Synthesis, $^1$H NMR Structure, and Activity of a Three-disulfide-bridged Maurotoxin Analog Designed to Restore the Consensus Motif of Scorpion Toxins*

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Maurotoxin (MTX) is a 34-residue toxin that has been isolated from the venom of the chactidae scorpion Scorpio maurus palmatus. The toxin displays an exceptionally wide range of pharmacological activity since it binds onto small conductance Ca$^{2+}$-activated K$^+$ channels and also blocks Kv channels (Shaker, Kv1.2 and Kv1.3). MTX possesses 53–68% sequence identity with HsTx1 and Pi1, two other K$^+$ channel short chain scorpion toxins cross-linked by four disulfide bridges. These three toxins differ from other K$^+$/Cl$^-$/Na$^+$ channel scorpion toxins cross-linked by either three or four disulfide bridges by the presence of an extra half-cystine residue in the middle of a consensus sequence generally associated with the formation of an αβ scaffold (an α-helix connected to an antiparallel β-sheet by two disulfide bridges). Because MTX exhibits an uncommon disulfide bridge organization among known scorpion toxins (C1-C5, C2-C6, C3-C4, and C7-C8 instead of C1-C4, C2-C5, and C3-C6 for three-disulfide-bridged toxins or C1-C5, C2-C6, C3-C7, and C4-C8 for four-disulfide-bridged toxins), we designed and chemically synthesized an MTX analog with three instead of four disulfide bridges ([Abu$^{19}$,Abu$^{34}$]MTX) and in which the entire consensus motif of scorpion toxins was restored by the substitution of the two half-cystines in positions 19 and 34 (corresponding to C4 and C8) by two isosteric α-aminobutyrate (Abu) derivatives. The three-dimensional structure of [Abu$^{19}$,Abu$^{34}$]MTX in solution was solved by $^1$H NMR. This analog adopts the αβ scaffold with now conventional half-cystine pairings connecting C1-C5, C2-C6, and C3-C7 (with C4 and C8 replaced by Abu derivatives). This novel arrangement in half-cystine pairings that concerns the last disulfide bridge results mainly in a reorientation of the α-helix regarding the β-sheet structure. In vivo, [Abu$^{19}$,Abu$^{34}$]MTX remains lethal in mice as assayed by intracerebroventricular injection of the peptide (LD$_{50}$ value of 0.25 μg/mouse). The structural variations are also accompanied by changes in the pharmacological selectivity of the peptide, suggesting that the organization pattern of disulfide bridges should affect the three-dimensional presentation of certain key residues critical to the blockage of K$^+$ channel subtypes.

MTX$^1$ is a toxin isolated from the venom of the chactidae scorpion Scorpio maurus palmatus (1). It is a basic, C-terminal amidated 34-mer peptide cross-linked by four disulfide bridges. The solid phase technique has been used to obtain synthetic maurotoxin (sMTX) and it was found that both the natural and synthetic MTXs are equally lethal to mice by intracerebroventricular injection (LD$_{50}$ of 80 ng/mouse). sMTX has been shown to be active in the nanomolar range on both voltage-gated K$^+$ channels (Shaker B, Kv1.1, Kv1.2, and Kv1.3) and onto rat brain apamin-sensitive small-conductance Ca$^{2+}$-activated K$^+$ channels (SK) (1). The solution structure of sMTX has been solved by $^1$H nuclear magnetic resonance technique (2). The three-dimensional structure shows that the toxin contains a bent helix from residues 6 to 16 connected by a loop to a two-stranded antiparallel β-sheet (residues 23–26 and 28–31), a conformation grossly similar to those of other scorpion toxins. The half-cystine pairings of sMTX were identified by enzyme proteolysis and found to be Cys$^3$-Cys$^{24}$, Cys$^9$-Cys$^{29}$, Cys$^{13}$-Cys$^{19}$, and Cys$^{31}$-Cys$^{34}$, consistent with experimental data obtained by Edman sequencing of the natural MTX (3). The structural and pharmacological features of MTX (less than 40 residues, 4 disulfide bridges, and binding onto K$^+$ channels) suggest that MTX belongs to a new class of natural K$^+$ channel blockers structurally intermediate between the Na$^+$ (60–70 residues and 4 disulfide bridges) and K$^+$ channel scorpion toxin families (less than 40 residues and 3 disulfide bridges) (4). This class also includes Pi1 and HsTx1 from the venoms of the scorpions Pandinus imperator (5) and Heterometrus spinifer (6), respectively. These K$^+$ channel-acting toxins share from 53 to 68% sequence identity with MTX but display slightly different pharmacological selectivities. For instance, Pi1 is inactive on rat Kv1.1 and Kv1.3 channels, whereas HsTx1 is inactive on apamin-sensitive SK channels in contrast to MTX (6).

The abbreviations used are: MTX, maurotoxin from the scorpion S. maurus palmatus; [Abu$^{19}$,Abu$^{34}$]MTX, a synthetic maurotoxin analog with α-aminobutyrate derivatives in positions 19 and 34; HPLC, high pressure liquid chromatography; HsTx1, toxin 1 from the scorpion H. spinifer; sMTX, synthetic maurotoxin; Pi1, toxin 1 from the scorpion P. imperator; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; DQF-COSY, double quantum-filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; Fnmoc, N$^-$fluorenlymethoxycarbonyl; SK, small-conductance Ca$^{2+}$-activated K$^+$ channels.

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interesting feature of MTX is that it has a unique disulfide bridge pattern among known scorpion toxins, including toxins from its own class such as Pi1 and HsTx1. In particular, it differs, first, from the classical three-disulfide-bridged toxins active on K⁺ channels by the presence of an extra disulfide bridge and, second, from all three- or four-disulfide-bridged toxins by a loss of the half-cystine pairings observed in classical three- or four-disulfide-bridged toxins (C1-C4, C2-C5, and C3-C6 pairings, or C1-C5, C2-C6, C3-C7, and C4-C8 pairings, respectively). The change concerns the two last disulfide bridges, and the pairings observed in MTX are of the type C1-C5, C2-C6, C3-C4, and C7-C8 instead.

It has been proposed that the α/β scaffold of scorpion toxins is determined by a consensus sequence of the type [C][C][CXXC][...]/[X][X][X][CXXX][...]/[X][X][X][CXXX][...]/[X][X][X][CXXX][...]/ for either three- or four-disulfide-bridged toxins, with two additional half-cystines located outside the motif in the latter case (2, 7, 8). From the α/β scaffold arises a great functional diversity of these scorpion toxins. The detailed structural basis for this variability is still poorly apprehended. We noticed that this consensus sequence is, however, altered in the case of MTX since the first half-cystine residue located at the N terminus is absent, whereas a new one is inserted in the central part of the motif (position Cys19, Fig. 1). Despite this change in the proposed consensus sequence, it was unexpectedly found that MTX still adopts the α/β scaffold (2). However, interesting differences result from this new disulfide bridge organization. In particular, it was found that, in this novel disulfide bridge pattern, the α-helix is connected by two disulfide bridges (Cys9-Cys29 and Cys15-Cys19) to two different strands of the β-sheet instead of connecting the α-helix to the same strand. These novel half-cystine pairings may nevertheless result in discrete conformational changes and/or positioning of certain residues of the toxin that could impact the pharmacological selectivity of MTX. To test this hypothesis, a three-disulfide-bridged structural analog of MTX was designed to fully restore the consensus sequence of the scorpion toxins. Accordingly, Cys9 and Cys34 that do not belong to the consensus sequence were replaced by isosteric α-aminobutyrate derivatives. Here, we report the chemical synthesis of [Abu19,Abu34]MTX, its half-cystine pairings, its three-dimensional structure in solution, and its biological and pharmacological activities. Our data indicate that there is indeed a rearrangement of the disulfide bridges in the MTX analog without a drastic alteration of the α/β scaffold, but with a marked reorientation of the α-helix regarding the β-sheet (angle of 50°) that may be sufficient to affect the pharmacological activity of the peptide.

EXPERIMENTAL PROCEDURES

Materials—N'-Fluorenlymethyloxycarbonyl (Fmoc)-L-amino acids, Fmoc-amide resin, and reagents used for peptide synthesis were obtained from Perkin-Elmer. Solvents were analytical grade products from SDS. Enzymes (trypsin and chymotrypsin) were obtained from Roche Molecular Biochemicals.

Chemical Synthesis and Physicochemical Characterization of [Abu19,Abu34]MTX—The [Abu19,Abu34]MTX was obtained by the solid phase technique (9) using a peptide synthesizer (model 433A, Applied Biosystems Inc.). Peptide chains were assembled stepwise on 0.25 meq of Fmoc-amide resin (0.65 meq of amino group/g using 1 mmol of (Fmoc) amino acid derivatives (10). The side-chain-protecting groups used for trifunctional residues were: trityl for Cys, Asn, and Gln; tert-butyl for Ser, Thr, Tyr, and Asp; pentamethylcyclohexyl for Arg, and tert-butyloxycarbonyl for Lys. The Fmoc-amino acid derivatives were coupled (20 min) as their hydroxybenzotriazole active esters in N-methylpyrrolidone (4-fold excess). The peptide resin (about 2.2 g) was treated for 2.5 h at room temperature with a mixture of trifluoroacetic acid/H₂O/thioanisole/ethanedithiol (88:5:5:2; v/v) in the presence of crystalline phenol (2.25 g). After filtration of the mixture, the peptide was precipitated and washed by adding cold diethyl ether. The crude peptide was pelleted by centrifugation (3,000 × g; 10 min) and the supernatant was discarded. The reduced peptide was then dissolved at 2 mCi in 0.2 M Tris-HCl buffer, pH 8.3, and stirred under air to allow folding (48 h, room temperature). The target product, [Abu19,Abu34]MTX, was purified by reversed phase high pressure liquid chromatography (HPLC) (Perkin-Elmer, C₁₈ Aqapolar ODS 20 μm, 250 × 10 mm) by means of a 60-min linear gradient of 0.05% (v/v) trifluoroacetic acid and 0–35% acetonitrile in 0.1% (v/v) trifluoroacetic acid/H₂O at a flow rate of 1 ml/min (λ = 230 nm). The homogeneity and identity of [Abu19,Abu34]MTX was assessed by: (i) analytical C₁₈ reversed phase HPLC, (ii) amino acid analysis after acidolysis, and (iii) mass determination by matrix-assisted laser desorption ionization-time of flight mass spectrometry.

Assignment of Half-cystine Pairings of [Abu19,Abu34]MTX by Enzyme-based Cleavage and Edman Sequencing Analysis—The [Abu19,Abu34]MTX (800 μg) was incubated with a mixture of trypsin and chymotrypsin at 10% (w/w) in 0.2 M Tris-HCl, pH 7.4, for 12 h at 37 °C. The peptide fragments were then purified by reversed phase HPLC (Vydac, C₁₈, 5-μm column, 4 × 150 mm) with a 60-min linear gradient of 0.08% (v/v) trifluoroacetic acid, 0–60% acetonitrile in 0.1% (v/v) trifluoroacetic acid/H₂O at a flow rate of 1 ml/min (λ = 230 nm), and freeze-dried prior to their analyses. The peptide fragments were hydrolyzed by acidolysis (6 N HCl/phenol), and their amino acid content was analyzed (Beckman, System 6300 amino acid analyzer). The peptide mixtures were further characterized by mass spectrometry analysis (RP-DE Voyager, Perseptive Biosystems), and Edman sequencing using a gas phase microsequencer (Applied Biosystems 470A). In standard HPLC conditions for analyzing phenylthiohydantoin-amino acid derivatives, diphenylthiohydantoin-cystine elutes at a retention time of 9.8 min.

Three-dimensional Structure Determination of [Abu19,Abu34]MTX in Solution by Bidimensional ¹H NMR—4.0 mg of [Abu13,Abu34]MTX was dissolved in 0.5 ml of H₂O/D₂O (90/10 v/v), pH = 3 uncorrected for isotope effects. Proton two-dimensional NMR spectra was first routinely recorded at 300 K. All the data were collected on a Bruker DRX 500. Clean total correlation spectra (TOCSY) (11, 12) were acquired with a spin lock of 80 ms. Phase sensitive two-dimensional nuclear Overhauser effect (NOE) spectra (NOESY) (13, 14) with watergate (15) composite were acquired using the time proportional phase increment method with mixing time of 100 ms. The solvent-OH resonance was suppressed either by low power irradiation in the relaxation delay and, for NOESY spectra, during the mixing time, or using a watergate 3–9–19 pulse train (15) using a gradient at the magic angle obtained by applying simultaneously x, y, and z gradients prior to detection.

For determination of amide proton exchange rates, the peptide was lyophilized twice and solubilized in 100% D₂O. Immediately after solubilizing, a series of NOESY spectra with a mixing time of 80 ms were recorded at 283 K, the first one during a time period of 1 h (1024 complex points with 256 experiments), followed by spectra of 10 h (1024 complex points with 512 experiments). All the data were processed using the Bruker software XWINNMR, running on a Silicon Graphic INDY R4000 workstation. The matrices were transformed with a zero filling to the next power of two in the acquisition dimension, and to 1024 points in the other. All the data were multiplied by a shifted sine-bell window, in both dimensions prior to Fourier transform, and a fifth-order polynomial base-line correction was applied. Spectra had finally a 12 ppm width with a digital resolution of 2.93 Hz/point in the w₁ dimension and 5.85 Hz/point in the w₂ dimension. The spectral analysis and structure calculation were performed as described previously (2).

Molecular Mechanics Calculations—Steric energy calculations were aimed at determining the most energetically favored half-cystine pairings of [Abu19,Abu34]MTX. These calculations were based on the three-dimensional structure of MTX obtained from the Protein Data Bank (2). Half-cystines in positions 19 and 34 were substituted by Abu derivatives, and full minimization was performed for each sterically possible half-cystine pairing arrangements (12 found possible out of 15, the disulfide bridge 29–31 being excluded from the calculations because of steric impossibility; see Table II for the various combinations used). Mimizations were achieved using the molecular modeling program Insight II (Molecular Simulations Inc.), the Discover-based minimization, and the CVFF force field. The mathematical method used for minimizations was the steepest descent gradient conjugate. Assignment of Half-cystine Pairings of [Abu19,Abu34]MTX in Mice—The peptide was tested in vivo for toxicity by determining the LD₅₀ after intracerebroventricular injection into 20 g C57BL/6j mice. Groups of six mice per dose were injected with 5 μl of [Abu19,Abu34]MTX solution containing 0.1% (w/v) bovine serum albumin and 0.9% (w/v) sodium chloride.

Binding Assay of ³H-I-Apin and Competition by [Abu19,Abu34]MTX onto Rat Brain Synaptosomes—Rat brain synaptosomes (P2 fraction)
were prepared as described by Gray and Whittaker (16). The protein content was determined by a modified Lowry method. $^{125}$I-Apamin (2,000 Ci/mmol) was obtained according to Seagar et al. (17). Aliquots of 50 µl of 0.1 mCi $^{125}$I-apamin were added to 400 µl of synaptosome suspension (0.4 mg of protein/ml). Samples were incubated for 1 h at 4 °C with 50 µl of a series of concentrations of [Abu19,Abu34]MTX (10$^{-6}$ to 10$^{-11}$ M) in 500 µl final volume. The incubation buffer was 25 mM Tris-HCl, 10 mM KCl, pH 7.2. The samples were centrifuged, and the resulting pellets were washed three times in 1 ml of the same buffer. Bound radioactivity was determined by counting (Packard Crystal II). The values expressed are the means of triplicate experiments ± S.D. Nonspecific binding, less than 10% of the total binding, was determined in the presence of an excess (10 nM) of unlabeled apamin.

Oocyte Preparation and Electrophysiological Recordings—Stages V and VI Xenopus laevis oocytes were prepared for cRNA injection and electrophysiological recordings as described (18). Briefly, oocytes were prepared by removing the follicular cell layer by enzymatic treatment with 2 mg/ml collagenase IA (Sigma) in classical Barth’s medium (in mM: 88 NaCl, 1 KCl, 0.82 MgSO$_4$, 0.33 Ca(NO$_3$)$_2$, 0.41 CaCl$_2$, 2.4 NaHCO$_3$, 15 HEPES, pH 7.4 with NaOH). The plasmids were cut with SmaI (Shaker B), NotI (rat Kv1.1), XbaI (rat Kv1.2), and EcoRI (rat Kv1.3). The linearized plasmids were transfected by means of a T7 or SP6 (mMessage mMachine kit, Ambion). The cRNA were stored frozen (Kv1.3). The linearized plasmids were transcribed by means of a T7 or SP6 (mMessage mMachine kit, Ambion). The cRNA were stored frozen.

**RESULTS AND DISCUSSION**

Fig. 1 illustrates the consensus sequence of the scorpion toxins independent of their length, primary structure, and pharmacological selectivity (7, 8). In three-disulfide-bridged toxins, the half-cystine pairings are so far of the type C1-C4, C2-C5, and C3-C6. In short chain four-disulfide-bridged toxins active on K$^+$ channels, one of the two additional half-cystine residues is located inside the consensus motif, between C3 and C4, whereas the other one is placed after the C-terminal end of the motif (after C6). Of note, the two additional half-cystine residues found in long-chain four-disulfide-bridged scorpion toxins active on Na$^+$ channels are covalently). In this analog, the two half-cystine residues were introduced by a modified Lowry method. $^{125}$I-Apamin (2,000 Ci/mmol) was obtained according to Seagar et al. (17). Aliquots of 50 µl of 0.1 mCi $^{125}$I-apamin were added to 400 µl of synaptosome suspension (0.4 mg of protein/ml). Samples were incubated for 1 h at 4 °C with 50 µl of a series of concentrations of [Abu19,Abu34]MTX (10$^{-6}$ to 10$^{-11}$ M) in 500 µl final volume. The incubation buffer was 25 mM Tris-HCl, 10 mM KCl, pH 7.2. The samples were centrifuged, and the resulting pellets were washed three times in 1 ml of the same buffer. Bound radioactivity was determined by counting (Packard Crystal II). The values expressed are the means of triplicate experiments ± S.D. Nonspecific binding, less than 10% of the total binding, was determined in the presence of an excess (10 nM) of unlabeled apamin.

- **FIG. 1.** Consensus motif and half-cystine pairings of three- and four-disulfide-bridged scorpion toxins. Top, consensus structural motif of short chain three-disulfide-bridged toxins active on K$^+$ channels and long chain four-disulfide-bridged toxins active on Na$^+$ channels. Corresponding half-cystine pairings are given in right panel for three-disulfide-bridged toxins. The half-cystines are numbered by order of appearance from the N to C termini. Middle, variant of the consensus motif for short chain four-disulfide-bridged toxins active on K$^+$ channels. The additional half-cystines (not numbered) are given in bold type. The case of MTX is illustrated showing complete primary structure. The two possible half-cystine pairings arising from this modified consensus motif are given in the right panel; top, for Pi1 and HsTx1; bottom, for MTX. Bottom, structural motif of [Abu19,Abu34]MTX after substituting the two additional half-cystines found in amino acid positions 19 and 34 by $\alpha$-aminobutyrate derivatives (Abu). Of note, the resulting motif resembles the structural motif for short chain three-disulfide-bridged or long chain four-disulfide-bridged toxins as shown on top figure. The half-cystine pairings (right) are defined in Fig. 2. Asterisks denote a C-terminal carboxylamide.
not engaged in the formation of the same disulfide bridge, and it can therefore be expected that there will be (i) a reduction in the number of disulfide bridges from four to three and (ii) a concomitant rearrangement in pairings of the remaining six half-cystine residues. These mutations aim to restore the entire consensus sequence and half-cystine pairings of three-disulfide-bridged scorpion toxins.

**Stepwise assembly of [Abu\textsuperscript{19},Abu\textsuperscript{34}]MTX.**

The crude peptide after folding/oxidation was purified by C\textsubscript{18} reversed phase HPLC (Fig. 2B, right panel). The amino acid ratios of [Abu\textsuperscript{19},Abu\textsuperscript{34}]MTX were in agreement with the deduced values (see Table I).

**Table I**

| Enzymes | Retention times (min) | Proteolytic fragments | Experimental \(M_r\) (M\textsubscript{1}H\textsubscript{1}) | Deduced \(M_r\) | Half-cystine pairings |
|---------|-----------------------|-----------------------|-----------------|---------------|---------------------|
| Trypsin | 16.8                  | TGAbuPN               | 473.6           | 472.5         | Cys\textsuperscript{19}-Cys\textsuperscript{21} |
| Chymotrypsin | 18.9                | VSGTGSK/CNK           | 601.9           | 600.6         | Cys\textsuperscript{29}-Cys\textsuperscript{31} |
|         | 19.9                  | DKY/CY                | 734.9           | 733.0         | Cys\textsuperscript{29}-Cys\textsuperscript{31} |
|         | 20.4                  | APCR/CA               | 729.0           | 727.9         | Cys\textsuperscript{29}-Cys\textsuperscript{31} |

The amino acid composition is given in brackets. Deduced and experimental \(M_r\) values are indicated. ND, not determined.

Next, we attempted to determine by computer-assisted molecular modeling which disulfide bridge organization among the 15 theoretically possible combinations was the most energetically favored for [Abu\textsuperscript{19},Abu\textsuperscript{34}]MTX. Three of these combinations, all based on the Cys\textsuperscript{29}-Cys\textsuperscript{31} pairing, were deliberately eliminated from the calculations because of the impossibility of this pairing to occur (the minimal pairing that can occur being the 14-membered disulfide ring requiring two residues between...
the two half-cystines). The most stable conformation (lowest steric energy) obtained was the one that can be found in scorpion toxins cross-linked by three disulfide bridges characterized so far, i.e. C1-C4, C2-C5, and C3-C6 (Table II).

To now formerly establish the half-cystine pairings of oxidized [Abu₁⁹, Abu₃₄]MTX, the analog was proteolized by a mixture of trypsin and chymotrypsin and the resulting peptide fragments were purified by HPLC. Amino acid analysis, mass spectrometry, and Edman sequencing techniques were used to identify the sequence of the peptides connected to each other by a disulfide bond. The results of the enzyme treatment are summarized in Fig. 2B. The half-cystine pairings were thereby mapped as Cys₃-Cys₂₄, Cys₉-Cys₂₉, and Cys₁₃-Cys₃₁ (Fig. 2B). These findings demonstrate that, as expected by introducing the two point mutations in positions 19 and 34 of MTX, the thiol functions were replaced by the oxidized residues, and a disulfide bond is formed between the amino acids. This is consistent with the results of the enzyme treatment.

Next, we determined by ¹H NMR whether the disulfide bonding distance (2 Å) may be due to the substitutions of the half-cystines 19 and 34 by the isosteric Abu derivatives themselves. Indeed, the potential differences in properties (hydrophobicity) between the side chains of Abu and Cys residues are likely to be the real cause of this structural rearrangement. Alternatively, it cannot be ruled out that disulfide bridges may not just simply stabilize pre-folded structures, as generally admitted, but could also affect the final three-dimensional structure of the molecule to a small extent, a possibility that is, however, difficult to address experimentally.

Next, we determined by ¹H NMR whether the disulfide bridge rearrangement observed in [Abu₁⁹, Abu₃₄]MTX induces significant changes in the three-dimensional structure of the peptide. Spin system assignment was carried out according to the 2-steps method. First, the spin systems were identified by their scalar connectivities. Cross-peaks between HN and Hα were identified by examination of the DQF-COSY spectrum (Fig. 3A). Spin systems were identified on the basis of DQF-COSY, and the TOCSY spectrum was used to correlate these side-chain spin systems with the HN-Hα cross-peaks. In a second assignment step, the spin systems were connected in medium range assignments. The sequential assignments are indicated by boxes, the sizes of which are related to the intensity of the corresponding sequential NOE.
and the axis of the β-sheet. This angle is 50° in MTX, whereas it is close to 0° in [Abu\(^{19},\) Abu\(^{34}\)]MTX. This structural difference could be explained by the fact that in MTX, the α-helix is connected by two disulfide bridges (Cys\(^2-Cys^{29}\) and Cys\(^{135}\)). Cys\(^{13}\) to each strand of the β-sheet, which forces the α-helix to orient with an angle of 50° as compared with the β-sheet. In contrast, in [Abu\(^{19},\) Abu\(^{34}\)]MTX, the two disulfide bridges (conserved Cys\(^2-Cys^{29}\) and novel Cys\(^{13}-Cys^{31}\)) connect the α-helix to the same strand (running from residues 28 to 31) of the β-sheet. This novel pattern of bridging now constrains the two secondary structures to the same axis orientation (Fig. 4). The relative orientation of the α-helix with regard to the main axis of the β-sheet measured on all available three-disulfide-bridged scorpion toxins from the Protein Data Bank files ranges from 0 to 70° independently of the pharmacological selectivity and sensitivity of these toxins. Therefore, this structural characteristic cannot be used to anticipate the pharmacological profile of any given toxin.

We next tested whether the structural rearrangement induced by the novel disulfide bridge pattern could result in changes in the pharmacological activity of the synthetic peptide. Intracerebroventricular injections of [Abu\(^{19},\) Abu\(^{34}\)]MTX still produced a lethal effect in mice with an LD\(_{50}\) value of 0.25 μg/mouse. This effect is thus 3 times less potent than the one observed with the four-disulfide-bridged MTX (1). However, the symptoms induced by the injection of the toxin analog closely resemble those of MTX itself (and other K\(^+\) channel scorpion toxins), suggesting that K\(^+\) channels are still likely the molecular targets of [Abu\(^{19},\) Abu\(^{34}\)]MTX. Because of the high relatedness of MTX and [Abu\(^{19},\) Abu\(^{34}\)]MTX primary structures, we compared their effects on the binding of \(^{125}\)I-apamin onto rat brain synaptosomes and on the K\(^+\) currents induced by the expression of Shaker\(_B\), rat Kv1.1, Kv1.2, and Kv1.3 into Xenopus oocytes. We first tested the ability of [Abu\(^{19},\) Abu\(^{34}\)]MTX to compete with \(^{125}\)I-apamin for binding onto rat brain synaptosomes. Fig. 5 shows an [Abu\(^{19},\) Abu\(^{34}\)]MTX-induced, concentration-dependent, inhibition of \(^{125}\)I-apamin binding with a half-effect of 100 nM. In comparison, unlabeled apamin produced a complete inhibition (IC\(_{50}\)) of \(^{125}\)I-apamin binding at a much lower concentration (100 pm). The disulfide bridge rearrangement produced by the Cys\(^{13}\) and Cys\(^{34}\) substitutions decreased the affinity of the peptide for SK-type channels by approximately 9-fold since an IC\(_{50}\) of 11 nM was found for sMTX, in agreement with previous data (1). Next, we examined the activity of [Abu\(^{19},\) Abu\(^{34}\)]MTX on Shaker\(_B\), rat Kv1.1, Kv1.2, and Kv1.3 expressed in Xenopus oocytes. We compared the dose-dependent inhibition of the currents associated to these channels by sMTX and [Abu\(^{19},\) Abu\(^{34}\)]MTX applications. Fig. 6A shows that 1 nM [Abu\(^{19},\) Abu\(^{34}\)]MTX potently inhibited rat Kv1.2 currents. The extent of inhibition was identical at various test depolarizations, suggesting that the toxin action was only slightly or not voltage-dependent. The reversibility of the inhibition is illustrated in Fig. 6B. 5 nM [Abu\(^{19},\) Abu\(^{34}\)]MTX induced a 95% inhibition after a 3-min application of the peptide, which was partially (up to 65%) reversed by washout of the toxin. Similar observations were made with Shaker\(_B\) and Kv1.3 under similar experimental conditions (data not shown). We performed the dose-response experiments for Shaker\(_B\), rat Kv1.1, Kv1.2, and Kv1.3 current inhibitions by [Abu\(^{19},\) Abu\(^{34}\)]MTX and compared the results to the dose-response data obtained using sMTX under identical experimental conditions (Fig. 6C). The IC\(_{50}\) values measured for [Abu\(^{19},\) Abu\(^{34}\)]MTX were 1.2 ± 0.9 nM (Shaker\(_B\), n = 26), 1.7 ± 2 nM (Kv1.2, n = 36), and 432 ± 23 nM (Kv1.3, n = 22). The toxin had no effect on Kv1.1 at concentrations up to 10 μM (n = 10). In comparison, we found IC\(_{50}\) values of 3.4 ± 2.2 nM (Shaker\(_B\), n = 26), 0.06 ± 0.1 nM (Kv1.2, n = 20), and 320 ± 51 nM (Kv1.3, n = 17) for sMTX. These data suggest that the substitution of Cys\(^{13}\) and Cys\(^{34}\) by Abu derivatives produces no significant change in peptide affinity for Shaker\(_B\) and Kv1.3, but a 20-fold decrease in affinity for rat Kv1.2. Notably, there was also a greater fraction of Kv1.3 current that could be blocked by the MTX analog, i.e. 53.2% for [Abu\(^{19},\) Abu\(^{34}\)]MTX versus 19.6% for sMTX. The IC\(_{10}\) values obtained with [Abu\(^{19},\) Abu\(^{34}\)]MTX for either the binding assay with \(^{125}\)I-apamin or the various K\(^+\) channel current blocks are within the concentration range observed for natural
three-disulfide-bridged scorpion toxins. For instance, SK channel-acting toxins compete with \textsubscript{125}I-sapamin for binding onto rat brain synaptosomes at concentrations ranging from 20 pm (P05) to over 1 \textmu M (P01). Similarly, for Shaker B current inhibition, IC\textsubscript{50} values reported for other toxins range from 0.16 nm (agitoxin) to 160 nm (noxiustoxin) (20). The only exception to the rule appears to be Kv1.3, which is much less sensitive to [Abu\textsubscript{19},Abu\textsubscript{34}]MTX than to other three-disulfide-bridged toxins. The IC\textsubscript{50} values so far reported range from 4 pm (agitoxin) to 1.7 nm (agitoxin 1), which is at least 2 orders of magnitude less than [Abu\textsubscript{19},Abu\textsubscript{34}]MTX. The wide range of toxin IC\textsubscript{50}s reported for any given channel and the difference in affinity observed between three-disulfide-bridged toxins and [Abu\textsubscript{19},Abu\textsubscript{34}]MTX for Kv1.3 suggest that the toxin primary structure is crucial to channel sensitivity and does not solely rely on the disulfide bridge framework.

Overall, it can be concluded that changes in the disulfide bridge organization do not significantly alter the pharmacological targets of the ligand but can be employed as an unique mean to change its selectivity. Unexpectedly, the conformational alterations in toxin structure that accompany the disulfide bridge reorganization do not systematically translate into decreased affinities of the peptide for its K\textsuperscript{+} channel target(s). These data suggest that the disulfide bridge reorganization can be used as a novel approach to tentatively improve the interaction surface of the ligand for its receptor site. For the channels in which reductions in ligand affinities were observed (i.e. Kv1.2), there must be a three-dimensional repositioning of key residues critical to the blockage of current. This could be associated with the novel orientation observed between the \alpha-helix and the \beta-sheet structures, suggesting that residues belonging to both the secondary structures could be involved in the recognition of the binding site. In contrast, for channels in which no or little change in affinity was observed (i.e. Shaker B), the key residues implicated in current blockade may exclusively involve amino acid residues of the \beta-sheet structure, shown to be involved in Kv-type recognition (21). These conclusions should be reinforced by a structure-activity relationship study based on an alanine-scanning approach that ultimately will reveal the intimate differences in the binding surface of MTX for interaction with Shaker B and rat Kv1.2 and Kv1.3 channels. A combination of amino acid substitution and disulfide bridge reorganization used in parallel may reveal itself a powerful approach to the specific increase in toxin selectivity.

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<FIG. 6. [Abu\textsubscript{19},Abu\textsubscript{34}]MTX is a high affinity blocker of various voltage-gated K\textsuperscript{+} currents. A, oocytes expressing rat Kv1.2 were recorded under two-electrode voltage clamp. K\textsuperscript{+} currents were obtained by depolarization from a holding potential of \textminus 90 mV to various potentials (from \textminus 40 to 70 mV in 10-mV steps). Upper panel, control currents; lower panel, currents during superfusion of [Abu\textsubscript{19},Abu\textsubscript{34}]MTX at 1 nm. In this cell, currents were inhibited by 77–83% depending on the voltage tested. B, transient perfusion with 5 nm [Abu\textsubscript{19},Abu\textsubscript{34}]MTX caused a 94.6% transient decrease in K\textsubscript{1} currents. C, dose-dependent inhibition curves of Shaker B, rat Kv1.1, Kv1.2, and Kv1.3 currents by sMTX (filled circles) and [Abu\textsubscript{19},Abu\textsubscript{34}]MTX (open circles). Data points are the mean ± S.E. The solid lines through the data are from the equation y = y\textsubscript{0} + (a1 + \textexp{-}\textsupscript{ic50},b)[x], with IC\textsubscript{50} values of 3.4 ± 2.2 nm (Shaker B, n = 26), 0.16 ± 0.11 nm (Kv1.2, n = 30), and 320 ± 51 nm (Kv1.3, n = 17) for MTX applications, or 1.2 ± 0.9 nm (Shaker B, n = 31), 1.7 ± 2 nm (Kv1.2, n = 36), and 443 ± 23 nm (Kv1.3, n = 22) for [Abu\textsubscript{19},Abu\textsubscript{34}]MTX. No detectable inhibition was observed on Kv1.1 for both peptides at concentrations up to 10 \textmu M (n = 10 for MTX and n = 10 for [Abu\textsubscript{19},Abu\textsubscript{34}]MTX). In B and C, currents were measured by depolarizing the membrane to 70 mV. When absent, error bars are within symbol size.>
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