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Thermal aggregation of bovine serum albumin studied by asymmetrical flow field-flow fractionation

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ABSTRACT

The use of asymmetrical flow field-flow fractionation (AsFlFFF) in the study of heat-induced aggregation of proteins is demonstrated with bovine serum albumin (BSA) as a model analyte. The hydrodynamic diameter (d_h), the molar mass of heat-induced aggregates, and the radius of gyration (R_g) were calculated in order to get more detailed understanding of the conformational changes of BSA upon heating. The hydrodynamic diameter of native BSA at ambient temperature was ~7 nm. The particle size was relatively stable up to 60 °C; above 63 °C, however, BSA underwent aggregation (growth of hydrodynamic diameter). The hydrodynamic diameters of the aggregated particles, heated to 80 °C, ranged from 15 to 149 nm depending on the BSA concentration, duration of incubation, and the ionic strength of the solvent. Heating of BSA in the presence of sodium dodecyl sulfate (1.7 or 17 mM) did not lead to aggregation. The heat-induced aggregates were characterized in terms of their molar mass and particle size together with their respective distributions with a hyphenated technique consisting of an asymmetrical field-flow fractionation device and a multi-angle light scattering detector and a UV-detector. The carrier solution comprised 8.5 mM phosphate and 150 mM sodium chloride at pH 7.4. The weight-average molar mass (M_w) of native BSA at ambient temperature is 6.6×10^4 g mol^{-1}. Incubation of solutions with BSA concentrations of 1.0 and 2.5 mg mL^{-1} at 80 °C for 1 h resulted in aggregates with M_w 1.2×10^6 and 1.9×10^6 g mol^{-1}, respectively. The average radius of gyration and the average hydrodynamic radius of the heat-induced aggregate samples were calculated and compared to the values obtained from the size distributions measured by AsFlFFF. For comparison static light scattering measurements were carried out and the corresponding average molar mass distributions of solutions with BSA concentrations of 1.0 and 2.5 mg mL^{-1} at 80 °C for 1 h gave aggregates with M_w 1.7×10^6 and 3.5×10^6 g mol^{-1}, respectively.

1. Introduction

In the native state, polypeptide chains of a protein are folded into a unique three-dimensional globular structure. Hydrophobic interactions and multiple hydrogen bonds stabilize these structures. In addition to peptide bonds, disulfide bonds occur in many proteins, when two cysteine residues lie close to each other [1–4]. Proteins are easily destabilized by heat, addition of salt, acidification, or treatment with hydrolytic enzymes or chemical reagents [5,6]. Under appropriate conditions, these destabilizing processes cause the protein to unfold and aggregate, and form associated intermolecular structures.

Thermal (heat-induced) aggregation starts from partially denatured conformations. At elevated temperature, the native form of the protein becomes more flexible and, upon cooling down to room temperature, large aggregates are formed via non-covalent or disulfide bonds or both [7]. The heat-induced aggregation of proteins has been studied by many techniques. These include two-dimensional gel electrophoresis [8], static and dynamic light scattering (SLS and DLS) [9–14], small angle X-ray scattering [15], nuclear magnetic resonance spectroscopy (NMR) [16], circular dichroism [17,18], Fourier-transform infrared spectroscopy [19,20], differential thermal scanning calorimetry [20], analytical ultracentrifugation [21,22], and flow assisted analytical separation techniques.

The flow assisted separation techniques include size exclusion chromatography (SEC) and field-flow fractionation (FFF). SEC is an established method, because it is easy to use and the repeatability of data is generally good [14,23,24]. However, the heat-induced protein aggregates are often too large to be effectively separated on typical SEC columns, and FFF is an alternative technique.

Asymmetrical flow FFF (AsFlFFF) and its variant hollow fiber flow FFF have been widely used for separation and characterization of protein aggregates [21,22,25–33]. The main advantages of

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AsFlFFF and hollow fiber flow FFF over SEC derive from the absence of column packing material and the ability to run samples over a wide range of sizes [22,25]. Zhu et al. [32] used programmed cross-flow with ceramic hollow fiber flow FFF to study heat-induced aggregation of β-lactoglobulin in aqueous solution. Large aggregates of β-lactoglobulin with particle sizes >100 nm and molar masses over 100 million were found when samples at concentrations of 10–100 mg mL^{-1} were heated at 65 °C for 2–24 h in the presence of 100 mM sodium chloride solution. In an earlier work [28] we used 2D FFF–liquid chromatography to study the effect of heat on white egg albumin in the presence of 150 mM sodium chloride and observed heat-induced aggregates with particle diameters of 130–150 nm due to unfolding of polypeptide bonds.

In the present work, we used bovine serum albumin (BSA) as a model biomolecule to evaluate the applicability of AsFlFFF, using UV and MALS detection, to the study of heat-induced protein aggregation. Native BSA has a weight-average molar mass (M_w) of 6.6×10^4 g mol^{-1}, determined by electrospray ionization mass spectrometry [34], and a hydrodynamic diameter of 7.0 nm, determined by 2D NMR [16]. In this work information will be given on the hydrodynamic diameter (d_h), the radius of gyration (R_g), and the weight-average molar mass (M_w) of heat-induced BSA aggregates.

2. Materials and methods

2.1. Materials

BSA (M_w 66 kDa, 98% purity, remainder mostly globulin) and sodium dodecyl sulfate (SDS) (M_w 288.4 g mol^{-1}) were from Sigma (St. Louis, MO, USA). According to the manufacturer, BSA contains 2% of γ-globulin (M_w of 155–160 kDa). The pI value of BSA is 4.9–5.1. The buffer solution used in the AsFlFFF work comprised 8.5 mM phosphate buffer (ionic strength of 20 mM), 150 mM sodium chloride, 0.02% sodium azide and pH was adjusted to 7.4 with 1.0 M sodium hydroxide.

2.2. Preparation of heat-induced aggregates

BSA samples were prepared by dissolving solid BSA in 8.5 mM phosphate buffer solutions with 0–2 M sodium chloride at pH 7.4. In addition, BSA samples (c = 10.0 mg mL^{-1}) in the presence of SDS (1.7 or 17 mM), dissolved in 8.5 mM phosphate, 150 mM sodium chloride at pH 7.4 were prepared. The concentration of BSA in the samples varied between 0 and 10.0 mg mL^{-1}. 1.0 mL of protein solution was placed in a preheated water bath and incubated at 50, 60, 65, 70, 75, and 80 °C, and the water bath was shaken horizontally at a speed of 200 cycles min^{-1}. The incubation times were 0–60 min. After the incubation, the samples were cooled down to room temperature, and AsFlFFF, DLS, or SLS were used to measure the particle sizes or the molar masses of the aggregates.

2.3. Methods

2.3.1. Asymmetrical flow field-flow fractionation

A self-constructed AsFlFFF device was used to study the effects of temperature, protein concentration, incubation time, ionic strength of the solvent, and concentration of SDS on the aggregation of BSA. A regenerated cellulose acetate ultrafiltration membrane with a molar mass cut-off of 10 kDa, DSS-RC70PP (Nakskov, Denmark) was laid on top of the porous frit. A Mylar^{TM} spacer with thickness of 500 μm, with the channel shape cut out, was placed between the ultrafiltration membrane and the upper Plexiglas plate. Dimensions of the AsFlFFF channel were 38 cm × 2 cm × 500 μm. An HPLC pump, model PU-980 (JASCO International, Tokyo, Japan) was used to move the carrier liquid. Samples (100–500 μL) were diluted from 1.0 mg mL^{-1} to a final volume of 5.0 mL and introduced to the channel at 1.0 mL min^{-1} for 1–5 min by another HPLC pump. During the injection–relaxation–focusing period, the carrier liquid was delivered from both the front and the backside of the channel at a flow rate of 3.3 mL min^{-1} for 12 min. The outlet flow from the channel was monitored with a UV/VIS detector, HP1050 model (Tokyo, Japan), set at 280 nm. Unless otherwise specified, the flow rates at the main and cross-flow outlets were 0.50 and 2.52 mL min^{-1}, respectively. Capillary Teflon tubes (i.d. 0.5 mm), restrictors, and three-way valves V101T Upchurch Scientific (Oak Harbour, WA, USA) were used to control the carrier liquid flow. Agilent ChemStation for LC and LC/MS (Palo Alto, CA, USA) was used for data acquisition. The channel thickness (w) was calibrated with BSA having a known diffusion coefficient of 6.21×10^{-7} cm^2 s^{-1} in a solution of 8.5 mM phosphate buffer and 150 mM sodium chloride (pH 7.4) at 20 °C [35]. Thus, the experimental channel thickness was 499 ± 6 μm.

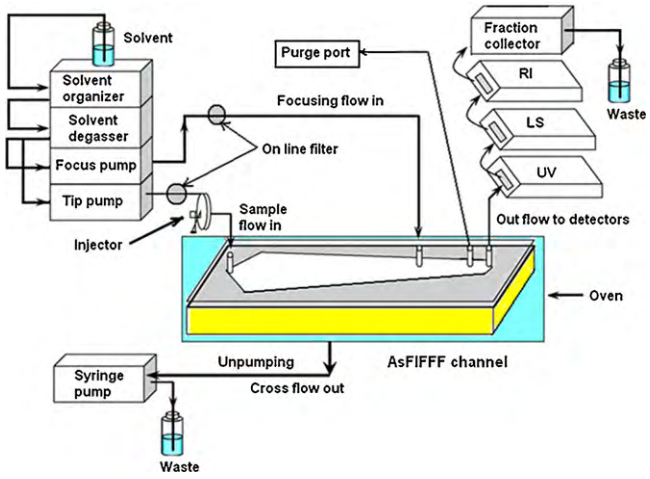

Fig. 1. Schematic drawing of the AsFlFFF/UV/MALS/RI system.

2.3.2. Asymmetrical flow field-flow fractionation connected to UV, multi-angle light scattering, and refractive index detectors

Connection of AsFlFFF to a multi-angle light scattering (MALS) detector makes the technique more suitable for absolute molar mass measurements, as well as for providing information on the radius of gyration and the structural conformation of the macromolecule. In the present work, AsFlFFF was used to determine the molar mass and radius of gyration of BSA aggregates using an AF2000 model instrument (Postnova Analytics, Landsberg, Germany). The instrument was equipped with a solvent organizer (PIN PN7140), a solvent degasser (PN7520), two isocratic HPLC pumps for generation of carrier flow (PN1130), a syringe pump (Kloehn v6) for cross-flow, a purging port (PN1610) for rinsing or slot operation, a manual injection valve (Rheodyne 9725i), an AsFlFFF separation channel, an oven for the AsFlFFF channel (PN4020), UV, MALS, refractive index (RI) detectors, and a fraction collector (Fig. 1). Control of all pumps, valves, data acquisition, and evaluation were made by Postnova software.

The UV-detector was a variable wavelength detector (PN3211) with a 10-mm path length and 12 μL volume. The MALS detector (PN3070, LS) had a 30 M_w light source, 60 nm diode laser with 635 nm wavelength, and a flow cell with 20 nL scattering volume. For a detailed MALS theory and principles, see Wyatt [37]. Calibration of the MALS detector (with UV and RI detectors) at 90° was performed with 66 kDa molar mass BSA in 0.9% sodium chloride, using a differential index of refraction (dn/dc) increment value of 0.165 mL mg^{-1}. The differential refractive detector output was set at 125 μRIU V^{-1}. Normalization of the rest of the angles was