A particularly labile Asp–Pro bond in the green mamba muscarinic toxin MTX2. Effect of protein conformation on the rate of cleavage

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Abstract The single Asp^{53}–Pro^{54} bond of the MTX2 toxin from the mamba snake *Dendroaspis angusticeps* is rapidly and efficiently cleaved in acidic solution (pH 1.5–2.5) at 45°C. Unfolding of the toxin slows down the cleavage reaction by several times. Modelling studies indicate that the native toxin conformation can catalyse the Asp^{53}–Pro^{54} bond cleavage. The implications of this study are: (i) cleavage of Asp–Pro bond for sequence determination may occur better in absence than in presence of denaturant, (ii) mild acid conditions, commonly used in NMR structure determinations, may irreversibly affect the structural integrity of Asp–Pro containing peptides and proteins.

Key words: Peptide bond cleavage; Asp–Pro bond; Protein conformation; Protein degradation; Peptide fragment; Muscarinic toxin

1. Introduction

Muscarinic toxins from snake mamba venoms are the only protein toxins known to bind to the muscarinic acetylcholine receptors (mAChR) [1–2]. Five such toxins (MTX1, MTX2, MTX3, MTX4 and m1-toxin) are now know from the green mamba *Dendroaspis angusticeps* [1–4] and two (MTα and MTβ) [1] from the black mamba *Dendroaspis polylepis*. Some of these toxins act selectively and irreversibly on individual subtypes of receptor, and some are antagonists while other activate the receptors.

The MTX2 toxin is one of the first isolated muscarinic toxins [3]. It was shown to be selective for the M1 pharmacological subtype of mAChR [2] and to act as an agonist in an inhibitory avoidance task on rats [5]. MTX2 has been sequenced by both conventional Edman degradation [6] and cDNA nucleotide sequencing [7]: it contains 65 amino acids (Fig. 1) and eight cysteines, forming four disulfide bridges. Its three-dimensional structure has been recently solved in solution by using ^{1}H-NMR and molecular modelling calculations [8] and shows a three-loop folding, as found in other snake toxins such as neurotoxins, cardiotoxins or fasciculins [9–11].

Different reports have pointed out the instability of the MTX2 protein: stockage at –20°C [1] and also storage in solution at pH below 6 [3] have been shown to inactivate the toxin. MTX2 contains six aspartyl bonds known to be acid sensitive, and in particular it contains the sequence Asp–Pro in position 53–54. Asp–Pro peptide bonds are known to be cleaved at low pH values, under conditions where other aspartyl bonds are stable [12–14], and cleavage of these bonds are used to produce large fragments useful for sequence determination. However, Asp–Pro bond cleavage requires strong denaturing conditions to occur and even in these conditions cleavage yields are generally poor [13].

In order to better understand the reasons of the apparent structural fragility of MTX2 and to find conditions which could preserve the integrity of the protein, we investigated the stability of this toxin in acid solutions by circular dichroism (CD) spectroscopy, high-performance liquid chromatography (HPLC) and capillary electrophoresis (CZE).

2. Materials and methods

2.1. Materials

The muscarinic MTX2 toxin was purified from the snake *Dendroaspis angusticeps* venom according to the published procedure [3]. The purity and identity of the toxin was checked by gel-electrophoresis, amino acid analysis and mass spectrometry. The reduced pyridylethylated (PE) form was prepared by 4-vinyl-pyridine alkylation after dithiotreitol reduction of the toxin denaturated in 6 M guanidine·HCl [15]. Analytical standard chemicals were from Merck and Sigma.

2.2. Chemical characterisation

Mass determination was performed on a Nermag R10-10 mass spectrometer, coupled to an Analitica of Bradford electrospray source.

Protein samples were hydrolyzed in evacuated, sealed tubes with 6 N HCl at 120°C for 16 h. The hydrolysates were analyzed on an Applied Biosystems model 130A automatic analyzer equipped with an on-line 420A derivatizer, for the conversion of the free amino acids into their phenylthiocarbamoyl derivatives.

Edman degradation was performed on an Applied Biosystems 477A sequencer equipped with an on-line ABI 120A phenylthiohydantoin (PTH) amino acid analyzer.

2.3. Chromatography and electrophoresis

All HPLC analyses were performed on a Spectra-Physics HPLC System, consisting of a binary P2000 pump, a UV2000 detector and a Chromjet recorder, by using a Vydac 218TP5415 column eluted at a 1.0 ml/min flow rate and with a gradient formed by solvent A (water containing 0.1% TFA) and solvent B (90% acetonitrile containing 0.09% TFA). The eluent was monitored at 214 nm.

CZE was carried out with an Applied Biosystems model 270A system. Separations were performed on a 50 cm silica capillary with 20 mM sodium phosphate buffer, pH 2.6 and detection at 200 nm.

2.4. Spectrometric measurements

Protein concentration was determined spectrophotometrically using the molar extinction coefficient of 9300 $M^{-1} \cdot cm^{-1}$ at 276 nm for the oxydized MTX2 [3]. The far-UV circular dichroism (CD) spectra were recorded at room temperature in 1 mm quartz cuvette with a protein concentration of 2×10^{-5} M on a Jobin-Yvon CD6 Dichrograph cali-

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Abbreviations: CZE, capillary zone electrophoresis; MTX2, *Dendroaspis angusticeps* muscarinic toxin 2; PE-MTX2, pyridine-ethylated MTX2.

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1 5 10 15 20 25 30
L T C V T T S I G G V T T E D C P A G Q N V C F K R W H Y V T P K

35 40 45 50 55 60 65
N Y D I I K G C A A T C P K V D N N D P I R C C G T D K C N D

Fig. 1. Amino acid sequence (single letter code) of MTX2 from *Dendroaspis angusticeps* venom.

brated with (+)-camphor-10-sulfonic acid. Each spectrum represents the average of four runs, obtained with an integration time of 0.5 s every 0.2 nm. Spectra were smoothed using the instrument's software by taking a sliding average over nine data points.

2.5. Kinetics experiments

For the oxydized MTX2 form, kinetic analyses of Asp–Pro cleavage were determined by incubating at 45°C 50 μg of toxin in 50 μl of buffered 0.2 M glycine · HCl solutions, with pH values varying between 1.5 and 5.0. Denaturant conditions were obtained by using 6 M guanidine · HCl in either 10% acetic acid or 0.2 M glycine · HCl buffered at pH 2.0. For the reduced form, analyses were performed by incubating the pyridylethylated toxin in 0.2 M glycine · HCl, pH 2.0, 45°C. At different times, 2 μg aliquots were directly analyzed by HPLC and CZE.

2.6. Structural analysis

To obtain a possible 'productive' conformation for Asp^{53}–Pro^{54} cleavage, the energy minimized average structure determined by NMR data and molecular modelling [8], has been used as a template and $\psi = -120°$ and $\chi_1 = +120°$ was imposed on the Asp^{53} residue (see section 4). The structure was then minimized using the Sybyl force field on an Evans and Sutherland PS 390 system, with Asp^{53}–Pro^{54} defined as the interesting region and a hot radius of 2 Å.

3. Results

CD spectroscopy was used to test the conformational stability of MTX2 toxin at acidic pH: the far-UV CD spectra recorded at pH 6.0 and 2.5 were superimposable (Fig. 2A), indicating that the secondary structure of the toxin is not significantly affected by the acidic environment, a property also common to other well-known snake curaremimetic neurotoxins, with which this toxin share a common three-loop β-sheet structure [8]. Even after 7 days incubation at 45°C, no measurable spectral variations were detected at pH 6.0 (Fig. 2B), while only a slight reduction in ellipticity was observed at pH 2.5 (Fig. 2B), indicating that no apparent gross structural change occurred in these conditions.

In contrast with these data, when the pH 2.5 solution was analyzed by HPLC, the toxin clearly resulted modified. To study this modification, MTX2 was incubated at 45°C and at different acidic pHs, between 1.5 and 5.0, and at different times these solutions were analyzed by HPLC and CZE. As shown in Fig. 3A-B, at pH 2,0 a modified species, lately eluting in RP-HPLC and early eluting in CZE, is already apparent after 1 h. This molecular form represents about 50% of the total protein after 6–7 h and is the major product after 24 h. The modified product, was purified by HPLC and analyzed by amino acid composition and sequencing. Four cycles of Edman degradation showed that the modified species contained, besides the original N-terminus, a new one, i.e. Pro-Ile-Arg-Cys, corresponding to the sequence 54–57. No other species was detected. Amino acid analysis (not shown) of the modified product before and after reduction, cysteine pyridine-ethylation and HPLC purification confirmed the sequence data and demonstrated that the new species was formed by two MTX2 fragments, 1–53 and 54–65, linked together by disulfide bonds and produced by Asp^{53}–Pro^{54} cleavage.

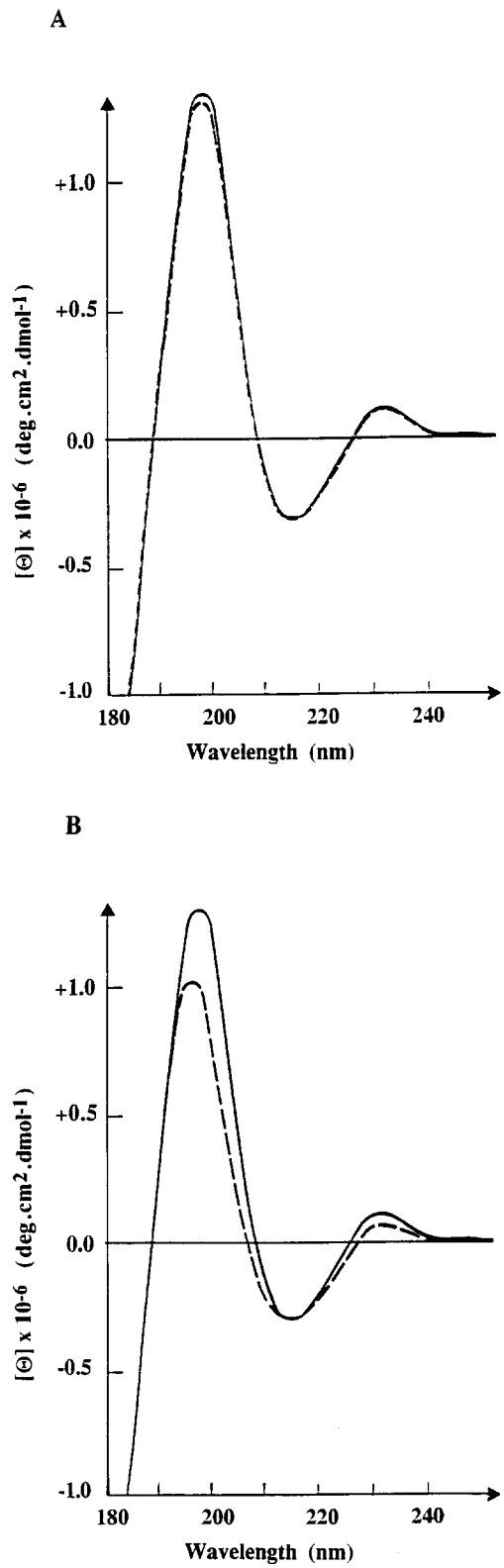

Fig. 2. Far-UV circular dichroism spectra of MTX2. (A) Spectra recorded with solutions at pH 6.0 (——) and pH 2.5 (- - -). (B) Spectra recorded after incubation at 45°C and at pH 6.0 (——) or pH 2.5 (- - -) for 7 days.

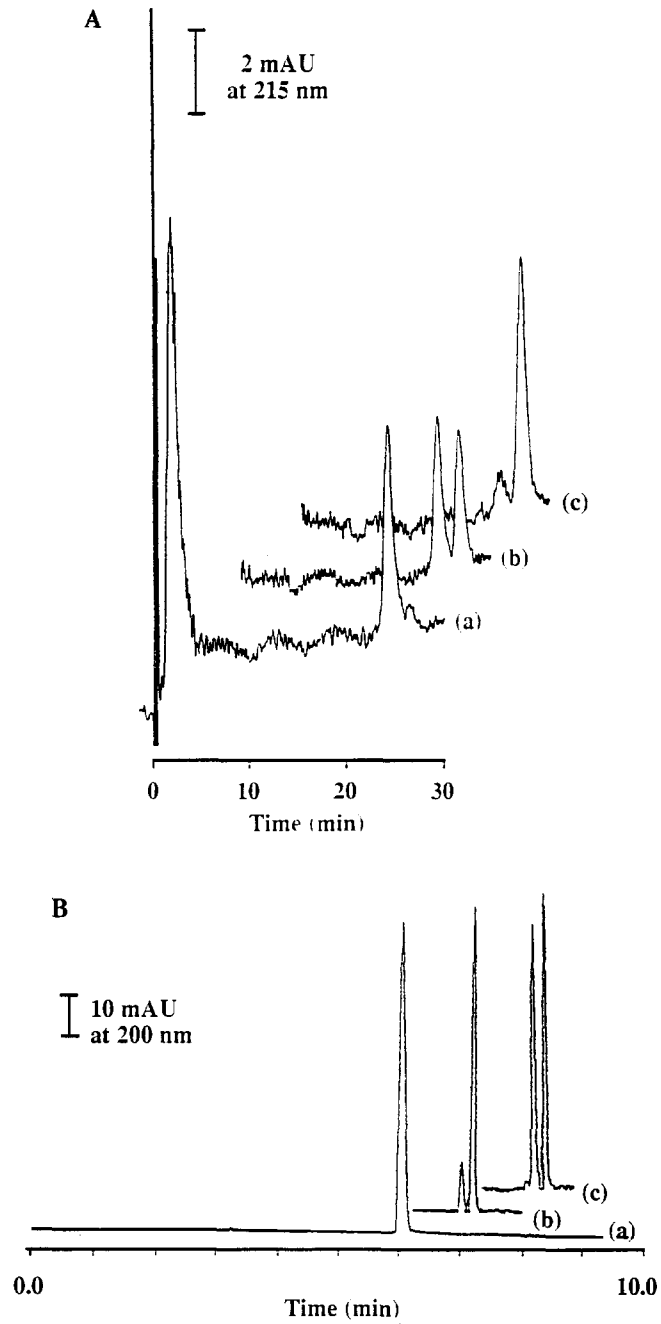

Fig. 3. RP-HPLC (A) and CZE (B) of MTX2 incubated at pH 2.0 and 45°C. HPLC traces were obtained after 1 h (a), 7 h (b) and 24 h (c). Gradient used is: 0–2 min, 5–10% B; 2–30 min, 10–30% B. CZE analysis was carried out as described in section 2 at 0 h (a), 1 h (b) and 7 h (c).

The pH dependence of the toxin Asp–Pro bond cleavage shown in Fig. 4A. Expressing the decrease of the HPLC peak area of native MTX2 as a function of time, we obtained first order kinetics at any pH values, consistent with the proposed mechanism of Asp–Pro cleavage [12]. The cleavage occurs at maximum rate at pH 1.5–2.0, with a half reaction time of less than 7 h, and is slowest at higher pHs, with half reaction times of 30 h at pH 2.5, 5 days at pH 3.0 and several days at pH 3.5–4.0. At pH 4.5–5.0, we could not detect formation of cleaved products even after 15 days. On the basis of these observations ^{1}H-NMR experiments on MTX2, used for three-dimensional structure determination, were performed at pH 5.2 and 35–45°C [8]. In order to reproduce the conditions suggested for optimal Asp–Pro cleavage [13], the protein was also incubated at 45°C in the presence of 6 M guanidine·HCl, buffered at pH 2.0 with either glycine·HCl or 10% acetic acid. Surprisingly, the rate of cleavage in denaturing conditions was much slower, with a half reaction time of 50–60 h (Fig. 4B), thus almost one order of magnitude slower than in absence of denaturant. In order to better test the role of the native conformation in the Asp–Pro cleavage reaction, we prepared a completely unfolded MTX2 sample by pyridine-ethylation of the eight cysteine residues. Unfolded PE-MTX2 was then incubated at pH 2.0 and at 45°C. HPLC analysis of this solution revealed the formation of two peaks presenting an amino acid composition corresponding to the two fragments 1–53 and 54–65, as expected (not shown). The monitoring of the decrease of the intact PE-MTX2 HPLC peak area vs. time (Fig. 4B) showed

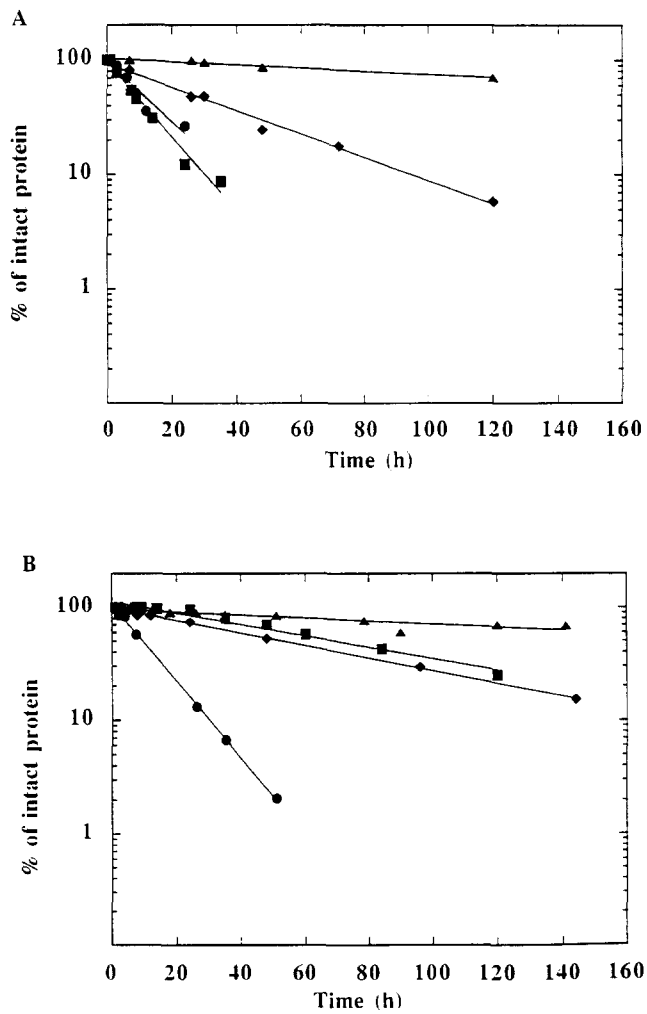

Fig. 4. Kinetics of the Asp–Pro cleavage. The decrease of the HPLC peak area corresponding to intact MTX2 is reported as a function of time. (A) MTX2 incubated at 45°C and in 0.2 M glycine buffer and at pH: 1.5 (●), 2.0 (■), 2.5 (♦) and 3.0 (▲). (B) MTX2 incubated at 45°C and in: 0.2 M glycine buffer, pH 2.0 (●); 0.2 M glycine buffer, pH 2.0, containing 6.0 M guanidine·HCl (■); 10% acetic acid, 6.0 M guanidine·HCl, pH 2.0 (♦). PE-MTX2 in 0.2 M glycine·HCl buffer, pH 2.0 (▲).

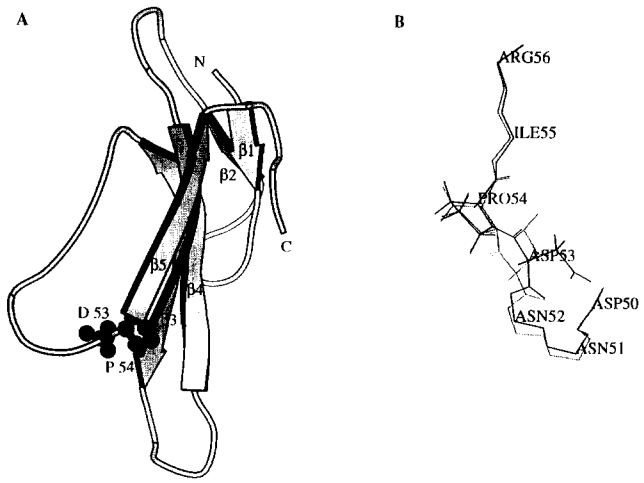

Fig. 5. Schematic representation of the three-dimensional MTX2 structure [8] showing the Asp^{53} and Pro^{54} residues in ball and stick representation. This picture was generated by the MOLSCRIPT program [19]. (B) Superposition of the 50–56 sequence in the native MTX2 (thick line) and in the modelled MTX2 (thin line). For clarity only the 50–56 backbone together with the Asp^{53}, Pro^{54} side chains are shown.

that the completely unfolded PE-MTX2 was cleaved at Asp^{53}–Pro^{54} bond at very slow rate with a half-reaction time of 170 h (7 days).

4. Discussion

MTX2 contains a unique Asp–Pro bond which we found particularly suspectible to cleavage in non-denaturing, acidic conditions. Specific cleavage at aspartyl-prolyl peptide bonds is well-documented [12–14]. Different conditions are, however, proposed in order to obtain cleavage: the most used ones employ temperatures of 37–45°C, reaction times of 24–120 h and acid solutions, including 70–90% formic acid and 7 M guanidine·HCl [13,14]. Formic acid and guanidine·HCl are required to unfold the protein, since it has been suggested that the native conformation may protect the protein from cleavage [14]. Yields of cleavage for some proteins, however, after several days are very low (10–20%), even in the most denaturing conditions [13].

In the case of the muscarinic toxin MTX2, the results of our HPLC and CZE analyses performed at 45°C in acidic solutions, show that a specific cleavage occurs between Asp^{53} and Pro^{54} and that, furthermore, it occurs rapidly, in high yields and without any need of denaturing conditions. The same reaction is considerably slowed down when performed in strong denaturing conditions or on the completely unfolded protein. This may suggest that the presence of a specific spatial organisation about the Asp–Pro bond in MTX2 may favour the cleavage reaction.

Selective hydrolysis of aspartyl bonds [16] involves intramolecular catalysis by aspartic carboxylate attack of the following carbonyl peptide bond with simultaneous donation of a proton to the peptide bond nitrogen and consequent anhydride formation resulting in peptide bond cleavage. The great lability of the aspartyl-prolyl bonds has been attributed to two factors: (i) the more basic nature of the proline nitrogen, leading to an increased protonation of the leaving imino group, (ii) an enhanced α–β isomerisation for aspartyl residues linked to proline, with the β-aspartyl form being more labile to acidic pH than the α-aspartyl one [12].

The pH dependence of the cleavage reaction observed for MTX2, with a great acceleration going from pH 3.5 to pH 2.0, is consistent with the protonation of a rather acidic group, probably the α-carboxyl group of the β-aspartyl form, thus suggesting the presence and the involvement of the β-aspartyl form in Asp^{53}–Pro^{54} bond cleavage.

To explain the high reactivity of the aspartyl-prolyl bond in MTX2, we examined the possibility that the Asp^{53} carboxylate and the Pro^{54} side chain could be in a favorable spatial disposition in native MTX2 to form the aspartimide intermediate, occurring in α–β aspartic isomerisation [12]. In the MTX2 three-dimensional structure [9] (Fig. 5A), the region 50–53 is very flexible in solution, while the region 54–59 is well defined, forming the β5-strand of the triple stranded β-sheet (β3-β4-β5). Pro^{54} is the first residue of the β5-strand, 54–59, and contributes to the formation and stabilisation of the β-sheet. In fact, the carbonyl of Pro^{54} provides hydrogen bonding interactions with the residues Trp^{28}, while the prolyl side chain shows multiple and defined long range interactions with Trp^{28} and Tyr^{30}. The Asp^{53} side chain, on the contrary, is ill-defined and very flexible in solution.

A 'productive' conformation about aspartyl residues favouring the imide formation has been defined by Clarke [17]. In order to form the aspartimide intermediate the dihedral angles ψ and χ_1, which define the rotation about the α-carbon/peptide carbonyl and the α-carbon/β-carbon should approach the values of –120° and +120°, respectively [17]. In MTX2 the experimentally determined dihedral angles of Asp^{53} are substantially different, $\psi = +118°$ and $\chi_1 = -45°$. However, given the high flexibility of the 50–53 region we examined what could be the structural consequences if the 'productive' conformation is imposed to Asp^{53}. Thus, these constraints, $\psi = -120°$ and $\chi_1 = +120°$, were imposed to Asp^{53} and the structure minimized by Sybyl program. When the hypothetical structure was compared with the native structure, we found that the 'productive' Asp conformation was compatible with the overall fold and that these changes were easily accomodated by minor conformational changes, limited to the 51–53 sequence (Fig. 5B), with the following rms distances for the Cα: Asn^{51} = 0.34 Å, Asn^{52} = 0.61 Å, Asp^{53} = 0.87 Å and Pro^{54} = 0.25 Å. Calculated weighted rms difference for the backbone was 0.193 Å. Furthermore, when we compared the interproton distances of the hypothetical structure in the modified region with the experimental distance constraints used to solve the native structure, we found that the only significant violation was for the Asp^{53} side chain.

Even in the absence of experimental evidence to comfirm the involvement of an α–β isomerisation in Asp^{53}–Pro^{54} bond cleavage, it is clear that it may occur in MTX2 with only minor conformational changes. Similar conclusions can also hold if the Asp^{53}–Pro^{54} bond splitting can occur without α–β isomerisation and with direct aspartic anhydride formation.

Furthermore, CD (Fig. 2B) and NMR spectroscopy (not shown) indicate that the cleaved and native form of MTX2 share similar secondary structures. Thus, the splitting of the Asp^{53}–Pro^{54} bond does not appreciably perturb the triple stranded β-sheet structure and Pro^{54} may still take part of the β-sheet. These data confirm that conformational changes larger than those required for the accomodation of the 'productive'

Asp conformation can be easily absorbed by the conformational flexibility present in determined regions of the structure.

The MTX2 case clearly shows that the native conformation of the protein may not protect it from Asp–Pro bond cleavage, but on the contrary may favour the cleavage. For example, the two fragments 1–53, 54–65 can be obtained in 90% yields by incubation at 45°C, pH 2.0 for 24 h. However, if the protein is first reduced and unfolded (PE-MTX2) the same two fragments cannot be obtained and extending the reaction time has as consequence only the production of other chemical modifications and cleavages. This case is probably not unique and in general when preparing large fragments for protein sequence determination, we suggest to treating the protein to be analyzed in acidic conditions (pH 2.0, 45°C) both in the presence and in the absence of denaturant: optimal conditions for cleavage may differ from protein to protein.

Finally, we note that conditions, 45°C and pH 2.5–3.0, most often used in NMR studies to slow down exchange rates of backbone amide protons and labile sidechain protons and to permit the NMR observation of these protons [18] may lead to almost complete bond splitting in proteins containing the Asp–Pro sequence, as we observed in MTX2. CD spectroscopy may not be a suitable analytical test for such modifications since, as shown here, Asp–Pro cleavage may occur without major conformational changes. HPLC and CZE are quite appropriate for such analysis.

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