Monitoring disappearance of monomers and generation of resistance to proteolysis during the formation of the activation domain of human procarboxypeptidase A2 (ADA2h) amyloid fibrils by matrix-assisted laser-desorption ionization–time-of-flight-MS

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The term ‘amyloidosis’ is used to represent a group of protein misfolding diseases characterized by the polymerization of normally innocuous and soluble proteins or peptides into insoluble proteinaceous deposits. One of the several questions that remain unclear regarding the process of amyloid fibril formation is related to the status of the protein when such a process begins. Protein engineering is one of the selected approaches to study amyloidosis. Characterization of many variants of a protein can give information about why a soluble protein aggregates to form fibrils. In the present study, we report information on the conformational changes that precede the formation of fibrils, monitored by the complementary use of exoproteolysis and matrix-assisted laser-desorption

ionization–time-of-flight-MS. This is a novel application of an easy and fast approach. In addition, we used it to evaluate the ability of the model protein ADA2h (activation domain of human procarboxypeptidase A2) and their mutants to generate amyloid fibrils. It could be a useful test to screen protein variants and to study to what extent some physicochemical parameters affect fibrillogenesis.

Key words: amyloid fibril, matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI–TOF MS), procarboxypeptidase A2 activation domain (ADA2h), protein aggregation, protein folding.

INTRODUCTION

A group of protein misfolding diseases, known as amyloidosis, are characterized by the polymerization of normally innocuous and soluble proteins or peptides into insoluble proteinaceous deposits [1]. No sequence or structural similarities are apparent between any of the proteins that display the ability to form amyloids [2]. Soluble precursors of amyloidogenic proteins do not have any obvious sequence or common folding patterns. However, structural models created from X-ray diffraction experiments indicate that all known amyloid fibrils share a characteristic cross- β structure. This suggests that the key elements of the fibrillation process may be common to all amyloidogenic proteins.

In recent years, proteins not related to any known human disease have been found to convert into higher-order structures *in vitro*, which also present a cross- β conformation and fulfil all the characteristics of amyloid fibrils [3–5]. This has suggested that the ability to form amyloids might be a property of the polypeptide backbone and sequence effects modulate this natural tendency. These results have supported the hypothesis that all proteins under appropriate conditions can form amyloid fibrils. However, several questions remain unclear regarding the process of fibril formation. One of them is related to the status of the protein when amyloid fibril formation begins. Protein engineering is a powerful tool to obtain rational variants of a protein with such a purpose. These variants would shed light on thermodynamic parameters that could be related finally to their ability to form amyloid fibrils [6]. A rapid and efficient approach to test these capabilities is desirable.

MS is becoming an essential tool for characterizing biomolecules [7]. The inherent sensitivity and high information content of MS have a strong impact on protein and peptide chemistry. Most of the work with MS and amyloids have been performed using electrospray ionization MS, probably due to its gentle ionization and quantitative capabilities [8]. Matrix-assisted laser-desorption ionization (MALDI)-MS is a rapid and sensitive analytical approach, which is well suited for obtaining molecular masses of peptides and proteins from complex samples. Until now, MALDI-MS has been applied to research on amyloids, only as a tool for protein identification. Recently, our research group has developed a new limited proteolysis methodology, based on the use of unspecific exoproteases coupled with MALDI–time-of-flight (TOF)-MS, to map quickly secondary-structure elements of a protein from both N- and C-terminal ends. The obtained proteolytic patterns (mass-spectra series) can be interpreted in the light of the conformation and local stability of the analysed proteins, a direct correlation being observed between the predicted and the experimentally derived protein secondary structure [9].

In the present study, we report on the use of MALDI–TOF-MS to study the conformational changes that occur when the activation domain of human procarboxypeptidase A2 (ADA2h) aggregates to form amyloid fibrils. This 81-residue domain has two α -helices packed against a four-stranded β -sheet [10] (Figure 1A). Its folding can be fitted into a two-state transition, showing no kinetic intermediate at pH 7.0 [11]. The structure of the transition state ensemble in the folding reaction was elucidated by protein engineering, showing a nucleation–condensation model [12]. The folding nucleus is made by the packing of α -helix 2 and the two

Abbreviations used: ADA2h, activation domain of human procarboxypeptidase A2; CPY, yeast carboxypeptidase Y; DM, double mutant; LAP, leucine aminopeptidase; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; PCI, potato carboxypeptidase inhibitor; WT, wild-type.

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Figure 1 Structural elements in the ADA2h domain

(A) Ribbon diagram depicting the three-dimensional structure of the ADA2h domain. t, terminus. (B) Amino acid sequence of the ADA2h domain and secondary-structure assignment taken from the crystallographic three-dimensional structure [10].

central β -sheets ($\beta 1$ and $\beta 3$), showing no relationship with topology ($\beta 1$, $\alpha 1$, $\beta 2$, $\beta 3$, $\alpha 2$, $\beta 4$). The wild-type (WT) protein when heated at pH 3.0 aggregates into a β -conformation, as shown by CD, rendering it highly structured, aggregated with all the characteristics of the amyloid fibrils [6]. Electron microscopy of the fibrils shows that the β -strands are orientated perpendicular to the long axis and have distinct X-ray reflections. The structural analyses also suggest the presence of subunits or protofilaments, arranged in a helical structure, similar to that found in other amyloid fibrils. On the other hand, stabilization of ADA2h through local stabilization of its α -helices [13,14] precludes amyloid formation [6]. NMR experiments suggest that a region involving residues of β -strands 2 and 3, as well as a part of α -helix 1, changes its conformation at acidic pH [15].

In the present study, we have used, exoproteolysis of WT-ADA2h, followed by MALDI-TOF-MS, to monitor the changes in the resistance to proteolysis of WT-ADA2h during the formation of the amyloid fibril. In addition, we present a simple, fast and sensitive semi-quantitative assay to screen the ability of proteins to form amyloid fibrils.

MATERIALS AND METHODS

Materials

Sinapic acid was purchased from Fluka (Neu-Ulm, Germany). Yeast carboxypeptidase Y (CPY) and leucine aminopeptidase (LAP) were obtained from Sigma. WT-ADA2h and its site-directed mutants were obtained as described previously [13,14].

Induction of amyloid fibril formation

All samples containing ADA2h at high concentration (2 mg/ml) and at pH 3.0, described in the present study, were heat-treated at 95 °C for 30 min, and equilibrated at 25 °C, before proteolysis treatment to induce amyloid fibril formation [6]. Once the fibril was formed and proteolysis analysis performed, the fibril was redissolved, by increasing the pH to 7.0, to visualize the complete monomer by MALDI-TOF-MS. On the other hand, diluted samples of ADA2h (0.1 mg/ml) were allowed to aggregate at room temperature (20 °C).

CPY and LAP exoproteolysis of WT-ADA2h, M1-ADA2h, M2-ADA2h and DM-ADA2h (where DM represents double mutant) at high concentrations

CPY proteolysis reactions with WT-ADA2h, M1-ADA2h, M2-ADA2h and DM-ADA2h at 2 mg/ml were performed with CPY

at pH 7.0 in 50 mM sodium phosphate or at pH 3.0 in 1 M glycine at 25 °C using an enzyme/substrate ratio of 1:50 (w/w). LAP proteolysis reactions with WT-ADA2h, M1-ADA2h, M2-ADA2h and DM-ADA2h were performed at pH 3.0 in 1 M glycine at 25 °C. Since LAP is obtained as a suspension, we cannot provide the enzyme/substrate ratio, but the activity units (activity that hydrolyses 1 μ mol of L-Leu-nitroanilide/min at 37 °C) per μ g of protein were 0.5 unit/8 μ g of substrate for ADA2h and their site-directed mutants. All proteolysis reactions were diluted in deionized water in the ratio 1:10 before its analysis. Time-course digestion of the different proteins was followed by MALDI-TOF-MS.

CPY exoproteolysis of WT-ADA2h at low concentration

Samples of diluted WT-ADA2h induced for amyloid fibril formation and allowed to aggregate for 0, 3 or 16 h were obtained. The WT-ADA2h concentration was 0.1 mg/ml and CPY proteolysis reactions were performed at pH 3.0 in 1 M glycine at 25 °C for 1 h using an enzyme/substrate ratio of 1:50 (w/w). All proteolysis reactions were diluted to one-third in deionized water before their analysis. Time-course digestion of the protein was followed by MALDI-TOF-MS.

ADA2h monomer disappearance during the formation of amyloid fibrils

A solution of 0.1 mg/ml WT-ADA2h was mixed with a solution of an internal standard, potato carboxypeptidase inhibitor (PCI), in such a way that both molecules give a similar MALDI-TOF ion intensity. A time-course monitoring of the ion intensities of the mixture was followed by MALDI-TOF-MS.

CPY proteolysis of WT-ADA2h at pH 7.0 followed by fibril induction

CPY proteolysis reactions with 2 mg/ml WT-ADA2h were performed at pH 7.0 in 50 mM sodium phosphate. Time-course digestion of the protein was followed by MALDI-TOF-MS. At different digestion time periods, the mixture was induced by decreasing the pH to 3.0 and heating at 95 °C for 30 min. Then, part of the aggregated mixture at pH 3.0 was allowed to refold by increasing the pH to 7.0. Finally, the original proteolytic mixture at pH 7.0, the aggregated mixture at pH 3.0 and the refolded mixture at pH 7.0 were analysed by MALDI-TOF-MS.

Preparation of samples for MALDI-TOF-MS

The matrix used in all the studies was a solution containing 5 mg/ml sinapic acid in 0.1 % trifluoroacetic acid/acetonitrile in the ratio 1:2 (v/v) at pH 2.5. The deionized-water-diluted proteolytic reactions (0.5 μ l) were placed on a sample probe spot containing 0.5 μ l of matrix solution. The mixture was mixed by pipetting, and then dried at room temperature. All dried samples were washed once with 1 μ l of 0.1 % trifluoroacetic acid to remove the salts. Once the samples were dried, they were transferred to the mass spectrometer.

MALDI-TOF-MS

All mass spectra were obtained from a Bruker Biflex TOF mass spectrometer equipped with a nitrogen laser with an emission wavelength of 337 nm. Spectra were obtained in the linear mode at an accelerating voltage of 19 kV. Deflection of the low-mass ions was used to enhance the target protein signal. An external (next spot) calibration using $M + H^+$ and $M + 2H^+$ ions was performed for each measurement with the entire ADA2h protein.

RESULTS AND DISCUSSION

We studied the formation of ADA2h fibrils using MALDI-TOF-MS. Different conditions have been used to slow down or speed up the formation of the amyloid fibril. To visualize the monomer before its aggregation, mild conditions were used (0.1 mg/ml protein concentration and/or several hours), and to visualize the already aggregated monomer, severe conditions were used (2.0 mg/ml protein concentration and/or several days) to form the fibril, and the fibril was then redissolved by increasing the pH before its analysis by MS.

Resistance to proteolysis of the WT-ADA2h amyloid fibril

Amyloid fibril formation of WT-ADA2h was induced at high concentrations by decreasing the pH and increasing the temperature, conditions where the production of fibrils is optimized [6] (see the Materials and methods section for the conditions to induce ADA2h amyloid fibril formation). Stabilized mutants M1 (α -helix 1 stabilized), M2 (α -helix 2 stabilized) and DM (the two helices stabilized) were subjected to the same treatment and used as negative controls in proteolysis-resistance experiments since they do not make amyloid fibrils [6]. Figure 2 shows the CPY proteolysis at pH 7.0 (upper panels) and at pH 3.0 (lower panels) of WT, M1, M2 and DM forms analysed by MALDI-TOF-MS. The WT protein at pH 3.0 is stable even after overnight incubation, whereas the stabilized mutants are trimmed just in 2 h to several fragments, in agreement with previous results obtained with pepsin treatment and reverse-phase HPLC analysis [6]. The fragment spanning β 1 and the N-terminus of α 1 accumulate in all the stabilized mutants after overnight proteolysis and consequently should be the end product of CPY digestion. Besides, the three stabilized mutants M1, M2 and DM are globally more stable at pH 7.0 than at pH 3.0, and under both conditions M2 and DM are more stable when compared with M1, as described previously [13–15]. Destabilization effects of mutations in helix α 1 in the mutant M1 at pH 3.0, reported previously [15], were also observed by LAP treatment of M1, M2 and DM (results not shown).

To check whether the N- or C-terminus of the WT-ADA2h monomer is outside the amyloid fibril, or whether they are integrated completely in it, we have treated amyloid-induced samples with CPY and LAP overnight. For both treatments, we did not

observe any partially digested product, suggesting that the two ends of the protein are incorporated into the fibril or are protected by it.

Sensibility of the WT to proteolysis during fibril formation

At room temperature, WT-ADA2h was incubated at a diluted concentration (pH 3.0) to slow down the induction of fibril formation (see the Materials and methods section). Aliquots were withdrawn at different time periods (at 0, 3 and 16 h) after fibrillogenesis induction and subjected to treatment with CPY for 1 h (Figure 3). At zero time, the degradation was very fast (Figure 3A). The main resultant peptide in the mass spectrum spans from the N-terminus of the protein to an internal part of α -helix 1, the end product of CPY digestion. This indicates that before fibril formation the protein unfolds partially, as has been postulated for other proteins with amyloid propensity [16–18]. In fact, we have demonstrated previously that when we changed the pH by dialysis from 7.0 to 3.0 in a diluted sample that was not submitted to high temperature, as is the case in this experiment, the CD spectrum corresponded to an α β -conformation with an increase in the random-coil conformation (the minimum at 208 nm is increased) [6]. Similarly, when dialysis was performed with a concentrated sample, the CD spectrum corresponded directly to a β -conformation whose population increases by increasing the temperature and/or by chemical denaturation [6]. Although the relationship between concentration and aggregation is established clearly, the increase in β -conformation obtained when denaturing the sample (by temperature or by urea) indicates that the unfolding of the initial conformation switches the protein to the aggregation-prone conformation. Several other peptides are found at zero time, mainly those spanning from the N-terminus of the protein to the β 3 and N-terminus of α 2 (Table 1). The absence of peaks corresponding to some structural elements of the ADA2h domain indicates that, in those regions, CPY activity is not slowed down by the presence of secondary structure [9]. Therefore the absence of mass spectra signals for β 4, the C-terminus of α 2 and β 2 indicate that these structural elements are quite unstable in the WT domain under amyloidogenic conditions.

When the sample was allowed to aggregate for 3 h, peaks were produced in the 5–3 kDa mass range of the spectrum, corresponding to β 1– α 1 fragments, but a group of high molecular-mass fragments (8–9.2 kDa) resistant to proteolysis also appeared (Figure 3B and Table 1). It is noteworthy that the peak representing the fragment corresponding to β 3 in Figure 3(A) has disappeared in Figure 3(B). This effect may be related to the different conformations of the protein during aggregation. When we allowed the aggregation to proceed for 16 h before CPY digestion, the complete protein was the main species observed (Figure 3C). However, still a fragment corresponding to β 4 is observed, suggesting that part of the C-terminus of the protein is exposed in the conformation prone to aggregation. This result is not in contradiction with the observation that the amyloid-induced samples (at high concentration and high temperature), treated with CPY and LAP overnight, do not produce any partially digested product, as mentioned in the preceding section. Instead, it suggests that long periods of incubation are required for a complete formation of the amyloid fibril. A similar result was observed by electron microscopy [6]: the fibrils incubated during short time periods were shorter and irregular, whereas those incubated for longer periods were longer and more regular, showing a ribbon-like pattern-twisting characteristic of the amyloid fibril. This is in agreement with the kinetics of formation observed for other amyloid fibrils [19–21]. Our results indicate that the amyloid

Figure 2 Limited exoproteolysis of WT-ADA2h and its site-directed mutants at pH 7.0 and 3.0

MALDI-TOF mass spectra of 1 h CPY exoproteolysis of WT-ADA2h, M1-ADA2h, M2-ADA2h and DM-ADA2h at pH 7.0 (upper panels) and at pH 3.0 (lower panels). $M + H^+$ and $M + 2H^+$ correspond to the single- and double-charged molecular ions respectively of the entire ADA2h domain molecule.

Figure 3 CPY exoproteolysis of WT-ADA2h at low concentration and at pH 3.0

Samples of diluted WT-ADA2h solution were induced for amyloid fibril formation and allowed to aggregate. At different aggregation times, 0 h (A), 3 h (B) and 16 h (C), aliquots were withdrawn, submitted to CPY proteolysis for 1 h and analysed by MALDI-TOF-MS. The molecular-ion signals, labelled α and β , correspond to the secondary-structure elements that act as stopping points in the C-terminal exoproteolysis of the fragment (see Figure 1). $M + H^+$ and $M + 2H^+$ correspond to the single- and double-charged molecular ions respectively of the entire ADA2h domain molecule.

fibril is capable of growing from solutions as dilute as $10 \mu\text{M}$, if the time allowed is long enough.

Correlation between aggregation propensity and polypeptide chain length of the monomer

To gain insight regarding the part of the polypeptide chain which is important for the formation of the amyloid fibril, proteolytic digestion of a concentrated solution of 2.0 mg/ml WT-ADA2h was performed at pH 7.0 under conditions rendering a representative population of all the secondary-structure elements (Figure 4A). The pH of this representative population of peptides

Table 1 List of m/z present in the CPY proteolysis of ADA2h and their relationship with protein sequence and secondary-structure elements

Fragments have been trimmed from their C-terminus to the region between two secondary structure elements. Their presence is due to the slowing down of CPY activity in the presence of a secondary-structure element (see [9]).

m/z	Fragment (amino acids)	Secondary-structure element involved
9210.35	1–81	WT (entire protein)
9082.67	1–80	WT- $\beta 4$
8983.60	1–79	WT- $\beta 4$
8625.40	1–76	$\beta 4$
7947.10	1–70	$\alpha 2$
7285.80	1–64	$\alpha 2$
6203.17	1–54	$\beta 3$
5848.42	1–51	$\beta 3$
5711.87	1–50	$\beta 3$
5640.83	1–49	$\beta 3$ – $\beta 2$
5410.74	1–47	$\beta 3$ – $\beta 2$
5255.67	1–45	$\beta 3$ – $\beta 2$
5154.30	1–44	$\beta 3$ – $\beta 2$
5053.58	1–43	$\beta 3$ – $\beta 2$
4869.37	1–41	$\beta 2$
4741.50	1–40	$\beta 2$
4051.08	1–35	$\alpha 1$
3938.99	1–34	$\alpha 1$
3102.61	1–27	$\alpha 1$
2989.53	1–26	$\alpha 1$

was lowered to 3.0 and denatured thermally for 30 min to assay the aggregation propensity of each peptide (Figure 4B). Fragments larger than 4 kDa disappeared (the small amount of peptides corresponding to 5–6 kDa present in the spectrum is negligible since they are the most populated peptides before aggregation). This effect is not due to the suppression of the larger peptides in the mixture by the pH of the matrix (as reported elsewhere [24]) but probably to their aggregation; equivalent peptides are visualized in Figure 2 (lower panels) where variants of ADA2h that do not form amyloid fibrils are proteolysed and analysed at pH 3.0. The aggregation is reversible when the pH is increased to 7.0, as described previously for the complete domain (Figure 4C) [6]. Peptides spanning $\beta 1$ – $\alpha 1$ do not aggregate (Figure 4B). Interestingly, a peptide corresponding to α -helix 2 strongly aggregates [13] and produces a β -sheet spectrum. Taken together, these results suggest that, probably, α -helix 2 plays an important role in the formation of the amyloid fibril. In this respect, it is interesting to point out that the recent study by Dobson and co-workers [22] has shown that only two small regions in a large protein affect the formation of amyloid fibrils and that designed short hexapeptides can form amyloid fibrils, which cannot be distinguished from the ones made by large proteins [25].

Disappearance of monomers during the formation of ADA2h amyloid fibril

The disappearance of monomers during amyloid fibril formation has been used recently to monitor fibril formation using electrospray ionization MS [23]. In the present study, we adapt this approach to MALDI-MS. The experiments were performed with a sample of diluted WT-ADA2h at pH 3.0, to which an internal reference (PCI, a proteinaceous inhibitor) that gives the same ion intensity when compared with ADA2h in the mass spectrum had been added. Samples were withdrawn at different time intervals. Figure 5(A) shows the results obtained at initial time, after 24 h and also after 12 days of incubation. Figure 5(B) shows the time course of the disappearance of the monomer compared with PCI,

Figure 4 Limited exoproteolysis of WT-ADA2h at pH 7.0 followed by fibril induction at pH 3.0

(A) MALDI-TOF mass spectrum of the exoproteolysis of WT-ADA2h (pH 7.0) at 7 h of the proteolytic reaction. (B) MALDI-TOF mass spectrum of the exoproteolysis of WT-ADA2h (pH 7.0) at 7 h of the reaction that was, subsequently, induced for amyloid fibril formation by lowering the pH to 3.0 and heating (see the Materials and methods section). (C) MALDI-TOF mass spectrum of the aggregated mixture shown in (B) was allowed to refold by increasing the pH to 7.0.

measured in relative areas. This curve follows a monoexponential equation, as described previously [23]. Once it has been demonstrated that a protein forms amyloid fibrils by classical experiments (i.e. electron microscopy), simple experiments like those monitoring the disappearance of monomers during fibril formation will be useful to test variants of a protein (i.e. site-directed mutants) or to study to what extent some physicochemical parameters affect fibrillogenesis. Advantages of performing these experiments by MALDI-MS are the extreme sensitivity, high tolerance to buffers, denaturants and contaminants (i.e. substances from cellular extracts), easy operation and automation.

Figure 5 Follow-up of ADA2h monomer disappearance during the formation of amyloid fibrils

(A) MALDI-TOF mass spectra of a mixture of WT-ADA2h and PCI (internal standard) at different aggregation time periods. M + H and M + 2H correspond to the single- and double-charged ions respectively of the entire ADA2h domain molecule. (B) Plot representing the disappearance of ADA2h monomer by measuring relative areas (WT-ADA2h/PCI) of mass spectra against time t .

CONCLUSIONS

Using a combination of exoproteolysis and MALDI-TOF-MS, we have been able to monitor the changes in the resistance to proteolysis of WT-ADA2h during the formation of amyloid fibrils. This methodology shows that α -helix 2 could be an important element in the formation of the fibril. Besides, MALDI-TOF-MS also allows us to monitor the disappearance of monomers during fibrillogenesis. Methods used in the present study could be of general use to test the fibrillogenic capability of variants of a protein and to study to what extent some physicochemical parameters affect fibrillogenesis. Furthermore, this methodology could be useful to test the effect of protein ligands and putative drugs on fibril formation.

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