Protein engineering as a strategy to avoid formation of amyloid fibrils

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Abstract

The activation domain of human procarboxypeptidase A2 (ADA2h) aggregates following thermal or chemical denaturation at acidic pH. The aggregated material contains well-defined ordered structures with all the characteristics of the fibrils associated with amyloidotic diseases. Variants of ADA2h containing a series of mutations designed to increase the local stability of each of the two helical regions of the protein have been found to have a substantially reduced propensity to form fibrils. This arises from a reduced tendency of the denatured species to aggregate rather than from a change in the overall stability of the native state. The reduction in aggregation propensity may result from an increase in the stability of local relative to longer range interactions within the polypeptide chain. These findings show that the intrinsic ability of a protein to form amyloid can be altered substantially by protein engineering methods without perturbing significantly its overall stability or activity. This suggests new strategies for combating diseases associated with the formation of aggregated proteins and for the design of novel protein or peptide therapeutics.

Keywords: ADA2h domain; amyloid fibrils; amyloid formation; protein engineering; protein misfolding; secondary structure propensity

Amyloid fibrils are highly ordered protein aggregates associated with a range of debilitating human conditions including Alzheimer's disease, type II diabetes, systemic amyloidosis, and the transmissible spongiform encephalopathies (Tan & Pepys, 1994; Ghetti et al., 1996; Kelly, 1997; Cohen & Prusiner, 1998). These fibrils are readily observed by electron microscopy, where they appear as long unbranched structures with a diameter of ~10 nm. They are characterized by their ability to bind dyes such as Congo red (Klunk et al., 1989a, 1989b) and thioflavine-T (Levine, 1993, 1995), and show a high content of β -structure when examined by FT-IR spectroscopy and X-ray fiber diffraction (Fabian et al., 1993a; Sunde et al., 1997). The soluble forms of the proteins associated with the various amyloid diseases can be converted into fibrils in

vitro, and there is increasing evidence that this occurs via at least partial unfolding of the native structures (Lai et al., 1996; Booth et al., 1997; Kelly, 1998).

Recently, proteins unrelated to any known human disease have been found to convert in vitro into higher order structures with all the characteristics of the disease-related amyloid fibrils (Guijarro et al., 1998; Litvinovich et al., 1998; Chiti et al., 1999). This observation has led to the suggestion that the ability to form amyloid fibrils is a common or generic property of polypeptide chains (Guijarro et al., 1998; Chiti et al., 1999; Dobson, 1999). Despite this, the propensity of proteins to form amyloid fibrils clearly varies substantially with the sequence of the polypeptide. At least part of this variation is associated with the stability of the native state, which results in the aggregation-prone polypeptide main chain and hydrophobic side chains being concealed within the cooperative globular fold. Thus, formation of amyloid is associated with solution conditions which disrupt the native fold, yet which do not inhibit the hydrogen bonding and other interactions necessary for intermolecular association (Chiti et al., 1999). The amyloid diseases are therefore likely to be associated, at least in part, with the loss of control and regulation of proteins within their biological environment. But the propensity to form fibrils must

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Abbreviations: ADA2h, activation domain of human procarboxypeptidase A2; WT, wild-type; DM, double mutant; M1, mutant with Helix-1 stabilized; M2, mutant with Helix-2 stabilized; FT-IR, Fourier transform infrared spectroscopy; CD, circular dichroism; RP-HPLC, reversed-phase liquid chromatography; UV, ultraviolet.

also be associated with properties of the polypeptide chain itself, such as its intrinsic conformational preferences, solubility, and charge state, and may also be associated with the ability of individual residues to pack into fibrillar structures (Westermarck et al., 1990; Turnell & Finch, 1992; Soto & Castaño, 1996; Soto et al., 1996). Kinetic features are also likely to be important because the formation of ordered aggregates has, at least in some cases, been shown to occur by a nucleation and growth mechanism analogous to that associated with the more familiar crystallization or gelation phenomena (Jarrett & Lansbury, 1993; Harper & Lansbury, 1997).

In this paper, we describe studies of an 81-residue protein, the activation domain of human procarboxypeptidase A2, ADA2h. This domain has two α -helices packed against a four-stranded β -sheet (Catasús et al., 1995; García Sáez et al., 1997; Aloy et al., 1998) (Fig. 1). The protein has been found to fold at neutral pH in a two-state manner through a compact transition state, possessing some secondary structure and a rudimentary hydrophobic core (Villegas et al., 1998). The study of peptides corresponding to both α -helices of the protein reveals that Helix-1 adopts a stable helical conformation in solution but Helix-2 readily aggregates (Villegas et al., 1996). Increasing the helical propensity, by improving solvent-exposed local interactions, results in a reduction in the tendency of Helix-2 to aggregate. Incorporation of these changes into the intact

protein by mutation of specific residues results in an acceleration of its folding rate (Villegas et al., 1996; Viguera et al., 1997). In the present work, we have found that the intact protein also aggregates under a variety of partially denaturing conditions and forms ordered structures with all the characteristics of amyloid fibrils. The availability of mutants whose properties have been characterized previously has allowed us to initiate an investigation of the factors that control the propensity of a protein to form amyloid under controlled laboratory conditions. In particular, we have compared the wild-type protein (WT-ADA2h) with variants in which one (Helix 1 in M1-ADA2h and Helix 2 in M2-ADA2h) or both α -helices (DM-ADA2h) have been stabilized by amino acid replacements (Fig. 1) (Villegas et al., 1996; Viguera et al., 1997).

Results and discussion

ADA2h thermal denaturation at low pH

Thermal denaturation at pH 7.0 of WT-ADA2h (Villegas et al., 1995) and the M1, M2, and DM-ADA2h mutants has been shown to be a reversible two-state transition (Viguera et al., 1997). At pH 3.0 the three mutants M1, M2, and DM again show fully reversible unfolding transitions (in a concentration range 10 to

Fig. 1. Structure of ADA2h. **A:** Tertiary structure of the domain. Red, mutations in α -helix 1. Blue, mutations in α -helix 2. The stabilizing mutations on the solvent-exposed face of ADA2h α -helices are the following: α -helix 1 (M1-ADA2h): N25/K, Q28/E, Q32/K, E33/K (Villegas et al., 1996); α -helix 2 (M2-ADA2h): Q60/E, V64/A, S68/A, Q69/H (Villegas et al., 1996). The double mutant (DM-ADA2h) contains the substitutions corresponding to both M1 and M2 (Viguera et al., 1997). **B:** Amino acid sequence of ADA2h and secondary structure assignment according to Kabsch and Sander (1983). Mutations included in the modified proteins are marked in red (mutant M1-ADA2h) and blue (mutant M2-ADA2h) respectively.

3 mM), and at high temperatures reveal a CD spectrum typical of a highly denatured protein (Fig. 2B–2E). The WT protein, however, undergoes a transition that is not reversible (Fig. 2A,E). The CD spectra at 95 °C, and after cooling to 25 °C, show that the protein has converted to a conformation with extensive β -sheet structure (Fig. 2A). Analytical centrifugation analysis indicates that the sample is highly aggregated (40% of the sample showed a $S_{w,20}$ of 35 S, indicating a value of M_r higher than 10^6 Da, corresponding to an aggregate that contains on average more than 100 protein molecules). The conversion of α -helical to β -sheet structure for the WT-ADA2h protein upon thermal denaturation is also indicated by FT-IR spectroscopy. Before heating, the amide I band shows two main components at 1,622 and 1,649 cm^{-1} (Fig. 2F), attributable to β -sheet and α -helical structure, respectively (Surewicz et al., 1993), consistent with the structure of the native protein. After incubating the sample at 90 °C two new bands appear, centered at 1,615 and 1,685 cm^{-1} , respectively, replacing the original bands (Fig. 2F). Such a pattern is characteristic of aggregated species with β -sheet structure (Fraser et al., 1992; Gasset et al., 1992; Fabian et al., 1993b). The protein, after heating, also produces a clear shift in the Congo red absorbance spectrum from 486 to 500 nm, together with an increase in absorption intensity (data not shown), and exhibits thioflavine-T binding (Table 1). All these properties are indicative of the formation of amyloid.

ADA2h urea denaturation at low pH

The behavior of the WT and DM proteins was also examined following incubation in the presence of a range of urea concentrations (4 to 7 M). At 7 M urea the CD spectra of both proteins are indicative of highly unfolded species. When samples of both WT and DM-ADA2h in 7 M urea were diluted rapidly to a concentration of 1 M urea, the proteins refolded to their native states. Incubation of the WT protein in 4 M urea for 1 h, followed by dilution to 1 M urea and incubation for 1 h, however, resulted in a CD spectrum indicative of extensive β -sheet structure (Fig. 3A). No such behavior was found with the DM protein (Fig. 3B) that recovered its native spectrum after the same successive equilibration steps that promote the appearance of β -sheet structure in WT-ADA2h. In accord with this, DM-ADA2h was found to be fully digested by pepsin after the successive equilibration steps in urea, as well as after thermal denaturation. The WT protein, however, showed a reduced susceptibility to digestion in samples where an increase in β -sheet structure had been detected (Table 2). Indeed, a clear correlation exists between the transition to β -structure revealed by CD and FT-IR and an increased resistance to proteolysis.

Analysis of WT-ADA2h aggregates

The study of the aggregated WT protein by electron microscopy shows clear evidence for fibrils that are long, unbranched, narrow (diameter 30–100 Å), and apparently quite flexible. These typically form tight networks of intertwined structures (Fig. 4A). Samples observed after long periods of incubation at 90 °C feature longer and more regular fibrils than those incubated for short times (Fig. 4B–D) and show more clearly a ribbon-like pattern twisting at irregular intervals (Fig. 4C,D, see arrows). The structural changes resulting from longer periods of incubation at high temperatures can be explained as a slow reorganization of the protofilaments that make up the fibrils. Such structural evolution has been observed previously in amyloid fibrils from a number of other pro-

teins (Bauer et al., 1995; Seilheimer et al., 1997; Jiménez et al., 1999). Fibrils are observable in aggregates formed from solutions containing WT-ADA2h concentrations as low as 20 μM . Fibrils with characteristics similar to those shown in Figure 4A were also observed in samples of WT-ADA2h subjected to chemical denaturation, a result consistent with the appearance of resistance to proteolysis under similar conditions. As expected, no fibrils could be detected from any sample of the DM mutant subjected to similar conditions of thermal and chemical denaturation. Fibrils from thermally denatured preparations of WT-ADA2h were also characterized by X-ray fiber diffraction (Fig. 5) and found to have the distinct cross- β X-ray diffraction pattern, characteristic of amyloid fibrils (Blake & Serpell, 1996; Sunde et al., 1997).

ADA2h, therefore, represents a clear example of a protein not associated with any known disease that is able to aggregate and form of amyloid fibrils. This aggregation, like that of the other nonpathological amyloid systems studied (Guijarro et al., 1998; Litvinovich et al., 1998; Chiti et al., 1999), takes place under conditions where the protein is at least partially denatured. This finding correlates well with conclusions from the study of proteins associated with in vivo formation of amyloid. In amyloidoses associated with lysozyme (Booth et al., 1997; Canet et al., 1999), immunoglobulin light chain (Hurle et al., 1994), and transthyretin (McCutchen et al., 1995) amyloidosis, the amino acid mutations giving rise to disease affect the overall stability of the proteins. Such decreased stability increases the population of unfolded and partially folded species essential for aggregation such that the latter can occur under relatively mild conditions. This might suggest, therefore, that the propensity for formation of amyloid is simply related to protein stability.

Secondary structure propensities and amyloid fibril formation

The inhibition of amyloid fibril formation by mutation of ADA2h is, however, not simply due to an increase in stability of the protein. Aggregation was initiated under conditions where both WT and mutant proteins are at least partially unfolded. Moreover, at pH 3.0 the variant protein with just the first helix stabilized (M1-ADA2h) is less stable overall than the WT protein, although the helical region is locally more stable, probably due to long-range electrostatic interactions (Villegas & Serrano, data not shown). For M1-ADA2h, as for DM-ADA2h and the protein having just the second helix stabilized (M2-ADA2h), both of which are more stable than the WT protein, the formation of amyloid fibrils is inhibited. The experimental CD data show that the denatured states of wild-type ADA2h and the variants studied here are all partially folded, and that the helical content is up to 45% of that of the native protein even at 95 °C (see Fig. 2). When repeating the same experiment under more diluted conditions and acquiring the spectra more rapidly, we can diminish WT aggregation. Under these conditions, we observe that the helicity of the mutant proteins is significantly greater than that of the WT protein (by nearly a 50% for the DM mutant as determined by fitting the CD spectra at 95 °C using different methods, data not shown). These observations are in agreement with an increase in stability of the native-like helical structure displayed by all the mutants. Another possibility to explain the absence of fibril formation in the mutant proteins is that the mutations have disrupted the packing in the fibers. However, this is very unlikely since fiber inhibition takes place not only in DM-ADA2h, but also in the individual mutants M1 and M2. Hence, we suggest that inhibition of amyloid formation could be caused

Fig. 2. Thermal denaturation analysis at pH 3.0 of WT-ADA2h and M1, M2m, and DM variants. **A–D:** Far-UV CD spectra of WT-ADA2h and variants at 25°C (open circles), 95°C (open squares), and at 25°C (filled circles) after cooling. **A:** WT. **B:** M1. **C:** M2. **D:** DM. **E:** WT, heating (open circles); WT, cooling (open squares); DM heating and cooling, (filled circles, the heating and cooling data are superimposable). The WT protein was monitored at 214 nm, whereas the DM variant was monitored at 222 nm. **F:** Amide I band in the FTIR spectra of WT-ADA2h samples before (solid line) and after (dashed line) thermal denaturation. The second derivatives of the spectra reveal peaks centered at 1,622 and 1,649 cm⁻¹ for the sample before heating, and at 1,615 and 1,685 cm⁻¹ after heating. The band at 1,615 cm⁻¹ is indicative of β -strands, whereas the one at 1,685 cm⁻¹ is commonly associated with a splitting in the amide I band due to antiparallel interstrand interactions (Krimm & Bandekar, 1986; Fabian et al., 1993a). Calculations of the residual helical content at 95°C were carried out from CD spectra as indicated in Materials and methods with the following results: WT retains 45% of the native helical structure (spectra were collected in a very dilute sample that was subjected to a very fast temperature change to prevent aggregation during data collection), M1 retains 63%, M2 retains 61%, and DM retains 68%. Therefore, the residual helical structure at 95°C for DM is about 50% greater than the amount retained by WT.

Table 1. Thioflavine-T binding of ADA2h samples, before and after thermal denaturation at pH 3.0^a

Sample conditions	Wild-type ^b	Double mutant
25 °C, 500 μ M	3.1	-0.8
90 °C, 500 μ M	520.1	-0.9
90 °C, 200 μ M	288.7 (721.8) ^c	—
90 °C, 100 μ M	111.5 (557.4)	2.3
90 °C, 50 μ M	64.3 (642.5)	—

^aAll the samples were prepared in 25 mM glycine, pH 3.0 and incubated for 30 min at the indicated temperatures.

^bResults show the increase in fluorescence (arbitrary units) after subtracting the Thioflavine-T contribution alone (45.2 units).

^cData in brackets represent the value normalized to a 500 μ M concentration.

by an increase in the stability of intramolecular interactions in the denatured state, and a concomitant decrease in the intermolecular interactions that give rise to aggregation and fibril formation.

Previous studies of amyloidogenic peptides have suggested that differing abilities of the various amino acids to stabilize intermolecular interactions within aggregated structures could be important in determining the propensity to form fibrillar structures (Westermarck et al., 1990; Hilbich et al., 1992; Jarrett & Lansbury, 1992; Turnell & Finch, 1992; Wood et al., 1995; Moriarty & Raleigh, 1999). It is possible that effects of this type might play a role in the differing behavior of wild-type ADA2h and the mutants described in this paper. Nevertheless, and in terms of protein engineering, these kinds of effects are very difficult to predict given that β -sheet formation seems to be mainly determined (with very few exceptions) by the tertiary context, and not by intrinsic secondary structure preferences (Minor & Kim, 1994a, 1994b). However, support for the suggestion that the increased stability of intramolecular interactions in the denatured state resulting from increased helical propensities is particularly important comes from studies of the formation of amyloid fibrils by acylphosphatase, a protein with a structure homologous to that of ADA2h. Fibril formation is stimulated by low concentrations of TFE that are sufficient to denature the protein, but it is inhibited by higher concentrations, that induce stable helices in the denatured state (Chiti et al., 1999). It is also consistent with observations which indicate that an A β 1–40 peptide mutant with increased helicity exhibits a reduced propensity to form amyloid fibrils in vitro, while another mutant with a decreased α -helical propensity aggregates faster than the original peptide (Soto et al., 1995).

Protein engineering to prevent amyloid fibril formation

The present study suggests, therefore, that mutations that stabilize regions of the secondary structure of ADA2h, rather than simply the overall global fold, inhibit its aggregation into amyloid fibrils. It is clearly essential for the native state of a protein to be disrupted to allow ordered aggregates to form. Thus, it is entirely reasonable that strong correlation will exist between mutations that destabilize a protein structure and its tendency to form fibrils. But the propensity for the aggregation process to take place must also depend on the intrinsic properties of the denatured state that results from the disruption of the native fold. Mutations that change the prop-

Fig. 3. Urea renaturation of WT-ADA2h and the DM variant at pH 3.0. **A:** Far-UV CD spectra of WT-ADA2h. **B:** Far-UV CD spectra of DM-ADA2h. Circles refer to samples prepared under the initial conditions. Squares refer to the protein after incubating the samples in 4 M urea, and then diluting to 1 M urea, see text. Data below 210 nm could not be collected due to the strong absorption of urea at low wavelengths.

erties of the denatured state are not expected to correlate in a simple manner with the structure or even the stability of the native protein, as is the case in the mutations shown here. This conclusion suggests an explanation for the existence of disease-related amyloidogenic mutants that do not simply cause a decrease in protein stability. There are clear examples of such mutations associated with the prion diseases (Riek et al., 1998; Swietnicki et al., 1998; Liemann & Glockshuber, 1999). Interestingly, at least some of these appear to decrease significantly the stability of individual helices in the protein structure (J. Zurdo & C.M. Dobson, unpubl. obs.).

It is well established that mutations to native proteins can increase amyloidogenic behavior. The present results with ADA2h show, for the first time, that aggregation of a native protein into amyloid fibrils can be reduced by modifications that maintain unchanged the native function, structure, and even stability of the protein. This suggests that there are opportunities for exploiting this phenomenon for rational design. For example, it suggests an alternative therapeutic approach toward avoidance of amyloidotic diseases to that based on the design of substances that inhibit the

Table 2. Resistance to proteolysis of the WT-ADA2h aggregates^a

	Temperature denatured	Urea denatured	Untreated
Severe proteolysis ^b	5.0%	0.7%	0%
Mild proteolysis ^c	39.7%	7.4%	1.5%

^aData are given as the percentage of the intensity in the chromatographic peak corresponding to the native protein remaining after treatment.

^bSevere conditions. Ratio ADA2h:pepsin 100:1 (W:W), 2 h digestion, 20 °C.

^cMild conditions. Ratio ADA2h:pepsin 400:1 (W:W), 15 min digestion, 0 °C.

aggregation of proteins into fibrils (Merlini et al., 1995; Salomon et al., 1996; Wood et al., 1996; Tagliavini et al., 1997). It might be possible to use gene therapy to engineer into an organism modified proteins with a reduced tendency to aggregate, or to produce transgenic animal strains that are resistant to prion diseases. In addition, polypeptides with industrial or medical importance, but having a high propensity to aggregate, such as insulin and calcitonin (Sluzky et al., 1992; Arvinte et al., 1993; Moriarty et al., 1998), could perhaps be engineered to be less aggregation prone by altering their secondary-structure propensities using a similar approach to that used with the ADA2h protein. The present demonstration that variant proteins can be designed with markedly reduced propensities to aggregate could therefore open up a range of novel avenues for future research.

Fig. 4. Electron microscopy of WT-ADA2h preparations. WT-ADA2h fibrils prepared by incubation of protein samples at 90 °C for (A) 1 h and (B–D) 48 h. Longer and straighter fibrils can be observed in the later preparations. Thin arrows point to possible crossover sites, whereas solid arrows indicate helical ribbon-like conformations. Bar is 1,000 Å.

Fig. 5. X-ray fiber diffraction pattern of WT-ADA2h fibrils. Reflections are observed at 4.7 Å (corresponding to the interstrand distance in the direction of the fibril axis) and 9.3 Å (corresponding to the distance between β -sheets in the direction perpendicular to the fibril axis). Anisotropy can be observed in the sharp 9.3 Å diffraction (the equatorial axis is the vertical axis in the display). Another faint reflection can be observed at 3.1 Å, with the anisotropy and sharpness of the 9.3 Å reflection, probably arising from a harmonic. A fourth weak reflection is observed at 3.8 Å with no apparent anisotropy. Reflections of this type have been observed previously in studies of amyloid fibrils from a variety of sources (Sunde et al., 1997).

Materials and methods

ADA2h expression and purification

The WT-ADA2h and its stabilized mutants were expressed and purified as previously reported (Villegas et al., 1995, 1996; Viguera et al., 1997). All of the recombinant proteins were examined by MALDI-TOF-MS and found to have the molecular weight anticipated from the sequence.

Circular dichroism

CD spectra of 20, 80, 160, and 200 μ M protein samples in 50 mM sodium phosphate (pH 7.0) or 25 mM glycine (pH 3.0) were recorded using a JASCO-710 spectropolarimeter at 278, 298, and 368 K in a 2.0 or 0.2 mm quartz cuvette. Measurements were averaged for 30 scans recorded at 50 nm min⁻¹. Thermally induced unfolding of 20 μ M protein samples was monitored in the temperature range of 278–368 K at a heating rate of 50 °C h⁻¹ by following their ellipticity at 222 or 214 nm. Calculations of residual helical structure at 95 °C were carried out by measuring $[\theta]$ at 220 nm (Chen et al., 1974). Secondary structure evaluation on the wild-type protein was carried out using different conditions to minimize artifacts due to protein aggregation. WT-ADA2h CD spectra were collected at a concentration of 5 μ M, and sample was heated from 25 to 95 °C at a rate of 150 °C h⁻¹. The sample was

then immediately placed at 25 °C and examination of its CD spectrum showed native-like features.

Sedimentation analysis

Sedimentation experiments were performed in a Beckman XLA analytical ultracentrifuge at 3,000 *g* with 20 and 200 μ M protein samples in a buffer solution at pH 3.0 containing 25 mM glycine. Samples were heated at a rate of 50 °C h⁻¹ from 5 to 95 °C and then left at 95 °C for 10 min before sedimentation experiments. A 200 μ M protein sample in 50 mM sodium phosphate at pH 7.0 was used as negative control.

Thioflavine-T and Congo red binding assays

Samples of WT-ADA2h and DM-ADA2h (at concentrations ranging from 20 to 500 μ M) were incubated for 30 min at 90 °C before the assay. A 2.5 mM Thioflavine-T stock solution was freshly prepared in 10 mM potassium phosphate, 150 mM NaCl, pH 7.0, and passed through a 0.2 μ m filter before use. Typically 10 μ L of sample was diluted in the reaction buffer (10 mM potassium phosphate, 150 mM NaCl, pH 7.0) containing 65 μ M Thioflavine-T (1 mL final volume). Samples were taken into and out of the pipette several times to facilitate fibril dispersion and to disrupt large aggregates. Data were collected in a Perkin-Elmer LS 50B luminescence spectrometer using a 440 nm (slit width 5 nm) excitation wavelength and 482 nm (slit width 10 nm) emission wavelength. Fluorescence values were obtained after 3–5 min to ensure thermal equilibrium had been achieved. Samples were continuously stirred to prevent signal oscillation due to the presence of large fibrillar aggregates and signals were averaged for 60 s to increase the signal-to-noise ratio. Congo red binding assays were performed according to Klunk et al. (1989a, 1989b). Aliquots of 10 μ L of sample were diluted in 10 mM potassium phosphate, 150 mM NaCl, pH 7.0 containing 5 μ M Congo red (1 mL final volume). Congo red solutions were prepared just before use and passed through a 0.2 μ m filter. Absorption spectra of samples in the reaction solution were collected together with negative controls (dye in the absence of protein and protein samples in the absence of dye) to subtract the signal associated with the absorption of the dye and the scattering contribution to the signal.

Proteinase resistance

Twenty micrometer samples of WT and DM-ADA2h in 25 mM glycine (pH 3.0) were incubated for 4 h in 4 M urea, then diluted to give an urea concentration of 1 M, and dialyzed before incubation with proteinase. Samples in the same buffer were also incubated for 10 min at 95 °C before proteolysis. These samples, together with untreated samples of the same proteins, were digested in the presence of pepsin at two different ADA2h:pepsin ratios (100:1 and 400:1) for 2 h at 20 °C or 15 min at 0 °C, respectively. Digested samples were then analyzed by RP-HPLC in a Vydac C4 column (214TP54, 5 μ m particle size, 300 Å pore, 1.0 $\times$ 25 cm) with a linear gradient from 10 to 52% of acetonitrile. Detection was carried out at 214 nm in a Waters 994 model.

Fourier-transform infrared spectroscopy

Infrared spectra were recorded in a Bio-Rad FTS 175C FT-IR spectrometer equipped with a liquid N₂-cooled MCT detector, and

purged with a continuous flow of N₂ gas. Five hundred micrometer WT-ADA2h samples were prepared in ²H₂O, glycine 25 mM, p²H 3.0 (the electrode reading was corrected for isotope effects), and spectra were collected at 25 °C before and after incubating the sample at 90 °C for 30 min. Protein solutions were placed between a pair of CaF₂ windows separated by a 12 μm Mylar spacer. For each sample, 256 interferograms were collected at a spectral resolution of 2 cm⁻¹. Spectra were collected under identical conditions for the buffer solution in the absence of protein and subtracted from the spectra of the protein samples. Second derivatives of the Amide I band spectra were produced to determine the wavenumbers of the different spectral components.

Electron microscopy

Samples were applied to Formvar-coated nickel grids (400 mesh), negatively stained with 2% uranyl acetate (w/v), and viewed in a JEOL JEM1010 transmission electron microscope, operating at 80 kV.

X-ray diffraction

Fibril suspensions were washed with Microcon-100 ultrafiltration tubes (Amicon, Beverly, Massachusetts), to eliminate salts and buffers that could interfere with the X-ray measurements. Samples were prepared by air-drying salt-depleted ADA2h-WT fibril preparations between two wax-filled capillary ends. The capillaries were separated slowly while drying, to favor fibril orientation along the stretching axis. A small stalk of fibrils protruding from one end of the capillaries was obtained. The sample was aligned in a X-ray beam, and diffraction images were collected in a Cu K_α rotating anode equipped with a 180 or 300 MAR-Research image plate (MAR Research, Hamburg, Germany) during 20–30 min. Images were analyzed by using IPDISP and MarView software.

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