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Binding of thioflavin T by albumins: An underestimated role of protein oligomeric heterogeneity

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ABSTRACT

Amyloid fibrils formation is the well-known hallmark of various neurodegenerative diseases. Thioflavin T (ThT)-based fluorescence assays are widely used to detect and characterize fibrils, however, if performed in bioliquids, the analysis can be biased due to the presence of other, especially abundant, proteins. Particularly, it is known that albumin may bind ThT, although the binding mechanism remains debatable. Here the role of low-order albumin oligomers in ThT binding is investigated using time-resolved fluorometry and size-exclusion chromatography. Under conditions used, the fraction of dimers in human (HSA) and bovine (BSA) serum albumin solutions is as low as ~7%, however, it is responsible for ~50% of ThT binding. For both albumins, the binding affinity was estimated to be ~200 and ~40 μM for monomeric and dimeric species, respectively. Molecular docking suggested that ThT preferentially binds in the hydrophobic pocket of subdomain IB of albumin monomer in a similar position but with a variable torsion angle, resulting in a lower fluorescence enhancement (~40-fold) compared to amyloid fibrils (~1000-fold). Dimerization of albumin presumably creates an extra binding site at the subunit interface. These results demonstrate the underestimated role of low-order albumin oligomers that can be highly relevant when analyzing drugs binding using fluorescence spectroscopy.

1. Introduction

Incorrectly folded proteins may lose their colloidal stability, resulting in aggregation. In certain cases, amyloid fibrils may form, which are protein aggregates characterized by a β-sheet-rich structure and needle-shaped geometry [1]. In the human organism, these structures may lead to amyloidosis by forming insoluble plaques, which accumulate in tissues and organs, leading to disruption of their normal functions [2]. Amyloidosis accompanies type II diabetes mellitus, atherosclerosis, Alzheimer's and Parkinson's diseases, etc. [3,4] Amyloidosis is also associated with the aging processes in the human organism [5,6].

Although specific amyloidogenic proteins, such as α-synuclein, β-amyloid, huntingtin, prion protein, etc., are known to be involved in neurodegenerative diseases, current understanding of fibril formation mechanisms implies that at certain (sometimes non-physiological) conditions almost every protein may form fibrils; however, the rate constant of fibril formation varies significantly depending on the presence of amyloidogenic motifs in a corresponding structure [1]. Numerous studies showed that fibrils can be produced *in vitro* as a result of external influences on various proteins, which are not prone to aggregation in its native state. For instance, fibril formation by serum albumin has been extensively studied, and it has been shown that glycation [7] and oxidative stress [8] may result in albumin fibrillation at physiological conditions. Hence, quantification of fibrillary structures in biological liquids, as well as investigation of mechanisms of their formation, are of interest for biomedical diagnostics [9].

Routine amyloid detection in tissues *ex vivo*, as well as investigations of fibril formation mechanisms and growth kinetics *in vitro*, mostly rely on the use of optical assays, including staining with specific dyes. One of the most commonly used markers for the detection of amyloid fibrils is thioflavin T (ThT) [10]. ThT is supposed to bind specifically to amyloid fibrils, and its spec-

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tral parameters, fluorescence lifetime and quantum yield, serve as indicators of the binding process [11].

These characteristic optical properties of ThT originate from its structure – ThT belongs to the class of molecular rotors [12]. The main channel of ThT fluorescence quenching is the torsional relaxation accompanied by intramolecular charge transfer [13], which results in formation of non-fluorescent state. Photophysical processes underlying the sensitivity of ThT's response to the parameters of microenvironment (viscosity, polarity, temperature, etc.) have been described both theoretically and experimentally [12–15].

Beta-sheets in amyloids are oriented perpendicular to the longer axis of the fibrils and form the so-called cross-beta structures and ThT incorporates parallel to the long axis of the fibril, in the grooves formed by the side chains of the protein secondary structure [11,16,17]. Fixation of the relative rotation of the rings in the ThT structure upon its binding to fibrils is considered as the main factor responsible for the manifold increase in the observed ThT fluorescence intensity [12]. At the same time, the geometry of ThT binding sites differs significantly depending on the source and type of fibrils, and the dissociation constant may vary in the submicromolar to hundred μM range [16].

It is generally believed that ThT binds specifically to amyloids and its interaction with globular proteins or even their amorphous aggregates can be neglected when compared to fibrils. This is based on the fact that even β-sheet rich proteins do not have a minimum required ThT binding motif, which was determined for amyloids [11,18]. This fact is of critical importance for the use of ThT for the detection of fibrils in biological liquids, as non-specific binding to a pool of background proteins may affect the results of a ThT-based fluorescence assay. For instance, Sen et al. investigated interaction between ThT and serum albumins and showed that the dissociation constant for the BSA-ThT and HSA-ThT complexes were 333 and 10 μM, respectively, and suggested the existence of a single binding site located in the hydrophobic cavity of these orthologs [19]. It was also shown by [20] that at least two binding sites for ThT are located on BSA with a 740 μM apparent dissociation constant. The presence of two binding modes for the ThT-albumin system characterized by different fluorescence lifetimes was also shown in the work of Maskevich et al. [21], and thermal denaturation of albumin led to an increase of ThT binding.

Despite the reported values for the albumin affinity towards ThT are relatively low, at high protein concentrations typical for biological liquids (e.g., ~60 mg/ml of proteins in blood plasma), the contribution of this relatively non-specific ThT binding could be significant, thus interfering with the signal from ThT complexes with fibrils. In this paper, we re-evaluate the role of this process by performing a detailed study of ThT binding with two albumins (HSA and BSA) by several independent methods, with a focus on the role of low-order protein oligomers, which are often present in a biological solution.

2. Materials and methods

2.1. Sample preparation

Human serum albumin (HSA, catalog no. A1887) and thioflavin T (ThT, catalog no. T3516) were purchased from "Sigma" (Germany), and bovine serum albumin (BSA, catalog no. 10775835001) was obtained from MP Biomedicals (USA). All measurements were performed in a 20 mM Tris-HCl buffer at pH 7.4. Ionic strength was adjusted to 0.1 M NaCl. A stock solution of albumin was prepared and then diluted to perform fluorescence titrations at a fixed ThT concentration. For BSA and HSA, extinction coefficients at 280 nm equal to 43824 M^{-1} cm^{-1} and 35700 M^{-1} cm^{-1} were used [22]. For ThT, 31600 M^{-1} cm^{-1} extinction coefficient at 410 nm was used [15].

2.2. Fluorescence measurements

Fluorescence spectra were obtained using a FluoroMax-4 spectrofluorometer (Horiba Jobin-Yvon, Japan-France) at a 410 nm excitation with the excitation and emission slits of 2 and 5 nm, respectively. The optical density of samples was controlled by a Lambda-25 spectrophotometer (Perkin-Elmer, USA).

Time-resolved measurements were performed on a custom-built setup, which is described elsewhere [23]. Briefly, excitation was performed by a 405 nm pulsed laser source (InTop, Russia), which provided 22-pJ, 40-ps pulses at a 10 MHz repetition rate. Fluorescence signal was collected at 90°, with a PMC-100 photodetector (Becker&Hickl, Germany), and time-correlated single photon counting (TCSPC) was performed by a SPC-130EM counting module (Becker&Hickl, Germany). A 450 nm longpass filter (ThorLabs, Germany) was used to cut excitation signal, allowing to detect ThT fluorescence, which is peaked at ca. 480 nm.

The best fits of fluorescence decay curves were obtained by using a three-exponential decay:

$$F(t) = a_1 \cdot e^{-t/\tau_1} + a_2 \cdot e^{-t/\tau_2} + a_3 \cdot e^{-t/\tau_3}, \quad (1)$$

where a_i and τ_i are amplitude and lifetime of the i-th component, and average lifetime was calculated as

$$\tau_{av} = (a_1 \cdot \tau_1 + a_2 \cdot \tau_2 + a_3 \cdot \tau_3)/(a_1 + a_2 + a_3). \quad (2)$$

Fluorescence decay curves were processed using the SPCImage 5.6 software (Becker & Hickl, Germany). Prior to processing, fluorescence decay curves for protein without ThT were subtracted from the protein-ThT kinetics to get rid of the background signal. A similar procedure was performed during processing of the steady-state fluorimetry data. ThT fluorescence decay curves obtained at different BSA concentrations were fitted using fixed lifetimes (τ_1, τ_2 and τ_3), and the discrepancy (χ^2) did not exceed 2.5. All the experiments were performed in a 1-cm quartz cuvette at 25 °C and repeated at least three times using independent protein preparations.

Dissociation constant was obtained by fitting the dependence of y, which corresponds to fluorescence intensity at the maximum, average fluorescence lifetime or amplitudes of components in fluorescence decay (depending on the experiment) on protein concentration:

$$y(C_P) = y_{max} \cdot \left(\frac{C_{ThT} + C_P + K_d}{2} - \sqrt{\left(\frac{C_{ThT} + C_P + K_d}{2} \right)^2 - C_P \cdot C_{ThT}} \right), \quad (3)$$

where C_P and C_{ThT} are the total concentrations of protein (BSA or HSA) and ligand (ThT) in solution, respectively, K_d is an apparent dissociation constant. In Eq. (3), y_{max} corresponds to the y value at saturation. We note that we changed protein concentration in the experiment at a fixed ThT concentration, and Eq. (3) doesn't allow for the assessment of the number of binding sites, and if the number of ThT binding sites exceeds 1, the affinity determined from Eq. (3) would be overestimated. To obtain fitting parameters, a nonlinear approximation of binding curves was performed using Origin Pro 9.0 software.

2.3. Size-exclusion chromatography (SEC) measurements

The samples (100 μl) with different protein content were preincubated for 10 min at room temperature and then subjected to a Superdex 200 Increase 10/300 column (GE Healthcare) equilibrated with a filtered and degassed 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl. The column was operated at 25 °C at a 1.2 ml/min flow rate using a chromatographic ProStar325 UV/Vis