrophobic surface; BmK, recombinant BmK; Tricine, N-2-hydroxy-1,1-bis(hydroxy-methylethyl)glycine; AaH, Androctonus australis Hector; Lqh, Leiurus quinquestriatus hebraeus.
EXPERIMENTAL PROCEDURES

Strains, Materials, and Animals—Plasmid pVT102Ua, Escherichia coli strain TG1, and Saccharomyces cerevisiae strain S-78 (Leu2, Ura3, Rep4) were used. Restriction endonucleases and T4 DNA ligase were obtained from Roche Applied Science (Mannheim, Germany). Primers were synthesized by Sangon (Shanghai, China). Taq DNA polymerase and Klenow fragment were obtained from MBI. CM32-cellulose cation-exchange and Sephasil® peptide C18 reversed-phase (12-μm ST4.6250) columns were from Whatman and Amersham Biosciences AB (Uppsala, Sweden), respectively. All other chemicals were at least analytical grade and were purchased from Merck or Sigma. The mice used for the bioassay were ICR mice from the Beijing Center for Experimental Animals.

Site-directed Mutagenesis of BmK M1—The cDNA of BmK M1 was previously cloned (14) and inserted into pVT102Ua (15). According to the sequence of pVT102Ua-BmK M1, two primers were designed: primer 1 (5'-CGCTCTAGATAAAAGAAATCTTCG-3', including a KEX2 protease linker and an XhoI restriction site) and primer 2 (5'-CCAGGCTTTTAAGGCCATTCCTGGTAC-3', with a HindIII site). The substitute residue for all aromatic residues was glycine. In addition, the more conservative substitutions of phenylalanine for Tyr5, the conservative aromatic residues

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Expression and Purification of Mutants—After digestion with XhoI and HindIII, the mutated CDNAs were inserted into plasmid pVT102Ua and transformed into E. coli TG1 competent cells. The recombinant plasmid pVT102Ua-mutant was extracted, sequenced, and transformed into S. cerevisiae S-78 using the LiCl method (18). The expression of the mutants was carried out using a described previously procedure (15). After fermentation, the supernatants of the culture were adjusted to pH 4.2 with acetic acid. The sample was directly applied to a CM32-cellulose cation-exchange column (2.8 × 14 cm), which was equilibrated with 0.1 M sodium acetate at a flow rate of 1 ml/min. Upon reaching a steady baseline, the column was washed by stepwise elution with 0.2, 0.3, and 0.5 M NaCl equilibration buffer. The 0.5 M NaCl fraction was directly applied to a Sephasil® peptide C18 reversed-phase column. Buffer A contained 0.1% trifluoroacetic acid in water; buffer B contained 0.1% trifluoroacetic acid in acetonitrile. The C18 column was eluted with a linear gradient of 0–80% buffer B for 15 column volumes. Reversed-phase chromatography was carried out using an ARKTA purifier chromatography system (Amersham Biosciences AB).

Molecular Mass Determination—The molecular masses of the purified mutants were obtained using a Finnigan LCQ ion-trap mass spectrometer (ThermoQuest, San Jose, CA) equipped with an electrospray ionization source. The spray voltage was 4.50 kV. Calculations were performed using the program provided by the manufacturer.

Bioassay—Using 0.9% NaCl as a negative control and rBmK M1 as a positive control, the toxicity of the mutants was determined in mice (male, specified pathogen free level, 18–20 g of body weight). Each group consisted of 10 mice. Various doses of toxin mutants were dissolved in 0.9% NaCl and injected into the mice through the tail vein. Survival times (times between injection and death), reaction, and doses were recorded. Evaluation of toxicity was based on the determination of LD50 (the dose capable of statistically killing 50% of the mice) according to the method of Meier and Theakston (19).

Expression in Xenopus Oocytes, Electrophysiological Recordings, and Analysis—The human Na+,1.5 gene was subcloned into pSP64T (20). For
in vitro transcription, pSP64T/Na v1.5 was first linearized by XbaI. Using the large-scale SP6 mMESSAGE mMACHINE transcription kit (Ambion Inc.), capped cRNAs were synthesized from the linearized plasmids. The in vitro synthesis of cRNA encoding histone H1 and the isolation of Xenopus oocytes were done as described previously (21). Oocytes were injected with 50 nl of Na v1.5 cRNA solution at a concentration of 1 ng/nl using a Drummond microinjector. Whole cell currents from oocytes were recorded using the two-microelectrode voltage clamp technique between 1 and 3 days after injection. Voltage and current electrodes were filled with 3M KCl. Resistances of both electrodes were kept as low as possible (~0.1–0.2 megohms). Experiments were performed using a GeneClamp 500 amplifier (Axon Instruments, Inc.) controlled by a pClamp data acquisition system (Axon Instruments, Inc.). Currents were sampled at 10 kHz and filtered at 5 kHz using a four-pole low-pass Bessel filter. Digital leak subtraction of the current records was carried out using a P/2 protocol. The bath solution composition was 96 mmol/liter NaCl, 2 mmol/liter KCl, 1.8 mmol/liter CaCl2, 2 mmol/liter MgCl2, and 5 mmol/liter HEPES (pH 7.4). This solution was supplemented with 50 mg/liter gentamycin sulfate for incubation of the oocytes. All experiments were performed at room temperature (20–22 °C).

Circular Dichroism Measurements—Samples used for analyses were dissolved in 20 mM phosphate buffer (pH 7) at a concentration of 1.0 mg/ml. Circular dichroism spectra were recorded on a Jasco J-720 spectropolarimeter. Spectra were run at 25 °C from 250 to 200 nm using a quartz cell 0.5 mm in length. Data were collected at 0.5-nm intervals with a scan rate of 50 nm/min. All CD spectra resulted from averaging four scans. The final spectrum was corrected by subtracting the corresponding base-line spectrum obtained under identical conditions. Spectra were smoothed by the instrument’s software. The secondary structure content was estimated by standard Jasco CD analysis.

RESULTS

Mutation, Expression, and Purification—Single point mutants Y14G, Y14F, Y21G, Y35G, Y35F, W38G, Y42G, W47G,

| Toxin             | Expression | Toxicity (LD50) | Relative toxicity | EC50  |
|-------------------|------------|-----------------|-------------------|-------|
| Wild-type rBmK M1| Trace      | 0.53            | 100               | 0.50  |
| Y5G               | Trace      | 0.75            | 100               | 3.4   |
| Y14G              | Trace      | 0.75            | 100               | 3.4   |
| Y14F              | Trace      | 0.25            | 100               | 3.4   |
| Y35G              | Trace      | 0.25            | 100               | 3.4   |
| Y35F              | Trace      | 0.25            | 100               | 3.4   |
| W38F              | Trace      | 0.25            | 100               | 3.4   |
| W38G              | Trace      | 0.25            | 100               | 3.4   |
| Y21G              | Trace      | 0.25            | 100               | 3.4   |
| Y21G              | Trace      | 0.25            | 100               | 3.4   |
| Y42G              | Trace      | 0.25            | 100               | 3.4   |
| Y42G              | Trace      | 0.25            | 100               | 3.4   |
| Y5F               | Trace      | 0.25            | 100               | 3.4   |
| Y5F               | Trace      | 0.25            | 100               | 3.4   |

FIG. 2. As an example, the entire purification process of Y42G is shown. A, Tricine/SDS-PAGE before and after purification. The protein bands were stained with Coomassie Brilliant Blue R-250. Lane 1, molecular mass markers; lane 2, fermentation supernatant of Y42G, lane 3, purified Y42G. B, purification of Y42G by reversed-phase chromatography. C, Finnigan LCQ ion-trap mass spectra of Y42G. The mass spectra of the other mutants are not shown. mAU, milliabsorbance units.
and W47F were created by three-step PCR. Mutants Y5G and Y5F were produced by one-step PCR. The target gene was expressed using the pVT102U/H9251 vector. Tricine/SDS-PAGE analyses of yeast cultures demonstrated that mutants Y5G, Y5F, Y14G, Y14F, Y21G, Y35G, Y35F, W38G, Y42G, and W47F were expressed and secreted into the medium. Mutant Y5G could not be used in the following characterization because its expression level was in a trace amount. Mutant W47G could not be expressed at all. The expression levels of the five glycine mutants Y14G, Y21G, Y35G, W38G, and Y42G were 1–2 mg/liter of culture medium. For three of the mutants with the conservative phenylalanine substitution (Y5F, Y14F, and Y35F), the expression levels were 3 mg/liter, comparable to that of unmodified rBmK M1 (3 mg/liter). Remarkably, the amount of W47F in the culture medium was 9–10 mg/liter, which is about three times the value of rBmK M1 (Table I).

The molecular masses of the purified variants were measured with the Finnigan LCQ ion-trap mass spectrometer. The individual peaks showed that the molecular masses of mutants Y5F, Y14G, Y14F, Y35G, Y35F, W38G, Y42G, and W47F were 7403, 7312, 7403, 7315, 7312, 7403, 7289, 7312, and 7380 Da, respectively (Y42G is shown as an example in Fig. 2C). This corresponded well with the estimated molecular masses of the mutants: 7404, 7313, 7404, 7313, 7313, 7404, 7290, 7313, and 7380 Da, respectively.

The CD spectra of rBmK M1 and its mutants in the UV range of 250–200 nm are shown in Fig. 3. Compared with native BmK M1, the CD spectra of Y14G and Y35G dramatically changed (Fig. 3A), indicating that there are apparent changes in the secondary structures of these two mutants. The secondary structure estimation (J-700 for Windows Secondary Structure Estimation, Version 1.1.0.0) indicates that mutation Y14G interrupts both the α-helix and β-sheet, whereas mutation Y35G interrupts only the β-sheet. In both cases, the estimated random coils show a significant increase.

For Y21G, W38G, and Y42G, the CD spectra show that the secondary structures have almost not changed compared with the native toxin (Fig. 3A). It seems that the loss of the aromatic side chains in these mutants does not alter the general struc-
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The inactivation kinetics of Na\(^{+}\) channels expressed in Xenopus oocytes. Current traces were evoked by a depolarization step to \(-20\) mV over \(25\) ms from a holding potential of \(-90\) mV in the absence (*) and presence of the following toxins: 5 \(\mu M\) rBmK M1 (A), 30 \(\mu M\) Y14G (B), 30 \(\mu M\) Y35G (C), 30 \(\mu M\) W38G (D), 50 \(\mu M\) Y42G (E), 5 \(\mu M\) Y21G (F), 10 \(\mu M\) Y14F (G), 7.5 \(\mu M\) Y35F (H), 100 \(\mu M\) Y5F (I), and 10 \(\mu M\) W47F (J).

**Bioassay**—The mice showed typical symptoms of envenomation after injection with rBmK M1. The LD\(_{50}\) determined by the method of Meier and Theakston (19) was \(-0.53\) mg/kg, which is consistent with that of native BmK M1 (16). Excluding W47G and Y5G, which were not expressed and expressed only in trace amounts, respectively, the other nine mutants (Y5F, Y14G, Y14F, Y21G, Y35G, Y35F, W38G, Y42G, and W47F) were used for bioassays. Each purified mutant was injected into the mice through the tail vein at different doses to determine the LD\(_{50}\) value (Fig. 4 and Table I).

Mutants Y14G, W38G, and Y42G showed no detectable toxicity even at a dose of 25 mg/kg, which is 47 times the LD\(_{50}\) of rBmK M1 (Table I). Mutant Y35G lost most of its toxicity (LD\(_{50} = 16.04\) mg/kg, which is 30 times the LD\(_{50}\) of rBmK M1). In contrast, the LD\(_{50}\) of Y21G was only twice that of rBmK M1 (Fig. 4 and Table I).

For the phenylalanine mutants, the toxicities of Y14F (71%) and Y35F (55%) were in the same order as that of unmodified rBmK M1. The toxicity of W47F displayed a certain decrease (23%). The toxicity of Y5F was dramatically reduced to 4% in comparison with unmodified rBmK M1 (Fig. 4 and Table I).

**Fig. 5. Effect of rBmK M1 and its aromatic amino acid mutants on the inactivation kinetics of Na\(^{+}\) channels expressed in Xenopus oocytes.** Current traces were evoked by a depolarization step to \(-20\) mV over \(25\) ms from a holding potential of \(-90\) mV in the absence (*) and presence of the following toxins: 5 \(\mu M\) rBmK M1 (A), 30 \(\mu M\) Y14G (B), 30 \(\mu M\) Y35G (C), 30 \(\mu M\) W38G (D), 50 \(\mu M\) Y42G (E), 5 \(\mu M\) Y21G (F), 10 \(\mu M\) Y14F (G), 7.5 \(\mu M\) Y35F (H), 100 \(\mu M\) Y5F (I), and 10 \(\mu M\) W47F (J).

**Effect of rBmK M1 and Its Aromatic Amino Acid Mutants on Voltage-gated Na\(^{+}\) Channels**—Fig. 5 displays the effects of rBmK M1 and mutants Y14G, Y35G, W38G, Y42G, Y21G, Y14F, Y35F, Y5F, and W47F on Na\(^{+}\) channels expressed in Xenopus laevis oocytes. The currents displayed were evoked by a depolarization step to \(-20\) mV from a holding potential of \(-90\) mV. The current traces recorded after the addition of the toxin reveal that rBmK M1 induced a slowing of the inactivation process of Na\(^{+}\) currents. This effect appeared a few seconds after the addition of the toxin and continued until reaching a steady state after 4–5 min. Under control conditions, the inactivation kinetics of Na\(^{+}\) currents were rapid, and almost no remaining currents were visible at the end of the traces, after \(25\) ms. The toxin-induced slowing of inactivation was evaluated by a single exponential fit (pClamp Version 8) of the current decay after the peak. The time window for each fit was manually set from the peak current to the end of the trace (25 ms). Under steady-state conditions, the time constant of inactivation (\(\tau\)) calculated by a single exponential fit increased from 1.4 ± 0.2 ms (\(n = 33\)) under control conditions to 4.4 ± 1.2 ms (\(n = 6\)) after the addition of 1 \(\mu M\) rBmK M1 and to 5.8 ± 0.8 ms (\(n = 3\)) after the addition of 5 \(\mu M\) rBmK M1. This represents an increase of \(-414\%\) in the time constant \(\tau\) in the presence of 5 \(\mu M\) rBmK M1. As shown in Fig. 5, all of the glycine mutants except Y21G were less efficient even at high concentrations (30 and 50 \(\mu M\)) in slowing the inactivation kinetics of Na\(^{+}\) channels compared with the wild-type toxin. Mutants Y14G and Y42G of rBmK M1 were the least effective in slowing the
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The kinetics of Na v1.5 channels compared with wild-type rBmK

slowing the inactivation of the Na

corresponding to 385, 507, and 414% of the time constants under

corresponding to 150 and 121% of the time constants under control

corresponding to 335% of the control value. Y35G was

corresponding to 165% of the control value. The

corresponding to 100 μM, corresponding to 165% of the control value. The
effects of rBmK M1 and some of its mutants on the peak Na

current and time to peak were somewhat variable (oocyte-de-

As shown in Fig. 5, all of the phenylalanine mutants except Y5F had about the same efficacy in Na•, as rBmK M1. The
time constants of inactivation were 5.4 ± 0.6 ms (n = 4), 7.1 ±

0.4 ms (n = 4), and 5.8 ± 0.6 ms (n = 3) after the addition of 10
μM W47F, 7.5 μM Y35F, and 10 μM Y14F, respectively, corre-

spiring to 385, 507, and 414% of the time constants under control conditions, respectively. Y5F was the least effective in
slowing the inactivation of the Na• channel. The time constant of
inactivation was only 2.3 ± 0.6 ms (n = 3) after the addition of 100 μM, corresponding to 165% of the control value. The
effects of rBmK M1 and some of its mutants on the peak Na

current and time to peak were somewhat variable (oocyte-de-

The slowing of inactivation induced by rBmK M1 and its mutants was concentration-dependent (Fig. 6). The EC50 val-

ues of rBmK M1, Y21G, Y35G, Y35F, Y14F, and W47F were
determined by a sigmoidal fit of the v-V relationship as dis-

played in Fig. 6. The EC50 values determined for rBmK M1,
Y21G, Y35G, Y35F, Y14F, and W47F were 0.50 ± 0.03,
1.02 ± 0.15, 5.05 ± 0.36, 0.71 ± 0.09, 3.36 ± 0.48, and 3.1 ±
0.3 μM, respectively. The EC50 value determined in this study
for rBmK M1 is slightly higher than the EC50 value deter-
mained for the native BmK M1 toxin (0.2 μM) in one of our pre-
vious studies (13). Y21G was comparable to native rBmK
M1. Y35G was ~30% less efficient in slowing the inactivation kinetics of Na•, 1.5 channels compared with wild-type rBmK
M1. The EC50 value of Y35G was at least 10 times higher than that of rBmK M1. The EC50 values of the phenylalanine

As shown in Fig. 6, the EC50 values of Y14G, Y42G, W38G, and Y5F could not be determined because the highest con-
centrations used did not reach a maximal effect in the dose-
response curve.

DISCUSSION

When the three-dimensional structures of the scorpion tox-
ins CsE V3 and AaH II were elucidated ~20 years ago, the
CHS, mainly including Tyr5, Tyr35, and Trp47/Yyr47, was pro-
tosed to be responsible for the pharmacological effect of these
toxins (17, 22, 23). Although the CHS is found in all scorpion
toxin structures known today, this assumption required exper-
imental identification. The individual residues in this cluster
e.g., Trp47 in AaH II and Tyr47 in Lqh (an insect toxin) have been
assessed by chemical modification (24) and mutagenesis analysis
(11, 25) and shown to play an important role in bioactivity. In
this study, seven aromatic residues, including three amino
acids of this cluster, were analyzed by site-directed mutagene-
sis. Correlating the high impact substitution of glycine to the
more conservative mutation of phenylalanine, our results
clearly indicate that these conserved aromatic residues are
specifically involved in either or both pharmacological function
and structural stability.

Aromatic Residues Possibly Involved in Pharmacological Function—The bioassay showed that the toxicity of W38G and
Y42G was dramatically reduced (Table I). In concordance, elec-
trophysiological analysis showed that W38G and Y42G were
the least effective in slowing the inactivation of the sodium channel. The EC50 could not be determined because the highest
concentration available could not induce a maximal effect in the
dose-response curves (Figs. 5 and 6). Simultaneously, the
CD spectra of these two mutants show the least alteration in
comparison with that of unmodified BmK M1 (Fig. 3A). These
results can be used to speculate that the conserved aromatic
residues Trp38 and Tyr42 are involved in the functional per-
formance of the toxin.

Tyr42 is located in the loop between the β2- and β3-sheets (Fig. 7). Considering that this loop is remarkably different in
sequence and structure between α- and β-toxins (7, 17), this
aromatic residue may be related to the preference for the distinct target site of α-toxins.

Aromatic Residues Possibly Involved in Structural Stability—The non-expression of Y35G and the extremely unstable
expression of Y5G indicate that these two residues are essential for the general structure of the toxin. The polypeptide
chain of the toxin cannot be folded correctly without these
aromatic side chains. Y35G could be expressed in an amount comparable to the wild type, but its toxicity was reduced dra-
matically (Table I). In agreement with the obtained LD50 value
in mice, the corresponding EC50 value of Y35G in Na•, 1.5 was
10 times higher than that of rBmK M1. The CD spectrum also
changed dramatically (Fig. 3A), indicating an alteration of the
secondary structure of Y35G. Interestingly, the conservative
phenylalanine substitution mutants were expressed very well.
Y5F and Y35F were present in the culture medium in an
amount of ~3 mg/liter, comparable to rBmK M1. The expres-
sion level of W47F was high (9–10 mg/liter) in comparison with
rBmK M1 (Table I). Compared with rBmK M1, alterations in
the CD spectra were milder for Y5F and Y35F, but severe for
W47F (Fig. 3B). In addition, the bioassay, in concordance with

FIG. 7. Possible role of the conserved aromatic residues ident-
ified by site-directed mutagenesis analysis. Tyr5 and Trp47
(Structural importance) and Tyr14 (Functional and struc-
tural importance), belonging to the CHS (Face A), are indicated in the upper left
model. Residues putatively involved in the functionality (Trp38 and
Yyr47) are shown in the upper right model. Tyr14 (Functional and structural importance) and Tyr35, belonging to Face B, are shown in the
lower left model. Tyr5 is also indicated in blue to show the interaction with Tyr14. The figures were produced using MOLMOL 2R.1 (27).
the electrophysiological characterization, showed that the bio-
activities of all of the phenylalanine mutants (although in
different degrees) were significantly higher than those of the
mutants that lost their aromatic side chains by substitution
with glycine (Table I). Hence, by constructing and thoroughly
comparing glycine and phenylalanine mutants of conserved
aromatic residues in BmK M1, we have shown that the ar-
omatic side chains of W47F and Y35F are indispensable for
maintaining the structure and pharmacological function of the
toxin. Interestingly, these residues do not have to be Trp47 or
Tyr35 because the aromatic side chain is the primordial com-
ponent. Also indicated by this study is the fact that residue 5
has to be a tyrosine because the phenylalanine mutant dis-
played a very low bioactivity.

The three-dimensional structures of BmK M1 and other
α-toxins reveal that Tyr5, Tyr35, and Trp47 are located on the
three-stranded β-sheet: β1–, β2–, and β3–strands, respectively.

The aromatic rings of these residues are positioned orthog-
nally one to the other in a so-called “herringbone” arrangement
(Fig. 7), which was identified as the lowest energy configura-
tion of relatively solvent-exposed aromatic rings (26). In this
way, this aromatic cluster plays an important role in the sta-
bilization of the three-stranded β-sheet. It is plausible to infer
that, due to the loss of the interactions between these aromatic
rings, the β-sheet will be interrupted and maybe even disinte-
grated. Trp47 is situated at the center of the cluster (Fig. 7),
and its aromatic ring resides in the vicinity of the side chains of
both Tyr5 and Tyr35 (distances of 3.5–4 Å). It can be hypothe-
sized that the disruption of the herringbone arrangement due
to the loss of the aromatic side chains in W47G and Y5G makes
the mutants unable to express. This conclusion is supported by
the fact that all of the phenylalanine mutants were very well
expressed. W47F, Y35F, and Y14F also displayed the essential
bioactivity (Table I). Instead of the non-expression for W47G,
the mutants unable to express. This conclusion is supported by
the fact that all of the phenylalanine mutants were very well
expressed. W47F, Y35F, and Y14F also displayed the essential
bioactivity (Table I).

The three-dimensional structures of BmK M1 and other
α-toxins reveal that Tyr14 is essential for stabilizing the
function with the voltage-gated sodium channel. The results show that Tyr14 is essential for stabilizing the
unique conformation of the toxin and is involved in the inter-
action with the voltage-gated sodium channel. To obtain a more
thorough insight in this matter, a more conservative pheny-
lalanine substitution for Tyr14 was constructed. Y14F was ex-
pressed in an amount comparable to that of Y14G. However,
in contrast to Y14G (no detectable toxicity and EC50 > 100 µM),
Y14F possesses a high bioactivity (71% relative toxicity and
EC50 = 3.36 ± 0.48 µM (Table I)). These data reveal that
the aromatic side chain of Tyr14 that mainly contributes to the
proper conformation and in turn affects the pharmacological
function of the toxin. This residue protrudes from the loop be-
tween the β1–sheet and the α-helix (Fig. 7). The structure
shows that its aromatic ring interacts with the side chain of
Ile6, which is also a crucial conserved residue in α-toxins.

The hydrophobic interactions between these two residues on
the surface may play an important role in stabilizing the unique
conformation of this loop so as to influence the β-sheet and the
α-helix of the toxin. The effect of mutation Y21G was milder on
bioactivity, on the EC50 in Na, and on the CD spectrum (Fig. 3A and Table I), indicating that the aromatic residue
Tyr21 is putatively not a crucial determinant for the structure
and pharmacological function of the toxin.

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