Maurotoxin Versus Pi1/HsTx1 Scorpion Toxins

TOWARD NEW INSIGHTS IN THE UNDERSTANDING OF THEIR DISTINCT DISULFIDE BRIDGE PATTERNS

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Maurotoxin (MTX) is a scorpion toxin acting on several K⁺ channel subtypes. It is a 34-residue peptide cross-linked by four disulfide bridges that are in an “uncommon” arrangement of the type C1-C5, C2-C6, C3-C4, and C7-C8 (versus C1-C5, C2-C6, C3-C7, and C4-C8 for Pi1 or HsTx1, two MTX-related scorpion toxins). We report here that a single mutation in MTX, in either position 15 or 33, resulted in a shift from the MTX toward the Pi1/HsTx1 disulfide bridge pattern. This shift is accompanied by structural and pharmacological changes of the peptide without altering the general α/β scaffold of scorpion toxins.

Maurotoxin (MTX), a toxin from the venom of the Tunisian chaetidae scorpion, Scorpio mauros palmaus, is a basic 34-residue peptide cross-linked by four disulfide bridges (1, 2). MTX exhibits an uncommonly wide range of pharmacological activities, since it binds onto apamin-sensitive small conductance Ca²⁺-activated K⁺ (SK) channels and also blocks voltage-gated K⁺ channels (Shaker B, Kv1.2 and Kv1.3) in the nanomolar concentration range (1–4). The three-dimensional structure of MTX in solution has been solved by ²H nuclear magnetic resonance (NMR) (5). It consists in a bent α-helix (residues 6–17) connected by a loop to a two-stranded antiparallel β-sheet (residues 22–25 and 28–31). Therefore, MTX adopts an α/β scaffold (6) that is similar to those of other scorpion toxins. Such a scaffold is thought to occur independently of the toxin chain length and ion channel pharmacological activity (7, 8). In three disulfide-bridged toxins acting on K⁺ channels, this scaffold is associated with the presence of a consensus sequence (6) of the type [...][C][...][CXX]C[...][G][...]. The same motif applies to four disulfide-bridged toxins acting on Na⁺ channels, the two extra half-cystine residues being located at either extremity of the consensus sequence. In the case of short-chain four disulfide-bridged scorpion toxins acting on K⁺ channels (e.g. MTX, Pi1, and HsTx1), a sequence variant of the type [...][C][...][CXX]C[...][C][...][G][...][C]C[...][C]X[...][C]C[...][X]C[...][C] is observed instead, which differs from the consensus sequence by the insertion of an extra half-cystine residue within the central part of the sequence (in bold). Also, the remaining half-cystine residue required for the fourth disulfide bridge formation is located at the C-terminal end of the motif as in the case of Na⁺ channel-specific toxins.

To maintain the integrity of the α/β scaffold, the α-helix is connected to the anti-parallel β-sheet by two disulfide bridges. The bridging pattern that is generally observed for three disulfide-bridged toxins is of the type C1-C4, C2-C5, and C3-C6. A similar half-cystine arrangement of the type C1-C5, C2-C6, C3-C7, and C4-C8 is also observed for Na⁺ channel-acting toxins cross-linked by four disulfide bridges and containing the consensus sequence. A similar pattern of half-cystine pairings is again observed in two recently characterized four-disulfide-bridged scorpion toxins, Pi1 (from Pandinus imperator (9)) and HsTx1 (from Heterometrus spinifer (10)), that contain the variant instead of the consensus sequence. These two toxins share high sequence identities (53–68%) with MTX and belong therefore to the same structural family, also referred to as the α-KTx6 subfamily (11). Although MTX is structurally related to Pi1 and HsTx1, it differs in its half-cystine pairings, which were unexpectedly “non-conventional” with disulfide bridges connecting C1-C5, C2-C6, C3-C4, and C7-C8. The two first disulfides are “conventional,” whereas the two others are rearranged to form short cyclic domains, one between Cys13 and Cys19 (C3-C4), and another between Cys31 and C-terminal amino acid residue Cys34 (C7-C8). At the structural level, these non-conventional pairings result in a significant difference, with the α-helix connected by two disulfide bridges (C2-C6 and C3-C4) to two different strands of the β-sheet instead of connecting the α-helix to the same strand as in Pi1 and HsTx1 (inferred from their three-dimensional structures (12, 13)). Possibly, this unique half-cystine pairing pattern of MTX may contribute to a conformation that could be slightly different from the one that would be exhibited with pairings of the Pi1/HsTx1 type. In
addition, the positioning of key residues in toxin required for the interaction with the ion channel receptor site(s), and therefore, crucial for toxin selectivity and/or affinity, could also be affected by the pattern of the disulfide bridges. It is worth noting that MTX exhibits a different pharmacological selectivity than Pi1 and HsTx1, although differences in pharmacology basically rely more on amino acid sequence variation than on disulfide bridge arrangement. For instance, Pi1 is inactive on rat Kv1.3 channels (14), whereas HsTx1 is inactive on rat brain apamin-sensitive SK channels (10), contrary to MTX (1, 2).

Here, we have investigated the structural basis of the non-conventional disulfide bridge pattern of MTX. The aim of our study was to introduce, by solid-phase peptide synthesis, targeted point mutations in the MTX amino acid sequence to obtain toxin analogs with Pi1/HsTx1-like disulfide bridging (C1-C5, C2-C6, C3-C7, and C4-C8 instead of C1-C5, C2-C6, C3-C4, and C7-C8). Our rationale in MTX mutagenesis was to introduce by substitution (i) Pi1-specific amino acid residue(s) between C3 and C4 aimed at mimicking Pi1 folding ([Q14]-MTX and [Gln15]MTX), and (ii) an amino acid residue that would produce steric hindrance and/or geometric constraints in order to prevent the connection between C7 and C8 ([Ala33]MTX). The corresponding folded MTX analogues were obtained by solid-phase chemical synthesis and were characterized for half-cystine pairings by enzyme-based cleavage. The consequences of a shift in disulfide bridging onto peptide structure and pharmacology were assessed using 1H NMR and electrophysiology on Kv channels expressed in Xenopus oocytes.

**EXPERIMENTAL PROCEDURES**

**Materials—N-α-Fmoc-1-amino acids, Fmoc-amide resin, and reagents used for peptide synthesis** were obtained from PerkinElmer Life Sciences. Solvents were analytical grade products from SDS. Enzymes (trypsin and chymotrypsin) were obtained from Roche Molecular Biochemicals.

**Chemical Synthesis and Physicochemical Characterization of [Gln15]MTX and [Ala33]MTX—** The [Gln15]MTX and [Ala33]MTX analogues were obtained by the solid-phase technique (15) using a peptide synthesizer (Model 433A, Applied Biosystems Inc.). Peptide chains were assembled stepwise on 0.25 millimolar equivalent of Fmoc-amide resin (0.65 millimolar equivalent of amino group) using 1 mmol of Fmoc-amino acid derivatives. The side chain-protecting groups used for trifunctional residues were: trityl for Cys, Asn, and Gln; butyl-tetrahydroxy-carbonyl for Lys. The Fmoc-amino acid derivatives were coupled (20 min) as their hydroxybenzotriazole active esters in N-methylpyrrolidone (4-fold excess). The peptide resins (2.2-2.5 g) were treated for 2.5 h at room temperature with a mixture of trifluoroacetic acid/H2O/thioglycerol/ethanedithiol (88:5:5:2, v/v) in the presence of crystalline phenol (2.5 g). After filtration of the mixture, the peptide was precipitated and washed by adding cold diethyl ether. The crude peptide was pelleted by centrifugation (3000 × g, 10 min), and the water was removed. The reduced peptides were then dissolved at 2 mEq in 0.2 mL tris/HCl buffer, pH 8.3, and stirred under air to allow folding (72 h, room temperature). The folded peptides, [Gln15]MTX and [Ala33]MTX, were purified by reversed-phase high pressure liquid chromatography (HPLC) (Perkin-Elmer Life Sciences, C18, Aquapore ODS 20 μm, 250 × 10 mm) by means of a 60 min linear gradient of 0.08% (v/v) trifluoroacetic acid, 0-35% acetonitrile in 0.1% (v/v) trifluoroacetic acid, H2O at a flow rate of 5 mL/min (λ = 230 nm). The homogeneity and identity of the MTX analogues were assessed by: (i) analytical C18 reversed-phase HPLC, (ii) amino acid analysis after acidolysis, (iii) Edman sequencing, and (iv) mass determination by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

**Assignment of Half-cystine Pairings of [Gln15]MTX and [Ala33]MTX by Spectral-based Cleavage and Edman Sequencing Analysis—** The two MTX analogues (800 μg) were each incubated with a mixture of trypsin and chymotrypsin at 10% (w/w) in 0.2 mL tris/HCl, pH 7.4, for 12 h at 37°C. The peptide fragments were then purified by reversed-phase HPLC (Vydac, C4, 5 μm, 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 h HCl/phenol), and their amino acid content was analyzed (Beckman, System 6300 amino acid analyzer). The peptides 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, diphenylthiocarbamoyl-cystine elutes at a retention time of 9.8 min.

**Three-dimensional Structure Determination of [Gln15]MTX and [Ala33]MTX in Solution by Two-dimensional 1H-NMR—** For sample preparation, 5 mg of [Gln15]MTX and 8 mg of [Ala33]MTX were individually dissolved in 0.5 mL of H2O/D2O (90/10 v/v), pH 3. For NMR spectroscopy, all 1H NMR spectra were recorded on a Bruker DRX 500 spectrometer equipped with a proton/carbon/azote probe and self-scanned triple axis gradients. The experiments were performed at 300 K. Two-dimensional spectra were acquired using step-state proportional phase increment method to achieve F1 quadrature detection (16). Water suppression was achieved using presaturation during the relaxation delay (1.5 s), as well as during the mixing time in the case of NOESY experiments. NOESY spectra were acquired using mixing times of 80 ms. Clean-TOSY was performed with a spin locking field strength of 8 kHz and a spin lock time of 80 ms. For spectral analysis, identification of amino acid residue spin systems and the sequential assignments were done using the standard strategy described by Wuthrich (17) applied with the graphical software XEASY (18). The TOSY spectra recorded in water gave the spin system signatures of the protein. The spin systems were then sequentially connected using the NOESY spectra.

For experimental restraints, NOE data were integrated by measuring the peak volumes. On the basis of known distances in regular

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**FIG. 1.** a, amino acid sequence (one-letter code) of MTX and comparison with those of Pi1 and HsTx1, two other related scorpion toxins. The amino acid sequences were aligned according to half-cystine residues. Gaps (—) have been introduced to maximize the alignment. The positions of half-cystines are indicated in shaded boxes (numbered C1 to C8). Amino acid sequence identities are enclosed in open boxes. The positions of the two MTX mutations studied are shown in bold letters (Q and A). b, the two patterns of disulfide bridging observed in MTX and Pi1/HsTx1 toxins.
secondary structures ($d_{H2-H2} = 0.23$ nm and $d_{HN-HN} = 0.33$ nm between two strands of an antiparallel $\beta$-sheet), these volumes were translated into upper limit distances by the CALIBA (a supporting program converting the measured nuclear Overhauser effects into upper distance limits) routine (19) of DIANA (20) software. The lower limit was systematically set at 0.18 nm.

For structure calculations, distance geometry calculations were performed with the variable target function program DIANA 2.8. A pre-

[Fig. 2. a and b, analytical C$_{18}$ reversed-phase HPLC profiles of [Gln$^{15}$]-MTX ([Q15]-MTX) and [Ala$^{33}$]-MTX ([A33]-MTX). Profiles of elution of the crude reduced peptides (left), crude peptides after folding/oxidation (middle), and purified folded peptides (right) are shown. c, assignment of the half-cystine pairings by analysis of the peptide fragments obtained by trypsin/chymotrypsin proteolysis of [Gln$^{15}$]-MTX and [Ala$^{33}$]-MTX (see “Experimental Procedures”). The resulting half-cystine pairing pattern of the two MTX analogues are compared with that of MTX.]
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![Image](255x31 to 554x239)

**Table I**

| Peptide     | Steric energy | MTX pattern | Pi1/HsTx1 pattern | Experimental pattern |
|-------------|---------------|-------------|-------------------|----------------------|
| MTX         | 885.6         | 912.0       | 885.8             | 914.1                |
| [Ala]MTX    | 882.7         | 907.0       | [Ala]MTX          | 731.3                |
| [Ala]MTX    | 865.7         | 907.8       | [Ala]MTX          | 731.3                |
| [Gln]MTX    | 845.9         | 821.9       | P1/HsTx1          | 845.9                |
| [Gln]MTX    | 639.7         | 628.1       | P1/HsTx1          | 845.9                |
| [Gln]MTX    | 705.4         | 691.5       | P1/HsTx1          | 845.9                |
| [Gln]MTX    | 921.8         | 905.2       | P1/HsTx1          | 845.9                |

Fig. 3. Three-dimensional solution structures of [Gln14]MTX and [Ala33]MTX. Amino acid sequence alignments and surveys of the sequential assignments of [Gln14]MTX (a) and [Ala33]MTX (b). The intensities of sequential NOEs, extracted from NOESY with mixing times of 80 ms, are represented by the bar thicknesses. Stereo views of the 25 best molecular [Gln14]MTX (c) and [Ala33]MTX (d) backbone structures are shown.
MTX-like disulfide bridge arrangement. In contrast, Pi1 contains the C-terminal sequence C7-YGC8, which is identical to that of MTX. Therefore, we focused on our study on the variable domains between MTX and Pi1 that are likely to be involved in their distinct half-cystine pairing patterns. First, we targeted the basic Lys15 of MTX (replaced by an uncharged Gln residue present in Pi1 at an homologous position) and synthesized a [Gln15]MTX analogue. Second, we targeted the MTX C terminus (residues 31–34) between the C7 and C8 connection. A prerequisite to the formation of such a cyclic structure (also referred to as the “14-member disulfide ring” (26–29)) is the presence of a glycine or proline within the ring (in position 32 or 33), according to geometric constraints. Such a glycine is indeed found at position 33 of MTX. Therefore, an MTX analogue without such a glycine, [Ala33]MTX, has been synthesized by a mixture of trypsin and chymotrypsin (Fig. 2c).

The results demonstrate that, contrary to MTX, both analogues exhibit half-cystine pairings between Cys4-Cys8, Cys5-Cys9, Cys12-Cys20, and Cys13-Cys15 (type C1-C5, C2-C6, C3-C7, and C4-C8, Fig. 1b). Thus, [Gln15]MTX and [Ala33]MTX differ from MTX by the two last disulfide bridges, a conventional pattern that is identical to that of both Pi1 and HsTx1. Interestingly, a single mutation of basic Arg14 (by a Gln residue), or dual mutations of both Arg14 and Lys15 (by two uncharged Gln residues), also resulted in MTX analogues with Pi1/HsTx1-like half-cystine pairing patterns (data not shown). In contrast, single mutations targeted toward another domain of MTX (substitution by alanine at positions 4, 6, 7, and 10) did not result in a disulfide bridge rearrangement of the peptides (data not shown). These data strongly suggest that the pattern of half-cystine pairings is tightly dictated by the nature of some key residues within MTX amino acid sequence.

**Table II**

| Disulfide Bridge Organization of Both MTX Analogues—To identify unambiguously the half-cystine pairings of the folded/oxidized peptides, both [Gln15]MTX and [Ala33]MTX were proteolyzed by a mixture of trypsin and chymotrypsin (Fig. 2c). The results demonstrate that, contrary to MTX, both analogues exhibit half-cystine pairings between Cys4-Cys8, Cys5-Cys9, Cys12-Cys20, and Cys13-Cys15 (type C1-C5, C2-C6, C3-C7, and C4-C8; Fig. 1b). Thus, [Gln15]MTX and [Ala33]MTX differ from MTX by the two last disulfide bridges, a conventional pattern that is identical to that of both Pi1 and HsTx1. Interestingly, a single mutation of basic Arg14 (by a Gln residue), or dual mutations of both Arg14 and Lys15 (by two uncharged Gln residues), also resulted in MTX analogues with Pi1/HsTx1-like half-cystine pairing patterns (data not shown). In contrast, single mutations targeted toward another domain of MTX (substitution by alanine at positions 4, 6, 7, and 10) did not result in a disulfide bridge rearrangement of the peptides (data not shown). These data strongly suggest that the pattern of half-cystine pairings is tightly dictated by the nature of some key residues within MTX amino acid sequence.

**Most Favorable Disulfide Bridging (MTX versus Pi1/HsTx1 Pattern)**

of MTX Analogues by Molecular Modeling and Steric Energy Calculations—We investigated by computer-assisted molecular modeling and steric energy calculations which disulfide bridge pattern, MTX versus Pi1/HsTx1 type, was the most energetically favored for a number of MTX analogues. As shown in Table I, the Pi1/HsTx1 type of half-cystine pairings was associated with the lowest steric energy (most stable conformation) for [Gln15]MTX and [Ala33]MTX, in full agreement with the half-cystine pairings observed experimentally (Fig. 2c). This observation also extends to other analogues such as [Q14]-MTX and [Q14,15]-MTX. Conversely, steric energies were found to converge minimally toward the MTX pattern for analogues that exhibited an experimental MTX disulfide bridging (analogues with a single mutation at position 4, 6, 7, or 10).

**¹H NMR Three-dimensional Structures of [Gln15]MTX and [Ala33]MTX—**The three-dimensional solution structures of [Gln15]MTX and [Ala33]MTX were solved by the ¹H NMR technique. These MTX analogues were selected because (i) they folded according to the Pi1/HsTx1 pattern of the disulfide bridges, and (ii) their point mutations were targeted toward...
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different domains of MTX, either in the α-helix or at the C terminus between C3-C4 or C7-C8, respectively.

Fig. 3 illustrates the different steps in the determination of the three-dimensional solution structures of [Gln15]MTX and [Ala33]MTX. The qualitative analysis of sequential NOE intensities for secondary structure determination, together with the pattern of medium range constraints, allowed us to predict a helical conformation between Ser6 and Thr17 for both analogues. There are some strong sequential HN(i)-HN(i+1), weak, or no Hα(i)-HN(i+1), and a stretch of medium range NOE in this region for both toxins. On the basis of the qualitative analyses of these analogues, we detected two extended fragments, one from Ala22 to Ile25 and another from Ser28 to Cys31 (strong Hα(i)-HN(i+1) together with weak HN(i)-HN(i+1) sequential NOE). These two strands are connected by a tight turn centered on Asn26 and Lys27. The N-terminal fragment is in an extended conformation from Val1 to Thr4. For structure calculations, sequential assignments of [Gln15]MTX and [Ala33]MTX were achieved first by the standard method of Wuthrich (17) (Fig. 3), and second, most protons were identified and the resonance frequencies determined for both analogues. The structures of the analogues were determined using (i) 348 NOE-based distance restraints (151 intra-residue, 114 sequential, 29 medium range, and 54 long range restraints) for [Gln15]MTX and (ii) 360 NOE-based distance restraints (154 intra-residue, 116 sequential, 23 medium range, and 67 long range restraints) for [Ala33]MTX. In addition, we used 26 ([Gln15]MTX) or 24 ([Ala33]MTX) hydrogen bond restraints and 12 distance restraints derived from the four Pi1/HsTx1-like disulfide bridges. Altogether, the final experimental set corresponds to 11.55 ([Gln15]MTX) and 11.66 ([Ala33]MTX) constraints per residue. The structures were calculated using a hybrid distance geometry simulated annealing protocol (the distance geometry algorithm for NMR (20) (DIANA) and crystallography and NMR system (21) (CNS)). Table II summarizes the structural statistics. All of the solution structures have a good nonbonded contact and covalent geometry. The best fits are shown in Fig. 3, c and d. The analysis of the structures, using TURBO-FRODO (22) and PROCHECK-NMR (23), indicates the presence of an α-helix running from Ser6 to Thr17 and a right-hand twist β-sheet from Ala22 to Ile25 and Ser28 to Cys31 for both MTX peptides. The two strands of the β-sheet are connected by a type 1-turn (31) formed by the residues Asn26 and Lys27 (Fig. 3, c and d).

The analysis of the three-dimensional solution structure of [Gln15]MTX reveals that the angle between the axis of the α-helix and the β-sheet is similar to that observed in MTX (45° versus 50°; Fig. 4a). We also found a root-mean square deviation value of 2.1 between [Gln15]MTX and MTX. The comparison of the HN and Hα chemical shifts of [Gln15]MTX and MTX showed no significant differences except for residues at positions 4, 5, 15, 17, and 33. These differences can be attributed to distinct disulfide bridging, in particular for the C terminus and the region from residues Glu15 to Thr17 (Fig. 4b). Of note, these two regions are located between the half-cystine residues Cys13-Cys19 (C3-C4) and Cys31-Cys34 (C7-C8) that are implicated in the disulfide bridge rearrangement. Also, the chemical shift observed for Thr4 and Gly2 may be due to the difference in geometry of the Cys8-Cys24 reticulation (C1-C5). Our data strongly suggest that the disulfide bridge organization in [Gln15]MTX does not control the angle between the axis of the α-helix and the β-sheet of the toxin. To explain the change in the disulfide bridge pattern (MTX toward Pi1/HsTx1 type) in the absence of striking structural modifications, we suggest that the substitution of basic Lys15 by uncharged Gln is likely to modify the local electrostatic fields of the Cys13 and Cys19 thiol groups. In MTX, the basic side chain of Lys15 is in the close vicinity of the thiol groups of Cys13 (11.27 Å) and Cys19 (13.14 Å), and in [Gln15]MTX, the uncharged side chain of Cys15 is at 11.99 and 14.68 Å from the thiol of Cys13 and Cys19, respectively. In a previous report, local variations in the electrostatic field were reported to affect the thiol pK_a values and thereby thiol reactivities (32) and presumably half-cystine pairings. Besides, mutation-induced conformational changes in [Gln15]MTX possibly modify the accessibility of either reactive thiol groups (Cys13/Cys19) in the aqueous environment leading to differential thiol reactivities and a Pi1/HsTx1-like pattern of the disulfide bridges (33). Indeed, calculations of solvent accessibility surfaces (MOLMOL) indicate a 2.3–18.3-fold increase in the accessibility of the γ-sulfurs of Cys13, Cys19, Cys31, and Cys34, which are involved in disulfide bridge rearrangement (data not shown).

The analysis of the three-dimensional solution structure of [Ala33]MTX reveals a significant difference in the chemical shifts between [Ala33]MTX and its natural counterpart (Fig. 5x). The main differences are located in region 8–23 and in the extreme C terminus. The change in the C-terminal region is obviously because of the substitution of Gly33. The structural modifications in the 8–23 region can be attributed to a marked reorientation of the α-helix; the latter being parallel to the β-sheet axis. This rotation is likely to be induced by the Gly23 mutation and not by the novel reticulation, because there is no significant reorientation of the α-helix as compared with the β-sheet in the [Gln15]MTX analogue, although it also adopts
the Pi1/HsTx1 disulfide bridge pattern. In MTX, the two α-protons of Gly33 are in close vicinity to the δ-protons of Pro20. In [Ala33]MTX, the Ala methyl group causes a steric hindrance that alters the Pro20 positioning, inducing backbone movement in the 8–23 region and thereby a novel α-helix orientation. One possible key determinant for this rotation may be the integrity of the 14-member disulfide ring itself. A similar observation is made for the [Abu19, Abu34]MTX analogue, which also displays the same angle rotation (4) (Fig. 5b). This peptide is a three disulfide-bridged MTX analogue, which was designed to restore the entire consensus motif of scorpion toxins by substituting the two half-cystine residues in positions 19 and 34 (corresponding to C4 and C8) by two isosteric α-amino butyrate derivatives. The three-dimensional structure of [Abu19, Abu34]MTX in solution shows that it adopts the α/β scaffold with conventional half-cystine pairings connecting C1-C5, C2-C6, and C3-C7. This novel bridging results in a reorientation of the α-helix regarding the β-sheet structure and is associated with changes in peptide pharmacology (4). It should be noted that in the case of [Ala33]MTX, the structural elements underlying the shift in disulfide bridging are totally unrelated to those implicated in the [Gln15]MTX shift. These elements remain to be investigated. However, we conclude with two points: (i) the formation of an MTX-like pattern is impossible in the case of [Ala33]MTX, and (ii) the Pi1/HsTx1 pattern appears to be more stable than any other potential combination in half-cystine pairings. There are no significant chemical shift differences between [Ala33]MTX and [Abu19, Abu34]MTX, with the exception of the C-terminal region, which can be attributed to the Gly/Ala33 and/or Cys/Abu34 mutation(s) (Fig. 5a). Also, the difference observed in the 17–22 region can be explained by

![Chemical shift differences between [Ala33]MTX and MTX (left) and [Abu19, Abu34]MTX and [Ala33]MTX (right). Chemical shift differences of the amide proton (gray) and of the α proton (black) are shown. A Molscript representation of [Abu19, Abu34]MTX is also shown for comparison (right). N-term, N-terminal; C-term, C-terminal.](image)

**Fig. 5.** a, chemical shift differences between [Ala33]MTX and MTX (left) and [Abu19, Abu34]MTX and [Ala33]MTX (right). Chemical shift differences of the amide proton (gray) and of the α proton (black) are shown. A Molscript representation of [Abu19, Abu34]MTX is also shown for comparison (right). N-term, N-terminal; C-term, C-terminal.

![Three-dimensional solution structure of [Ala33]MTX (left). A Molscript representation of [Abu19, Abu34]MTX is also shown for comparison (right).](image)

**Fig. 5.** b, three-dimensional solution structure of [Ala33]MTX (left). A Molscript representation of [Abu19, Abu34]MTX is also shown for comparison (right). N-term, N-terminal; C-term, C-terminal.
(i) the Abu19 mutation and/or (ii) the lack of a fourth disulfide bridge. Interestingly, the additional difference observed in the 2–4 region may be attributed to a distinct C1-C5 disulfide bridge geometry, as found in the case of [Gln15]MTX. This observation stems from the fact that the geometry of the C1-C5 disulfide bridge of [Abu19,Abu34]MTX is equivalent to that of MTX itself (data not shown).

**Pharmacology of [Gln15]MTX and [Ala33]MTX**—The MTX analogues were tested in vivo for neurotoxic activity by intracerebroventricular inoculations in C57/BL6 mice. The two peptides were lethal in mice with an LD50 of 0.25 ( [Gln15]MTX) and 2.5 mg ( [Ala33]MTX). In comparison, the LD 50 values of MTX (2), Pi1 (14), and [Abu19,Abu34]MTX (4) are 0.08, 0.20, and 0.25 mg/mouse, respectively. These data indicate that, as for [Abu19,Abu34]MTX, both analogues remain active but with some changes in affinity and/or specificity toward K1 channels.

To address the pharmacological activity of both peptides, we tested, first, their ability to compete with 125I-apamin for binding onto SK channels of rat brain synaptosomes and, second, the effects on currents resulting from Kv-type channel expression in Xenopus oocytes. The binding experiments illustrate that there is an 8- and 33-fold decrease in affinity for apamin-sensitive SK channels for [Gln15]MTX and [Ala33]MTX, respectively (Fig. 6). The IC50 values of inhibition were 98 ± 20 ( [Gln15]MTX) and 395 ± 122 nM ( [Ala33]MTX) compared with 12 ± 12 nM for MTX and 0.52 ± 0.11 pM for apamin. A similar 9-fold decrease in affinity was also observed for [Abu19,Abu34]MTX (4), suggesting that the structural alterations induced by mutations and disulfide bridge rearrangements in these MTX analogues affect the toxin sensitivity for SK channels. In contrast, it should be noted that Pi1 has a much higher affinity for SK channels (14) (IC50 of 55 pM) and that the Pi1 disulfide bridging type adopted by [Gln15]MTX and [Ala33]MTX is not accompanied by an increased affinity toward SK channels. This observation, along with the fact that HsTx1 is reportedly inactive on SK channels (10), demonstrates that the amino acid sequence is basically far more important than the pattern of disulfide bridges per se for SK channel recognition.

Next, we tested the effects of [Gln15]MTX and [Ala33]MTX onto Shaker B, rat Kv1.2, and Kv1.3 expressed in Xenopus oocytes (Fig. 7). Fig. 7a illustrates an example of Shaker K+ channel current block by 100 nM [Ala33]MTX, and washout effect. Currents were elicited by a voltage stimulation to 70 mV every 15 s. a–d, dose-dependent inhibition curves of Shaker B, rat Kv1.2, and Kv1.3 currents by [Gln15]MTX (gray circles) and [Ala33]MTX (open circles). Data points are the mean ± S.D. The solid lines through the data are from the Hill equation y = yo + (axIC50 + x)/IC50, with IC50 values of 2.3 ± 2.7 ( [Gln15]MTX, n = 32) and 0.8 ± 0.3 nm ( [Ala33]MTX, n = 28) for Shaker B channels, and 74 ± 83 ( [Gln15]MTX, n = 16) and 142 ± 157 pm ( [Ala33]MTX, n = 21) for rat Kv1.2 channels. No significant inhibition was observed on rat Kv1.3 for both peptides at concentrations up to 10 μM (n = 14). Currents were measured by membrane depolarization to 70 mV. When absent, error bars are within symbol size. Fits were performed using the same algorithm as in Fig. 6.
extent of blockage by this peptide is 61%, whereas it is 83% for [Ala-33]MTX and [Abu-19,Abu-34]MTX (4) and 92% for MTX (3). A similar but opposite modulation in the maximal extent of blockage had already been observed for the block of rat Kv1.3 channels by [Abu-19,Abu-34]MTX (4). Such a partial block remains complex to interpret, but an incomplete permeability block, possibly because of an imperfect pore occlusion, can tentatively be proposed. For rat Kv1.2 channels, the blockage by both peptides is above 80% with IC_{50} values of 74 ± 83 ([Gln-15]MTX, n = 16) and 142 ± 157 pm ([Ala-33]MTX, n = 21). Compared with MTX, these values correspond to slight affinity reductions of 1.4-fold ([Gln-15]MTX) and 2.4-fold ([Ala-33]MTX). In contrast, [Abu-19,Abu-34]MTX, which displays the same α-helix/β-sheet orientation as [Ala-33]MTX, presented a 28-fold reduction in affinity for rat Kv1.2 under the same experimental conditions. There is thus at least a 10-fold difference in affinity between [Ala-33]MTX and [Abu-19,Abu-34]MTX. [Abu-19,Abu-34]MTX and [Ala-33]MTX on rat Kv1.3 is clearly independent of (i) the relative orientation between the α-helix and the β-sheet, because these peptides display very different angles (one MTX-like and the other [Abu-19,Abu-34]MTX-like; both Kv1.3 active), and (ii) the presence (or not) of a cyclic C-terminal domain, because [Abu-19,Abu-34]MTX and HsTx1, which lack C-terminal cyclic domains, are nevertheless active on Kv1.3 channels.

Our data demonstrate that two single mutations within the MTX amino acid sequence can result in a shift in the disulfide bridge pattern, which is accompanied by structural and pharmacological changes. The disulfide bridge organization contributes to the spatial distribution of key residues that are implicated in ion channel recognition. In the case of MTX, the shift in half-cystine pairings results in an increased selectivity of the toxin for rat Kv1.2 channels. Our data illustrate that the affinity of the MTX analogues for Kv1.2 is maintained, whereas there is a loss and a reduction of activity on rat Kv1.3 and SK channels, respectively. Additional mutagenesis on these analogues aimed at further decreasing the affinity on SK channels may render the toxin Kv1.2 selective.

Acknowledgments—We thank R. Oughchedi for technical support.

REFERENCES

1. Kharrat, R., Mansuelle, P., Sampieri, F., Crest, M., Oughchedi, R., Van Rietschoten, J., Martin-Eauclaire, M. F., Rochat, H., and El Ayeb, M. (1997) FEBS Lett. 400, 294–299
2. Kharrat, R., Mabrouk, K., Crest, M., Darbon, H., Oughchedi, R., Martin-Eauclaire, M. F., Jacquet, G., El Ayeb, M., Van Rietschoten, J., and Sabatier, J. M. (1996) Eur. J. Biochem. 242, 491–498
3. Carlier, E., Avdonin, V., Geib, S., Fajloun, Z., Kharrat, R., Rochat, H., Sabatier, J. M., Hoshi, T., and De Waard, M. (2000) J. Peptide Res. 55, 419–427
4. Fajloun, Z., Ferrat, G., Carlier, E., Fatollahi, M., Leconte, C., Sandou, G., di Lucio, E., Mabrouk, K., Legros, C., Darbon, H., Rochat, H., Sabatier, J. M., and De Waard, M. (2000) J. Biol. Chem. 275, 13605–13612
5. Blance, E., Sabatier, J. M., Kharrat, R., Meunier, S., El Ayeb, M., Van Rietschoten, J., and Darbon, H. (1997) Proteins 29, 321–333
6. Bontems, F., Roumeandest, C., Gilquin, B., Ménez, A., and Toma, F. (1991) Science 254, 1521–1523
7. Ménez, A., Bonlems, F., Roumeandest, C., Gilquin, B., and Toma, F. (1992) Proc. R. Soc. Edinb. Sect. B (Biol. Sci.) 209, 99–103
8. Darbon, H., Blance, E., and Sabatier, J. M. (1999) in Perspectives in Drug Discovery and Design: Animal Toxins and Potassium Channels (Darbon, H., and Sabatier, J. M., eds) Vols. 15/16, pp. 41–60, Kluwer Academic Publishers, Dordrecht, The Netherlands
9. Olamendi-Portugal, T., Gomez-Lagunas, F., Gurrola, G. B., and Possani, L. (1996) Biochem. J. 315, 977–981
10. Lebrun, B., Romi-Lebrun, L., Martin-Eauclaire, M. F., Yasuda, A., Ishigura, M., Oyama, Y., Pongs, O., and Nakajima, T. (1997) Biochem. J. 328, 321–327
11. Tytgat, J., Handy, G., Garcia, M. L., Gutman, G. A., Martin-Eauclaire, M.-F., van der Walt, J. J., and Possani, L. D. (1999) Trends Pharmacol. Sci. 20, 444–447
12. Delepierre, M., Prochnicka-Chalufour, A., and Possani, L. D. (1997) Biochemistry 36, 2649–2658
13. Savarir, R., Romi-Lebrun, L., Martin-Eauclaire, M. F., Yasuda, A., Ishigura, M., Oyama, Y., Pongs, O., and Nakajima, T. (1997) Biochem. J. 328, 321–327
14. Lebrun, B., Romi-Lebrun, L., Martin-Eauclaire, M. F., Yasuda, A., Ishigura, M., Oyama, Y., Pongs, O., and Nakajima, T. (1997) Biochem. J. 328, 321–327
15. See all references in the above text.