Characterization of the native and fibrillar conformation of the human N^{α} -acetyltransferase ARD1

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Abstract

ARD1 (arrest-defective protein 1), together with NAT1 (N^{α} -acetyltransferase protein 1), is part of the major N^{α} -acetyltransferase complex in eukaryotes responsible for α -acetylation of proteins and peptides. Protein acetylation has been implicated in gene expression regulation and protein–protein interaction. We characterized the native folded and misfolded conformation of hARD1. Structural characterization of native hARD1 using size exclusion chromatography, circular dichroism, and fluorescence spectroscopy shows the protein consists of a compact globular region comprising two thirds of the protein and a flexible unstructured C terminus. In addition, hARD1 forms protofilaments under physiological conditions of pH and temperature, as judged by electron microscopy and staining with the dyes Congo red and thioflavin T. The process is accelerated by thermal denaturation and high protein concentrations. Limited proteolysis of aggregated hARD1 revealed a resistant fragment whose sequence matched a region contained within the acetyl transferase domain.

Keywords: ARD1; protofilaments; amyloid fibers

N^{α} -acetylation is one of the most common protein modifications. It has been estimated that as many as 80%–90% of eukaryotic proteins are N^{α} -terminally acetylated. N^{α} -acetylation occurs during protein synthesis and involves the transfer of an acetyl group from acetyl-coenzyme A to the protein α -NH₂ group (Bradshaw et al. 1998; Arnold et al. 1999). Human ARD1 (arrest-defective protein 1, hARD1) and human NAT1 (N^{α} -acetyltransferase protein 1, NATH), and their homologs in other organisms, are the subunits of the major N^{α} -terminal acetyltransferase complex, NatA (Park and Szostak 1992; Arnesen et al. 2005a). hARD1 consists of 235 residues with a predicted acetyltransferase domain comprising amino acids 44–130,

and a putative nuclear localization signal (NLS) between residues 78 and 83 (Fig. 1). The first 60 N-terminal residues of hARD1 mediate heterodimerization with NATH (Fig. 1) (Arnesen et al. 2005a). In yeast, the NatA complex modifies about 50% of all yeast proteins and regulates proteins involved in cell cycle regulation. Mutations in the yeast *ard1* gene cause defects in mating, and impede entry into stationary phase in response to nitrogen deprivation and sporulation (Whiteway and Szostak 1985). In eukaryotes, ARD1 is thought to play a role in cell proliferation, tissue development, and cancer (Sugiura et al. 2003; Fisher et al. 2005). Additionally, a mouse ARD1 variant (mARD1²²⁵) containing a conserved acetyltransferase domain but different C terminus, has also been shown to mediate ϵ -acetylation of Lys 532 of HIF-1 α enhancing its degradation (Jeong et al. 2002; Kim et al. 2006). On the other hand, Bilton et al. (2005) demonstrated that overexpression or silencing of hARD1 has no impact on HIF-1 α stability and in vitro experiments using purified recombinant proteins show that hARD1 does not catalyze the acetylation of HIF-1 α ODD domain (oxygen-dependent degradation domain) (Arnesen et al. 2005c; Murray-Rust et al. 2006). Thus,

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Figure 1. Amino acid sequence of human ARD1 showing the functional domains and secondary structure content. Secondary structure prediction was made using the JNET server (Cuff and Barton 1999). (put NLS) putative nuclear localization signal; (H) helical; (E) β -sheet; (-) random coil.

present evidence in the literature suggests that not all ARD1 proteins are capable of both α - and ϵ -protein acetylation, but all ARD1 proteins are N^α -acetyltransferases.

Many proteins are able to adopt a generic aggregated conformation known as amyloids. All amyloid fibers, independent of the protein from which they were formed, have very similar morphology: long and unbranched, a few nanometer in diameter, and they all exhibit a cross-beta X-ray diffraction pattern (Sunde and Blake 1997). Amyloidoses are a group of protein misfolding disorders associated with extracellular protein deposition and include Alzheimer's and Parkinson's disease (Dobson 1999). The ability of structurally and functionally diverse proteins, some not associated with amyloid-deposition diseases, to form amyloids suggest this property is common to all polypeptides (Guijarro et al. 1998; Dobson 2004). The formation of fibers can be described as a nucleation-dependent process. The earliest species visible by electron microscopy resemble small bead-like structures. These early "pre-fibrillar aggregates" subsequently form species with more distinctive morphologies described as "protofilaments." These structures are short, thin, sometimes curly species that are thought to subsequently assemble into mature fibers (Caughey and Lansbury 2003).

Characterization of hARD1 showed that it consists of a compact globular region and a flexible unstructured C terminus. In contrast to proteins capable of forming fibers at acidic pH, hARD1 readily formed protofilament

aggregates under physiological conditions of pH and temperature. The process was accelerated by thermal denaturation and high protein concentrations. Limited proteolysis of hARD1 protofilaments revealed a resistant fragment of 4 kDa whose sequence matched a region contained within the acetyl transferase domain.

Results

hARD1 is a globular protein with an extended C-terminal region

The size exclusion chromatography elution profile of purified recombinant hARD1 (95% purity) contained two peaks (Fig. 2). Analysis by SDS PAGE electrophoresis showed that hARD1 was present in both peaks (Fig. 2B). The elution volume of the first peak corresponded to a greater apparent molecular weight than that of the molecular weight marker ferritin (440 kDa). The apparent molecular weight of the protein contained in the second peak corresponded to 47.7 kDa compared to 26.5 kDa obtained from its amino acid sequence and by

Figure 2. (A) Size exclusion chromatography elution profile of hARD1 (solid line) and hARD1 1-212 (dashed line) monitored at 220 nm. Asterisks denote the position of the molecular weight standards, from left to right: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease (14 kDa). (B) 10% SDS-PAGE gel stained with Coomassie Brilliant Blue R-250 of the different fractions from the hARD1 size exclusion chromatographic separation.

mass spectrometry (data not shown), but similar to that of 52.2 kDa expected for a hARD1 dimer. This higher apparent molecular weight could also be consistent with the protein being partially unstructured monomers. Secondary structure prediction for hARD1 (Fig. 1) suggests that the protein may have an unstructured C terminus. The size exclusion chromatography elution profile of a construct lacking the last 23 C-terminal residues, hARD1 1–212, exhibited some similarities to that of the full-length (Fig. 1A). This protein also separated into two peaks; in the first, the protein also behaved like a molecule with a high apparent molecular weight, but the protein in the second peak had an apparent molecular weight of 21.8 kDa. This value is similar to that expected for the monomeric protein of 24.2 kDa as calculated from its amino acid sequence. These results suggest that, at least for the concentrations used in the size exclusion experiments (initial concentration of 100 μ M), hARD1 is most probably a monomer and its behavior as a protein of high molecular weight may be due to an extended region at the C terminus of the protein.

To confirm that hARD1 is a monomeric globular protein with some unstructured regions, we performed further studies using circular dichroism (CD) and chemical denaturation equilibrium unfolding analysis. The denaturation of hARD1 and hARD1- Δ C (residues 1–178) was measured by monitoring the changes in emission fluorescence at 350 nm upon addition of increasing amounts of urea at 10°C. Both denaturation spectra show a sigmoidal shape (data not shown) suggesting the presence of well-defined tertiary structure in hARD1 and hARD1- Δ C. A summary of the numerical results obtained from the fit to a two-state transition model is given in Table 1. All three parameters for both constructs are the same within the margin of error. The obtained m values of 1.6 and 1.8 appeared too small for proteins of the size of hARD1 and hARD1- Δ C, 235 and 178 residues respectively. Factors known to lower the m value include electrostatic repulsion between charges, which results in a more extended unfolded state, the presence of disulfide bonds and deviation from a two-state unfolding mechanism (Myers et al. 1995). hARD1 and hARD1- Δ C stabilities were analyzed assuming a two-state transition process, although the validity of this assumption required further monitoring of the denaturation

Table 1. Stability parameters of hARD1 and hARD1- Δ C obtained from the chemical denaturation experiments

	m (kcal mol ⁻¹ M ⁻¹)	[Urea] _{50%} (M)	$\Delta G_{D-N}^{H_2O}$ (kcal mol ⁻¹)
hARD1	1.8 $\pm$ 0.06	2.5 $\pm$ 0.02	4.5 $\pm$ 0.1
hARD1- Δ C	1.6 $\pm$ 0.07	2.7 $\pm$ 0.03	4.3 $\pm$ 0.1

Measurements were made using a 3 μ M protein concentration in PBS buffer at 10°C.

Figure 3. (A) Far UV-CD spectra of native hARD1, hARD1 denatured after heating the sample at 85°C and hARD1- Δ C (residues 1–178). (B) Temperature dependence of the molar ellipticity of hARD1 followed at 222 nm. The black trace corresponds to the heating of the sample from 10°C to 80°C; the gray trace corresponds to the cooling of the sample after heating.

process using other methods. The $\Delta G_{D-N}^{H_2O}$ value of 4.5 kcal/mol calculated for hARD1 at 10°C is marginally smaller than the expected average value of 5–15 kcal/mol at 25°C (Fersht 1998). It is probable that hARD1 would be even more destabilized at physiological temperature.

Circular dichroism was employed to investigate the secondary structure content and thermal stability of hARD1. The far UV-CD spectrum of hARD1 and hARD1- Δ C (Fig. 3A) displayed features associated with the presence of α -helices. Although similar, the two spectra showed subtle differences. Both minima at 208 and 222 nm are more pronounced for hARD1- Δ C, the minimum at 208 nm for hARD1 is shifted to 210 nm in the spectrum for hARD1- Δ C, and the signal at 200 nm reaches higher positive values in the spectrum of hARD1- Δ C. The increase in the signal at 200 nm upon removal of residues 179–235 suggests that the

fragment removed consisted of a random coil whose hallmarks are a weak signal at 225 nm and a strong negative band at 200 nm. Deconvolution of hARD1 and hARD1- Δ C far-UV spectra using the CDNN program predicted a random coil content of 44% and 40%, respectively. In addition, the CD spectrum of the isolated hARD1 C terminus (residues 168–235) displayed only a negative signal at 200 nm, as is observed for random coil polypeptides (data not shown).

Thermal denaturation of hARD1 monitored at 222 nm exhibited a cooperative transition with increasing temperature (Fig. 3B). Surprisingly, the signal became more negative upon denaturation, whereas loss of helical structure would give a change in the opposite direction. Thermal denaturation of hARD1 was not a reversible process (Fig. 3B), although there was no apparent aggregation and the signal of the photomultiplier tube did not ramp off as happens when proteins aggregate. The apparent temperature of unfolding of hARD1 corresponded to $\sim 45^{\circ}\text{C}$. The CD spectrum of hARD1 at 85°C (Fig. 3A) was consistent with an increase β -sheet content.

hARD1 forms protofilaments under physiological conditions

The results presented above suggest that hARD1 exists as more than one structural conformation in equilibrium: (1) Size exclusion chromatography provided evidence of the presence of soluble aggregates of high molecular weight; (2) chemical denaturation showed the protein has an unusually low m value; and (3) upon thermal denaturation, instead of becoming a random coil, hARD1 acquired new secondary structure content characteristic of β -sheets. All these pointed to the possibility that hARD1 was forming fibers whose characteristics, among others, are those of large aggregates with an increased β -sheet content.

Recombinant hARD1 was incubated with constant agitation at 37°C in PBS buffer for ~ 4 d or until there were no further changes in the absorbance value at 300 nm, and subsequently stored at 4°C for further analysis. hARD1 underwent a profound change after prolonged incubation at 37°C . Aggregates of hARD1 bound both Congo red and thioflavin T, a common indication of the presence of amyloid fibers (Fig. 4A,B) (Klunk et al. 1999; LeVine 1999). A solution of Congo red containing hARD1 aggregates showed a red shift of visible light absorbance with a point of maximal spectral difference between the fibril-containing solution and dye-only solution corresponding to 521 nm, the same as that observed for $\beta 2$ -microglobulin fibrils (Ivanova et al. 2003). Moreover, the thioflavin T fluorescence emission spectrum was clearly enhanced in a dose dependent-manner in the presence of hARD1 aggregates. Higher initial concentration of proteins resulted in bigger increases in the fluorescence signal, suggesting the presence of higher amounts of fibers in the sample. Finally,

electron micrographs of hARD1 aggregates revealed unbranched elongated fibrils with an average diameter of 14 nm, comparable to amyloid fibers formed by the yeast prion

Figure 4. Characterization of hARD1 aggregates. (A) Fluorescence emission spectra of thioflavin T in the presence of different concentrations of aggregated hARD1. (B) Absorbance spectrum of a Congo red solution in the absence and presence of aggregated hARD1. The difference spectrum was obtained by subtracting the Congo red spectrum in the absence of aggregated hARD1 from the Congo red spectrum in the presence of aggregated hARD1. (C) Electron micrograph of aggregated hARD1. Bar, 100 nm. In all cases, aggregated samples were obtained by incubation at 37°C in PBS buffer with constant agitation for 4 d.

protein Ure2p. The hARD1 fibers were shorter than those observed for Ure2p (Bousset et al. 2004) or α -synuclein (Miake et al. 2002), but similar in length to the protofilaments formed by a mutant transthyretin under physiological conditions (Lashuel et al. 1999). They were only a few hundreds of nanometers in length (150–400 nm) and were not totally straight (Fig. 4C). These results point to hARD1 forming protofilaments under physiological conditions.

Limited proteolysis with proteinase K was used to identify the specific fragment in hARD1 responsible for protofilament formation (Fig. 5). Proteolysis of aggregated hARD1 resulted in a predominant resistant fragment of ~ 4 kDa. This well-defined fragment was absent in the digestion profile of the soluble form in which only a smear could be observed. Another band of ~ 6 kDa was populated in both cases; for the soluble form, however, it disappeared after 16 min of digestion while in the aggregated form small amounts could still be detected after 26 min. The 4 kDa-resistant peptide was purified by SDS-PAGE and analyzed by N-terminal Edman microsequencing, revealing the sequence AVKRSHR at its N terminus. This region corresponds to residues 76–82 of the hARD1 primary sequence that exactly coincides with the predicted nuclear localization signal and is next to the Acetyl-CoA binding domain (Fig. 1). Given that the peptide product of digestion is ~ 4 kDa, it seems reasonable to conclude that the core of the protofilaments comprises residues 76–102 and is contained within the acetyltransferase domain of hARD1.

Figure 5. Proteinase K time course digestion of soluble (A) and aggregated (B) hARD1. Proteolysis fragments were analyzed by SDS-PAGE electrophoresis stained with Coomassie Brilliant Blue R-250. Lanes from left to right: Molecular weight markers, hARD1 input and digested samples taken every 2 min.

Unstructured regions have long been recognized as features common to proteins capable of forming fibers. Unstructured regions in amyloid-forming proteins can range from the totally unfolded such as α -synuclein (Li et al. 2002), to proteins which share a two-domain organization consisting of a globular domain and an unstructured N or C terminus, such as the yeast prion protein Ure2P (Perrett et al. 1999; Thual et al. 2001), or the fungal HET-s prion protein (Balguerie et al. 2003). hARD1 also has a two-domain organization and is capable of forming fibers. To confirm further the limited proteolysis results that suggest that the structured region of the protein is the one responsible for forming the fibers, recombinant hARD1- Δ C was incubated under the same conditions as the full-length protein and further examined. After incubation at 37°C for 4 d, hARD1- Δ C showed visible aggregation. A Congo red solution displayed the characteristic red shift at the wavelength of maximum absorbance upon binding to hARD1- Δ C aggregates (Fig. 6A). Electron microscopy examination of hARD1- Δ C aggregates revealed unbranched elongated fibers similar to the ones observed for the full-length hARD1 (Fig. 6B). They were 20 nm in diameter but considerably shorter (100–250 nm). Aggregates with spherical and oblong shapes were also observed. In contrast, similar experiments performed with a fragment of hARD1 comprising residues 168–235 (hARD1 C terminus) lacked the typical red shift in the wavelength of maximal absorbance of Congo red and electron micrographs from them show only amorphous aggregates (Fig. 6C,D). These results suggest that the region of hARD1 forming the fibers is indeed contained within the structural region of the protein and coincides with that responsible for the catalytic activity of the enzyme.

Discussion

ARD1 and NAT1 (NATH for the human homolog) are the subunits of the major N^{α} -acetyltransferase complex in eukaryotes. We showed by biophysical characterization of hARD1 using size exclusion chromatography, CD, and fluorescence spectroscopy that it consisted of a folded globular region comprising two thirds of the N-terminal portion of the protein and a flexible unstructured C terminus. The C-terminal portion of hARD1 adopted an unstructured conformation both in isolation and in the context of the full-length protein. Under physiological conditions, hARD1 was capable of assembling into protofilaments as judged by CD and electron microscopy. The aggregates bind the diagnostic dyes Congo red and thioflavin T, characteristic of amyloid fibers formed by other proteins. Deletion of the C-terminal region did not affect the ability to form fibers nor the stability of the N-terminal globular domain. Analysis of the amino acid

Figure 6. Characterization of hARD1-ΔC and hARD1 C terminus aggregates. (A) Absorbance spectrum of a Congo red solution in the absence and presence of aggregated hARD1-ΔC. (B) Electron micrograph of aggregated hARD1-ΔC. (C) Absorbance spectrum of a Congo red solution in the absence and presence of aggregated hARD1 C terminus. (D) Electron micrograph of aggregated hARD1 C terminus. The difference spectra were obtained by subtracting the Congo red spectrum in the absence of aggregated protein from the Congo red spectrum in the presence of aggregated protein. In the electron micrographs the scale bar corresponds to 100 nm. Aggregated samples were obtained by incubation at 37°C in PBS buffer with constant agitation for 4 d.

composition of the hARD1 C terminus (residues 179–235) indicated that it is enriched in the disorder-promoting residues Ser, Glu, Asp, Lys, Ala, and Pro (Dunker et al. 2001) which account for 70% of the total amino acid composition of this region. In addition, the PONDR predictor of intrinsically disordered regions (Romero et al. 2001) predicted two disordered regions between residues 153–173 and 179–235, while the JNET server suggests a random-coil region spanning residues 178–235 (Fig. 1). All these predictions are in agreement with the observations presented here. In addition, the flexible nature of hARD1 C terminus may facilitate the previously observed caspase-dependent cleavage of this region (Arnesen et al. 2005a). The biological function of the hARD1 C terminus is not yet completely understood. Sequence alignment of ARD1 proteins from yeast and higher eukaryotes show that this C terminus is not present in yeast and may be the result of an insertion in higher eukaryotes (Polevoda and Sherman 2003; Arnesen et al. 2005a). The C terminus of yeast ARD1 contains a potential coiled-coil domain, which may mediate heterodimerization with NAT1. In the case of the human

protein, the N-terminal region performs this function. Recently, Kim et al. (2006) identified three variants of the mouse ARD1 (mARD1) protein and two variants of the human ARD1, which share a well-conserved *N*-acetyltransferase domain but differ in their C terminus. These variants modulate HIF-1α stability differently: mARD1²²⁵ negatively regulates HIF-1α, while hARD1²³⁵, the subject of this study, does not. In agreement with this observation it is unlikely that the unstructured C terminus of hARD1²³⁵ mediates the interaction with the unstructured HIF-1α ODD domain (Sánchez-Puig et al. 2005). There is no evidence, so far, as to whether the C terminus of the other variants are also unstructured, and further research is needed to clarify their structures and functions. hARD1 C terminus has also been shown to associate with the cytoplasmic domain of APP (amyloid precursor protein) suppressing the secretion of the Aβ40 amyloidogenic peptide (Asaumi et al. 2005).

So far, hARD1 has not been associated with any protein deposition disease. Its aggregates, similarly to those of many proteins not associated with amyloid diseases, are

indistinguishable from those found in pathological conditions. This evidence supports the proposal that the ability to form amyloids is a general property of polypeptide chains (Dobson 2001, 2004). hARD1 protofilaments were produced under physiological conditions, but so far there is no evidence that demonstrates they are also formed *in vivo* and thus further experiments are needed. Limited proteolysis experiments showed that the protofilaments are being formed upon structural rearrangements of the acetyltransferase domain. It is tempting to speculate that fiber formation abrogates the enzymatic activity of hARD1 and, on the contrary, binding to its substrates or heterodimerization with NATH may increase hARD1 stability and avoid the fibril formation pathway. In agreement with this idea, it has recently been shown that in all tumors in which NATH was down-regulated compared to non-neoplastic tissue, hARD1 levels were also reduced (Arnesen et al. 2005b).

Conditions or mutations that destabilize the native protein and populate partially unfolded states are associated with the amyloid formation of several proteins, including transthyretin (Lai et al. 1996; Lashuel et al. 1999), lysozyme (Booth et al. 1997), β 2-microglobulin (McParland et al. 2000), and SH3 domains (Guijarro et al. 1998). Some proteins such as transthyretin require dissociation of the native tetrameric form to an alternatively partially folded monomer, which then self-assembles into protofilaments and ultimately into amyloid fibrils. Partially unfolded intermediates and mature fibers were not observed for hARD1 and additional studies need to be done to confirm that the mechanism followed by hARD1 is similar to that already described for other amyloidogenic proteins.

Materials and methods

Plasmids

Wild-type human *ard1* (arrest-defective 1 protein) was amplified by PCR from a human thymus cDNA library (Clontech) and cloned into the pGEMT vector (Promega). Plasmids expressing the full-length protein and different fragments of it were constructed in order to carry out a detailed characterization of hARD1. The full-length gene and regions of it corresponding to the different fragments were re-amplified by PCR and digested with BamHI and EcoRI. The amplified fragments were subsequently purified and ligated in frame into the expression plasmid pRSETHisLipo-TEV (Sánchez-Puig et al. 2005) at the BamHI and EcoRI sites immediately downstream of the TEV protease site.

Protein expression and purification

The plasmids expressing hARD1 full-length (hARD1), hARD1 1–212 (residues 1–212) and hARD1- Δ C (residues 1–178) were transformed into *Escherichia coli* C41 (Miroux and Walker 1996) for protein overexpression. Cultures were grown in 2 x TY medium containing 100 μ g/mL ampicillin to an optical density at 600 nm of $\sim$ 0.8, induced with 1 mM isopropyl-1-thio- β -D-

galactoside (IPTG) and further incubated for 16 h at 20°C. The purification of hARD1, hARD1 1–212, and hARD1 C terminus was done as described (Sánchez-Puig et al. 2005). The purification of hARD1 Δ C was identical to the procedure followed for hARD1 and hARD1 1–212 up to the digestion with TEV protease. Subsequently, the salt concentration was halved and the sample was loaded onto a column packed with 30 mL of Resource 30S media (Amersham) pre-equilibrated with 30S buffer A (20 mM phosphate buffer [NaP_i] at pH 6.4, 150 mM NaCl, and 10 mM 2-mercaptoethanol). The protein was eluted with a gradient of 0%–50% of 30S buffer B (20 mM NaP_i at pH 6.4, 500 mM NaCl, 10 mM 2-mercaptoethanol) over 15 column volumes. The fractions containing the proteins were dialyzed against PBS buffer and concentrated using Centrprep centrifugal concentrating devices. Samples were flash frozen in liquid nitrogen and stored at -80°C .

Size exclusion chromatography

Purified hARD1 and hARD1 1–212 (initial concentration of 100 μ M) was injected onto an analytical Superdex 200 HR10/30 column (Pharmacia) through a 200 μ L loop in an ÄKTA design XT Explorer 900 Kit (Pharmacia) instrument equipped with a Monitor UV-900 detector and the Unicorn 3.10 software package. The shorter construct hARD1- Δ C was not assayed because of the low solubility of the protein (15 μ M). The buffer used consisted of 25 mM NaP_i (pH 7.2), 150 mM KCl, and 10 mM 2-mercaptoethanol. Molecular weight standards (Pharmacia) were run under the same conditions. Their experimental elution volumes and theoretical molecular weights were used to create a calibration curve from which the apparent molecular weight (M_r) of hARD1 and hARD1 1–212 was calculated.

Far UV circular dichroism

Temperature dependence of the ellipticity was followed with a JASCO J-720 spectropolarimeter equipped with a JASCO PTC-348WI temperature controller. CD spectra were recorded using a 1 mm pathlength cuvette and protein concentration of 10 μ M. Thermal denaturation was followed at 222 nm, with an increase of 1° per min, a time response of 10 sec and a bandwidth of 1 nm. Circular dichroism wavelength scan measurements were followed with an AVIV 2025F stopped flow circular dichroism spectrometer equipped with a Peltier temperature controller. CD spectra were recorded using a 1 mm pathlength cuvette at the same protein concentrations as for thermal denaturation. Scan wavelength was followed from 260 to 190 nm, with an increase of 0.5 nm per step, an averaging time of 5 sec, and a bandwidth of 1 nm. All samples were dialyzed against PBS buffer, 1 mM dithioerythritol (DTE). The CDNN Deconvolution software (version 2; <http://Bioinformatik.biochemtech.uni-halle.dee/cdnn>) was used to calculate the secondary structure content.

Urea equilibrium denaturation

The intrinsic fluorescence of hARD1 was monitored using a Perkin Elmer LS 50B luminescence spectrometer, at an excitation wavelength of 270 nm (4 nm bandwidth) and the emission scanned from 300 to 400 nm (4 nm bandwidth). Samples consisted of 3 μ M protein and different concentrations of urea in PBS buffer, 10 mM 2-mercaptoethanol. All samples were incubated at 10°C for 10 h before the fluorescence

was measured in a 1 mL cuvette in thermostatted cuvette holders at the same temperature. The data were analyzed according to a two-state transition model as described previously (Bullock et al. 1997) and the m value was calculated as $m = \Delta G_{D-N}^{H_2O} / [Urea]_{50\%}$.

Thioflavin T assay

Thioflavin T solution was made up to 65 μ M in PBS buffer. Measurements were performed as described (LeVine 1993) at 25°C in an Aminco-Bowman SLM series 2 luminescence spectrometer (SLM Instruments, Urbana, IL). The fluorescence spectra were measured at an excitation wavelength of 440 nm (4-nm slit width) and emission was recorded from 460 to 650 nm (8-nm slit width). Two milliliters of solutions containing different concentrations of ARD1 were incubated at 37°C for 4 d in PBS buffer with constant agitation. Samples were diluted sixfold with Thioflavin T solution and pre-incubated for 5 min before fluorescence measurement. A strong increase in fluorescence emission at 482 nm was diagnostic of fiber formation.

Congo red assay

A 300 μ M stock solution of Congo red was prepared in PBS buffer and filtered through a 0.22 μ m filter. Measurements in solution were done as described (Klunk et al. 1989, 1999). The exact concentration of the solution was determined spectrophotometrically by measuring the absorbance of a diluted aliquot at 505 nm ($\epsilon_{505} = 59,300 \text{ cm}^{-1} \text{ M}^{-1}$). Suspensions of protein precipitates from earlier fiber growth experiments ($\sim 5 \mu$ M) were mixed with the Congo red solution (10 μ M) and the absorbance was recorded from 700 to 300 nm. A red shift in the wavelength of maximum absorbance of the Congo red was diagnostic of fiber formation.

Electron microscopy

Negatively stained samples were adsorbed onto air glow-discharged carbon-coated grids. A 3- μ L drop containing protein aggregates was applied to the grid for 1 min and subsequently washed with 50 μ L of PBS buffer. Staining was performed using 50 μ L of 2% (w/v) uranyl acetate, before blotting dry. Grids were examined with an FEI 208 electron microscope (Philips Electron Optics) at 80 kV.

Limited proteolysis

Soluble and aggregated hARD1 (20 μ M) were digested at 37°C with 10 μ g/mL proteinase K. Reactions were stopped by addition of one volume of SDS-PAGE loading buffer and were immediately heated at 100°C for 5 min. Aliquots of 15 μ L were analyzed by SDS-PAGE followed by Coomassie Blue staining and transferred to a PVDF membrane for N-terminal microsequencing.

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