Evolution of maurotoxin conformation and blocking efficacy towards *Shaker B* channels during the course of folding and oxidation *in vitro*

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Maurotoxin (MTX) is a 34-mer scorpion toxin cross-linked by four disulphide bridges that acts on various K⁺ channels, including the voltage-gated *Shaker B* subtype. In the present study, we have investigated over 80 h: (1) the time-course of folding of synthetic MTX (sMTX) by CD analysis; (2) the kinetics of disulphide bridge formation by MS; and (3) the potency of MTX in blocking *Shaker B* currents during the combined process of its *in vitro* folding and oxidation. From the CD data, we show that stable secondary structures of sMTX evolve sequentially over time, with the appearance of the α -helix within 5 h, followed by the formation of the β -sheet within 22 h. Using MS analysis, the sMTX intermediates were also found to appear sequentially from the least (one-disulphide-bridged sMTX) to the most oxidized species (native-like, four-disulphide-bridged sMTX). The time course of formation of secondary structures coincides mainly with the occurrence of one-disul-

phide-bridged sMTX for the α -helix and two- or three-disulphide-bridged sMTX for the β -sheet. On-line electrophysiological recordings, which measure sMTX blocking efficacy on K⁺ currents during its folding and oxidation, were performed on *Shaker B* channels expressed in *Xenopus* oocytes. Unexpectedly, the results demonstrate that sMTX is highly potent at the initial stage of oxidation, whereas its blocking activity can be transiently and dramatically reduced at later stages during the course of folding/oxidation before it reaches full bioactivity. These data suggest that formation of disulphide bridges can both physically stabilize and alter the bioactive three-dimensional structure of sMTX.

Key words: circular dichroism, half-cystine pairing, K⁺ channel, mass spectrometry, scorpion toxin.

INTRODUCTION

Maurotoxin (MTX) is a scorpion toxin from the venom of *Scorpio maurus palmatus* [1,2]. This basic 34-mer peptide (VSC₁TGS-KDC₂YAPC₃RKQTGC₄PNACK₅INKSC₆KC₇YGC₈-NH₂) is cross-linked according to a C1–C5, C2–C6, C3–C4 and C7–C8 disulphide-bridge pattern [2]. MTX in solution [3] adopts a classical α/β scaffold [4], which is composed of a bent α -helix (residues 6–17) connected by a loop to a two-stranded antiparallel β -sheet (residues 22–25 and 28–31). This toxin exhibits a wide spectrum of pharmacological activity by blocking, at nanomolar concentrations, both apamin-sensitive small conductance Ca²⁺-activated K⁺ channels and voltage-gated K⁺ channels, such as *Shaker B* (*ShB*) [1,2,5,6].

Small toxins from 20–40 amino acid residues, such as MTX, possess ideal characteristics as peptide models to investigate the process by which a protein folds and oxidizes to acquire its bioactivity [7–10]. Firstly, large quantities of the reduced synthetic MTX (sMTX) can be obtained by chemical synthesis, which is almost impossible to achieve by purifying the natural toxin from its biological source. The chemically synthesized MTX is directly recovered in its reduced/unfolded state after peptide-chain cleavage from its solid support, and folds/oxidizes to reach a biologically active conformation approx. 3 days after

solubilization in a suitable oxidation buffer. Secondly, we noticed that changes in the three-dimensional (3-D) structure and in the pattern of disulphide bridges of sMTX produce significant changes in its pharmacological activity. This observation suggests that sMTX is a convenient and adequate model to monitor the changes in bioactivity during the course of peptide folding and oxidation. Moreover, the *in vitro* oxidation process of sMTX has a slow time scale, which should permit a careful kinetic analysis of the pharmacological modification associated with the incremental reticulation of sMTX. Indeed, we have demonstrated in a previous study [11] that the intermediates of sMTX oxidation appear sequentially over time, from the least (one-disulphide-bridged sMTX) to the most oxidized species (native-like, four-disulphide-bridged sMTX).

In the present study, we have used reduced sMTX to investigate the kinetics of formation of its stable secondary structures, which was correlated with its degree of reticulation and efficacy to block the *ShB* current. We show that (1) the α -helix of MTX is formed before its β -sheet structure, and (2) both the oxidation process and the bioactivity of the toxin still evolve after apparent acquisition of the main structural features. The unexpected observation that the reduced form of sMTX has a higher blocking potency than some of the oxidation intermediates strongly suggests that oxidation proceeds by stages through successive

Abbreviations used: Abu, α -aminobutyrate; Fmoc, N²-fluorenyl-9-methyloxycarbonyl; MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; MTX, maurotoxin; sMTX, synthetic MTX; [Abu3,9,13,19,24,29,31,34]-sMTX, Cys-free sMTX; P_{max}, period of maximal sMTX blocking potency; P_{min}, period of minimal sMTX blocking potency; *ShB*, *Shaker B*; TFA, trifluoroacetic acid; 3-D, three-dimensional.

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acquisition of active native-like and inactive non-native-like domains that are involved in *ShB* channel recognition and blockage.

EXPERIMENTAL

Materials

N^z-fluorenyl-9-methoxycarbonyl (Fmoc)-L-amino acid derivatives, Fmoc-amide resin and reagents used for peptide synthesis were purchased from PerkinElmer (Shelton, CT, U.S.A.) and Neosystem Laboratoire (Strasbourg, France). Solvents were analytical-grade products from SDS (Peypin, France). α -Cyano-4-hydroxycinnamic acid and iodoacetamide were obtained from Sigma (St. Louis, MO, U.S.A.).

Chemical synthesis and physicochemical characterization of sMTX

Reduced and oxidized sMTX were obtained by the solid-phase technique [2] using a peptide synthesizer (Model 433A; Applied Biosystems, Foster City, CA, U.S.A.). The sMTX peptide chain was assembled stepwise on 0.25 mmol of Fmoc-amide resin (0.65 mmol of amino group/g) using 1 mmol of Fmoc amino acid derivatives as described previously [2]. The reduced peptide was purified to > 99% homogeneity by reversed-phase HPLC (PerkinElmer Life Sciences, C₁₈ Aquapore ODS 20 μ m, 250 mm $\times$ 10 mm) by means of a 70-min linear gradient of 0.08% (v/v) trifluoroacetic acid (TFA)/0–35% acetonitrile in 0.1% (v/v) TFA/water at a flow rate of 2 ml/min (λ = 230 nm). The homogeneity and identity of the reduced and oxidized sMTX species were assessed by: (1) analytical C₁₈ reversed-phase HPLC; (2) amino acid content determination after acidolysis; (3) Edman sequencing; and (4) MS. After the purified reduced sMTX was freeze-dried, the fully reduced state was verified by iodoacetamide-based alkylation of the eight Cys residues, analytical C₁₈ reversed-phase HPLC and MS. The Cys-free structural analogue of sMTX, [Abu3,9,13,19,24,29,31,34]-sMTX (Cys-free sMTX; where Abu is α -aminobutyrate), was synthesized and characterized using similar experimental procedures.

Folding and oxidation of reduced sMTX

The freeze-dried reduced peptide was dissolved at 0.4 mM in 30–100 μ l aliquots of oxidation buffer [20 mM sodium phosphate buffer (pH 8.2)] and stirred for 80 h at 25 °C under air to allow folding/oxidation. Under our conditions, the pH value was set at 8.2 to approach the pK_a value of thiol groups of Cys residues. In theory, this corresponds to an optimal pH value, since equivalent proportion of thiolate anions and thiol groups should be present; a condition presumably appropriate for an efficient formation of the disulphide bridges.

Secondary-structure analysis by CD

CD spectra were obtained from 260–182 nm on a Jobin-Yvon UV CD spectrophotometer (MARK VI; Long-Jumeau, France) by using a 100 μ m pathlength cell. The instrument was calibrated with ($\pm$)-10-camphorsulphonic acid. A ratio of 2:1 was found between the positive CD band at 290.5 nm and the negative band at 192.5 nm. Data were collected at intervals of 0.5 nm with a scan rate of 4 nm/min. CD spectra were reported as the absorption coefficient ($\Delta\epsilon$) per amide. An absorption spectrum was also measured with the same apparatus (MARK VI) to check for light transmission of samples in the far UV (182–260 nm) and to determine the peptide concentration of the sample. The samples were prepared in 20 mM sodium phosphate

buffer (pH 8.2). The concentration of reduced sMTX was 0.4 mM. After recording the CD spectrum, the lid of the cell was opened to allow exposure to air. The secondary-structure calculations were achieved using the method of Manavalan and Johnson [12].

Alkylation of free thiol groups and MS analysis of the peptide samples

Samples (5 μ l) of the folding/oxidation medium were alkylated by using 5 μ l of an 85 mM iodoacetamide solution for 30 s at 25 °C. The MS analyses were carried out in the linear mode using a matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS (Voyager DE-RP; Perseptive Biosystems Inc., Framingham, MA, U.S.A.). The parameters fixed before MS analyses were: 20 kV as accelerating voltage, 92% and 0.1% as grid and guide wire voltages respectively, 100 ns as delayed extraction time and 256 average scans. The data were analysed by using the GRAMS/386 software. The matrix used to prepare the samples was α -cyano-4-hydroxycinnamic acid (10 mg/ml) in TFA/acetonitrile/water (0.4:49.8:49.8, by vol.). The peptide sample (0.5 μ l at 1 μ M) was placed on a 100-well plate, and 0.5 μ l of matrix solution was added. This mixture was allowed to dry prior to MS analysis. For each sMTX folding/oxidation assay, samples were collected at different times (0–80 h), alkylated and the peptide species were characterized by their respective masses after external calibration with appropriate peptide standards. The peptides in the samples were then accurately quantified by the total counts of ionized molecules detected (areas of peaks) as previously described [11].

Oocyte preparation and electrophysiological recordings

Stage 5 and 6 *Xenopus laevis* oocytes were prepared for cRNA injection and electrophysiological recordings. The follicular cell layer was removed by enzymic treatment with 2 mg/ml collagenase IA (Sigma) in classical Barth's medium. The *ShB* was linearized with *Sma*I and subsequently transcribed with T7 RNA polymerase (mMessage mMachine kit; Ambion, Austin, TX, U.S.A.). The cells were micro-injected 2 days later with 40 nl of cRNA (0.05 μ g/ μ l). To facilitate channel expression and cell survival, oocytes were incubated at 16 °C in a defined nutrient oocyte medium [13] for 2–6 days before current recordings. K⁺ currents were then recorded at 25 °C by standard two-microelectrode techniques using a voltage-clamp amplifier (GeneClamp 500; Axon Instruments, Foster City, CA, U.S.A.) interfaced with a 16-bit AD/DA converter (Digidata 1200A, Axon Instruments). Electrodes, filled with 140 mM KCl, had an electric resistance of 0.3–0.8 M Ω . Voltage pulses were delivered every 15 s to 70 mV from a holding potential of –80 mV. Current records were sampled at 10 kHz and low-pass filtered at 2 kHz using an eight-pole Bessel filter and stored on computer for subsequent analysis. The extracellular recording solution contained 88 mM NaCl, 10 mM KCl, 2 mM MgCl₂, 0.5 mM CaCl₂, 0.5 mM niflumic acid, 5 mM Hepes and 0.1% BSA (pH 7.4). Leak and capacitive currents were subtracted on-line by a P/4 protocol. Residual capacitive artefacts were removed for display purposes. The toxin samples were solubilized in 20 mM sodium phosphate buffer (pH 8.2) and kept at 25 °C to allow folding and oxidation. Toxin applications were performed every 20 or 30 min over an 80 h period by direct dilution into the recording chamber (10 μ l of toxin was added to a recording volume of 190 μ l). Under these experimental conditions, sMTX was incubated in the extracellular recording solution over a period of 12 min (versus 80 h of total experimental time), which did not significantly affect the oxidation state of the toxin (see the Results and Discussion

section). Currents were analysed using pCLAMP 6.03 software (Axon Instruments). The results are presented as means $\pm$ S.D.

RESULTS AND DISCUSSION

Rationale

In the classical view of protein folding and oxidation, it is generally assumed that the final 3-D structure of the protein is first acquired followed by its stabilization through disulphide bonding. The overall prediction of such a scheme would thus be that a protein should progressively acquire its bioactivity during the course of the folding process and until completion. The final stabilization of the protein 3-D structure through the formation of disulphide bonds should thus play a minor role *per se* in the acquisition of its bioactivity. The aim of this study was to determine whether these generally admitted assumptions on protein folding and oxidation also apply to sMTX *in vitro*. We followed, under strictly similar experimental conditions, the time course of sMTX folding by CD, oxidation by MS and acquisition of bioactivity by electrophysiology through the measurements of peptide-induced *ShB* current blockage.

Synthesis and characterization of reduced sMTX

The reduced sMTX was obtained by solid-phase peptide synthesis using Fmoc chemistry [2]. After cleavage from the solid support, the reduced sMTX was purified to homogeneity (> 99%) by using C_{18} reversed-phase HPLC under acidic conditions (pH 2.5) to avoid partial oxidation. The product was then aliquoted, freeze-dried and stored at -80°C prior to use. The reduced state of sMTX was verified by MALDI-TOF MS immediately after solubilization in oxidation buffer and alkylation of free Cys residues, as described in the Experimental section. Figure 1 demonstrates that, using this procedure, sMTX was indeed in its fully reduced form. Equivalent peptide samples were then used for CD and MS analyses as well as for electrophysiological testing (see below).

Time course of sMTX folding *in vitro*

The CD technique was used to assess the evolution of secondary structures during the course of sMTX folding and oxidation. A

Figure 1 MALDI-TOF MS characterization of the reduced sMTX

MALDI-TOF mass spectra of samples collected from the oxidation milieu after immediate solubilization (0 h) of 0.4 mM reduced sMTX in 20 mM sodium phosphate buffer (pH 8.2), and after 80 h of folding/oxidation. In each case, the sample collected was alkylated by iodoacetamide prior to MS analysis. 0 S-S and 4 S-S correspond to the fully reduced and oxidized sMTX forms respectively.

Figure 2 CD analysis of sMTX folding

(a) CD spectra recorded at various times after the start of sMTX folding/oxidation in 20 mM sodium phosphate buffer (pH 8.2). (b) Superimposed CD spectra recorded after 24 and 80 h of sMTX folding/oxidation and comparison with the CD spectrum of native MTX. At 80 h and thereafter, the CD spectrum of sMTX is stable and considered as native-like. (c) Absorption spectra of native MTX and sMTX at various times of folding/oxidation.

sample of the reduced sMTX was solubilized in the oxidation buffer at a peptide concentration of 0.4 mM and immediately added to the CD cell for secondary-structure assessment. Subsequently, a number of CD spectra were recorded at various times after the start of the folding and oxidation processes (Figure 2a). Measurements were performed at a wavelength ranging from 182–260 nm, and the data obtained corresponded essentially to $\pi-\pi^*$ and $n-\pi^*$ transitions of the amide chromophore located in the polypeptide chains [14]. In the reduced state of sMTX (0 h), the CD spectrum was characterized by the presence of a marked negative band between 195–200 nm. This band was associated with a $\pi-\pi^*$ transition, which is typical of a random coil [15]. After 5 h of oxidation, a positive band was

detected on the spectrum at 190 nm. The latter corresponded to a $\pi-\pi^*$ transition that is characteristic of an α -helical structure. Two negative bands were also observed at 208 and 222 nm representing the $\pi-\pi^*$ and $n-\pi^*$ transitions of an α -helix respectively. In the spectra at 24 and 48 h, a red shift of the positive band was observed from 190–195 nm and a negative contribution from 208–212 nm. These changes are likely to result from the formation of a β -sheet. The CD spectrum of this secondary structure is characterized by the negative and positive bands at 215 and 200 nm respectively, owing to $n-\pi^*$ and $\pi-\pi^*$ transitions. The additivity of α -helix and β -sheet signals, a property that is inherent in the CD technique [14], explains why the red shift was observed after 24 h on the CD bands. After 24 h of folding/oxidation, the recorded CD spectra of sMTX were not yet fully superimposable with the CD spectrum of native sMTX, suggesting that discrete, but significant, changes in overall secondary structures still occur after that time (Figure 2b). The main differences in CD spectra after 24 h were a positive contribution at 190 nm and a negative contribution at 210 nm. These two contributions cannot be attributed to an increase in the CD signal of the α -helix, since a similar contribution should also have been detected at 222 nm. In contrast, they could be attributed to the signal originating from a type I β -turn [14]. It should be emphasized that, in native sMTX, the α -helix is connected to two distinct strands of the antiparallel β -sheet by two disulphide bridges (Cys-13–Cys-29 and Cys-13–Cys-19). Of note, the α -helix is separated from the β -sheet structure by a type I β -turn located between residues 17 and 21, as revealed by consecutive NN (i, i + 1) nuclear Overhauser effects in the ^1H -NMR study of sMTX [3]. It is therefore likely that disulphide-bridge formation may result in a relative repositioning of the α -helix with regard to the β -sheet structure, thereby potentially affecting the global content of type I β -turn(s). Indeed, structural analogues of sMTX, in which half-cystine pairing patterns are modified ([Abu19,34]-sMTX, [Q¹⁵]-sMTX and [A³³]-sMTX) as compared with native sMTX, present a reorientation of the α -helix with respect to the β -sheet structure [16,17]. Alternatively, it cannot be ruled out that disulphide bridging within the α -helix might induce some structural changes that may affect the CD signals. We assume that sMTX folding/oxidation reaches completion before 80 h, since the CD spectrum of sMTX at this time superimposed with that of native MTX (Figure 2b). According to the Manavalan and Johnson program [12], we estimated the secondary structures of sMTX at 80 h to represent 32.3% α -helix, 23.5% β -sheet, 22.0% β -turn and 22.2% random coil. These values are similar to those obtained for native MTX, and in close agreement with the 3-D structure of sMTX in solution [3]. To validate the results of this kinetic investigation, we checked that the sMTX concentration in the sample remained constant over 80 h by measuring the absorption spectrum at various times of folding/oxidation (Figure 2c). This provides evidence to suggest that no peptide precipitation, resulting from aggregation, occurred during the course of sMTX folding/oxidation.

In conclusion, sMTX has clearly no stable secondary structures in its reduced state. Modifications of the CD spectra during sMTX folding/oxidation suggest that structural changes of sMTX molecules proceed grossly via three main steps. During the first step of folding/oxidation, which lasts approx. 5 h, sMTX molecules tend to form an α -helical structure. In the second step, after approx. 7 h, a stable β -sheet structure starts to appear, with an apparent complete formation within 22 h. In the third step, after approx. 24 h, the secondary structures of sMTX molecules resemble those of their native counterparts and apparently undergo only discrete changes, which may be associated

with the formation of a type I β -turn through disulphide bridging. It should be emphasized that these steps reflect the global structural tendencies of the sum of all sMTX populations present at a given time during the folding/oxidation process. Such a relative 'heterogeneity' in the sMTX populations has been demonstrated by MS [11], and shown below (see Figure 4b).

In an attempt to assess the contribution of oxidation to the folding process, we synthesized a full-length sMTX analogue in which all eight Cys residues were replaced with isosteric Abu derivatives, i.e. Cys-free sMTX. The formation of stable secondary structures of Cys-free sMTX was then evaluated by CD analysis (Figure 3). Interestingly, under similar oxidative conditions, the CD spectrum of Cys-free sMTX was superimposable with that of reduced sMTX. A large negative band between 195 and 200 nm was observed, suggesting that Cys-free sMTX has a random coil structure. In contrast with sMTX, the CD spectra recorded at various times following peptide solubilization remain similar, highlighting the inability of Cys-free sMTX to form stable secondary structures over time. In the case of sMTX, it appears from such data that disulphide bridging may favour the formation of stable secondary structures. This interpretation should obviously be taken with caution, since it is also likely that the substitution of Cys residues by Abu derivatives may itself alter peptide conformation by introducing changes in structural constraints.

Next, using MS, we estimated the degree of reticulation of sMTX as a function of folding and oxidation time to tentatively correlate the various levels of oxidation of sMTX with the appearance of its stable secondary structures.

Kinetics of formation of the sMTX disulphide bridges

We have recently reported [11] that the MALDI-TOF MS technique can be used successfully as an accurate and sensitive method of individually analysing the kinetics of appearance and disappearance of the various sMTX intermediates during the course of the folding/oxidation process *in vitro*. Figure 4(a) shows the MS spectra after 0, 0.2, 29 and 80 h of sMTX folding/oxidation in 20 mM sodium phosphate buffer (pH 8.2). The peaks corresponding to each sMTX oxidation intermediate were separated well by iodoacetamide-based alkylation of the free thiol groups of Cys residues. Figure 4 also shows the progressive disappearance of the reduced form of sMTX and appearance of the various oxidation intermediates. Increasing the time of oxidation resulted in the appearance of the most oxidized forms of sMTX. Under these experimental conditions, 80 h was sufficient to oxidize sMTX fully. Of note, all the sMTX oxidation intermediates were transiently formed and irreversibly converted into the four-disulphide-bridged form. As expected, analysis of sMTX at the end of the folding and oxidation processes demonstrated that it was homogeneous and had native-like half-cystine pairings (results not shown) [1,2]. As shown previously [11], comparison of the area of each peak can be used as a quantitative method to estimate the relative proportion of each sMTX molecular species. In order to investigate the kinetics of transitions between the various oxidation intermediates of sMTX, we plotted the percentage of each population of sMTX species as a function of the folding/oxidation time (Figure 4b). The data show that the oxidation intermediates appeared sequentially over time, from the least (one-disulphide-bridged sMTX) to the most oxidized species (native-like, four-disulphide-bridged sMTX). The process of oxidation is not formally sequential, since an overlap exists in the appearance of two sMTX intermediates that differ by a single disulphide bridge. Consequently, each sMTX oxidation intermediate with either one, two or three

Figure 3 CD analysis of Cys-free sMTX folding

CD spectra recorded at various times after the start of Cys-free sMTX folding in 20 mM sodium phosphate buffer (pH 8.2). The CD spectrum of reduced and unfolded sMTX is also shown for comparison.

disulphide bridges does not exceed 50% of the total molecules present at any given time. An important observation is that the kinetics of appearance and disappearance of each sMTX species are not identical, suggesting distinct orders of complexities for disulphide bridging. For instance, under these experimental conditions, the life span of the one-disulphide-bridged sMTX was approx. 30 h, whereas that of the three-disulphide-bridged sMTX was over 70 h. This observation also suggests the occurrence of greater orders of complexities for sMTX intermediates with higher degrees of oxidation. An interdependence appears to occur between the various sMTX oxidation intermediates, which is best evidenced for the three- and four-disulphide-bridged sMTX. There is a long-lived and exclusive co-existence (after approx. 40 h) of these two sMTX species at the end of the folding/oxidation reaction. It is noteworthy that the time scale of appearance of the one-disulphide-bridged sMTX (< 7 h) coincided with the kinetics of the formation of the α -helix (after approx. 5 h) as assessed by CD analysis. This is consistent with the concept that the first step of sMTX oxidation might be essential to stabilize the α -helical structure. Such a hypothesis would also explain why the Cys-free sMTX is unable to form a stable α -helix. Also, it is striking that the formation of the β -sheet structure reaches apparent completion at approx. 22 h, which corresponds to a higher degree of sMTX reticulation. It is therefore tempting to speculate that the appearance of the stable α -helical and β -sheet structures are closely associated with the formation of the first two disulphide bridges. In both cases, it should be mentioned that disulphide bridging clearly occurs before complete stabilization of these two secondary structures. Finally, the CD analysis suggests that the later detection of a type I β -turn in sMTX is associated with some disulphide bridging. Indeed, the MS data demonstrate that disulphide bridging still occurs at a late stage of the sMTX folding/oxidation process (> 24 h). In that respect, there is an apparent contradiction between the fact that disulphide bridging is faster during the formation of the main secondary structures of sMTX (α -helix and β -sheet) than at later stages, when only minor structural changes are observed. Overall, our experimental data

Figure 4 Analysis of sMTX oxidation by MALDI-TOF MS

(a) MS spectra after 0, 0.2, 29 and 80 h of folding/oxidation of 0.4 mM sMTX in 20 mM sodium phosphate buffer (pH 8.2). The sMTX oxidation intermediates are resolved by iodoacetamide-based alkylation of the free thiol groups of Cys residues. 0 S-S and 4 S-S correspond to the fully reduced and oxidized sMTX forms; 1 S-S, 2 S-S and 3 S-S are partially oxidized sMTX intermediates. (b) Evolution in relative percentages of sMTX species over time during folding/oxidation of reduced sMTX.

suggest that the oxidation process of short peptides is a more complex phenomenon than previously thought. These data seem to differ from the simple view that disulphide bridging between pairs of Cys residues only serves to lock a preformed 3-D structure of the reticulated polypeptide.

With regard to bioactivity, it would be expected that, during the acquisition of the structural features of sMTX, the peptide should progressively acquire the ability to recognize the ionic pore of *ShB* channels and block K^+ currents during the activation of these channels. We therefore tested the blocking potency of sMTX towards *ShB* channels expressed in *Xenopus* oocytes during the combined processes of folding and oxidation.

ShB current blocking properties of sMTX during folding/oxidation

To evaluate the effect of sMTX on *ShB* currents during the course of folding/oxidation *in vitro*, the experimental procedures

Figure 5 Inhibition of *ShB* currents by 'reduced', native-like and Cys-free sMTX

(a) Representative current blocks induced by the application for 12 min of 100 nM native-like (left panel), 'reduced' (middle panel) and Cys-free sMTX (right panel). (b) Average concentration-dependent inhibition of *ShB* currents by native-like (●), 'reduced' (○) or Cys-free sMTX (▲). Data points are means $\pm$ S.D. The lines through the data are from the Hill equation [$y = y_0 + (ax^b/IC_{50}^b + x^b)$]; where y_0 is the basal current inhibition; x , the sMTX concentration; b , the Hill coefficient; and a , the maximum inhibition by sMTX] with IC_{50} values of 2.4 ± 0.6 (native-like sMTX, $n = 23$) and 149.1 ± 44.4 nM ('reduced' sMTX, $n = 23$). A linear regression is used for Cys-free sMTX data ($n = 19$). Currents are measured by membrane depolarization to 70 mV. When absent, error bars are within symbol size.

used require that the toxin be added for 12 min to an extracellular solution suitable for recording. Under these experimental conditions, this time of toxin testing had no detectable impact on sMTX folding and oxidation at later stages of the experiment (after 1 h of initiating oxidation). However, oxidation occurred to some extent with reduced sMTX, i.e. at the start of the experiment (Figure 4b), with approx. 10% of one-disulphide-bridged sMTX being formed [11]. For the functional assays, we used mostly chemically non-modified sMTX to avoid experimental bias, such as the sterical hindrance that could result from alkylation of the Cys residues. We next evaluated the effects of variable concentrations of reduced sMTX applied to *ShB* channels expressed in *Xenopus* oocytes. Figure 5(a) shows representative current blocks induced by the application of either 100 nM fully oxidized sMTX (native sMTX), 'reduced' sMTX or Cys-free sMTX. The average *ShB* current blocks observed at this concentration were $92 \pm 1.1\%$ (native sMTX), $41.7 \pm 3.3\%$ ('reduced' sMTX) and $1.2 \pm 2.1\%$ (Cys-free sMTX). Average dose-response curves are shown in Figure 5(b). The calculated IC_{50} were $2.4 \pm 0.6\%$ for native sMTX, $149.1 \pm 44.4\%$ for 'reduced' sMTX, whereas Cys-free sMTX had no effect up to 40 μ M. Thus there was an almost 60-fold reduction in blocking potency of sMTX in its 'reduced' form, whereas Cys-free sMTX was inactive. The behaviour of fully Cys-alkylated sMTX was similar to that of Cys-free sMTX (results not shown). At this level, we are unable to conclude whether the blocking effect of 'reduced' sMTX was due to the reduced form or to the one-disulphide-bridged sMTX (approx. 10%) that forms during testing. In any case, these data suggest that at least 1–2% of the total molecules applied in the recording chamber are active and that a number of molecules fold according to structural domain(s) that allow *ShB* recognition and blockage. It is noteworthy that it would not be possible to detect such a small percentage of bioactive molecules by our CD analysis. It is likely that the blocking activity observed with the 'reduced' sMTX is due to the small level of oxidation which occurs to some extent during testing, since Cys-free sMTX is unable to block *ShB* currents even at high peptide concentrations. This is also in agreement with our view that disulphide bridging is somehow required to stabilize sMTX secondary

structures. Of note, toxin activity on voltage-gated K⁺ channels (e.g. *ShB*) has generally been attributed to the presence of the β -sheet structure within the α/β scaffold, which is common to known scorpion toxins [18]. We can therefore only speculate that a small percentage of this structure is present during the initial step of oxidation following the application of reduced sMTX to the recording chamber.

Next, we investigated the evolution of sMTX potency to block *ShB* currents during folding and oxidation (Figure 6). Representative current blocks by 200 nM sMTX are shown at various times after the start of the folding/oxidation process (Figure 6a). Interestingly, there was a marked variation in the blocking efficacy of sMTX over time. For instance, 92 and 91% blocks were observed at 1 and 30 h respectively, whereas smaller blocks of 13 and 20% were measured at 10 and 50 h. These data indicate that sMTX evolves through various structural states by which it is able to successively block, or not, *ShB* currents during the course of folding/oxidation. To more accurately describe this evolution, we followed the blocking efficacy of sMTX every 20–30 min over a period of 80 h (Figure 6b). Several distinct stages of sMTX blocking ability are shown by this on-line analysis. We depict four evident periods of maximal blockage (from $P_{max}1$ to $P_{max}4$; where P_{max} is the period of maximal sMTX blocking potency) that are interspersed by four periods of significant reduction in blocking potency (from $P_{min}1$ to $P_{min}4$; where P_{min} is the period of minimum sMTX blocking potency). The P_{max} are characterized by a weak variability with regard to sMTX blocking efficacy, whereas a much greater variability exists in the P_{min} . Such a large variability in P_{min} is expected for sMTX concentrations close to the toxin IC_{50} . The initial phase of $P_{min}1$, which is characterized by a higher blocking efficacy, may correspond to an additional $P_{max}0$. However, this period was too short to be conclusively termed in this way. Testing sMTX at longer periods of folding/oxidation (over 80 h) did not reveal any additional variation in blocking efficacy (results not shown). The toxin remained at maximal efficacy ($P_{max}4$) and is therefore considered as native-like during this period. A similar experiment was performed twice at 40 nM sMTX, which revealed identical periods of blocking potency, though with less well-defined P_{max} ,

Figure 6 Evolution of sMTX ability to block *ShB* currents during folding/oxidation

(a) Representative current blocks by 200 nM sMTX are shown at various times (1, 10, 30 and 50 h) after the start of the folding/oxidation process. Traces are shown 2.50 min before, and 1.25, 2.5, 5, 7.5, 9.75 and 10 min after sMTX application. The inhibition increases with time and the percentage current inhibition is shown. Maximal current inhibition was obtained within a 10 min sMTX application time. (b) Time course of *ShB* current blockage by 200 nM sMTX during the course of its folding/oxidation. The percentage K^+ current inhibition as a function of folding/oxidation time of reduced sMTX (○) is measured at 12 min application time. $P_{\min}$ (light grey shading) and $P_{\max}$ (dark grey shading) are numbered according to their consecutive order of appearance.

suggesting that 40 nM is close to the IC_{50} (results not shown). A sub-maximal sMTX concentration of 40 nM is normally required to efficiently block *ShB* currents if we consider that 100% of the molecules are bioactive (native-like sMTX; see Figure 5b). Therefore these data suggest that more than 20% of the molecules have a bioactive conformation in the various $P_{\max}$ at 200 nM sMTX. In fact, according to the CD data, it is only at the end of $P_{\max 1}$ (approx. 22 h) that 100% of the molecules should theoretically exhibit an α -helix and a β -sheet structure. In the subsequent periods ($P_{\min 2} - P_{\min 4}$, and $P_{\max 2} - P_{\max 4}$), we therefore assume that the formation of both secondary structures should have reached completeness. In that respect, it is interesting to note that the formation of the α -helix and β -sheet in sMTX is clearly not necessarily associated with full toxin activity, since several $P_{\min}$ occur at later stages. These data highlight that some structural changes are needed in sMTX before it can reach full activity. The toxin activity appears to rely on the formation of an adequate α/β scaffold, rather than simply on the formation of the elementary and constitutive secondary structures. As such, the slow acquisition of other structural elements, e.g. type I β -turn(s), seems to be pivotal for sMTX to gain full bioactivity. In that sense, the relative positioning of the α -helix with regard to the β -sheet structure might be essential to toxin activity. In addition, the formation of an α/β scaffold is mainly conditioned by disulphide bridging between the α -helix and the β -sheet. Once formed, the α/β scaffold, which is not necessarily in its exact native arrangement, may evolve further during additional reticulation. Abnormal patterns of sMTX reticulation, possibly altering the α/β scaffold, could be responsible for the transient phases of decreased toxin activity. In support of this interpretation, we have recently demonstrated [16,17] that a reorientation of the α -helix with regard to the β -sheet structure is accompanied by some modifications in sMTX pharmacology. This has been interpreted as an alteration of sMTX's 3-D structure, leading to a novel spatial distribution of key residues that are critical to toxin pharmacology. Before 40 h, it is difficult to reasonably correlate the various $P_{\max}$ and $P_{\min}$ with the states of sMTX reticulation, since there are considerable overlaps in the transition

states between the various sMTX oxidation intermediates. For instance, at 20 h, all five species of sMTX (reduced and from one–four disulphide bridges) co-exist in the oxidation milieu. The changes in sMTX bioactivity occurring after 40 h are more informative, since only the three- and four-disulphide-bridged species are present. Interestingly, there are two $P_{\min}$ that occur after this time in spite of the fact that the four-disulphide-bridged sMTX represents approx. 85% of the total molecules present. If all of these molecules were correctly folded and oxidized, one would thus have an effective native-like sMTX concentration of 170 nM (0.85×200 nM), at which full *ShB* blockage is expected to occur (Figure 5b). Since this is not the case during $P_{\min 3}$ and $P_{\min 4}$, we assume that considerable disulphide bridge reshuffling and/or structural changes occur even with all four disulphide bridges formed. It would be unlikely that these changes are limited to these $P_{\min}$ periods. They presumably also occur during the interspersed periods ($P_{\max 3}$ and $P_{\max 4}$), in spite of a higher sMTX bioactivity, either to a lesser extent or because they implicate structural domains that are not directly involved in *ShB* recognition. This would represent the strongest apparent divergence from the concept that sMTX first folds into its native-like conformation, then appropriately connects the facing half-cystine pairs to allow stabilization of its 3-D structure. In fact, the structural changes associated with disulphide bridging probably differ in complexity with the number of disulphide bridges and structural domains involved. It is noteworthy that $P_{\max 1}$ occurs during the final acquisition of the β -sheet structure, a period at which we observe mainly one- and two-disulphide-bridged sMTX intermediates. The molecular models depicting an interaction between a toxin and a voltage-gated K^+ channel suggest that the recognition of *ShB* by sMTX occurs through the toxin β -sheet structure. High level of sMTX bioactivity during the formation of the β -sheet structure is a strong indication of a native-like structuration of this domain. The existence of $P_{\min 2}$, just after complete formation of the β -sheet structure, coincides with the appearance of three-disulphide-bridged sMTX and indicates that disulphide bridging is responsible for a decreased bioactivity associated with this particular secondary structure.

In conclusion, the present study highlights the complexity of sMTX oxidative folding *in vitro*. In a pictorial view of the process, derived from the diffusion-collision model of Karplus and Weaver [19,20], one would predict that microdomains of sMTX (e.g. portions of secondary structures) randomly move and eventually collide, leading to coalescence into multi-microdomains following either a single or several lowest energy barrier pathways (downhill folding). In the case of sMTX, we show that: (1) disulphide bridging occurs before complete acquisition of the stabilized secondary structures; (2) the acquisition of a stable 3-D structure is slowed down by the transient formation of non-native disulphide bridges during the course of oxidation; and (3) disulphide-bridge reshuffling finally yields normal folding and half-cystine pairings. Such a reshuffling represents a further delaying element of the folding process. Overall, the present results highlight the meaning of the unusually high degree of reticulation observed in short-chain scorpion toxins, since their disulphide bridging contributes to secondary structure and α/β scaffold stabilization. Additional investigations will be required to understand further the favoured sequential route in the folding and oxidation of MTX.

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REFERENCES

- Kharrat, R., Mansuelle, P., Sampieri, F., Crest, M., Oughideni, R., Van Rietschoten, J., Martin-Eauclaire, M. F., Rochat, H. and El Ayeb, M. (1997) Maurotoxin, a four disulfide bridge toxin from *Scorpio maurus* venom: purification, structure and action on potassium channels. *FEBS Lett.* **406**, 284–290
- Kharrat, R., Mabrouk, K., Crest, M., Darbon, H., Oughideni, R., Martin-Eauclaire, M. F., Jacquet, G., El Ayeb, M., Van Rietschoten, J. and Sabatier, J. M. (1996) Chemical synthesis and characterization of maurotoxin, a short scorpion toxin with four disulfide bridges that acts on K⁺ channels. *Eur. J. Biochem.* **242**, 491–498
- Blanc, E., Sabatier, J. M., Kharrat, R., Meunier, S., El Ayeb, M., Van Rietschoten, J. and Darbon, H. (1997) Solution structure of maurotoxin, a scorpion toxin from *Scorpio maurus*, with high affinity for voltage-gated potassium channels. *Proteins* **29**, 321–333
- Bontems, F., Roumestand, C., Gilquin, B., Ménez, A. and Toma, F. (1991) Refined structure of charybdotoxin: common motifs in scorpion toxins and insect defensins. *Science (Washington, D. C.)* **254**, 1521–1523
- Carlier, E., Avdonin, V., Geib, S., Fajloun, Z., Kharrat, R., Rochat, H., Sabatier, J. M., Hoshi, T. and De Waard, M. (2000) Effect of maurotoxin, a four disulfide-bridged toxin from the chactoid scorpion *Scorpio maurus*, on *Shaker* K⁺ channels. *J. Pept. Res.* **55**, 419–427
- Avdonin, V., Nolan, B., Sabatier, J. M., De Waard, M. and Hoshi, T. (2000) Mechanisms of maurotoxin action on *Shaker* potassium channels. *Biophys. J.* **79**, 776–787
- Sali, A., Shakhnovich, E. and Karplus, M. (1994) How does a protein fold? *Nature (London)* **369**, 248–251
- Eaton, W. A., Munoz, V., Hagen, S. J., Jas, G. S., Lapidus, L. J., Henry, E. R. and Hofrichter, J. (2000) Fast kinetics and mechanisms in protein folding. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 237–259
- Brockwell, D. J., Smith, D. A. and Radford, S. E. (2000) Protein folding mechanisms: new methods and emerging ideas. *Curr. Opin. Struct. Biol.* **10**, 16–25
- Menéz, A., Bouet, F., Guschlbauer, W. and Fromageot, P. (1980) Refolding of reduced short neurotoxins: circular dichroism analysis. *Biochemistry* **19**, 4166–4172
- di Luccio, E., Azulay, D. O., Regaya, I., Fajloun, Z., Sandoz, G., Mansuelle, P., Kharrat, R., Fathallah, M., Carrega, L., Estève, E. et al. (2001) Parameters affecting *in vitro* oxidation/folding of maurotoxin, a four disulfide-bridged scorpion toxin. *Biochem. J.* **358**, 681–692
- Manavalan, P. and Johnson, W. C. (1987) Variable selection method improves the prediction of protein secondary structure from dichroism spectra. *Anal. Biochem.* **167**, 76–85
- Eppig, J. J. and Dumont, J. N. (1976) Defined nutrient medium for the *in vitro* maintenance of *Xenopus laevis* oocytes. *In Vitro* **12**, 418–427
- Johnson, W. C. (1985) Circular dichroism and its empirical application to biopolymers. *Methods Biochem. Anal.* **31**, 61–163
- Waterhous, D. V. and Johnson, W. C. (1994) Importance of environment in determining secondary structure in proteins. *Biochemistry* **33**, 2121–2128
- Fajloun, Z., Ferrat, G., Carlier, E., Fathallah, M., Lecomte, C., Sandoz, G., di Luccio, E., Mabrouk, K., Legros, C., Darbon, H. et al. (2000) Synthesis, ¹H-NMR structure, and activity of a three-disulfide-bridged maurotoxin analog designed to restore the consensus motif of scorpion toxins. *J. Biol. Chem.* **275**, 13605–13612
- Fajloun, Z., Mosbah, A., Carlier, E., Mansuelle, P., Sandoz, G., Fathallah, M., di Luccio, E., Devaux, C., Rochat, H., Darbon, H. et al. (2000) Maurotoxin versus P1/HsTx1 scorpion toxins. Toward new insights in the understanding of their distinct disulfide bridge patterns. *J. Biol. Chem.* **275**, 39394–39402
- Darbon, H., Blanc, E. and Sabatier, J. M. (1999) Three-dimensional structure of scorpion toxins: towards a new model of interaction with potassium channels. In *Perspectives in Drug Discovery and Design: Animal Toxins and Potassium Channels*, vol. 15/16, (Darbon, H. and Sabatier, J. M., eds.), pp. 41–60, Kluwer Academic Publishers, Dordrecht, The Netherlands
- Karplus, M. and Weaver, D. L. (1976) Protein-folding dynamics. *Nature (London)* **260**, 404–406
- Karplus, M. and Weaver, D. L. (1994) Protein folding dynamics: the diffusion-collision model and experimental data. *Protein Sci.* **3**, 650–668

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