Parameters affecting *in vitro* oxidation/folding of maurotoxin, a four-disulphide-bridged scorpion toxin

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Maurotoxin (MTX) is a 34-mer scorpion toxin cross-linked by four disulphide bridges that acts on various K⁺ channel subtypes. MTX adopts a disulphide bridge organization of the type C1–C5, C2–C6, C3–C4 and C7–C8, and folds according to the common α/β scaffold reported for other known scorpion toxins. Here we have investigated the process and kinetics of the *in vitro* oxidation/folding of reduced synthetic L-MTX (L-sMTX, where L-MTX contains only L-amino acid residues). During the oxidation/folding of reduced L-sMTX, the oxidation intermediates were blocked by iodoacetamide alkylation of free cysteine residues, and analysed by MS. The L-sMTX intermediates appeared sequentially over time from the least (intermediates with one disulphide bridge) to the most oxidized species (native-like, four-disulphide-bridged L-sMTX). The mathematical formulation of the diffusion-collision model being inadequate to accurately describe the kinetics of oxidation/folding of L-sMTX, we have formulated a derived mathematical description that better fits the experimental data. Using this mathematical description, we have compared for the first time

the oxidation/folding of L-sMTX with that of D-sMTX, its stereoisomer that contains only D-amino acid residues. Several experimental parameters, likely to affect the oxidation/folding process, were studied further; these included temperature, pH, ionic strength, redox potential and concentration of reduced toxin. We also assessed the effects of some cellular enzymes, peptidylprolyl *cis-trans* isomerase (PPIase) and protein disulphide isomerase (PDI), on the folding pathways of reduced L-sMTX and D-sMTX. All the parameters tested affect the oxidative folding of sMTX, and the kinetics of this process were indistinguishable for L-sMTX and D-sMTX, except when stereospecific enzymes were used. The most efficient conditions were found to be: 50 mM Tris/HCl/1.4 mM EDTA, pH 7.5, supplemented by 0.5 mM PPIase and 50 units/ml PDI for 0.1 mM reduced compound. These data represent the first report of potent stereoselective effects of cellular enzymes on the oxidation/folding of a scorpion toxin.

Key words: mass spectrometry, half-cystine pairing.

INTRODUCTION

Maurotoxin (MTX, also referred to as L-MTX because it contains only L-amino acid residues) is a toxin isolated from the venom of the Tunisian chactidae scorpion *Scorpio maurus palmatus* [1,2]. This basic 34-residue peptide has the sequence VSC₁TGSKDC₂YAPC₃RKQTGC₄PNAKC₅INKSC₆KC₇YG C₈-NH₂ and is cross-linked according to the uncommon C1–C5, C2–C6, C3–C4 and C7–C8 pattern of disulphide bridging [2]. The normal type of bridging is C1–C5, C2–C6, C3–C7 and C4–C8 for other scorpion toxins [3,4]. In spite of this unconventional disulphide-bridge organization, L-MTX still adopts the classical α/β scaffold [5]. The three-dimensional (3D) structure of synthetic L-MTX (L-sMTX, equivalent to natural MTX) in solution [6] shows that it 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 displays a large spectrum of pharmacological activity since it blocks apamin-sensitive small-conductance Ca²⁺-activated K⁺ channels and voltage-gated K⁺ channels (*Shaker* B, Kv1.2 and Kv1.3) at nanomolar concentrations [1,2,7,8].

There is evidence from recent structure–activity studies on L-MTX that the type of half-cystine pairings alters 3D structure

and spatial distribution of key residues that are critical to pharmacological selectivity [9–11]. For instance, point-mutated L-MTX analogues ([Q¹⁵]MTX and [A³³]MTX) with C1–C5, C2–C6, C3–C7 and C4–C8 pairings, a pattern normally adopted by the toxins P11 (toxin 1 from the scorpion *Pandinus imperator*) and HsTx1 (toxin 1 from the scorpion *Heterometrus spinifer*), exhibit decreased binding affinities for small-conductance Ca²⁺-activated K⁺ channels without any loss of blockage efficacy for voltage-gated *Shaker* B and Kv1.2 channels [10]. A similar observation was made for a three disulphide-bridged [Abu¹⁹, Abu³⁴]MTX analogue (where Abu indicates α -aminobutyrate) that was designed to restore the entire consensus motif of scorpion toxins [9]. This analogue oxidizes/folds according to the C1–C5, C2–C6 and C3–C7 pattern (C4 and C8 being replaced by isosteric α -aminobutyrate derivatives), which mainly results in a reorientation of the α -helix with regard to the β -sheet structure. These structural changes are also accompanied by some modifications in peptide pharmacology. Although it could be argued that the structural and pharmacological variations observed with these analogues might be inferred by the mutation itself, these examples suggest the importance of peptide-backbone reticulation for final toxin 3D structure and pharmacology. L-MTX represents an ideal candidate for studying the oxidation/folding

Abbreviations used: MTX, maurotoxin; L-MTX, natural MTX, containing only L-amino acid residues; sMTX, synthetic MTX; L-sMTX, synthetic L-MTX; D-sMTX, sMTX containing only D-amino acid residues; Fmoc, N⁹-fluorenylmethyloxycarbonyl; MALDI-TOF, matrix-assisted laser desorption/ionization–time of flight; PDI, protein disulphide isomerase; PPIase, peptidylprolyl *cis-trans* isomerase; 3D, three-dimensional; TFA, trifluoroacetic acid.

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process of scorpion toxins since (i) it belongs to a class of highly cross-linked toxins (the α -KTx6 subfamily) [12] that can easily be chemically synthesized, (ii) slight amino acid sequence variations are able to modify its disulphide bridge organization and (iii) preliminary evidence suggests that its *in vitro* oxidation/folding is slow enough to allow a careful analysis of the oxidation intermediates transiently formed during the process. Although L-MTX is used as a model to investigate the process of disulphide bridge formation in highly reticulated polypeptides, such a study will certainly be beneficial to the general understanding of oxidation/folding of other proteins.

Here we present compelling evidence that MS can be used successfully as an accurate and sensitive method to individually analyse the kinetics of appearance and disappearance of the various synthetic MTX (sMTX) intermediates during the course of the oxidation/folding process *in vitro*. We found that a large number of parameters can affect the kinetics of such a process. Notably, the most important parameters for an efficient and complete oxidation of reduced L-sMTX are the temperature, pH, ionic strength and the presence of cellular enzymes involved in protein folding *in vivo* (peptidylprolyl *cis-trans* isomerase, PPIase, and protein disulphide isomerase, PDI). Except when stereospecific enzymes are used, the effects of the various parameters are indistinguishable for L-sMTX and D-sMTX (sMTX containing only D-amino acid residues).

EXPERIMENTAL PROCEDURES

Materials

N^ε-Fluoren-9-ylmethoxycarbonyl (Fmoc)-L- or -D-amino acid derivatives, Fmoc-amide resin and reagents used for peptide synthesis were purchased from Perkin-Elmer (Shelton, CT, U.S.A.) and Neosystem Laboratoire (Strasbourg, France). Solvents were analytical-grade products from SDS (Peypin, France). Human recombinant PPIase (also referred to as FK-binding protein, EC 5.2.1.8), bovine liver PDI (EC 5.3.4.1), α -cyano-4-hydroxycinnamic acid and iodoacetamide were obtained from Sigma (St. Louis, MO, U.S.A.).

Chemical synthesis and physicochemical characterization of L-sMTX and D-sMTX

The peptides were obtained by the solid-phase technique [13] using a peptide synthesizer (model 433A, Applied Biosystems). Peptide chains were 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. The procedure used for the chemical syntheses of both peptides was as described previously [2]. The reduced peptides were purified to > 99% homogeneity by reversed-phase HPLC (Perkin-Elmer 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 reduced L-sMTX and D-sMTX were assessed by: (i) analytical C₁₈ reversed-phase HPLC; (ii) amino acid content determination after acidolysis; (iii) Edman sequencing and (iv) MS. After lyophilization of the purified reduced peptides, the fully reduced state of the peptides was verified by iodoacetamide-based alkylation of the eight cysteine residues, analytical C₁₈ reversed-phase HPLC and MS.

Alkylation of free thiol groups and MS analysis of the samples

The lyophilized reduced peptide was dissolved in 100 μ l of oxidation buffer (for conditions, see the Results and discussion

section) at a final peptide concentration of 0.4 mM (except in enzyme assays; 10 μ l of oxidation buffer at 0.1 mM peptide) and exposed to air by stirring to allow folding (72 h). Samples of 5 μ l were alkylated by using 5 μ l of a 85 mM iodoacetamide solution (except in enzyme assays; samples of 0.5 μ l were alkylated by using 0.5 μ l of 1.4 M iodoacetamide) 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) mass spectrometer (Voyager DE-RP, Perseptive Biosystems, 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 oxidation/folding assay, samples were collected at different times (0–72 h), and the peptide species were characterized by their respective masses after external calibration with appropriate peptide standards. The peptides in the sample were then accurately quantified by the total counts of ionized molecules detected (areas of peaks). When high-ionic-strength buffers (> 400 mM salt concentration) were used for the assays, the samples collected were desalted using C₁₈ ZipTip (Millipore, Bedford, MA, U.S.A.) prior to MS analyses. The accuracy of the relative proportions between the oxidized and reduced species of sMTX was verified using control samples in which mixtures of known quantities of purified peptides were preformed. These peptides correspond to alkylated (reduced sMTX and partially oxidized sMTX intermediates) and non-alkylated (sMTX with all four disulphide bridges formed) products.

RESULTS AND DISCUSSION

Rationale

The study of protein oxidation/folding is of fundamental importance to understand how a protein gets its final 3D structure and bioactivity [14–16]. *In vitro* models, including of venomous animal toxins, have been used extensively to approach protein folding experimentally [17]. Small peptide toxins, from 20 to 40 amino acid residues, possess the ideal characteristics to investigate the process by which a protein oxidizes and folds. First, high yields of the reduced forms of these peptides can be obtained by chemical synthesis; which is often difficult to achieve by purifying natural or recombinant proteins from biological sources. Working on chemically synthesized proteins also presents the advantage that the peptide is directly in its reduced/unfolded state after peptide-chain cleavage from its solid support. Second, small peptide toxins are highly structured and reticulated proteins that are readily amenable to structure–function studies. We here studied various experimental parameters that are likely to affect the oxidation/folding of reduced sMTX. We evaluated the effects of these parameters on the sequential events of the oxidative folding, the kinetics of appearance and disappearance of each sMTX oxidation intermediate and the extent of final oxidation. At term, the aim of this study is to better understand the molecular determinants involved in sMTX oxidation/folding.

Technical approach to the study of the *in vitro* oxidation of sMTX

There are two main technical limitations in studying the oxidation process of reduced sMTX by MALDI-TOF MS. First, it is

Figure 1 MS-based analysis of the various L-sMTX species

(a) MALDI-TOF mass spectrum of an oxidation milieu sample collected after 5 h of oxidation/folding of 0.4 mM reduced L-sMTX in 0.2 M Tris/HCl buffer, pH 8.3 (25 °C). 0 S-S and 4 S-S correspond to the fully reduced and oxidized (native-like) L-sMTX forms; 1 S-S, 2 S-S and 3 S-S are partially oxidized L-sMTX intermediates. *m/z* is the ratio of the molecular mass over the number of charges of the ionized species. (b) Similar to (a) except that the oxidation milieu was treated with 85 mM iodoacetamide (alkylation of the free thiol groups of cysteine residues) prior to MS analysis. For conditions, see the Experimental procedures section. (c) MALDI-TOF mass spectrum of a preformed mixture containing equivalent amounts (1 pmol) of fully oxidized (Ox) and 8 Cys-alkylated (Alk) L-sMTX. The complete alkylation of reduced L-sMTX was achieved using iodoacetamide. The quantification of the purified products was performed by amino acid analyses. (d) Evaluation of the MALDI-TOF MS technique for the quantification of the various L-sMTX species. The example shown relies on MS data obtained using a number of preformed mixtures containing various proportions of fully oxidized and alkylated L-sMTX. Both peptides in sample were then accurately quantified by the total counts of ionized molecules detected (areas of peaks) and the proportion of oxidized/alkylated L-sMTX is represented by filled circles. A fit through the experimental data demonstrates the linearity in the proportion between the two L-sMTX species studied and validates the quantification method.

difficult to individualize the peaks corresponding to the various sMTX forms (from the reduced to the fully oxidized species). Figure 1(a) illustrates the MS spectrum of several sMTX intermediates after 5 h of oxidation in 0.2 M Tris/HCl, pH 8.3 (standard buffer). With only ≈ 2 Da difference in molecular mass between each of the closest oxidation intermediates, the MS spectrum poorly discriminates the various molecular species present in the sample analysed. Another drawback of studying oxidation intermediates of sMTX at the start of the oxidation process is the fact that these intermediates are unstable and rapidly convert from one form to another. We therefore sought to improve both the resolution of the MS spectrum peaks and the stability of the transient oxidation intermediates. As shown in Figure 1(b), iodoacetamide-based alkylation greatly improved the separation between the peaks. In the sample shown (5 h of oxidation in standard buffer), all oxidation intermediates were present (reduced sMTX, and sMTX with 1–4 disulphide bridges).

These peptides are now stabilized by rendering unreactive each available thiol group of cysteine residues by the addition of an alkyl moiety [18]. One should also note that, under our experimental conditions (here and thereafter), there are no additional molecular species that would result from the partial alkylation of the sMTX intermediates. These molecules could theoretically be present, half-cystines being connected by pair, only one out of two half-cystines could be alkylated. Therefore, we assume that our alkylation conditions are optimal. To estimate the relative proportion of each molecular species, we first determined whether a comparison of the area of each peak could be used as a quantitative method. The peak area corresponds to the total counts of the peak as measured by the ion MS detector. To be fully quantitative, the laser-induced desorption of the various compounds should be equivalent for each molecule contained in the sample. At least for sMTX, the MALDI-TOF MS technique can be used for quantitative analy-

Figure 2 MALDI-TOF mass spectra of samples collected from the oxidation milieu after immediate solubilization (0 h) of 0.4 mM reduced L-sMTX in 0.2 M Tris/HCl buffer, pH 8.3 (25 °C), and after 0.1–72 h of oxidation/folding

In each case, the sample collected was alkylated by iodoacetamide prior to MS analysis.

ses (Figure 1c). A mixture of 50% alkylated reduced L-sMTX and 50% fully oxidized L-sMTX produces two peaks of equivalent areas (number of counts), suggesting that desorption is identical for both molecules. A similar observation was made for a mixture of 50% reduced L-sMTX (non-alkylated) and 50% fully oxidized L-sMTX (results not shown). Figure 1(d) illustrates that the estimate of molecule proportions (alkylated reduced L-sMTX versus fully oxidized L-sMTX) by peak counts accurately reflects the actual percentage of each molecule as initially fixed prior to MS quantification.

Kinetics of appearance and disappearance of L-sMTX species

Next, we used MS to analyse the kinetics of appearance and disappearance of the various L-sMTX species during oxidation/folding. Figure 2 shows the progressive disappearance of the reduced form of L-sMTX and appearance of the various oxidation intermediates. Increasing the time of oxidation results in the appearance of the most oxidized forms of L-sMTX. Oxidation for 8 h is sufficient for a complete disappearance of reduced L-sMTX, whereas four-disulphide-bridged L-sMTX could be detected as early as 2 h after the start of the oxidation process. Under these experimental conditions, about 72 h are needed to fully oxidize L-sMTX. Most importantly, all the L-sMTX

oxidation intermediates are transiently formed and irreversibly converted to the four-disulphide-bridged form. As expected, analysis of the final synthetic L-sMTX demonstrates that it is homogeneous and has native-like half-cystine pairings and bioactivity (results not shown) [2]. In order to determine the kinetics of transitions between the various oxidation intermediates of L-sMTX, we plotted the percentage of each population of L-sMTX species as a function of oxidation time (Figure 3). This study was not aimed at determining the selective order of connection (if any) between pairs of half-cystines, something that could not be achieved with the MS technique anyway. In contrast, our first goal was to get kinetic data on the appearance and disappearance of each oxidation intermediate to allow a thorough comparison between various experimental oxidation conditions. However, some basic conclusions could be obtained from a preliminary analysis of the data.

First, the oxidation intermediates seem to appear sequentially over time, from the least (one disulphide bridge) to the most oxidized species (native-like, four-disulphide-bridged L-sMTX). A similar sequential order of oxidation was observed for hen egg white lysozyme [18]. However, this order of appearance is not strictly sequential since an L-sMTX intermediate with a certain degree of oxidation does not start to appear when the immediately preceding intermediate has fully disappeared. This is clearly shown in Figure 3 by the facts that (i) an overlap exists between

Figure 3 Evolution in relative percentages of L-sMTX species over time, as assessed by MALDI-TOF MS, during oxidation/folding of 0.4 mM reduced L-sMTX in 0.2 M Tris/HCl buffer, pH 8.3 (25 °C)

The samples were alkylated by iodoacetamide prior to MS analyses. (a) ■, 0 S-S L-sMTX; (b) △, 1 S-S L-sMTX; (c) ▼, 2 S-S L-sMTX; (d) ◇, 3 S-S L-sMTX; (e) ●, native-like 4 S-S L-sMTX; (f) superimposition of the curves obtained from (a)–(e).

curves describing the kinetics of appearance of two L-sMTX intermediates that differ by only one disulphide bridge and (ii) each oxidation product does not reach 100 % of the molecules (e.g. intermediates with either one, two or three disulphide bridges do not exceed 41 % of the total molecules present at any given time).

Second, the shapes of the curves of appearance and disappearance are not identical for each L-sMTX species, suggesting different orders of complexity for the formation of disulphide bridges. The fact that L-sMTX intermediates of a higher degree of oxidation have longer lifetimes is evidence for higher orders of complexity. For instance, L-sMTX with one disulphide bridge

has a lifetime of about 15 h, whereas that with three disulphide bridges remains present in the oxidation milieu for ≈ 60 h.

Third, a complex interdependence exists between the various L-sMTX species during oxidation. This is difficult to demonstrate between L-sMTX with one, two and three disulphide bridges, but appears more clearly between L-sMTX with three and four disulphide bridges. Notably, long-lived three-disulphide-bridged L-sMTX seems to stabilize to a plateau value of about 13%; a plateau that maintains itself from 30 to 60 h after the start of oxidation when L-sMTX with one and two disulphide bridges have disappeared from the oxidation milieu. Of note is that a concomitant plateau occurs at 87% of the total molecules for the four-disulphide-bridged L-sMTX. This suggests that there is a long latency in which both three- and four-disulphide-bridged toxins do co-exist. After 55 h of oxidation an interconversion occurs between these two molecular species. The slow kinetics of interconversion represent a rate-limiting step in the obtention of 100% native-like four-disulphide-bridged L-sMTX. Delayed transition could be associated with more complex molecular events, such as *cis*-to-*trans* isomerization of prolyl imidic peptide bonds in L-sMTX [19,20]. Accordingly, this is in close agreement with the generally assumed probability that about 10–30% of the prolyl imidic peptide bonds are in the *cis* configuration in any given unfolded polypeptide chain [21]. Indeed, there are two proline residues in L-sMTX at positions 12 and 20, adjacent to Cys-13 and Cys-19, themselves forming a disulphide bridge. The 3D structure of L-sMTX in solution shows that both prolyl imidic peptide bonds are in the *trans* configuration [6]. One could speculate therefore that formation of the four disulphide bridges of L-sMTX highly disfavour the *cis* configuration of the Xaa-Pro bonds, which normally should represent $\approx 6\%$ of the total Xaa-Pro bonds in native proteins [22]. Also, isomerization of either one or both of the prolyl imidic peptide bonds may be a prerequisite to the formation of the last disulphide bridge due to geometric constraints. At present, it is not possible to precisely identify which of the four disulphide bridges is the last to be formed, but one could suggest that the Cys-13–Cys-19 connection is the most likely candidate due to its close proximity to both proline residues. Another argument in favour of this particular bridge is the fact that Cys-13 and Cys-19 can most easily associate with other half-cystines in point-mutated L-sMTX analogues, including [A¹²]sMTX and [A²⁰]sMTX [11].

In order to compare the kinetic parameters of the appearance and disappearance of the various sMTX species, we first investigated whether our experimental data could appropriately be described by the diffusion-collision model of Karplus and Weaver [23,24]. This model is commonly used and accepted for a quantitative analysis of protein folding, whereas other models only provide pictorial views of the process. It would predict that microdomains of sMTX (e.g. portions of secondary structures) randomly move and collide, eventually leading to coalescence into multi-microdomains. In such a view, folding of sMTX would proceed through a series of coalescence steps that might follow either a single or several lowest-energy barrier pathways (downhill folding). For a kinetic description, the diffusion-collision model is based on single exponential functions of the type:

$$f_j(t) = e^{a_j t}$$

in which j is the number of disulphide bridges (0–4) and t is the folding/oxidation reaction time. The coefficient a_j is the eigenvalue of a 5×5 matrix. The matricial formulation of this model is a weighted sum of these functions and represents each plot used to fit the experimental data. It is described as follows:

$$P'(t) = \begin{bmatrix} X_{0,0} \\ \cdot \\ \cdot \\ \cdot \\ X_{4,0} \end{bmatrix} f_0(t) + \dots + \begin{bmatrix} X_{0,5} \\ \cdot \\ \cdot \\ \cdot \\ X_{4,5} \end{bmatrix} f_5(t) + \begin{bmatrix} B_0 \\ \cdot \\ \cdot \\ \cdot \\ B_4 \end{bmatrix}$$

in which X is a 5×5 matrix, each coefficient $X_{i,j}$ being a weight. The function for each oxidation intermediate is found at row i of the vector P ; B is a vector containing the constant terms for each equation P'_i . There are as many equations as there are sMTX intermediates (thus i ranges from 0 to 4).

Using these equations, we were unable to adequately fit the experimental data (results not shown). In particular, we could not properly describe (i) the delay periods observed for the appearance of sMTX species with three and four disulphide bridges, (ii) the inflexion of these curves after the start of the oxidation/folding process and (iii) the final plateaux of these two species (Figure 3). One of the peculiar problems faced with the highly reticulated sMTX is that it may undergo oxidation before completion of the folding process. We tentatively speculate that non-native disulphide bridges that may form before complete folding could themselves slow down the normal folding process described by Karplus and Weaver's model. Evidence for transient non-native disulphide bridging during the course of oxidation has been provided in the case of ribonuclease A [25]. This would ultimately be overcome by disulphide bridge reshuffling to finally yield normal folding and half-cystine pairings. Such a reshuffling could represent the delaying element observed in our kinetics. Therefore, we sought to mathematically modify the model of Karplus and Weaver to the case of sMTX. In particular, we replaced the exponential function $f(t)$ with a sigmoidal function $g(t)$ that better describes the experimental phenomena. This function is defined as follows:

$$g_j(t) = \frac{1}{1 + e^{(v_j - t)/S_j}}$$

The function is defined by the instant when the plot reaches 50% of its maximum (the half-life coefficient v_j) and its acceleration (the slope s_j). We then referred to the sigmoidal function as the basis for our model and we reproduced the plots of all L-sMTX species thanks to this elementary function. The sum of six sigmoidal functions ($j = 0, \dots, 5$) was introduced; one for each L-sMTX species (reduced, partly oxidized and native-like L-sMTX) and the last sigmoid to fit the plateaux observed for L-sMTX with three and four disulphide bridges. The mathematical function implemented to fit a plot for i disulphide bridges ($i = 0, \dots, 4$) for any given experimental condition (here and thereafter) was therefore:

$$P_i(t) = \sum_{j=0}^5 \frac{H_{i,j}}{1 + e^{\frac{v_j - t}{S_j}}} + B_i$$

We define for each probability $P_i(t)$ a constant term B_i , a slope s_j and a half-life coefficient v_j for each sigmoid j , and a height value $H_{i,j}$ for each couple (i, j) . Only part of the 30 $H_{i,j}$ coefficients were determined to lead to solvable calculations. The matricial formulation lets the common terms between all the plots appear:

$$P(t) = \begin{bmatrix} H_{0,0} \\ \cdot \\ \cdot \\ \cdot \\ H_{4,0} \end{bmatrix} g_0(t) + \dots + \begin{bmatrix} H_{0,5} \\ \cdot \\ \cdot \\ \cdot \\ H_{4,5} \end{bmatrix} g_5(t) + \begin{bmatrix} B_0 \\ \cdot \\ \cdot \\ \cdot \\ B_4 \end{bmatrix}$$

Table 1 Time course of appearance and disappearance of sMTX species during the oxidative folding of reduced L- or D-sMTX under various experimental conditions

These kinetic data result from theoretical fits through experimental values (see the Results and discussion section). $T_{50\% \downarrow}$, $T_{50\% \uparrow}$ and $T_{\max}$ values correspond, respectively, to times (expressed in h) of half-appearance, half-disappearance and maximum production of each alkylated sMTX intermediate. %_{max} corresponds to the maximum percentage of production of each sMTX intermediate under any given condition. Experimental buffer conditions were: buffer A, 0.2 M Tris/HCl, pH 8.3; buffer B, 0.2 M Tris/HCl, pH 7.0; buffer C, 0.2 M Tris/HCl, pH 5.0; buffer D, 0.4 M Tris/HCl, pH 8.3; buffer E, 0.01 M Tris/HCl, pH 8.3; buffer F, 0.05 M Tris/HCl/1.4 mM EDTA, pH 7.5. The L- and D-sMTX added in each oxidative folding assay (left-hand column) are in their reduced forms. U, units of enzyme activity; ND, not determined.

Kinetic parameters	0 S-S		1 S-S			2 S-S				3 S-S				4 S-S	
	$T_{50\% \downarrow}$		$T_{50\% \uparrow}$	$T_{\max}$	% _{max}	$T_{50\% \downarrow}$	$T_{50\% \uparrow}$	$T_{\max}$	% _{max}	$T_{50\% \downarrow}$	$T_{50\% \uparrow}$	$T_{\max}$	% _{max}	$T_{50\% \downarrow}$	$T_{100\%}$
Buffer A + 0.4 mM L-sMTX	0.6		0.5	1.5	38	5.1	1.5	3.6	30	10	5.1	12	29	27	8.7
Buffer A + 4 mM L-sMTX	0.6		0.6	2.0	40	7.0	0.8	3.0	40	22	2.0	4.0	40	22	23
Buffer A + 4 μ M L-sMTX	2.0		12	21	19	80	3.0	9.0	32	100	5.0	15	48	150	> 200
Buffer B + 0.4 mM L-sMTX	0.7		0.6	2.2	37	5.2	1.5	3.6	36	10	2.2	8.0	39	17	12
Buffer C + 0.4 mM L-sMTX	2.0		0.6	5.1	44	18	4.4	17	41	43	17	45	48	71	54
Buffer A (4 °C) + 0.4 mM L-sMTX	0.7		0.7	7.3	39	16	2.9	11	31	24	13	22	42	28	26
Buffer A (37 °C) + 0.4 mM L-sMTX	0.4		0.5	0.7	22	1.5	0.7	2.2	11	2.7	0.9	2	47	5.1	3.0
Buffer D + 0.4 mM L-sMTX	0.6		0.6	3.7	41	6.6	2.2	7.0	28	13	7.3	17	30	40	11
Buffer E + 0.4 mM L-sMTX	2.2		0.7	4.4	45	16	7.3	18	50	26	20	26	49	47	28
Buffer A + 0.4 mM L-sMTX + 1 mM GSH + 1 mM GSSG	0.5		0.4	0.7	31	16	4.4	21	28	ND	2.2	11	20	ND	ND
Buffer F + 0.1 mM L-sMTX	0.7		0.6	1.5	47	5.2	2.2	4.4	44	7.0	4.0	7.0	48	14	10
Buffer F + 0.1 mM L-sMTX + 10 U PDI	0.4		0.4	0.7	38	2.3	0.6	2.2	34	3.6	0.8	2.2	41	7.3	3.0
Buffer F + 0.1 mM L-sMTX + 0.5 mM PPlase	0.7		0.6	1.5	48	5.1	2.1	4.4	44	7.0	4.0	8.0	46	17	11
Buffer F + 0.1 mM L-sMTX + 10 U PDI + 0.5 mM PPlase	0.1		0.1	0.2	25	0.3	0.3	0.6	27	2.4	2.3	3.0	30	5	5.0
Buffer F + 0.1 mM D-sMTX	0.7		0.6	1.5	47	5.1	2.2	4.4	43.7	7.0	4.0	7.3	48	14	10.3
Buffer F + 0.1 mM D-sMTX + 10 U PDI	0.6		0.5	1.4	46.8	5.2	2.1	4.3	44.1	6.9	3.0	15	35	52	9.7
Buffer F + 0.1 mM D-sMTX + 0.5 mM PPlase	0.7		0.6	1.5	47.1	5.3	2.2	4.3	44	7.1	4.4	14.8	38	51	10.1
Buffer F + 0.1 mM D-sMTX + 10 U PDI + 0.5 mM PPlase	0.6		0.5	1.4	46.7	5.2	2.1	4.2	43.6	6.8	3.5	14.7	39	52	9.6

Figure 4 Parameters affecting *in vitro* oxidation/folding of 0.4 mM reduced L-SMTX

(a) Same as Figure 3(f) shown for comparison; the buffer used was 0.2 M Tris/HCl, pH 8.3, at 25 °C. (b) As in (a) but with the addition of 1 mM GSH/1 mM GSSG. (c) As in (a) except at 4 °C (left-hand panel) and 37 °C (right-hand panel). (d) pH-dependence at 25 °C, with buffer as in (a) except at pH 5.0 (left) and pH 7.0 (right). (e) Ionic-strength-dependence at 25 °C, with buffer as in (a) except for Tris/HCl concentrations of 10 mM (left) and 400 mM (right). All symbols are as in Figure 3.

This mathematical model permits a graphical representation of all the experimental data. With this equation, we can reproduce the exponential behaviour of any kinetic process, the latency period observed before the appearance of an intermediate L-sMTX species, and the plateau occurring at the final step of the oxidation/folding process. From this equation, we extracted significant values to allow data comparison: these include half- and maximal-appearance times, half-disappearance time and maximal percentage of each L-sMTX species. These data are compiled in Table 1.

Experimental parameters affecting oxidation/folding of reduced sMTX

Next, we tested a number of experimental conditions that were likely to affect oxidation/folding: redox potential, pH, temperature and ionic strength. As shown in Figure 4, all the parameters affect oxidation/folding to some extent as compared with the standard conditions (0.2 M Tris/HCl, pH 8.3; Figure 4a). First, we tested the effect of a mixture of 1 mM reduced and 1 mM oxidized glutathione (Figure 4b). The results obtained with glutathione were unexpected considering that it is used widely by many peptide chemists as a catalyst for the oxidation process (presumably by increasing the rate of thiol–thiol interchange) [26]. To some extent, glutathione behaves as a catalyst for disulphide-bridge formation since the kinetics of appearance of all oxidation intermediates were greatly accelerated (Table 1). However, the main flaw of using glutathione is that the oxidation process is incomplete with regard to the formation of L-sMTX with four disulphide bridges. For instance, less than 40% final L-sMTX was obtained after 72 h of oxidation. Furthermore, the L-sMTX intermediates with one, two and three disulphide bridges persisted in the milieu together with fully oxidized L-sMTX. Therefore, these experimental conditions are not recommended for the chemical synthesis of reticulated peptides. Similar results were also obtained by varying the ratios of reduced and oxidized glutathione in the oxidation milieu (e.g. 0.1 mM GSH/1 mM GSSG or 1 mM GSH/0.1 mM GSSG; results not shown).

Second, we tested the effect of temperature on oxidation/folding. We expected that the oxidation/folding reaction would be slowed or accelerated with decreasing or increasing temperature, respectively. Indeed, compared with our standard conditions (25 °C, Figure 4a), the oxidation/folding reaction was significantly slowed at 4 °C with a better separation between individual L-sMTX species at any given time (Figure 4c, left-hand panel). Of note was that three- and four-disulphide-bridged L-sMTX still formed plateaux at 14% and 86% of the total molecules, respectively, similar to standard conditions at 25 °C (Figure 4a). Because of the slowing in kinetics, the final conversion from three to four disulphide bridges was not observed by 72 h of oxidation. Conversely, increasing the temperature of oxidation to 37 °C markedly increased the oxidation/folding process. In this case, 100% fully oxidized L-sMTX was formed within 15 h of oxidation. Interestingly, there was no detectable plateau for either three- or four-disulphide-bridged L-sMTX, suggesting that the final interconversion between both molecules occurred much more rapidly with increased reaction temperature. One may suggest that the rate-limiting step represented by the prolyl imidic peptide bond *cis*-to-*trans* isomerization is an energy-dependent spontaneous event and that temperature significantly facilitates the transition between peptide-bond isomers. This is in agreement with the concept that Xaa-Pro bonds that are in the non-native geometry (*cis* configuration) in the unfolded state of a protein must overcome a ≈ 20 kcal/mol activation energy

barrier during folding for transition to the *trans* configuration [27].

Despite the fact that 37 °C represents an optimal temperature for L-sMTX oxidation/folding, the separation between the various L-sMTX species was not discriminatory enough to reliably study different experimental conditions. We therefore tested the effects of pH value, buffer ionic strength and enzyme addition in reaction milieu at 25 °C for a better comparative analysis with our standard conditions (Figure 4a). The effects of pH value on oxidation/folding are shown in Figure 4(d). In our standard conditions, the pH value was set at 8.3 to approach the pK_a value of thiol groups in cysteine residues. In theory, this corresponds to an optimal pH value since equivalent proportions of thiolate anions and thiol groups should be present, a condition presumably appropriate for efficient formation of disulphide bridges. First, we decreased the pH value to 7.0 (Figure 4d, left-hand panel). Except for slight variations in the kinetics of some L-sMTX species (two-disulphide-bridged L-sMTX), we did not notice any striking difference in kinetic behaviour for the formation of the four-disulphide-bridged L-sMTX (Table 1). This may stem from the fact that pK_a values of individual thiol groups from cysteine residues in L-sMTX may vary greatly as a function of the local chemical environment within L-sMTX and during folding [28]. For these reasons, we also tested an acidic pH value of 5.0 (Figure 4d, right-hand panel), which initially was not expected to allow productive disulphide bridging. Contrary to our expectations, we still observed oxidation of reduced L-sMTX at pH 5.0 albeit at a reduced rate (Table 1). At 72 h, 75% fully oxidized L-sMTX was obtained with only the three-disulphide-bridged L-sMTX still detectable in the oxidation milieu.

Another physico-chemical condition that was tested on L-sMTX oxidation/folding was buffer ionic strength (Figure 4e). Compared with standard conditions (0.2 M Tris/HCl, pH 8.3), reducing the ionic strength with 10 mM Tris/HCl (pH 8.3) resulted in a lower oxidation/folding rate, whereas a higher ionic strength (400 mM) increased the observed rate (Table 1). Salts can affect the peptide oxidation/folding process by (i) weakening electrostatic interactions through charge screening [29] (which is generally ion-type-independent but ionic-strength-dependent) and (ii) changing the peptide hydration state by acting mainly on hydrophobic interactions [30]. Such an effect of ionic strength has been reported for the oxidative folding of RNase A with differences in kinetics observed when using either kosmotropes or chaotropic salts [29]. We have also tested the effect of reduced L-sMTX concentration (4 μ M–4 mM) on the kinetics of oxidation/folding and found marked differences. Notably, formation of 100% four-disulphide-bridged L-sMTX takes 65 h under standard oxidative conditions (0.4 mM reduced L-sMTX), whereas it takes about 35 h and an estimated 200 h with concentrations of 4 mM and 4 μ M, respectively (Table 1). These data indicate that increasing thiol density results in a faster oxidation of the molecule, presumably by accelerating thiol–thiol interchange and reshuffling of disulphide bonds. In contrast to the addition of a mixture of reduced/oxidized glutathione, which reportedly increases thiol–thiol interchange, elevating reduced toxin concentration at the start of the oxidation/folding process produces a greater (100% versus 47%) proportion of final L-sMTX (results not shown). This observation is of practical interest to the peptide chemist dealing with the synthesis of reticulated peptides since highly diluted reduced peptide concentrations (below the micromolar range) are generally used for the oxidation/folding of these peptides [31].

Our experimental data do not discriminate between the folding of the molecule and the oxidation process itself. Formerly, these

Figure 5 Stereo-selective effects of cellular enzymes (PDI or PPase) on *in vitro* oxidation/folding of reduced sMTX

(a and b) Comparative oxidation/folding of 0.1 mM reduced L-sMTX (a) and D-sMTX (b) in standard enzyme-active conditions (50 mM Tris/HCl/1.4 mM EDTA, pH 7.5, at 25 °C). (c and d) Effect of 50 units/ml PDI on oxidation/folding of 0.1 mM reduced L-sMTX (c) and D-sMTX (d). (e and f) Effect of 0.5 mM PPase on oxidation/folding of 0.1 mM reduced L-sMTX (e) and D-sMTX (f). All symbols are as in Figure 3.

Figure 6 Stereo-selective effects of a mixture of PDI and PPIase on *in vitro* oxidation/folding of reduced sMTX

(a and b) Comparative oxidation/folding of 0.1 mM reduced L-sMTX (a) and D-sMTX (b) in the presence of 50 units/ml PDI and 0.5 mM PPIase. Buffer and temperature conditions were as in Figure 5(a); all symbols are as in Figure 3.

MS-based data could only be interpreted in terms of the oxidation process, although one can assume that with completion of the final oxidation step (i.e. formation of the fourth disulphide bridge) the folding process is completed. Also, due to the fact that both processes are intimately linked to each other, parameters that affect folding of L-sMTX should also affect oxidation. For instance, most parameters tested (such as temperature and pH value) are likely to affect both folding and oxidation. In contrast, buffer ionic strength is more likely to primarily affect folding and, indeed, a concomitant change in the kinetics of oxidation of reduced L-sMTX was observed. Conversely, concentration of reduced toxin and the presence of a glutathione-based redox mixture are more likely to affect oxidation first. In any case, the process of oxidation is obviously affected by all these parameters. We expect that a combination of favourable parameters that optimize both folding and oxidation would improve yields of the chemical synthesis of reticulated peptides.

There are at least two types of cellular enzyme present in the endoplasmic reticulum whose function is to contribute to the folding and oxidation of proteins. PDI is reported to catalyse the breakage and re-formation of disulphide bridges [26,32]. By promoting rapid exchanges in half-cystine pairings, PDI helps the protein to reach the disulphide bridge pattern that is the most compatible with its stably folded conformation. The second enzyme of interest is PPIase, which favours the isomerization between the *cis* and *trans* configurations of prolyl imidic peptide bonds; a rate-limiting step in protein folding [33,34]. As both enzymes seemed of interest in our case, we tested their effects on the oxidation/folding of L-sMTX. The action of these enzymes being stereo-selective, we also tested their effects on the oxidation/folding of D-sMTX. Figure 5(a) illustrates the oxidation/folding of reduced L-sMTX with slightly different control-buffer conditions (50 mM Tris/HCl/1.4 mM EDTA, pH 7.5), optimized for enzyme activity. We first studied the oxidation/folding of reduced D-sMTX (Figure 5b). Interestingly, we found that the kinetics of oxidation/folding of this

stereoisomer were similar to that of L-sMTX. For instance, the times of half-appearance of fully oxidized L-sMTX or D-sMTX were 10.0 and 10.3 h, respectively (Table 1). Similar results were obtained under the standard conditions as defined in Figure 4(a), i.e. (200 mM Tris/HCl, pH 8.3, 25 °C; results not shown). These data strongly suggest that both peptides oxidized/folded according to a similar pattern of sequential molecular events.

The effect of 10 units of PDI on the oxidation/folding of L-sMTX and D-sMTX are shown in Figures 5(c) and 5(d). PDI markedly increased the rate of oxidation/folding of L-sMTX, whereas it had no significant effect on D-sMTX. Half-appearance of the four-disulphide-bridged L-sMTX was 3.0 h with PDI, instead of 10.0 h in its absence (Table 1). In contrast, the values observed for the D-sMTX were 9.7 and 10.3 h in the presence and absence of PDI, respectively. These data therefore represent the first experimental evidence of the effects of PDI and stereo-selectivity on the oxidation/folding of a peptide toxin *in vitro*. Next, the effects of PPIase on the oxidation/folding of both stereoisomers were investigated. We expected an effect of this enzyme on the occurrence of plateaux, observed at 13 and 87% of total molecules, for the three- and four-disulphide-bridged L-sMTX, respectively. A disappearance of these plateaux is expected if they are indeed associated with *cis-trans* isomerization of the prolyl imidic peptide bonds [19,20]. Whereas the overall kinetics of oxidation/folding of L-sMTX remained similar in the absence and presence of 0.5 mM PPIase, we noticed that both plateaux, normally associated with three- and four-disulphide-bridged L-sMTX species, were lacking in the presence of the enzyme (Figure 5e). Again, this enzyme acted in a stereo-selective manner since it had no effect on the oxidation/folding of reduced D-sMTX (Figure 5f). Together, the data indicate that the plateaux we observed are indeed associated with *cis-to-trans* isomerization of prolyl imidic peptide bond(s).

Next, we tested a combination of both enzymes (PDI and PPIase) to determine whether their effects were additive or not. As shown in Figure 6(a), a mixture of PDI and PPIase produced

both a marked increase in the oxidation/folding of L-sMTX and a disappearance of the usual plateaux. These conditions for oxidation/folding appear to have been optimal among those tested since 100% native-like L-sMTX was formed within 10 h. As expected, the mixture of enzymes was without effect on the oxidation/folding of D-sMTX (Figure 6b).

Concluding remarks

Here, for the first time, we have used a MALDI-TOF MS technique to investigate the kinetics and process of *in vitro* oxidation/folding of reduced L-sMTX or D-sMTX by varying one of a number of experimental parameters. The data indicate that there is temperature-, pH-, ionic strength-, redox-, peptide concentration- and enzyme-dependence of the kinetics of peptide oxidation. Except when stereo-selective enzymes were used, the effects of the different parameters were indistinguishable for L-sMTX and D-sMTX. The oxidation intermediates were found to appear sequentially over time, from the least (one disulphide bridge) to the most oxidized species (native-like sMTX with four disulphide bridges). The most efficient oxidation/folding milieu tested was found to be 50 mM Tris/HCl/1.4 mM EDTA, pH 7.5, supplemented with 0.5 mM PPIase and 50 units/ml PDI, at a peptide concentration of 0.1 mM. These findings provide new insights into the oxidation/folding of reticulated peptides that should be of general interest for peptide chemists.

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