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Thermal aggregation of glycated bovine serum albumin

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ABSTRACT

Aggregation and glycation processes in proteins have a particular interest in medicine fields and in food technology. Serum albumins are model proteins which are able to self-assembly in aggregates and also sensitive to a non-enzymatic glycation in cases of diabetes. In this work, we firstly reported a study on the glycation and oxidation effects on the structure of bovine serum albumin (BSA). The experimental approach is based on the study of conformational changes of BSA at secondary and tertiary structures by FTIR absorption and fluorescence spectroscopy, respectively. Secondly, we analysed the thermal aggregation process on BSA glycated with different glucose concentrations. Additional information on the aggregation kinetics are obtained by light scattering measurements. The results show that glycation process affects the native structure of BSA. Then, the partial unfolding of the tertiary structure which accompanies the aggregation process is similar both in native and glycated BSA. In particular, the formation of aggregates is progressively inhibited with growing concentration of glucose incubated with BSA. These results bring new insights on how aggregation process is affected by modification of BSA induced by glycation.

1. Introduction

Because of their high relevance for biomedical and for biotechnological fields, protein aggregation processes represent a growing interest in several studies. In medical field, fibrillar aggregates are strongly associated with the manifestation of many neurodegenerative pathologies such as Parkinson, Alzheimer and Creutzfeld-Jacob's diseases [1,2]. Indeed, it was observed that all these pathologies are accompanied by formation of senile plaques in the brain and intraneuronal deposits of amyloid fibrils [2,3]. Regarding to the biotechnological aspect, comprehension of the proteins aggregation mechanism is relevant for processes in pharmaceutical applications and in food industry. Knowledge and characterization of aggregation pathway of specific proteins, such as the whey proteins, reveal to be crucial in food technologies. Indeed, these proteins, whose bovine serum albumin (BSA) is one of the main components, are widely used as emulsifiers, gelling or foaming agents [4]. Finally, ordered protein aggregates can be of great interest in technological applications as a new potential nano-materials that can be exploited by research, industry and medicine [5].

As described in many studies, aggregation phenomena result from a partial unfolding of the tertiary structure of the protein and from the conformational changes of secondary structure as well [6–8]. Indeed, from recent studies on thermal aggregation process, we have concluded that the first step of aggregation consists on a partial opening of the protein native conformation. Consequently, some specific regions such as hydrophobic sites or free −SH groups become more exposed to new intermolecular interactions and so contribute to the formation of aggregates [9,10]. In parallel, for most proteins, the conformational changes at the secondary structure, in favour of higher β-sheet structure content, may promote the formation of intermolecular bonds, which is the first step for the growth of ordered aggregates as the amyloid fibrils [11].

Among these proteins, albumin is well known to be able of self-assembling in aggregates under particular conditions [6,10,12]. Besides, the different structural levels of bovine (BSA) and human (HSA) albumins contribute to significant modifications in the shape and the size of aggregates in response to changes in different experimental conditions (pH, temperature, concentration and p*I*) [10,13]. In addition, albumin has a particular interest because of being the most abundant in the circulatory system, and for its multi-beneficial biological properties, among them, the antioxidant activity is the most relevant in oxidative stress field [14,15]. Most of antioxidant properties of albumin can be attributed to its different

Abbreviations: AGE, advanced glycated end; ANSA, 1-anilino-8-naphthalene sulfonic acid; BSA, bovine serum albumin; DNPH, *2,4-dinitrophénylhydrazine*; MES, 2-(*N*-morpholino)ethane sulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FTIR, Fourier transform infrared; DLS, dynamic light scattering

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levels of structure and it has previously shown that these properties could be totally impaired by aggregation phenomenon [12].

As a circulating protein, serum albumin is also likely undergo glycative alteration in case of diabetes pathology and hyperglycemia [16,17]. For instance, antioxidant activities of serum albumin were impaired in patients with diabetes [18]. In fact, the glycated proteins give rise *in vivo* to deleterious advanced glycation end (AGE) products which affect the antioxidant properties. In addition, we showed that *in vitro* glycated albumin could enhance oxidative damages in many types of cells [19,20].

Similarly to aggregation phenomenon, there is also a large interest in studying the glycated protein resulted from the Maillard reaction in many biological fields. First of all, as we described above, there is a relevance for investigating glycation of long-lived proteins, such as serum albumin or collagen in case of diabetes mellitus [21]. On the other hand, from the standpoint of food technology, non-enzymatic glycation of proteins through the Maillard reaction has been widely investigated and reported in order to improve their functional properties, such as emulsifying, foaming properties and thermal stability [22]. During industrial processing or prolonged storage, the Maillard reaction occurs frequently, improving organoleptic properties of food (color, aroma and flavor) [23]. For instance, acceleration of Maillard reaction at 60 °C for β-lactoglobulin improves significantly the functional properties of the protein, which could be attributed to the sugar added [24]. Glycation of BSA has also been investigated in the improvement of the foaming properties of this protein [25].

With regard to various alterations suffered by proteins, it is necessary to consider that proteins can undergo simultaneously both glycation and aggregation. Previous results, obtained with PAGE electrophoresis had shown the possible role of glycation in the prevention of BSA aggregation [12]. To get further insights on the aggregation mechanisms in glycated proteins, here we report spectroscopic studies on thermal aggregation of native and modified BSA with different glucose concentrations. We also study the specific changes on antioxidative properties of this modified protein.

2. Materials and methods

2.1. Preparation of AGEs

Advanced glycation end products (AGEs) are modifications of proteins or lipids that become nonenzymatically glycated and oxidized in presence of aldose sugars [26,27]. Glycation is based on Maillard reaction and corresponds to a condensation between a carbonyl compound, usually a reducing sugar, and a free amino group of specific residues of protein (lysine or arginine) [28]. Early glycation processes result in the formation of Schiff's bases and Amadori products. Further oxidation and rearrangement of glycated proteins give rise irreversibly to advanced glycated end products (AGE) [29]. The term "glycoxidation" denotes these processes and refers to AGEs that require both glycation and oxidation for their formation. AGEs are fluorescent products, form covalent crosslinks and produce intracellular reactive oxygen species (ROS) when binding to specific cell surface receptors [26,27].

For preparation of glycoxidized albumins, non-recombinant bovine serum albumin (BSA) (Sigma cat# A9647) was used. AGEs were prepared as previously described [30] by incubating 0.5 mM BSA with increasing concentrations of glucose (0 , 5 , 25 , 100 and 500 mM) in phosphate-buffered saline (PBS) at pH 7.4, under sterile conditions and nitrogen gas in capped vials at 37 °C for 7 weeks. The proteins were dialyzed against PBS, pH 7.4 and sterile-filtered with 0.2 μm Millipore. Endotoxin content as assessed by in vitro toxicology assay kit (E-TOXATE, Sigma) was bellow detectable level (0.03 endotoxin U/ml).

ANSA (A 1028), anti-dinitrophenyl (DNP) antibody (D 9656) and TMB (T 0440) on one hand and D_2O (151882) and AAPH(44091) on another hand were purchased from sigma and Aldrich, respectively.

2.2. Oxidative modification of SH

Thiol groups in native, modified albumin were measured by Ellman's assay using 5, 5′-dithiobis, 2-nitrobenzoic acid (DTNB) as described previously [30]. A standard curve was performed for each assay and various concentrations of L-cysteine (10 to 100 nmol) (Sigma) were used. The content of thiol groups for each BSA sample was measured in duplicate on two different quantities of BSA by reading the absorbance at 412 nm and with the help of the standard curve. Results are expressed as the number of free −SH groups per mol of BSA.

2.3. Carbonyl contents by ELISA

The degree of carbonylation of modified albumin was determined by the carbonyl ELISA assay developed in our labs based on recognition of protein-bound DNPH in carbonylated proteins with an anti-DNP antibody.

Protein derivatization was carried out in 1.5 ml reaction tubes, with 10 μl of DNPH solution 10 mM and 5 μL SDS 12% added to 5 μl of sample (5 μg/μl). Samples were incubated at 25 °C for 30 min and the reaction was neutralized and diluted in coating buffer (10 mM sodium carbonate buffer, pH 9.6) to give a final protein concentration of 0.25 μg/μl. Growing volumes (0–100 μl) of derivatized samples (in the range of 25 ng to about 250 ng of protein) completed until 200 μl with coating buffer were added to wells of an Immuno Plate Maxisorp. In this ELISA experiment, native and glycated treated DNPH proteins were used as standards. Plates were incubated 3 h at 37 °C then washed five times with PBS between each of the following steps: blocking with 1% BSA in PBS / TWEEN 20 overnight at 4 °C; addition of anti-DNP antibody (1:2000 dilution in PBS/TWEEN 0.1%/BSA 1%) and incubation for 2 h at 37 °C; addition of horseradish peroxidase-conjugated polyclonal anti-rabbit immunoglobulin (1:4000 dilution in PBS/TWEEN 0.1%/BSA 1%) and incubation for 1 h at 37 °C; and addition of 100 μl of TMB substrat solution and incubation for 30 min before stopping the coloration with 100 μl of 2 M sulfuric acid. Absorbances were read at 490 nm against the blank (DNP reagent in coating buffer without protein) with the FLUOstar microplate reader. The data were plotted as a function of increasing amount of protein (0 to 250 ng) and the degree of oxidation (carbonylation) of modified albumin was calculated as follows: $\%\ \text{carbonyl} = [(B-A)/A] \times 100$, where A and B are the slopes of the unmodified standard (native BSA) and modified protein, respectively, as determined from the absorbance data at 490 nm in the linear part.

2.4. Absorption, fluorescence and Rayleigh scattering measurements

Absorptions measurements of treated albumin were carried out with a Shimadzu UV 2401PC spectrophotometer in the UV-Vis range 190–600 nm. The absorption spectra were corrected for the scattering contribution and normalized to the maximum value to take into account the different contributions of the sample concentrations. Fluorescence spectra and Rayleigh scattering measurements were carried out on Jasco FP-6500 equipped with a Jasco ETC-273T peltier-thermostat. BSA samples at a concentration of 10 μM in PBS were positioned in a cuvette of 1 cm. All emission spectra were recorded with 0.5 nm wavelengths intervals. For the aggregation kinetics, measurements were started after thermal equilibration and performed for 20 h every 6 min. The tryptophans emission spectra were obtained in the range of 280–430 nm under excitation at 270 nm [6,31]. All fluorescence spectra were corrected for the respective different absorption.

The Rayleigh scattering at 90° was also measured as the maximum of the elastic peaks of excitation light due to particles much smaller than the excitation wavelength.

The 1-anilino-8-naphthalenesulfonic acid (ANSA) dye was dissolved in 5 μM albumin sample with a ratio ANSA/albumin of approximately