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Effect of Cu^{2+} on the Oxidative Folding of Synthetic Maurotoxin *in Vitro*

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Running title: Cu^{2+} SPEEDS UP MAUROTOXIN OXIDATIVE FOLDING

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Abbreviations

Fmoc, Nα-(9-fluorenyl)methyloxycarbonyl;

HPLC, high pressure liquid chromatography;

IKCa, intermediate-conductance Ca^{2+}-activated K^+ channel;

Kv, voltage-gated K^+ channels;

MTX, synthetic maurotoxin, a scorpion toxin from *Scorpio maurus* palmatus venom;

MALDI-TOF, matrix-assisted laser desorption ionization-time of flight;

MS, mass spectrometry;

SKCa, small-conductance Ca^{2+}-activated K^+ channel.

Abstract:

Maurotoxin (MTX) is a 34-mer scorpion toxin cross-linked by four disulphide bridges that acts on various K^+ channel types. It folds according to an α/β scaffold, i.e. a helix connected to a two stranded β-sheet by two disulphide bridges. In a former study, various parameters that affect the oxidation and folding of the reduced form of synthetic MTX were investigated *in vitro*. It was found that MTX achieves its final 3-D structure by evolving over time through a series of oxidation intermediates, from the least to the most oxidised species. MTX oxidative intermediates can be studied by iodoacetamide alkylation of free cysteine residues followed by mass spectrometry analysis. Here, we have analysed the effect of Cu^{2+} on the kinetics of MTX oxidative folding and found that it dramatically speeds up the formation of the four-disulphide bridged, native-like, MTX (maximal production within 30 minutes instead of > 60 hours). Cu^{2+} was also found to prevent the slow transition of a three disulphide-bridged MTX intermediate towards the final four disulphide-bridged product (12% of total MTX). The data are discussed in light of the potential effects of Cu^{2+} on MTX secondary structure formation, disulphide bridging and peptidyl prolyl *cis-trans* isomerisation.

Keywords: scorpion toxin, maurotoxin, oxidative folding, mass spectrometry, disulphide bridging, Cu^{2+}.

Introduction

Maurotoxin (MTX) is a scorpion toxin isolated from the Tunisian *chactidae Scorpio maurus palmatus* [1-3]. This basic 34-residue peptide (amino acid sequence: $VSC_1TGSKDC_2YAPC_3RKQTGC_4PNAKC_5INKSC_6KC7YGC_8$-$NH_2$) is cross-linked according to a C1-C5, C2-C6, C3-C4 and C7-C8 pattern of disulfide bridging [1] and adopts the classical α/β scaffold of most scorpion toxins [4]. The 3-D solution structure of MTX has been solved [2]; it is composed of a bent α-helix (residues 6 to 17) connected by a loop to a two-stranded antiparallel β-sheet (residues 22 to 25 and 28 to 31). This toxin exhibits a wide range of pharmacological activity since it potently blocks voltage-gated Kv channels (*Shaker* B and Kv1.2), small- (SKCa) and intermediate-conductance (IKCa) Ca^{2+}-activated K^+ channels [1,3,5,6]. Previous studies have evidenced the relationship that exists between disulphide bridging, 3D-structure and spatial distribution of toxin amino acid residues key to pharmacology [6-8]. To gain its full pharmacological activity, the reduced form of chemically produced MTX needs to undergo a full process of folding and oxidation *in vitro* [9,10]. Small but highly reticulated peptides, such as MTX, possess ideal characteristics to investigate peptide folding and oxidation. First, large quantities of MTX can be obtained by chemical synthesis. Second, chemically produced MTX can be directly recovered in its reduced and unfolded state after final acidolytic treatment. It requires an appropriate folding/oxidation buffer to reach its biologically active 3-D structure. In a classical oxidative buffer (e.g. air-exposed 0.2 M Tris/HCl buffer, pH 8.3), oxidation of MTX *in vitro* has a slow time scale, which allows for a detailed kinetic analysis of its incremental reticulation [9,10]. In previous studies, we were able to monitor the sequential formation of each MTX oxidation intermediates, from the less (1 disulphide-bridged MTX species) to the most oxidised form (native-like, 4

disulfide-bridged MTX) [9,10]. This was achieved by iodoacetamide alkylation of free cysteine residues (not engaged in disulphide bridges) of the reduced form and oxidation intermediates of MTX. This treatment allowed one to discriminate between the various MTX oxidation intermediates using mass spectrometry (MS) analysis.

Various parameters were evaluated such as temperature, pH, ionic strength, redox potential, presence or not of some enzymes (protein disulphide isomerase (PDI) or peptidylprolyl *cis-trans* isomerase (PPIase)), that all affected to some extent MTX folding and oxidation. The best experimental conditions allowed for the formation of 100% of four-disulphide-bridged MTX in ca. 10 h and relied on the combined use of PDI and PPIase [9]. Addition of PPIase to the oxidation buffer was found to be particularly relevant to favour isomerisation between the *cis* and *trans* configurations of prolyl imidic peptide bonds; a rate limiting step in protein folding [11,12]. In approximately 85% of the cases, the two prolyl imidic peptide bonds of MTX (CO-N bonds between residues 11-12 and 19-20) are in a favourable configuration (presumably *trans*) for the formation of the four disulphide-bridged MTX, whereas the remaining 15% allows for the formation of (a) three disulphide-bridged intermediate(s) only. The conversion between the three-disulphide-bridged intermediate(s) towards the four disulphide-bridged end product is rate-limiting and shown to be PPIase-sensitive [9]. Whether this last step implies reshuffling of disulphide connections among the three-disulphide-bridged intermediate(s) remains an open question.

From previous data, one may suggest the existence of a complex interplay between folding and oxidation of MTX, which was evidenced during the progressive acquisition of its stabilised secondary structures and disulphide bridges. In particular, the helical structure appears at the same time than the one disulphide-bridged species

of MTX, whereas the β-sheet structure appears later on when the two- or three-disulphide-bridged MTX intermediates are detected [10]. With the aim of accelerating the process of MTX oxidative folding, we searched for an agent that has the capability to speed up peptide folding and oxidation, such as a redox-active metal. Because copper ions easily cycle between Cu^{2+} and Cu^{+}, copper complexes could participate in non-enzymatic redox processes. Indeed, copper behaves as a potent oxidant of thiol groups [13] and is beneficial for both protein folding [14-19] and oxidation [13,20]. We therefore investigated the potential effects of copper ions on the oxidative folding of reduced MTX *in vitro*. Since both processes of folding and oxidation of MTX are linked, addition of copper ions in the oxidation milieu is expected to increase the rate of formation of highly reticulated MTX species.

Materials and Methods

Materials. N-α-Fmoc-L-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). Iodoacetamide and copper ions were obtained from Sigma (St. Louis, MO, U.S.A.).

Chemical synthesis and characterisation of MTX. The peptide was obtained by the solid-phase method using a peptide synthesiser (Model 433A, Applied Biosystems Inc.) as previously described [1]. Briefly, the 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 activated as their hydroxybenzotriazole active esters. The reduced MTX was purified to > 99% homogeneity by reversed-phase high-pressure liquid chromatography (HPLC) (Perkin-Elmer Life Sciences, C_{18} Aquapore ODS 20 μm, 250 mm x 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/H_2O at a flow rate of 2 ml/min (λ = 230 nm). The homogeneity and identity of reduced MTX was assessed by: (i) analytical C_{18} reversed-phase HPLC, (ii) amino acid analysis, (iii) Edman sequencing and (iv) MS analysis. After lyophilisation of the purified reduced MTX, its fully reduced state was verified by iodoacetamide-based alkylation of the eight free cysteine residues, analytical C_{18} reversed-phase HPLC, and MS analysis.

MS analyses of samples after alkylation of the free thiol groups of MTX during its process of oxidative folding. The lyophilized reduced MTX was dissolved in 100 µl of oxidation buffer (200 mM Tris/HCl buffer, pH 8.3 with or without 10 mM $CuCl_2$) at 0.4 mM peptide concentration and exposed to air to allow oxidation. Samples of 5 µl were alkylated by using 5 µl of a 85 mM iodoacetamide solution for 30 s at 25°C. The MS analyses were carried out in the linear mode using a 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 samples (1 µl) were placed on a 100-well plate, and 1 µl of matrix solution was added. This mixture was dried prior to MS analysis. For each condition assay, samples were collected at different times (0-80 h), and the peptide species were analysed by their respective masses after 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). The accuracy of the relative proportions between oxidized and reduced MTX species was demonstrated using control samples in which mixtures of known quantities of purified peptides were preformed (see [9]).

Results and Discussion

Analysis by MALDI-TOF MS of the oxidation process of reduced MTX in vitro

The reduced MTX was obtained by solid-phase peptide synthesis using Fmoc/t-butyl chemistry [9,21]. After cleavage from the solid support, the reduced MTX was purified to homogeneity (> 99%) by means of C18 phase-reversed HPLC in acidic conditions (pH 2.5) to prevent oxidation. The reduced state of MTX was further verified by MALDI-TOF MS immediately after solubilisation in oxidation buffer (0.2 M Tris/HCl, pH 8.3) and alkylation of free cysteine residues, as described in *Materials and Methods*.

The Tris/HCl oxidation buffer (0.2 M Tris/ HCl, pH 8.3) is commonly used for an oxidative folding of reticulated peptides. This buffer condition was therefore used to investigate the oxidation process of reduced MTX. Studying the oxidation process of reduced MTX by MALDI-TOF MS poses two problems that need to be overcome. First, because MTX species differ at most by 8 Da (reduced *versus* fully oxidised MTX), the MS technique is not discriminative enough to allow an adequate separation and accurate quantification of each of its species. The use of iodoacetamide alkylation, which adds 57 Da to each free thiol group, permits a much better peak separation and quantification. In this case, each MTX species is separated from the closest one by 114 Da, instead of 2 Da in the absence of alkylation. Second, although the MALDI-TOF MS is not always considered as a quantitative technique, we previously demonstrated that it could be used to quantify properly the various MTX species [9,10]. The kinetics of disappearance of reduced MTX and appearance of the oxidized MTX intermediates, including the fully oxidized form, were followed over time by MS analysis after alkylation of the remaining free thiol groups. As shown in

Figure 1, reduced MTX disappears within 10 h of folding/oxidation to convert to oxidised species. The one-, two-, three- and four-disulphide bridged MTX species appear sequentially over time from the least (intermediates with one disulphide bridge) to the most oxidised species (native-like, four-disulphide-bridged MTX). Maximal productions of these MTX intermediates are 2.5 h (one-disulphide-bridged), 4.5 h (two-disulphide-bridged), 11 h (three-disulphide-bridged) and 65 h (four-disulphide-bridged). Of note, plateaux of disappearances (two- and three-disulphide-bridged MTX) and appearance (four-disulphide-bridged MTX) of oxidation intermediates are observed. As previously demonstrated, these plateaux correspond to the rate-limiting isomerisation from *cis* to *trans* of peptidylprolyl bonds [9]. Noteworthy, all reduced molecules are converted to the fully oxidised, native-like, bioactive form of MTX at the end of the oxidation process.

Effect of copper ions on the kinetics of oxidation of reduced MTX in vitro

Copper ions (Cu^{2+}) have been described to markedly speed up the oxidative folding of peptides [13-20]. When they are added (at 10 mM concentration) to the oxidation buffer, the kinetics of oxidative folding is dramatically increased (Figure 2). The process is so fast that we were unable to monitor the disappearance of reduced MTX, as well as the appearance and disappearance of the one- and two-disulphide-bridged MTX species. This suggests that these oxidation intermediates have very short half-lives. Indeed, the reduced MTX apparently evolves directly towards either three-disulphide-bridged intermediate(s) (12%) or the four-disulphide-bridged, native-like, MTX (88%). A maximal production of these two species is obtained in ca. 30 min *in vitro*, indicating that the addition of copper ions increases the kinetics of oxidation by a factor of 22- (three-disulphide-bridged MTX) to 130-fold (fully

oxidised MTX). These plateaux of three- and four-disulphide-bridged MTX species remain stable up to 80 h. Therefore, no interconversion between both species could be detected. Interestingly, the plateau level (12%) of the three-disulphide-bridged MTX in the presence of copper ions is identical to the long-lasting plateau level observed in the absence of copper ions (Figure 1). This is coherent with the *cis* configuration of Xaa-Pro bonds, which represents ca. 10% of the total Xaa-Pro bonds in proteins [22]. Although copper ions dramatically increase the kinetics of MTX oxidative folding, one drawback of its use appears to be the apparent lack of full interconversion of 12% of the three-disulfide-bridged species towards the final MTX product. Characterisation of the four-disulphide-bridged MTX reveals that it is indistinguishable from its native counterpart both in the absence or presence of copper ions (data not shown).

Concluding remarks

Oxidation of reduced MTX is intimately linked to its folding. In other terms, the correct connection pattern between half-cystine residues requires also the proper folding of MTX, in this case the acquisition of its helical and β-sheet structures that form an α/β-scaffold. It would be expected that if folding occurs rapidly with the adequate positioning of toxin secondary structures, then the oxidation process would also proceed rapidly, allowing the final native fold to be stabilised early. However, the folding/oxidation pathway is probably more complex and it is likely that the peptide undergoes a series of "trial/error" steps by following a downhill favourable energetic slope (the low-energy conformation being favoured). Also, some of the folding steps are slow ones, such as the peptidylprolyl *cis-trans* isomerisation. It is therefore conceivable that the formation of all four native-like disulphide bridges of

MTX requires the occurrence of *cis* to *trans* isomerisation. Non-native-like disulphide bridges likely undergo a process of reshuffling that would progressively yield native-like disulphide bridging and maintain the most favourable energetic state of MTX conformation. It has been shown that molecules are greatly aided by chaperone proteins in their search for a bioactive conformation. In the case of MTX, catalytic activities of PDI and PPIase have been evidenced [9]. Previous reports hint that copper ions may play a similar role by favouring the acquisition of secondary structures and by catalysing disulphide bridging [12-20]. Our data show that copper ions do indeed catalyse formation of MTX disulphide bridges. By favouring disulphide bridge formation, copper ions also seem to lock MTX in two stable oxidation states, i.e. three- and four-disulphide-bridged species. The use of copper ions prevents the slow interconversion between some of the three-disulphide bridged MTX species (12% of the total species that are likely to possess one or two *cis* configurations of Xaa-Pro bonds) towards the four-disulphide-bridged MTX. The end products obtained with copper ions in oxidation buffer appear to represent "naturally" occurring conformational states of MTX, as the same are present in the absence of copper ions, albeit produced with slower kinetics. From the data, we speculate that once MTX disulphide bridges are formed in the presence of copper ions, reshuffling is prevented. This could possibly block the final conversion of the remaining 12% three-disulphide-bridged molecules towards fully oxidised MTX, if reshuffling is concerned. Alternatively, copper ions may prevent the *cis* to *trans* isomerisation of peptidylprolyl bonds by stabilising some structural elements, and possibly secondary structures. This effect would not be detected for one- and two-disulphide-bridged species since, at this stage, MTX is in the process of acquiring its stabilised secondary structures [10]. Finally, it was shown that metal ions like copper could act on the *cis*

to *trans* isomerisation equilibrium of proline residues in peptides [23]. Copper has been reported to bind on the ionised amide nitrogen, which inhibits the *trans* configuration of the Xaa-Pro bond. It is therefore possible that copper ions stabilise the *cis* configuration of the Xaa-Pro bond. The results obtained with copper ions on the oxidative folding of reduced MTX *in vitro* suggest that this method could be beneficial to peptide folding/oxidation, and of general interest to the peptide chemist dealing with the chemical production of reticulated peptides.

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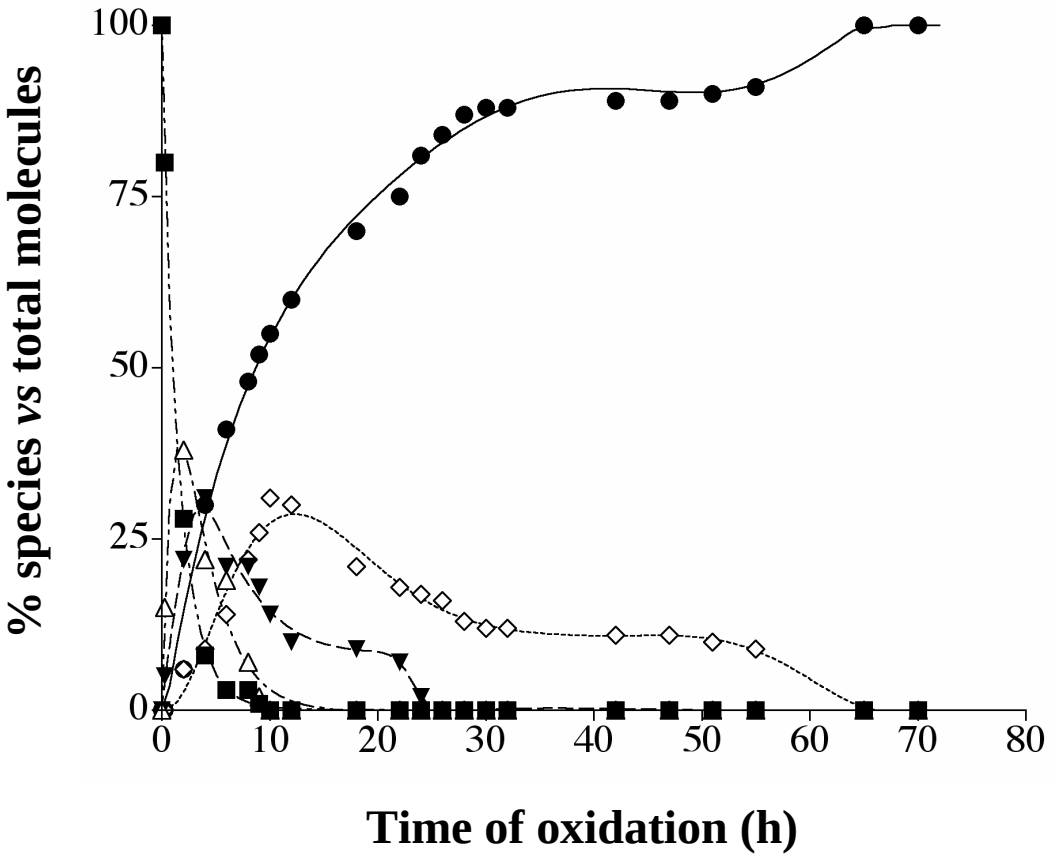
Figure legends

Figure 1 Evolution of MTX oxidation in relative percentages of the various MTX species as a function of time of oxidative folding, as assessed by MALDI-TOF MS. MTX species from the samples were Cys-alkylated by iodoacetamide prior to MS analyses. The oxidation process was started at 25°C with 0.4 mM reduced MTX in oxidation buffer without copper ions. Symbols: filled squares (reduced MTX, thus without any disulphide bridge (0 S-S)); open triangles (one-disulphide-bridged MTX species (1 S-S)); filled inverted triangles (two-disulphide-bridged MTX species (2 S-S)); open diamonds (three-disulphide-bridged MTX species (3 S-S)); and filled circles (native like, four-disulphide-bridged MTX (4 S-S)).

Figure 2 Effect of copper ions (Cu^{2+}) on the evolution of MTX oxidation over time. Same experimental conditions as in Figure 1, except that 10 mM $CuCl_2$ was added to the oxidation buffer.


% species *vs* total molecules
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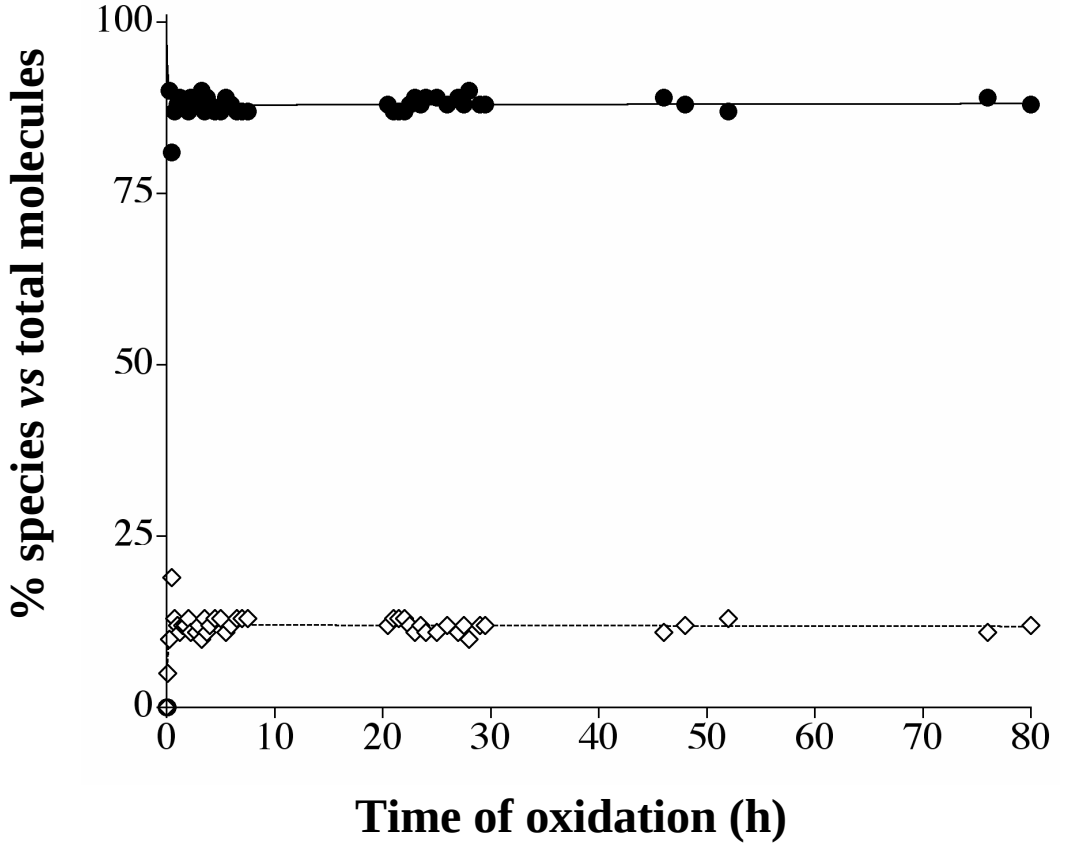
75
50
25
0
0
10
20
30
40
50
60
70
80
Time of oxidation (h)


% species *vs* total molecules
100
75
50
25
0
0
10
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50
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70
80
Time of oxidation (h)