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Different conditions of fibrillogenesis cause polymorphism of lysozyme amyloid fibrils

Anna I. Sulatskaya [a], Natalia P. Rodina [a, b, *], Olga I. Povarova [a], Irina M. Kuznetsova [a], Konstantin K. Turoverov [a, b]

[a] Laboratory of Structural Dynamics, Stability and Folding of Proteins, Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia
[b] Institute of Physics, Nanotechnology and Telecommunications, Peter the Great St.-Petersburg Polytechnic University, St. Petersburg, Russia

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ABSTRACT

Structural differences of lysozyme amyloid fibrils prepared under different conditions were examined with the use of electron microscopy, CD spectroscopy together with a specially developed approach based on the absorption and fluorescence spectroscopy of solutions of amyloid fibrils with a specific fluorescent probe thioflavin T, prepared by equilibrium microdialysis. It was shown that the amyloid fibrils differ in their photophysical properties, morphology, parameters of thioflavin T binding. Furthermore, characteristic of the dye bound to fibrils obtained in various conditions are different. These results lead us to conclude that the conditions of fibrillogenesis can influence the rate of formation as well as the properties and structure of investigated amyloid fibrils.

1. Introduction

Amyloidosis is a large group of diseases (Alzheimer's disease, Parkinson's disease et al.) characterized by the formation in different organs and tissues of intra- and extracellular protein deposits in the form of insoluble fibrils. Investigation of amyloid fibrils formed by different fibril-forming proteins (globular proteins, intrinsically unstructured, rich in β-sheet, α-helix, or containing both β-sheets and α-helix) showed great similarity in their structure: all of them contain a cross-β spine with β-strands perpendicular to the fibril axis [1,2]. At the same time later investigations showed that amyloid fibrils formed by different proteins are not identical. Furthermore, even single amino acids differences lead to distinctions of amyloid fibrils. For example, this was shown for amyloid fibrils on the basis of a) wild-type alpha-synuclein and its familial Parkinson's disease mutants [3,4]; b) mutants of beta2-microglobulin which are detected during the hemodialysis amyloidosis [5]; different mutants with changes in prionization domain of yeast prion protein Sup35p [6].

An interesting fact is that environment-induced polymorphism of amyloid fibrils also occurs. It was revealed for amyloid fibrils formed from alpha-synuclein [7], insulin [8], serum albumin [9], prion protein [10,11], Abeta-peptide [12,13], human [14] and the hen egg white lysozyme [15]. For example, amyloid fibrils formed by commonly known small protein lysozyme obtained in pH = 2 are non-toxic for the human neuroblastoma cells, while amyloid fibrils obtained in neutral pH lead to death of these cells [14]. Lysozyme is an interesting object for investigation because the accumulation of amyloid fibrils of this protein in human organism leads to the development of a hereditary systemic amyloidosis. The disease occurs on average at the age of 20–40 years and is accompanied by proteinuria, slow progression of chronic renal failure, hepatosplenomegaly and petechiae [16,17].

The aim of the present work was to study the polymorphism of amyloid fibrils formed from lysozyme obtained in different conditions of fibrillogenesis. For our work the most representative, common used and well studied hen egg white lysozyme was chosen. Amyloid fibrils formed from this protein were characterized with methods including the approach based on examination of their interaction with a fluorescent probe thioflavin T (ThT). ThT is a common used diagnostic agent for amyloid fibrils detection. It does not interact with globular proteins in a native state (other than with acetylcholinesterase [18] and serum albumins [19]), with molten

* Corresponding author. Laboratory of Structural Dynamics, Stability and Folding of Proteins, Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia.
E-mail address: natalia240994@gmail.com (N.P. Rodina).

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globule and unfolded states or amorphous aggregates of proteins. At the same time this dye has a unique property to form highly fluorescent complexes with amyloid and amyloid-like fibrils [20–22].

In the recent works it was suggest, that the spectral characteristics of ThT bound to amyloid fibrils formed by different proteins and ThT bound to different binding modes may be different [23–25]. It can be assumed that characteristics of the dye bound to amyloid fibrils formed from the one protein under different conditions can also be different and thereby indicate the polymorphism of these fibrils. Therefor one of the main tasks of the present work was the examination of ThT – amyloid fibrils binding parameters and the characterization of binding sites. To obtain this information we propose to use absorption spectrophotometry of solutions prepared by equilibrium microdialysis [26–30]. The determination of the ThT – amyloid fibril binding parameters are utterly important in connection with new data about the therapeutic amyloid fibrils effect of ThT [31,32].

2. Materials and methods

2.1. Materials

The samples of "UltraPure Grade" thioflavin T (ThT) from AnaSpec (USA) were used without further purification. The stock solution of ThT was prepared in 2 mM Tris-HCl buffer (pH 7.7) with 150 mM NaCl. In experiments where interaction of ThT with lysozyme amyloid fibrils was studied the dissolved from stock in solutions in which fibrillogenesis proceeds was used. Fluorescent dye ATTO-425 from ATTO-TEC (Germany), lysozyme, and buffer components from Sigma (USA) were used without after-purification.

2.2. Preparation of lysozyme amyloid fibrils

For the preparation of lysozyme amyloid fibrils protein were incubated with constant agitation for 1 day (500 rpm) in a TS-100 Thermo-Shaker (Biosan) in two different conditions: in 100 mM KH2PO4-NaOH in the presence of 3 M GdnHCl (pH 7) at 57 °C and in 20% acetic acid solution in the presence of 100 mM NaCl (pH 2.0) at 37 °C. It was shown that different conditions in which amyloid fibrils were prepared do not influence to photophysical properties of ThT.

2.3. Spectroscopic studies

The absorption spectra were recorded using a U-3900H spectrophotometer (Hitachi, Japan). For the experiments with a wide range of concentrations, Helma cells (Germany) with different optical path lengths (0.1, 0.2, 0.5, 1, and 5 cm) were used. The amyloid fibril absorption spectra were analyzed along with the light scattering using a standard procedure [33]. The recoded absorption spectra of ThT in the presence of amyloid fibrils ($A(\lambda)$) represent the superposition of the absorption spectra of free ThT, ThT bound to fibrils and the apparent absorption determined by light scattered by the fibrils ($A_{scat}(\lambda)$). The dependence of apparent absorbance, determined by fibril light scattering, on λ is determined by equation: $A_{scat} = a\lambda^{-m}$. Coefficients a and m were determined from the linear part of the dependence $A(\lambda)$, where there is no active dye absorption plotted in logarithmical coordinates ($lg(A_{scat}) = f(lg(\lambda))$).

Fluorescence spectra and fluorescence excitation spectra were measured using a Cary Eclipse spectrofluorimeter (Varian, Australia). A PBS solution of fluorescent dye ATTO-425, whose fluorescent and absorption spectra were similar to that of ThT, was taken as a reference for determining the fluorescence quantum yield of ThT bound to fibrils. Fluorescence of ThT and ATTO-425 was excited at 450 nm. The spectral slits width was 5 nm in most of experiments. Changing the slit widths did not influence the experimental results. The fluorescence quantum yield of ATTO-425 was taken as 0.9 (ATTO-TEC Catalogue 2009/2010 p. 14).

2.4. Fluorescence intensity correction for the primary inner filter effect

For a fluorescent substance, the nonlinearity of the concentration dependence of the fluorescence intensity is caused by the so-called primary inner filter effect. The reasons for this effect include both the attenuation of the excitation light flux along its path through an absorbing solution (Beer–Lambert law) and the difference between the area that is illuminated by the excitation light and the working area from which the fluorescence light is gathered (that is in the most wide used spectrofluorimeters with a vertical slits). It is generally accepted that for solutions of low concentration (low absorbance), the fluorescence intensity is proportional to the concentration of the fluorescent substance, and the primary inner filter effects are negligible. However, we earlier showed that this assumption is not valid and fluorescence intensity values always require correction [34].

In the present work we used Cary Eclipse spectrofluorimeter (Varian, Australia) with horizontal slits where the area illuminated by the excitation light coincides with the working area from which the fluorescence light is gathered. The correction factors were determined with the use of equations obtained in work [34].

2.5. Electron microscopy

To obtain electron micrographs, the method of negative staining with a 1% aqueous solution of uranyl acetate was used. Amyloid fibrils were placed on copper grids coated with a collodion film-substrate.

2.6. Preparation of tested solutions by equilibrium microdialysis and their use to investigate the ThT-amyloid fibril interaction

Equilibrium microdialysis was performed using a Harvard Apparatus/Amika (USA) device that consists of two chambers (500 μL each) that are separated by a membrane (MWCO 10000) that is impermeable to particles larger than 10 000 Da. Lysozyme amyloid fibrils in the buffer solution in which their fibrillogenesis proceeded were placed in chamber #1; at an initial concentration C0, the ThT solution in the same buffer was placed in chamber #2. After equilibration, the ThT concentrations in chambers #1 and #2 become equal (C_f), and the total ThT concentration in chamber #1 exceeded that in chamber #2 by the concentration of the bound dye (C_b). These conditions, along with the identical volumes of chambers #1 and #2, yield the following equation:

$$C_b = C_0 - C_f. \quad (1)$$

The initial concentration of ThT in chamber #2 (C_0) and the dye concentration in this chamber after equilibration (C_f), which equals the concentration of free dye in chamber #1, were determined by absorption spectrophotometry. The concentration of the dye bound to fibrils (chamber #1) was determined using Eq. (1); consequently, the parameters describing the binding of ThT to amyloid fibrils could be evaluated using the following equation [35–37]:

$$C_b = \frac{n_i C_p C_f K_{bi}}{1 + C_f K_{bi}}, \quad (2)$$

where C_p is the concentration of lysozyme at which fibrils form, and

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