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Aggregation and fibrillation of bovine serum albumin

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Abstract

The all-α helix multi-domain protein bovine serum albumin (BSA) aggregates at elevated temperatures. Here we show that these thermal aggregates have amyloid properties. They bind the fibril-specific dyes Thioflavin T and Congo Red, show elongated although somewhat worm-like morphology and characteristic amyloid X-ray fiber diffraction peaks. Fibrillation occurs over minutes to hours without a lag phase, is independent of seeding and shows only moderate concentration dependence, suggesting intramolecular aggregation nuclei. Nevertheless, multi-exponential increases in dye-binding signal and changes in morphology suggest the existence of different aggregate species. Although β-sheet content increases from 0 to ca. 40% upon aggregation, the aggregates retain significant amounts of α-helix structure, and lack a protease-resistant core. Thus BSA is able to form well-ordered β-sheet rich aggregates which nevertheless do not possess the same structural rigidity as classical fibrils. The aggregates do not permeabilize synthetic membranes and are not cytotoxic. The ease with which a multidomain all-α helix protein can form higher-order β-sheet structure, while retaining significant amounts of α-helix, highlights the universality of the fibrillation mechanism. However, the presence of non-β-sheet structure may influence the final fibrillar structure and could be a key component in aggregated BSA's lack of cytotoxicity.

Keywords: Albumin; Fibrillation; Aggregation; Amyloid; Proteolysis; Nucleation

1. Introduction

Under various conditions, many proteins can aggregate to regular arrays of β-sheet rich filaments or fibres of indefinite length, often coiled together in higher-order structures [1,2]. The ability to fibrillate is independent of the original native structure of the protein, whose amino acid sequence primarily appears to play a role in terms of filament arrangement [3], fibrillation kinetics [4] and overall yield and stability of the fibrils [5,6]. It typically occurs under conditions that stabilize partially folded or unfolded states [7]. For example, the all-α protein myoglobin fibrillates under conditions (pH 6.5, 65 °C, heme factor removed) where the denatured state dominates [8,9]. Although fibrillation is associated with the development of neurodegenerative diseases, cytotoxicity appears to be associated with prefibrillar aggregated states, either because of their ability to permeabilize cell membranes to general ion flux [10–12] or because the rather diffuse hydrophobic surface may catalyze unwanted reactions [13,14]. The fibrils themselves appear to be relatively innocuous, except when they associate to very high levels in e.g. systemic amyloidoses, causing organ failure [15]. In addition, fibrils are associated with beneficial functions such as the formation of bacterial biofilm that allows microbial organisms to adhere to cell surfaces [16].

A typical fibrillation time profile, usually monitored with the help of the fibril-specific dyes Thioflavin T or Congo Red, commences with a long lag time followed by a relatively short

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elongation phase where fibrils accumulate [17]. The length of the lag time is thought to be determined by the formation of the fibrillation nucleus, a thermodynamically and kinetically unfavourable state that functions as a template for fibril growth. In such cases, addition of sonicated fibrils (which are assumed to be equivalent to nuclei) can reduce or abolish the lag phase [17]. However, other models including fragmentation, branching and heterogeneous nucleation can also give rise to the characteristic sigmoid appearance [18]. Some proteins, such as acyl phosphatase, commence aggregation without a lag phases in the rise of the ThT signal [19]; however, in this case the first aggregate species to accumulate are relatively amorphous and *bona fide* fibrils only form much later [20].

Here we describe fibrillation of the ubiquitous bovine serum albumin (BSA). BSA is a 583-residue protein consisting of three homologous all-α domains, organized in a heart-shaped structure [21]. It constitutes ca. 60% of all plasma protein and binds and transports a large number of physiological and non-physiological ligands [22]. Although its aggregation properties have been studied for a number of years, we are not aware of any reports of actual fibrillation. BSA contains 17 disulfide bonds and one unpaired cysteine (Cys34), which facilitates dimerization and also influences higher-order association, since the rate of aggregation is slowed down if Cys34 is covalently bound to another compound [23]. Aggregation of 1 g/l BSA at elevated temperatures proceeds through the formation of an intermediate with an increased content of β-sheets [24]; irreversible intermolecular β-sheet formation only occurs at 70 °C and above [25]. Secondary structural changes are essentially complete within the first 70 min of incubation at 70 °C, whereas tertiary changes occur over longer time scales [23]. In view of the fact that aggregation requires access to non-native states, it is not unexpected that aggregation is inhibited by the addition of ligands such as Tween 80, tryptophan and caprylate which stabilize the native state.

We show that BSA forms aggregates with amyloid characteristics within minutes upon incubation at neutral pH at elevated temperatures. The reaction proceeds without a lag phase, is only linearly dependent on protein concentration and is not accelerated by seeding, nor do the ensuing aggregates show any cytotoxicity. The ease with which BSA fibrillates highlights once again the ubiquitous nature of fibrillation, which does not exempt even multi-domain all-α proteins; at the same time it serves to illustrate that fibrils can tolerate a high amount of non-β structure, presumably due to their ability to relegate α-helix structure to parts outside the fibrillar core.

2. Materials and methods

2.1. Materials

Bovine Serum Albumin fraction V (BSA), Congo Red (CR) and Thioflavin T (ThT) were from Sigma-Aldrich (St. Louis, MO). Trypsin was from Promega (Madison, WI). 1,2-Dioleoyl-*sn*-Glycero-3-phosphocholine (DOPC) and the sodium salt of 1,2-Dioleoyl-*sn*-Glycero-3-[Phospho-rac-(1-glycerol)] (DOPG) were from Avanti Polar Lipids (Alabaster, AL). Calcein (sodium salt) was from Merck (Darmstadt, Germany). All chemicals were of the highest purity available. The buffer used was in all cases 20 mM Tris–HCl pH 7.4 unless otherwise stated.

2.2. Preparation of BSA aggregates

BSA was dissolved in buffer to a final concentration of typically 1–5 mg/ml as indicated in the text. Before incubation the solution was filtered through a 0.2-μm filter into sterile test tubes. The samples were incubated at a specified temperature (60–75 °C) in a water bath without agitation. Samples were taken out at intervals and stored on ice before adding CR or ThT. For longer storage before measurement, tubes with samples were removed and frozen at −20 °C at 0 h, 2 h and 96 h.

2.3. Thioflavin T spectroscopy

Typically, 5 μl BSA sample was mixed with ThT to a final concentration of 20 μM ThT in buffer. Fluorescence was measured in an LS55 fluorimeter (Perkin Elmer, Wellesley, MA) with excitation and emission wavelengths of 450 and 482 nm, respectively.

2.4. Protease digestion experiments

2.5 g/l BSA was incubated at 70 °C in buffer for 4 days, after which they were diluted out to 0.25 g/l in a 96-well plate in the presence of 40 μM ThT and 1% trypsin (w/w). Recordings were started within a few minutes on a SpectraMaxGeminiXS plate reader (Molecular Devices, Sunnyvale CA). Excitation was at 450 nm with emission at 485 nm at 37 °C. Recordings were carried out every 20 min, preceded by 5 s of shaking. Between measurements, the plate was shaken for 180 s.

2.5. X-ray fiber diffraction

Fibrils were formed by incubating a 2-mg/ml BSA solution in Tris buffer at 70 °C for 4 days. The solution was centrifuged at 32,000×*g* for 30 min and drops of sample from the bottom of the Eppendorf tube were placed between two end-to-end glass pipettes sealed with wax. After several days at 5 °C, one drop had turned into a thin, gel-like fibre. Diffraction data were collected at beamline I911-3, MAX-lab, Lund, Sweden, at 1.00 Å. During the 6-min exposure, the fibre was rotated 1°. Diffraction intensity, in general, was low in accordance with the gel-like nature of the fibre. Intensity profiles were made by integrating from 30 to 150° and between 150 and 240°. The intensity profiles show a sharp peak at 4.65 Å and several peaks at 9.7–10.8 Å. The fact that several sidechain peaks are present could indicate that several fibril morphologies are present in solution.

2.6. Calcein experiments

Vesicles containing 80% DOPC and 20% DOPG by weight were prepared essentially as described [26]. Briefly, after dissolution in methanol and evaporation of methanol, the lipid mixture was resuspended to 12.5 mg/ml in buffer containing 40 mM calcein, subjected to freeze–thawing and extruded through a 100-nm filter. Free calcein was removed by desalting over a pre-equilibrated PD10 column, leading to an approximately 2-fold dilution in the most concentrated eluted fraction of vesicles. The vesicle solution was then diluted out an additional 100-fold in buffer in the presence of 0.22 mg/ml BSA. The BSA was diluted from a 2.5-mg/ml solution which had been incubated in buffer at 70 °C for 0–120 min. At 40 mM inside the vesicles, calcein quenches its own fluorescence, and fluorescence will increase if it is diluted by exiting the vesicle [27]. As positive control we used the antimicrobial peptide Novispirin [26], where we observed up to 90% leakage compared to full vesicle rupture upon addition of 2% Triton X-100. The fluorescence emission at 515 nm was recorded every 4 s in the LS55 fluorimeter using excitation at 490 nm and slit widths of 2.5 nm for both excitation and emission.

2.7. Congo Red absorption

50 μl sample was mixed with CR in PBS-buffer (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 pH 7.4) to a final CR concentration of 20 μM and a total volume of 1 ml. The absorbancies at 403 and 541 nm were