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Thioflavin T fluorescence anisotropy: An alternative technique for the study of amyloid aggregation

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Abstract

The process of amyloid polymerisation raises keen interest in particular because of the biomedical impact of this process. A variety of analytical methods have been developed to monitor amyloid formation. Thioflavin T (ThT) is the most commonly used dye for detection of amyloid aggregation. Nevertheless, ThT fluorescence enhancement is strongly dependent of fibril morphology. In this study using the HET-s prion fibril model, we show that amyloid formation can be monitored by measuring ThT fluorescence anisotropy. Kinetic parameters obtained by this method are identical to those determined by CD spectrometry. We propose that ThT anisotropy represent an interesting, simple and alternative technique to analyze the amyloid formation process.

Keywords: Thioflavin T; Anisotropy; Amyloid; Prion; Kinetic aggregation; Dye binding; Fibrillogenesis; *Podospora anserina*; Protein aggregation; Fibril polymerisation

Amyloid fibrils are β-sheet rich protein polymers responsible for a number of fatal human protein deposition diseases [1]. Thioflavin T (ThT) is the most commonly used dye to diagnose amyloid fibril formation, both *ex vivo* and *in vitro* [2,3]. In spite of this wide spread use, the exact mechanism underlying specific and rapid binding to amyloid fibrils remains largely unknown. Generally, ThT undergoes a strong increase in fluorescence quantum yield (around 480 nm, when excited at 450 nm) upon binding to amyloid fibrils [3]. However, fibril induced ThT-fluorescence varies greatly depending on the considered amyloid peptide and can be extremely modest for certain amyloid models [3–5]. The ThT molecule consists of a pair of benzothiazole and benzaminic rings freely rotating around a shared C–C bond [6]. ThT behaves as a rotor molecule; it is believed that the increase in quantum yield upon binding results from the inhibition of this free rotation of the rings. In free ThT, when the rotation is not hindered, excited ThT molecules can undergo a torsional relaxation which effectively competes with the radiative transition [7]. It is thus possible that the enhancement of fluorescence emission depends dramatically of the capacity of a given amyloid fibril to hinder the ThT ring free rotation [4]. In addition, in all of ThT binding reaction to macromolecules, there is an alteration of the ThT global Brownian tumbling that can be detected by an increase in fluorescence anisotropy.

Reliable characterisation of the fibrillogenesis kinetics of amyloid proteins is of uppermost importance for instance to study the effect of inhibitor molecules of potential pharmacological importance or of familial disease-associated mutations [8]. Aggregation kinetics can be monitored by absorbance and scattering determinations [9,10], sedimentation assays, circular dichroism (CD) [11,12], dynamic and static light scattering [13], Congo Red binding [14], ThT binding [15,16], electron microscopy (EM), atomic forces microscopy (AFM) [17] or Trp/Tyr anisotropy [18,19]. All of these instrumental techniques suffer some associated technical limitations. For instance, absorbance and scattering depend on the fibril morphology (in a

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non-obvious way) [20], CD only determines soluble species and amyloid fibrils usually undergo rapid precipitation, Congo Red is not a specific dye and it is a kinetic inhibitor molecule [21], the quantum yield of ThT fluorescence depends dramatically of amyloid fibril type and there is a number of amyloid fibrils with a very modest and unappreciable ThT fluorescence [4]. Obviously, Trp/Tyr anisotropy can only be applied to peptides and proteins containing such residues.

In the present study, we used HET-s prion fibrils as a model of amyloid fibrils leading to a low ThT fluorescence enhancement and show that amyloid aggregation kinetic can conveniently be monitor by following the increase in ThT fluorescence anisotropy. We propose that ThT anisotropy represents an interesting, simple alternative method to follow amyloid formation.

Materials and methods

HET-s expression and purification. HET-s(218–289) protein expressed as a C-terminal histidine-tagged construct in *Escherichia coli* was purified under denaturing conditions (50 mM Tris at pH 7.2, 300 mM NaCl, and 6 M GuHCl buffer) by affinity chromatography on Talon histidine-tag resin (ClonTech). Buffer was exchanged by gel filtration on Sephadex G-25 column (Amersham) for 175 mM acetic acid. The protein was conserved at 4 °C. For expression of HET-s(218–289), 2 l DYT medium were inoculated with an overnight culture of BL21(DE3) bearing the plasmid to be expressed at 37 °C. When an OD600 of 0.8–1.0 was reached, the bacteria were induced with 1 mM of isopropyl-1-thio-β-D-galactopyranoside for 2 h at 37 °C, then the culture were centrifuged and the cell pellet were frozen at −20 °C [11].

ThT anisotropy determination and ThT relative fluorescence. ThT anisotropy measurements were recorded using a Perkin-Elmer LS50 fluorescence spectrometer with an excitation wavelength of 450 nm and emission at 480 nm. ThT and protein concentration of 10 μM at pH 7 and 37 °C were used. The anisotropy values were calculated using the following equation: $A = (I_{VV}\text{-}GI_{VH})/(I_{VV}+2GI_{VH})$, where A is the anisotropy and $G = I_{HV}/I_{HH}$, and V and H in the subscript represent the vertical or the horizontal position of the excitation and the emission polarizers. For a rigid system the maximum anisotropy value is 0.4 whereas for a freely rotating small molecule the anisotropy values are considerably smaller [22]. ThT relative fluorescence has been determined with an excitation wavelength of 450 nm and emission range from 470 to 570 nm and the emission at 480 nm was recorded. ThT and protein concentration of 25 and 10 μM, respectively, at pH 7 and 37 °C were used.

CD spectroscopy determination. CD spectra measured at a spectral resolution of 1 cm^{-1}, and a scan rate of 15 nm min^{-1} was collected from the wavelength range 200–250 nm at 37 °C using a Jasco 810 spectropolarimeter with a quartz cell of 0.1 cm path length. The transition from non-aggregated (random coil) to amyloid aggregated form (principally β-sheet) can be conveniently followed by circular dichroism. The CD spectra were recorded every 5 min after a simple cell inversion and the ratio of two wavelengths (217 and 210 nm) were recorded [4].

Treatment and fitting of data. HET-s(218–289) aggregation process may be studied as an autocatalytic reaction using the equation $f = (\rho\{\exp[(1+\rho)kt]-1\})/\{1+\rho^*\exp[(1+\rho)kt]\}$ under the boundary condition of $t = 0$ and $f = 0$, where $k = k_n a$ (when a is the protein concentration) and ρ represents the dimensionless value to describe the ratio of k_e to k By non-linear regression of f against t, values of ρ and k can be easily obtained, and from them the rate constants, k_e (elongation constant) and k_n (nucleation constant). The extrapolation of the growth portion of the sigmoid curve to abscissa ($f = 0$), and to the highest ordinate value of the fitted plot, afforded two values of time (t_0 and t_1), which correspond to the lag time and to the time at which the aggregation was almost complete. The half-aggregation time ($t_{1/2}$) is the time at which half of HET-s monomers were aggregated ($f = 0.5$) [9].

ThT fixation determination. ThT–amyloid HET-s(218–289) fixation was determined according to a centrifugation method. ThT at 25 μM was mixed with a range from 0 to 20 μM of protein for 30 min. In order to precipitate the ThT–HET complex a centrifugation at 10,000*g* for 30 min was used. ThT absorbance at 411 nm (λ_{max}) was recorded at 37 °C before and after centrifugation using a Perkin-Elmer Lambda Bio 20 UV/vis spectrophotometer using a matched pair of quartz cuvettes of cm optical length placed in a thermostated cell holder, at 37 °C [23].

Electron microscopy. For negative staining, samples were adsorbed to freshly glow-discharged carbon-coated grids, rinsed with water, and stained with 1% uranyl acetate. Samples of pH 7 fibrils were usually sonicated shortly (5 s on a Kontes sonicator at about 60 W) to assure optimal particle size. Micrographs were recorded on a Philips CM120 microscope.

Results and discussion

The HET-s(218–289) fungal prion protein undergoes a spontaneous transition from a random coil to a β-sheet rich structure at pH 7 in 1:1 mixture of 175 mM acetic acid and 1 M Tris pH 7.2 (high ionic strength) [24]. In this buffer, ordered bundles of laterally-associated fibrils are detected by EM (Fig. 1A). The elementary fibrils are ~5 nm in

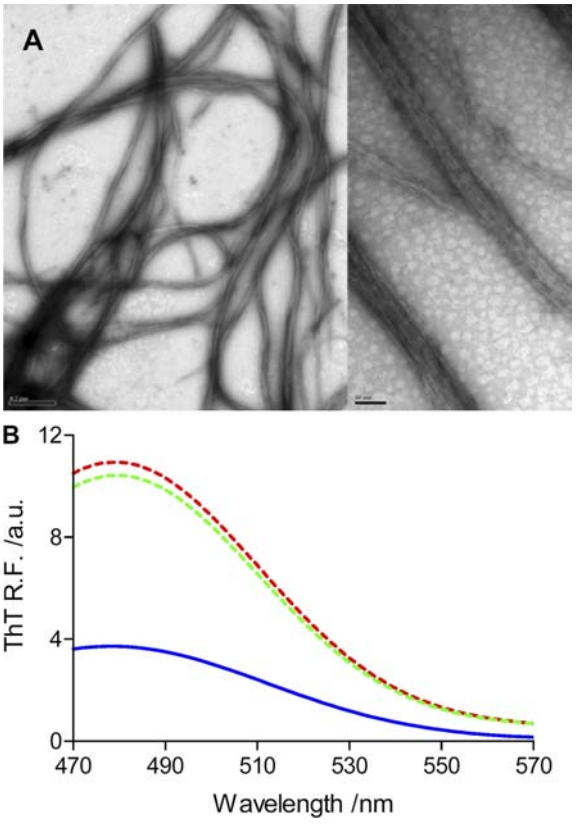

Fig. 1. Electron micrographs and ThT fluorescence of pH 7 HET-s(218–289) fibrils. (A) Electron micrographs showing amyloids formed at pH 7 at 37 °C in high ionic strength consisting of ordered bundles containing variable number of 5 nm fibrils. (B) ThT fluorescence without (blue continuous line) and with 10 μM of HET-s in random coil form (green dotted line) and assembled pH 7 fibrils (red continuous line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)