Amyloid fibrils from the mammalian protein prothymosin α

Nikolai A. Pavlov[a,b], Dmitry I. Cherny[a,c], Gudrun Heim[a], Thomas M. Jovin[a,*], Vinod Subramaniam[a,*,1]

[a] *Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany*
[b] *Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899 Moscow, Russia*
[c] *Institute of Molecular Genetics, Russian Academy of Sciences, Kurchatov's Square, 123182 Moscow, Russia*

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Abstract Mammalian prothymosin α, a small (12 kDa) and extremely acidic protein (p*I* 3.5), is a member of the growing family of 'natively' unfolded proteins. We demonstrate that at low pH (∼3) and high concentrations, prothymosin α is capable of forming regular elongated fibrils with flat ribbon structure 4–5 nm in height and 12–13 nm in width as judged from scanning force and electron microscopy. These aggregates induced a characteristic spectral shift of thioflavin T fluorescence and their circular dichroism spectra were indicative of significant β-sheet content, suggesting formation of classical amyloid. Our findings indicate that natively unfolded proteins may have a general propensity to form amyloid fibrils under conditions inducing partially folded conformations.

Key words: Natively unfolded; Protein aggregation; Amyloid fibrils; Scanning force microscopy; Electron microscopy

1. Introduction

A number of human diseases are accompanied by deposition of normally soluble proteins in the form of stable fibrils called amyloids [1–3]; mature fibrils and/or their precursors are believed to have a pathogenic effect. About 20 different proteins have been found to be involved in various types of amyloidoses, and most are related neither structurally nor at the level of primary sequence. X-ray diffraction data suggest that all amyloid proteins form a cross-β structure, with continuous β-sheets running parallel and β-strands perpendicular to the fiber axis [4,5]. Despite large differences in the size of aggregating peptides and proteins, ranging from 5 [6] to 415 [7] amino acids, the overall morphology of fibrils is strikingly common: all the fibrils have similar diameter (6–12 nm) and substructure, consisting of four to eight protofilaments organized in a symmetric tubular or ribbon-like array [6,8,9]. It has been suggested that the ability of a protein to form amyloid is not determined by its sequence but rather reflects a general property of the protein backbone [10]. In support of this conclusion is the fact that several proteins not involved in amyloidoses aggregate into fibrils under certain conditions [7,10–13]. In most cases, the aggregation process was induced by partial unfolding of normally globular proteins, that is, by maximizing the population of intermediate conformations arising during the unfolding transition.

Here we report the formation of fibrils by the 'natively unfolded' protein prothymosin α (pTα). This 109 amino acid-long protein is very acidic, containing ∼50% aspartic and glutamic acid, no aromatic and sulfur residues, and very few large hydrophobic aliphatic amino acids [14,15]. pTα adopts a random coil-like conformation with no regular secondary structure, characteristic of the family of 'natively unfolded' proteins [16,17]. An important member of this family is α-synuclein, which forms fibrillar aggregates, and whose aggregation is associated with many neurodegenerative diseases including Parkinson's Disease [18,19]. We have found that pTα aggregates into typical amyloid fibrils under acidic conditions that presumably decrease the repulsive intermolecular forces caused by the high negative charge of the protein at neutral pH and thereby induce its partial folding. pTα is only the second known example of a natively unfolded protein forming fibrillar aggregates, and supports the argument that the determinants of aggregation are the nature and population of intermediate conformations in the folding–unfolding transitions.

2. Materials and methods

2.1. Protein purification and amyloid fibrils formation

pTα was isolated from *Escherichia coli* BL21(DE3)/pHP12 (gift of Dr. Alexandra Evstafieva) by a hot phenol extraction procedure [20], further purified by anion exchange chromatography on a HiLoad Q-Sepharose column (Amersham Pharmacia Biotech, Sweden), concentrated using Centricon-3 cartridges (Amicon, USA), and extensively dialyzed against distilled water. There was no evidence of aggregation in the resulting stock solution. For fibrillization, 100–200 μl solutions of 1–5 mg/ml pTα in 100 mM glycine–HCl buffer, pH 2.5–3.5, were incubated in glass bottles with stirring at 37 °C.

2.2. Circular dichroism (CD) spectroscopy

CD measurements were performed with a Jasco J715 spectropolarimeter. The CD spectra of samples diluted to a pTα concentration of 8.3 μM (4 s response time, 50 nm/min scan rate, 7–20 scan averages) were obtained using a 1 mm pathlength cuvette. Experimental data were converted to units of differential molar circular dichroic absorption. The spectra were deconvoluted using the CDPro software package [21].

2.3. Fluorescence spectroscopy

Fluorescence measurements were made with a Cary Eclipse fluorescence spectrophotometer (Varian, Australia) in 100 μl sample volume

*Corresponding authors. Fax: (49)-551-2011467.
[1] Present address: Advanced Science and Technology Laboratory, AstraZeneca R&D Charnwood, Bakewell Road, Loughborough LE11 5RH, UK. Fax: +44-1509-645519.
E-mail addresses: tjovin@gwdg.de (T.M. Jovin), vinod.subramaniam@astrazeneca.com (V. Subramaniam).

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cuvettes, using excitation and emission slit widths of 5 nm. For the thioflavin T (ThT) binding assay, a 1 μl aliquot was withdrawn from the fibril suspension/solution containing 100–333 μM pTα and added to 100 μl of freshly made 10 μM ThT solution (at pH 2.5 or 4.5). ThT emission and excitation fluorescence spectra were obtained using 446 nm excitation (325 nm for the free dye) and emission at 480 nm (465 nm for the free dye). The kinetics of fibrillogenesis were followed by measuring the ThT fluorescence emission at 480 nm using excitation at 446 nm. For the 1-anilino-8-naphthalene sulfonate (ANS) binding assay, a 1 μl aliquot of the fibril solution was added to 100 μl of 100 μM ANS solution containing 100 mM glycine–HCl, pH 2.5. Emission spectra were recorded from 460 to 600 nm, using 370 nm excitation. The fluorescence emission maximum shifted from 520 nm to 485 nm during the course of the fibrillization, and was used to monitor the kinetics of the process.

2.4. Electron microscopy (EM)

A 1 μl aliquot was taken from the reaction mixture, diluted 10-fold in the incubation buffer, and immediately placed onto glow-discharged carbon film as described [22]. After 2–5 min of adsorption, the samples were rinsed with 2% aqueous uranyl acetate for 10–15 s and blotted with a filter paper. Where indicated, the samples were unidirectionally shadowcast at an angle of 8° with tungsten, using the electron gun of an Edwards Auto 306 apparatus. The samples were examined with a CM12 electron microscope (Philips, The Netherlands). Measurements of the micrographs were done using NIH Image software modified for Windows (Scion Corporation, Frederick, MD, USA)

2.5. Atomic force microscopy (AFM)

An aliquot from the fibril suspension was diluted with the incubation buffer and immediately deposited onto freshly cleaved mica (W. Plannet GmbH, Germany), and imaged in tapping mode in a fluid cell of the Nanoscope IIIa AFM (Digital Instruments, Santa Barbara, CA, USA). A silicon nitride cantilever (NP-S, Digital Instruments) with a force constant of 0.32 N/m was used at an oscillation frequency of ~8.4–8.8 kHz.

3. Results and discussion

Human pTα was produced in *E. coli* and purified by the hot phenol extraction method developed by Evstafieva et al. [20], which is based on the unique tendency of pTα to accumulate in an aqueous phase rather than in an organic phase, like all bacterial proteins. The protein was purified further by anion exchange chromatography to more than 99% purity and showed characteristic features reported previously [7,23,24]. Thus, in the course of gel-filtration chromatography, pTα eluted as a protein with an apparent molecular mass of 58.4 ± 0.7 kDa (not shown); the molecular mass calculated from its amino acid composition is 12 kDa. The CD spectrum of the protein at pH 7.5 exhibited a minimum at 198 nm (Fig. 1A). Both experiments suggested that under neutral pH conditions pTα exists in a random coil conformation. At pH 2.5 the shape of the CD spectrum changed, indicating the adoption of α-helical conformation.

There was no evidence of further conformational perturbations after prolonged incubation of 8.3 μM pTα under acidic conditions. However, overnight incubation of the protein at higher concentration (100–333 μM) in 100 mM glycine–HCl, pH 2.5, at 37°C resulted in a dramatic CD change, indicating a predominant (~70%) β-strand conformation of the polypeptide chain (Fig. 1A). Such a behavior is characteristic for the formation of amyloid fibrils that have high β-strand content. The rate of fibril formation increased with the concentration of the incubated protein.

Further evidence of pTα fibrillization was obtained with a ThT binding assay. It is known that most amyloids induce a

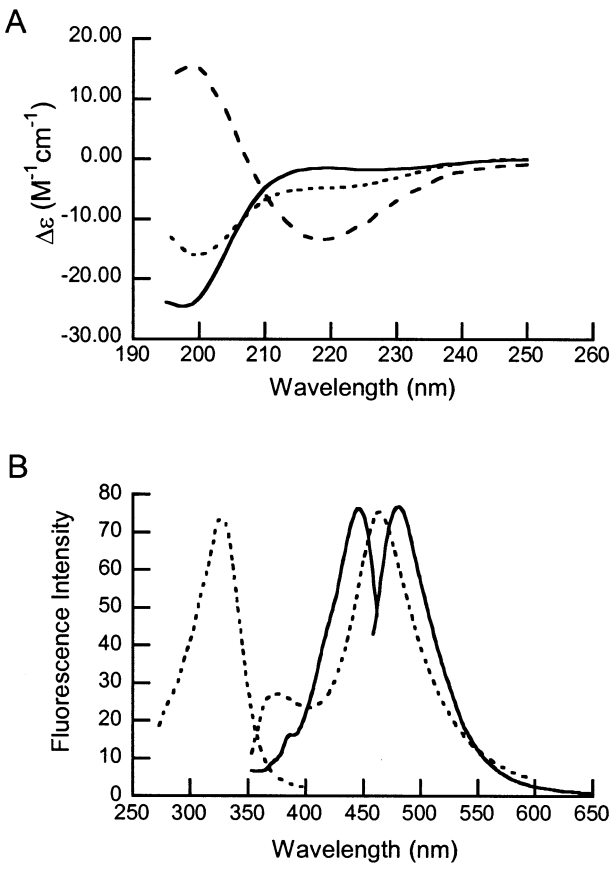

Fig. 1. CD spectra of pTα and fluorescence spectra of bound ThT. A: CD spectrum at protein concentration 8.3 μM, pH 7.5 (solid line), and pH 2.5 (short-dashed line). Protein concentration, 333 μM in 100 mM glycine–HCl, pH 2.5, after overnight incubation at 37°C with stirring (long-dashed line). B: Fluorescence excitation and emission spectra of 10 μM ThT in the presence of pTα aggregates (solid line) and of free 10 mM ThT (short-dashed line).

strong red shift of the ThT fluorescence excitation spectrum and increase the fluorescence quantum yield of the dye [25]; pTα aggregates demonstrated these properties very clearly (Fig. 1B). Since it has been suggested that the apparent affinity for ThT decreases with lower pH due to the quaternary positively charged benzothiazole ring nitrogen [26], a pH ≥ 6 is normally used for ThT binding. We indeed observed a five-fold increase of fluorescence intensity at 480 nm (using excitation at 446 nm) for fibrils studied at pH 4.5 compared to pH 2.5 (not shown). However, despite the lower sensitivity, we performed fluorescence assays at pH 2.5 in order to preserve conditions optimal for fibrillization.

We further analyzed the morphology of the aggregates, which showed a high signal in the ThT assay, by means of transmission EM and scanning force microscopy. Both techniques showed the presence of amyloid fibrils of lengths ~50–500 nm with similar structures, although minor differences could be discerned (Fig. 2). From the images of negatively stained samples it was clear that fibrils were not uniform in width; i.e. some short regions were narrower (~5 nm) than the typical width (~11 nm) of the majority of the fibrils. Sometimes these narrow segments were clustered, with a periodicity of ~100 nm (Fig. 2A). Unidirectionally shadowed samples showed that the height of the fibrils was not uniform (Fig. 2B). That is, the height was generally ~4–5 nm,

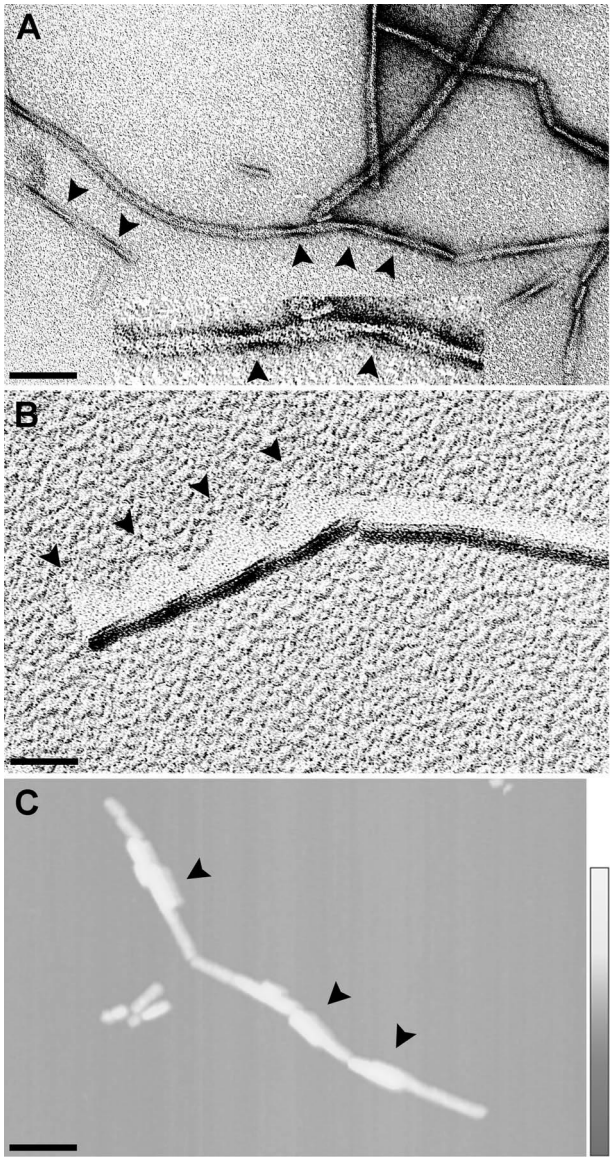

Fig. 2. Structure of pTα fibrils. A: Transmission electron micrograph of negatively stained sample. Arrowheads indicate narrow regions of fibrils; inset, magnified view of fibril segment with modulated width. B: Transmission electron micrograph of unidirectionally shadowed sample. Arrowheads indicate regions of fibrils with increased height. C: Scanning force microscopy image (height). Arrowheads indicate regions of fibrils with increased height and width. Color bar, height 30 nm. Scale bars, 100 nm.

although a strong modulation from 4–5 to 11–13 nm was clearly seen with a period of ~120 nm. We conclude that the fibrils exhibited a flat ribbon structure, ~4–5 nm in height and ~11–13 nm in width. Modulations in width or height were most probably due to twist of the fibrils. SFM imaging revealed a minimal height of fibrils of ~4–6 nm consistent with the values determined by EM. However, contrary to EM data, segments with approximately double height had a significantly wider appearance (Fig. 2C). The different morphologies observed depended on the conditions and imaging technique.

The time course of pTα fibrillization at pH 2.5 was monitored simultaneously with several techniques (Fig. 3A). Both ThT and ANS fluorescence, as well as CD at 218 nm (a measure of β-strand content) demonstrated a sigmoidal time dependence with a lag phase characteristic of nucleation-dependent processes. All methods yielded almost coincidental signal progressions suggesting that the various signals detected synchronously arising features. In contrast, turbidity measurements showed a delayed response, most likely related to the formation of larger aggregates (not shown). CD spectra col-

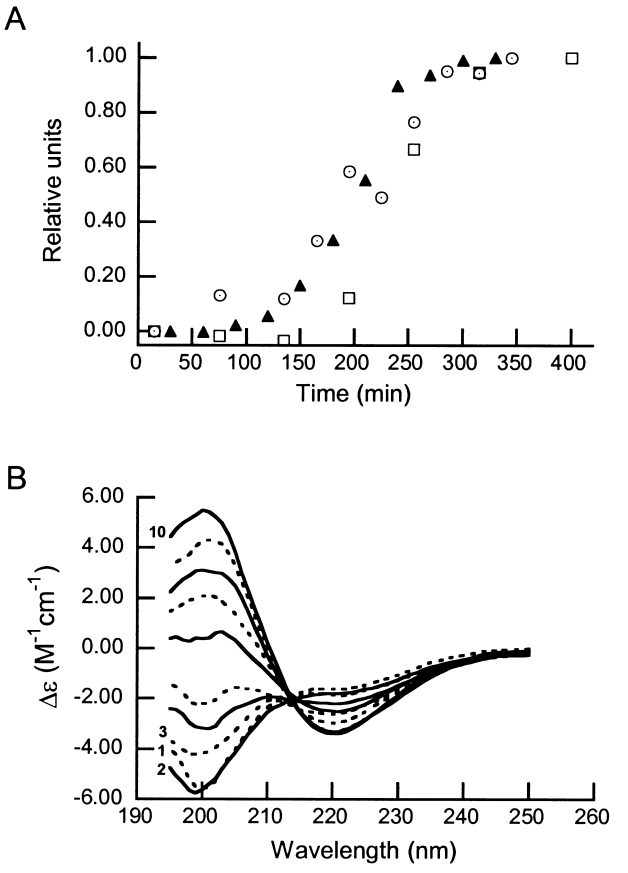

Fig. 3. Kinetics of pTα fibrillization. A: ThT binding assay (▲), ANS binding assay (□) and molar ellipticity at 218 nm (⊙). All measures are given in relative units. B: Far-UV CD spectra of pTα acquired during the course of fibrillization as a function of incubation time (curves increasing in number). Spectra 1–10 were acquired after 15, 75, 135, 165, 195, 225, 255, 285, 315, and 345 min of incubation respectively. Read spectra from bottom to top (i.e. spectrum 10 corresponds to the longest incubation time), except for spectra 1 and 2, which correspond to 15 and 75 min of incubation, respectively.

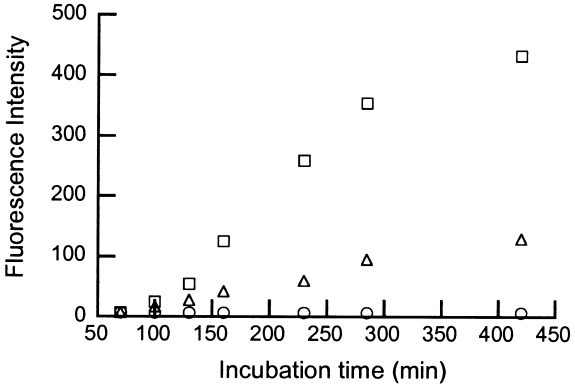

Fig. 4. Kinetics of pTα fibrillization assessed by ThT binding assay. pH 2.5 (□), pH 3 (△), and pH 3.5 (○).

lected during fibrillization exhibited an isodichroic intersection at 212 nm, suggesting a two-state transition of pTα from a partially folded to the β-strand conformation (Fig. 3B).

Fibrils formed by pTα demonstrated high sensitivity to pH, being fairly stable upon increasing pH up to 4.5 but immediately breaking down at pH 5. The ability to fibrillize was inhibited at even lower pH (Fig. 4). It has been reported previously that pTα partially folds under acidic conditions, demonstrating a sigmoidal pH dependence of α-helicity with a midpoint at pH 4.4 [24]. It was hypothesized that the structure of the folding product (10% α-helix at pH 2.4) represents the premolten globule (compact denatured) state, based on decreased hydrodynamic volume of the protein and enhancement of ANS fluorescence indicative of solvent exposed hydrophobic clusters.

We hypothesize that the extreme instability of pTα fibrils at $pH > 4.5$ originates from strong repulsive forces between negatively charged molecules, which also account for the total unfolding of isolated protein molecules. It is also possible that the pTα fibrillogenic behavior at $pH \sim 3$ is facilitated by the transformation of a random coil into a partially folded conformation characterized by the presence of hydrophobic patches and lack of rigid tertiary structure. At high pTα concentration these properties could favor both hydrophobic and hydrogen bond-mediated intermolecular contacts and lead to the formation of oligomers slowly transforming into the β-strand-rich nuclei required for amyloid formation.

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