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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

0.4 (mol/mol) in order to be sure that the dye was linked to hydrophobic sites of albumin. ANSA–protein complexes fluorescence was measured at 440 nm by using a microplate fluorometer FLUOstar OPTIMA (BMG Labtech, France) with an excitation wavelength of 355 nm.

2.5. FTIR measurements

Thermal aggregation kinetics of native and glycated albumins were followed by acquiring FTIR spectra at different times and through a Bruker Vertex 70 spectrometer, equipped with an MIR light globar source (i.e. a U-shaped silicon carbide piece), with spectral resolution of 2 cm^{-1}. Each spectrum was averaged over 100 scans. The absorption spectrum of the empty beam line was subtracted from the spectrum of each sample. The resultant spectra were smoothed with a 13-point Savitzky–Golay function. D_2O solutions were used in BSA samples to avoid the spectral overlaps between Amide I band and strong absorption band of water at about 1640 cm^{-1} and to monitor the H–D exchanges between protein and solvent by Amide II and Amide II′ bands. Samples freshly concentrated with Microcon tube (Millipore) were dissolved at about 60 mg/ml^{-1} in PBS prepared in D_2O. Then, samples were placed between two CaF_2 windows, with a 0.05 mm Teflon spacer. The investigated infrared zones are the regions of the Amide I and of the Amide II bands. Amide I band, that in D_2O is called Amide I′ for the shifts towards 1650 cm^{-1}, is due to an out of phase combination of the C=O and C–N stretching modes of amide groups. Generally, it has a composite profile consisting of several spectral components related to the different types of secondary structures [32,33–35]. The main band in the Amide I region at 1656 ± 1 cm^{-1} was assigned to α-helices, the band at 1681 ± 1 cm^{-1} was assigned to β-turns, and the band at 1636 ± 1 cm^{-1} was assigned to β-sheets. These bands can endure a shift in different solutions. Further information on the intermolecular aggregation (β-aggregates) can be obtained from two shoulders at about 1620 and 1680 cm^{-1} [6,36–38] due to vibrations of strongly bound intermolecular between parallel and anti-parallel β-sheets, respectively [39]. Amide II (or II′) band (1400–1580 cm^{-1}) is predominantly associated with the N–H (or D) in-plane bending of the peptidic groups [40]. When an H–D exchange occurs, a simultaneous increase of Amide II′ and decrease of Amide II is observed. In order to identify the time evolution of each spectral component under the broad amide bands, difference spectra were obtained by subtracting to the spectrum at a generic time t, the spectrum at t_0 (where t_0 was 7 min after the beginning of the experiment to reach thermal equilibrium):

$$\Delta Abs(t,\upsilon) = Abs(t,\upsilon) - Abs(t_0,\upsilon)$$

2.6. Dynamic light scattering measurements

On one hand, modifications in the protein size were qualitatively studied by means Rayleigh scattering at 90° measured as the maximum of the elastic peak of excitation light during fluorescence measurements, as described above. On another hand, quantitative information on the growth of macromolecular assemblies in solution during the aggregation pathway was measured by dynamic light scattering (DLS) employing a Nano S Zetasizer (Malvern Instruments). It was equipped with a light source from a 4 mW He-Ne laser ($\lambda = 633$ nm) and a scattering angle of 173°. Protein concentration was about 1 mM in PBS buffer in 1 ml cuvette and in a sample compartment self enclosed, in which the temperature was automatically controlled. The most important information on the aggregation state of proteins obtained with DLS is given by the scattered light intensity, the Z-average diameter and the diameter size distribution of the particles in solution. Two approaches are utilized to obtain size information from the correlation function: (a) fitting a single exponential to the correlation function to obtain the mean size (z-average diameter), that is the weighted mean hydrodynamic size of the ensemble collection of particles measured by DLS (an increase of Z-average value is an indication of particles aggregation); (b) fitting a multiple exponential to the correlation function to obtain the distribution of particle sizes (such as CONTIN). The size distribution obtained is a plot of the relative intensity of light scattered by particles in various size classes and is therefore known as an intensity size distribution (see details in reference 41). Each kinetic measurement was performed on protein sample kept at 58 °C for aggregation process.

2.7. Hemolysis test of red blood cells

The antioxidant properties of AGEs were examined with the free radical-induced blood hemolysis test [42]. Human blood samples were obtained from the Biochemistry department of the local hospital center (CHD Felix Guyon) and were taken on EDTA substrate as anticoagulant. Then, plasma was removed and erythrocytes were washed with an isotonic solution (NaCl 0.15 M). Each well of a 96-well plate was filled with 100 µl (about 1.10^8 erythrocytes, 400,000 cells/µL final concentration) of diluted solution of red blood (1/10 in 0.15 M NaCl). Different albumin samples (10 µM final concentration) were added in triplicates. Hemolyses were started by adding 0.45 M of 2, 2′-azobis (2-amidinopropane) (AAPH) in each well. Turbidimetry at 450 nm was recorded every 10 min, using a 37 °C-thermostated microplate reader. Results were expressed as 50% of maximal hemolysis time (HT_{50} in min), which represents the total defense against free radicals in human and animal models submitted to oxidative stress [43].

2.8. Statistical analysis

Data were expressed as the means ± standard deviation (SD) from a minimum of three experiments. Statistical significances were determined using one-way ANOVA (followed by the Tukey test).

3. Results

3.1. Characterization of glycoxidized BSA

Different samples of modified BSA were characterized at room temperature in order to obtain information about the effects induced on the BSA structure by glycation and formation of AGE products. In particular, we have studied samples named as BG0, BG5, BG25, BG100 and BG500, where the number identifies the amount of glucose (mM) used to induce glycation of BSA (0.5 mM).

First of all, we have characterized these glycated samples by measuring the absorption spectra. In Fig. 1a, the spectra normalized for the concentration are reported, while the spectral line shape of the main absorption of the protein, centered at about 280 nm, is substantially unmodified, a significant increase of absorbance at about 300–380 nm was observed in albumin incubated with increasing concentrations of glucose; in particular, this effect is more pronounced in BG500 sample. This spectral contribution is attributed to the absorption of the glycophore, formed by the link between protein and glucose molecules [44]. We have also obtained the fluorescence spectra on all the samples as reported in Fig. 1b, in which the typical band at about 340 nm ($\lambda_{ex} = 270$ nm) is attributed to the tryptophans fluorescence (Trp 134 and Trp 214 [6,49]). Similarly as done in previous works [45–48], the production of AGE products was monitored by measuring the glycophore fluorescence ($\lambda_{ex} = 360$ nm), as shown in Fig. 1c. This last shows that the band amplitude increases when the glucose content used for the glycation increases; further, a blue-shift of the maximum is evident, from 460 to

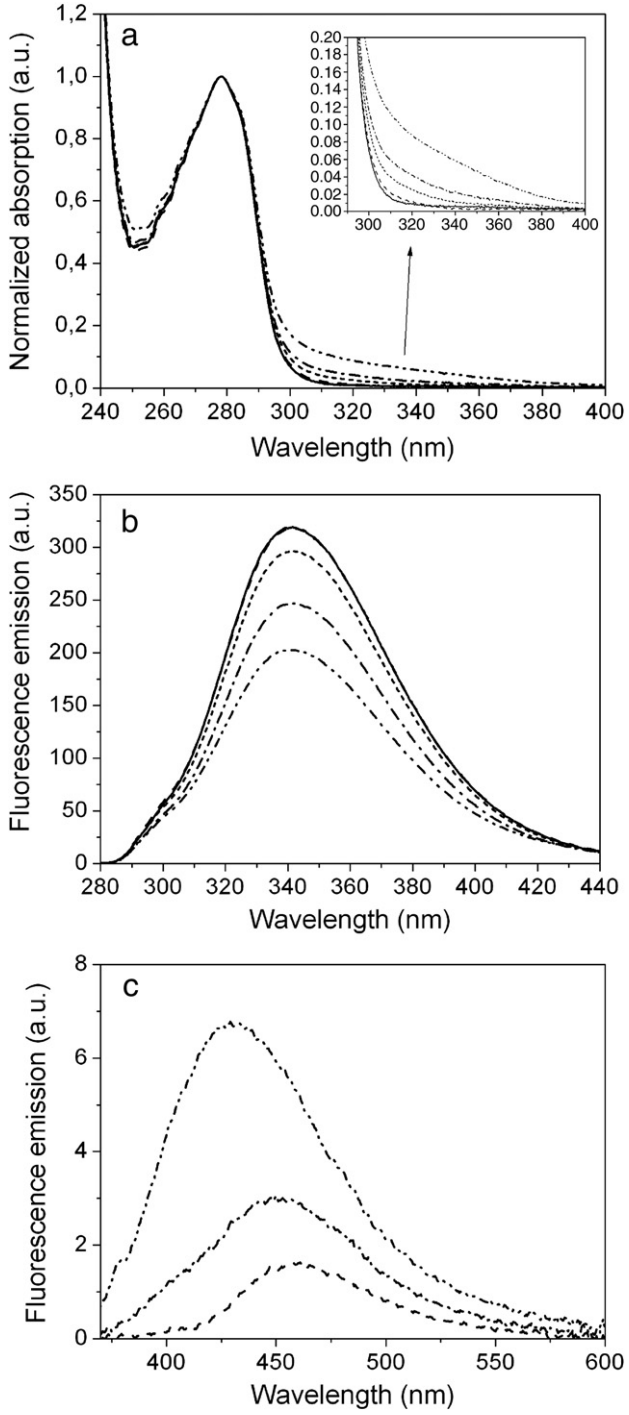

Fig. 1. (a) Normalized absorbance spectra; the inset shows a zoom in the range 290–400 nm. (b) Fluorescence spectra of tryptophans with excitation wavelength at 270 nm of BSA glycated with different concentrations of glucose: BG0 (—), BG5 (— —), BG25 (– –), BG100 (– · –), BG500 (– · · · –). (c) Fluorescence spectra of glycophore with excitation wavelength at 360 nm in BG5 (— —), BG100 (– · –) and BG500 (– · · · –). Absorption spectra of all samples are shown after having normalized the data to the tryptophan absorption maximum and to have eliminated the contribution of the scattered light.

425 nm. These two spectral effects are due respectively to an increased number of glycophores in BG500 sample with respect to the other samples and to a decreased polarity of the glycophore environment as more glucose molecules are bound to the protein. In other words, the more glucose molecules are linked to BSA, the more the intensity of glycophore emission increases; contemporarily, the more BSA molecules are glycated (for example in BG500), the more the glycophore environment becomes apolar. In general, we remind that the emission spectra are widely used to monitor the exposure degree to the solvent of the chromophores caused by conformational changes [6,49–51]. As evident in Fig. 1b, the fluorescence spectra of native BG0 and progressively of glycated BSA show that the modified proteins undergo a dose-dependent decrease of quantum yield of the fluorescence, suggesting different changes in tertiary structure with increased exposure degree of the tryptophans in respect to the native molecules. This effect clearly indicates that conformational changes are a consequence of glycation.

From these results, glycated BSA is a less polar molecule than the native one. To confirm these data, the accessibility of the hydrophobic regions in the protein was detected by the binding of fluorescent probe ANSA. As reported in Fig. 2a, the glycated BSA binds this hydrophobic probe more efficiently than the native albumin. The number of hydrophobic sites in the protein results to be different for the same experimental conditions; it increases by increasing the glucose concentration. This result confirms again that the link between protein and glucose molecules changes the native conformation of the tertiary structure, while the secondary structure remains unchanged, as obtained by data of circular dichroism not shown here.

The antioxidative activity is one of the numerous functions of the serum albumin and the glycation pathway associated with oxidative phenomenon could affect the albumin integrity [19]. As shown in Fig. 2b, glycation of BSA with glucose induces a dose-dependent decrease in free thiol groups contained in the protein, as assessed by Ellman's method. Moreover, the monitoring of the carbonyl content of the protein, measured by ELISA using a phenylhydrazine formation reaction, provides a convenient way to assess oxidative modification with glycation pathway. Results of carbonyl accumulation are given in Fig. 2c. When BSA was incubated with increasing glucose concentrations, a pronounced increase in the carbonyl content was observed. Finally, with the oxidative and glycation processes leading to the production of AGEs, a decrease of intrinsic antioxidative properties of BSA is expected. To determine whether both glycation and oxidation processes of albumin, affect these beneficial properties, the free radical-induced hemolysis test has been studied and reported in Fig. 2d. As already observed in a previous paper by Bourdon et al. (1999), the sole glycoxidation of BSA with increasing concentration of glucose (up to 500 mM glucose) did not affect the free radical-trapping capacities of albumin [46].

3.2. Aggregation pathway of glycated BSA

In this section, we focused on glycated BSA susceptibility for the aggregation. It is well known that native BSA is prone to the aggregation with production of different aggregates, from the oligomers to the amyloid fibrils, according to the experimental conditions [6,9,10]. The involvement of glycation state in the aggregation process is now determined by monitoring the modifications in the secondary and tertiary structures accompanying the aggregates formation. The main aim is to understand how the structural modifications in BSA, due to the advanced glycation process will affect its ability to form aggregates under heating conditions. In particular, we follow the thermal aggregation of the BSA at 58 °C, as done in other studies [9,41]. Moreover, by fluorescence spectroscopy, FTIR absorption, static and dynamic light scattering, we follow the time evolution of the aggregation process and of the simultaneous conformational changes.

In a previous study, we hypothesized that aggregation susceptibility of BSA at 58 °C may be progressively reduced with the extent of glycation [12]. The kinetics of aggregation was monitored by following the tryptophan fluorescence emission. Fig. 3a shows tryptophan emission spectra of the extreme samples BG0 (a) and BG500 (b) acquired at different incubation times at 58 °C. The Rayleigh scattering

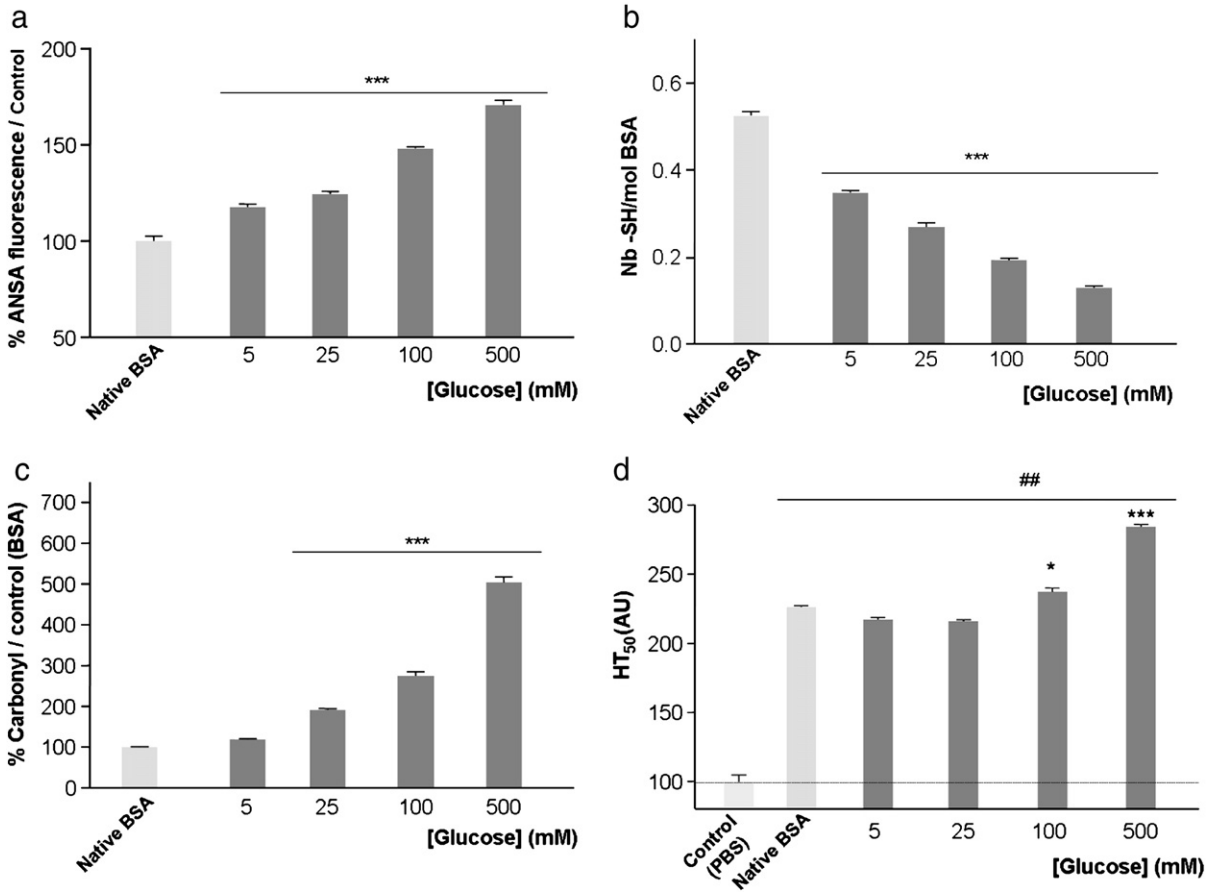

Fig. 2. (a) ANSA emission spectra of AGEs at 440 nm under excitation at 355 nm. (b) Thiol groups contents in the different preparations of albumin evaluated with Ellman's method. (c) Percentage increase of carbonyl content modification of glycated BSA (compared with native BSA) as assessed by the ELISA carbonyl assay. (d) Impairment in the antioxidant activities of BSA as assessed by the free radical-mediated blood hemolysis test. Histograms represent half time hemolysis (HT_{50} in min) expressed as means ± SD of three independent experiments. The doted line facilitates comparison with control (no albumin). All data are expressed as mean ± SD ($n = 3$) and statistical analyses were performed using one-way ANOVA for multiple comparisons. *Effect of the glycoxidation of BSA (vs. BG0): $^{***}p < 0.001$, $^{*}p < 0.05$. $^{\#}$Effect of BSA (vs. PBS): $^{\#\#}p < 0.01$.

peaks at the excitation wavelength are reported in the insets in order to monitor simultaneous changes in the mean dimension of the aggregates in solution. As seen, in both cases the intensity of the emission decreases with time (arrow in the figures) but with lower extent at the increasing of number of AGEs formed, indicating that the rise of the exposure degree to the solvent is progressively smaller. A little and progressive blue-shift is also revealed; this blue-shift is due to a less polar environment of the tryptophans and it has also been attributed to the formation of more tightly packed structures [52]. Formation of similar structures was also found and shown in the previous section through the decreased intensity of tryptophans fluorescence and the relative blue-shift of the glycophore as a function of the glucose concentrations (Fig. 1b and c). The same effects are also observed for glycated albumins when incubated at 58 °C. Indeed, tertiary structure and apolarity of glycated BSA, already modified by glycoxidation process, are then altered with heating treatment.

The aggregation process, qualitatively monitored by the Rayleigh scattering data, is reported in Fig. 3c. These results put in evidence that the extent of the aggregation process progressively decreases and suggest that the conformational changes are not strictly related to an eventual aggregates growth. In particular, it is evident that in BG0 and BG500, the modifications in the tertiary structure are similar, while aggregation is not observed in BG500 (molecules keep the same size during the heating process), as probed by unchanged Rayleigh peak intensity as a function of time, shown in inset of Fig. 3b. The more the protein is glycated, the less susceptible it is to aggregation. Recently, we have also shown that the incubation of BSA with glucose in the concentration ratio 1:1 for 7 weeks led to 59% of albumin in a glycoxidized state [30]. This could mean that in BG500 sample in which the glycation process has reached the saturation (all protein molecules are involved in the formation of AGEs), the aggregation does not occur.

In order to quantify the extent of the aggregates growth induced by heating at 58 °C in glycated BSA, dynamic light scattering (DLS) measurements were performed. In Fig. 4a, the time evolution of the total scattered intensity (up) and of the Z-average diameter (bottom) of modified albumin aggregates is reported. The increase of the scattered light simultaneous to the increase of the mean diameter of the molecules in solution indicates that aggregation is induced by heating. Fig. 4a shows that the increase of both parameters is less intense for highly glycated albumin. The scattered intensity by molecules of BG0 sample achieves the highest value, indicating that the efficiency of the aggregation process reaches its maximum; further, we note that after about 200 min, the scattered intensity suddenly decreases because of the formation of precipitates at the bottom of the cuvette. Conversely, in BG500 sample, no significant variation is observed. These results are in agreement with the ones shown for BG500 by the unchanging of the Rayleigh scattering intensity as a function of the time, at lower protein concentration (insets in Fig. 3a and c). The course of the mean diameter as a function of the time indicates that the heating induces instantaneous formation of oligomeric structures with typical dimensions lower than 25 nm for BG0 sample; this dimension value is progressively lower when increasing concentrations of the glucose were used for the glycation. For BG500 sample, the molecular size remains around a value of 8 nm. Analysis of data, using the CONTIN procedure, allows us to obtain the

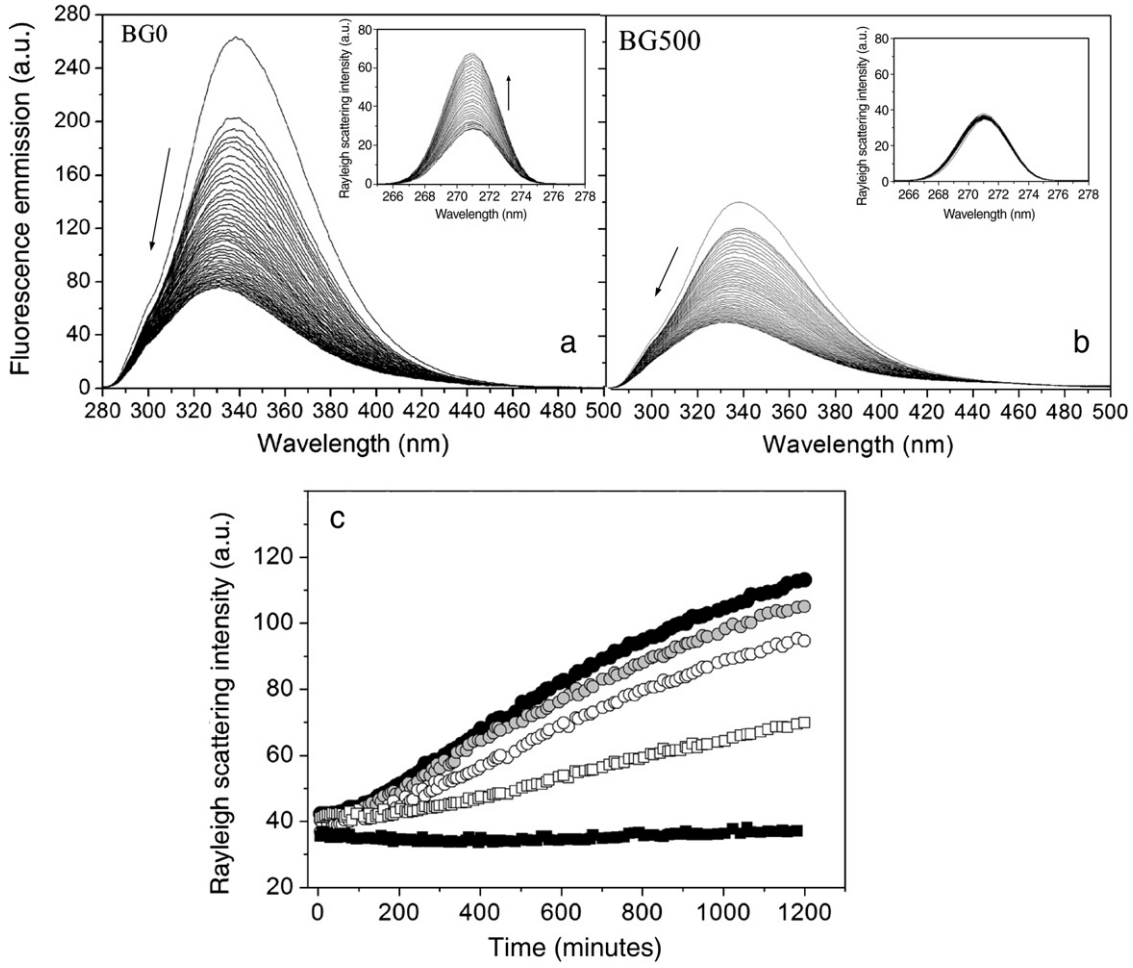

Fig. 3. Kinetics of tryptophans emission for BG0 (a) and BG500 (b) at 58 °C; the insets show the Rayleigh peak evolution measured at the excitation wavelength of 270 nm; the arrows indicate the changes of the bands as a function of the time (0–1200 min). (c) Time evolution of the Rayleigh peak intensity for BG0 (●), BG5 (◍), BG25 (○), BG100 (□) and BG500 (■).

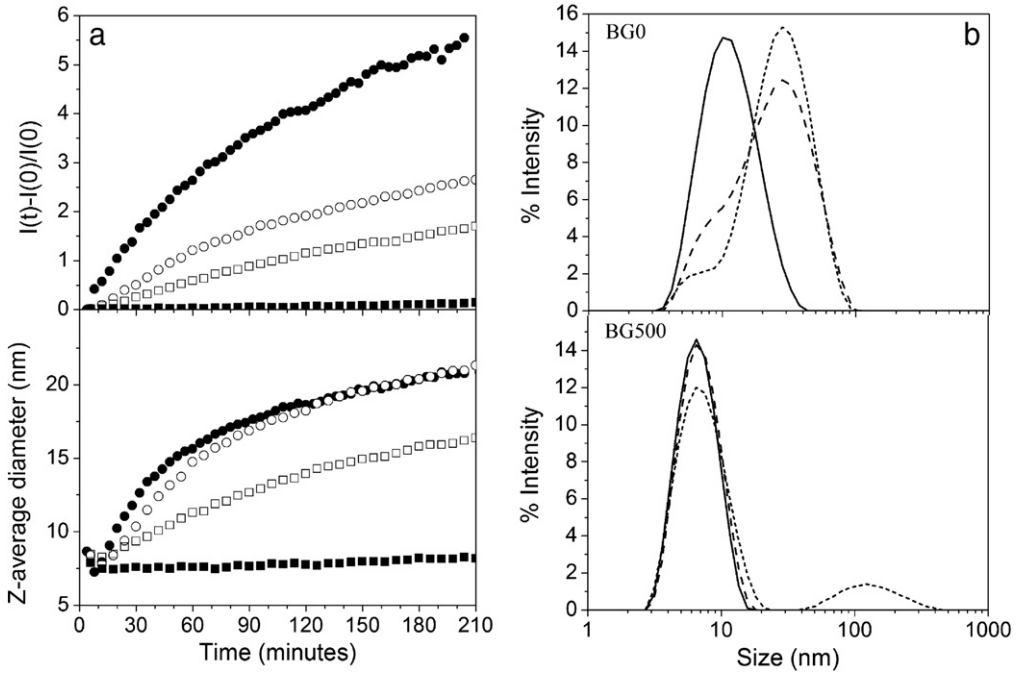

Fig. 4. (a) Time evolution of normalized scattered intensity (upper) and of the average diameter (lower) for BG0 (●), BG25 (○), BG100 (□) and BG500 (■) at 58 °C. (b) Size distribution of the aggregates dimensions for BG0 and BG500, based on the light scattered intensity by the particles present into solution, at three different times: after thermal equilibration (—), after 120 min (— —) and after 210 min (- -).

distribution of the diameter values with respect to the scattered intensity and at different times of the whole kinetics. In Fig. 4b, we report this distribution at $t=0$, $t=120$ and $t=210$ min only for BG0 and BG500 samples. The analysis of the distribution allows us to reveal the formation of larger species also when present in low number. The comparison of the distribution in BG0 (Fig. 4b, top) and BG500 (Fig. 4b, bottom) shows that only for BG0 a marked growth of a species with diameter of about 30 nm is revealed at 120 and 210 min, indicating that most part of the native molecules (monitored at $t=0$) have auto-assembled, forming mainly oligomers. Conversely, in BG500 the dimension of the molecules in solution is unchanged, as it is evident from the position of the peak at about 10 nm, which remains unchanged as a function of the time. The Gaussian profile in this graph is determined by the peak mean and standard deviation of the distribution, as derived from an non-negatively constrained least squares (NNLS) deconvolution CONTIN algorithm; hence, the little differences showed in the native dimension of BG0 and BG500 samples (8–10 nm in the maximum peak) must be considered within the analysis error. The existence of a larger species (100 nm), negligible in respect to the main species, is revealed in the final times of the kinetics. By following a longer time evolution, these species could be investigated in the future. The results obtained by light scattering are in accordance with the previous data attesting a lower susceptibility to aggregation for BSA incubated with higher glucose content.

In order to extend the structural study and remembering that the native BSA has 18.1% turns, 60.3% α-helix, 13.7% β-sheet and 7.9% random coil [32], we investigate the effect of glycation on secondary structure (Amide I′), through kinetics of infrared absorption. Actually, this technique gives also specific information on the modifications induced by heating in the tertiary structure (Amide II and Amide II′). Once again, we find different results for BG0 and BG500, while the remaining samples show intermediate behaviors.

In Fig. 5a, the differential infrared absorption spectra of BG0 (up) and BG500 (bottom), acquired at different times and normalized in respect to the maximum absorption of the different samples are reported. The most evident result is the decrease of a component at about 1660 cm^{-1} in favour of a component at about of 1640 cm^{-1}. This effect suggests that, in native and in BSA modified by AGEs, the heating induces conformational change of secondary structure, through a conversion of α-helix and random coil structures in intramolecular β-sheets [9,32,39,41]. Moreover, the increase of a negative component at 1682 cm^{-1} indicates that the simultaneous decrease of turn structures occurs [53]. The most important difference among the samples consists in the absence of a positive peak at about 1620 cm^{-1} in BG500. This peak is progressively lost in the remaining glycated samples with a strong difference between BG25 and BG100. This band is attributed to the vibration of CONH in intermolecular β-sheets; hence, it constitutes a marker of the formation of β-aggregated structures [9,39,41,54]. Thus, in agreement with the previous techniques used to characterize the glycation, we observe that the aggregation is progressively decreased up to inhibit the formation of β-aggregated structures in BG500. The time evolution of the components at 1640 and 1660 cm^{-1} reported in Fig. 5b evidences the effect of the occurring aggregation on the modification of the secondary structure. The behavior of the component at 1660 cm^{-1} probing the decrease of α-helix structures as a function of the time is the same in native and glycated albumins, while a lower amount of intermolecular β-sheets (1640 cm^{-1}) is revealed in BG500 compared with BG0. This result indicates that only when an aggregation process is activated, a part of β-sheets deriving by conversion of α-helix structures is engaged to form β-aggregated structures. In addition, it is important to note that in the first minutes, the course of the components at 1640 cm^{-1} is very similar in all investigated samples, while only after about 40 min, the aggregates growth is detectable.

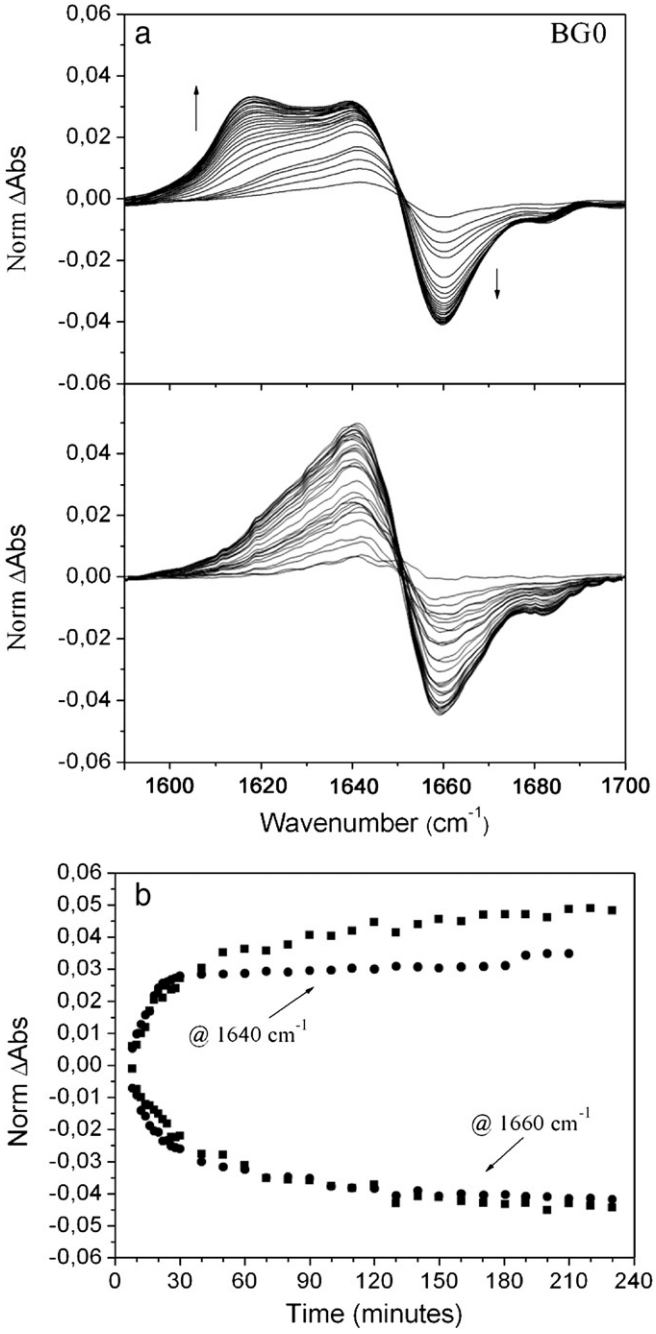

Fig. 5. (a) Normalized differential absorption spectra in the Amide I′ region of BG0 and BG500. (b) Time evolution of differential absorption intensity of Amide I′ components at 1640 and 1660 cm^{-1} for BG0 (●) and BG500 (■), respectively.

We previously reported thermal aggregation of BSA at the same pH but in different buffers (PB or MES buffer) [9,41,55]. Here, the aggregation (monitored by dynamic light scattering) was faster because the protein underwent the most important changes in the first 20 min [41,55]; moreover, the conformational changes of secondary structures (monitored by FTIR spectroscopy) were very different: the modification of secondary structures was characterized by the change of α-helix structures (~1650 cm^{-1}) in β-sheets of intermolecular structures (~1615 cm^{-1}) directly [9,41,55]. Here the same native protein in PBS (phosphate buffer with NaCl) is less inclined to the thermal aggregation compared with BSA in PB (it's well known the stabilizing effect of the salt).

Finally, the kinetics of the differential absorption spectra of Amides II and II′ bands have been analysed between 1400 and 1580 cm^{-1}. The

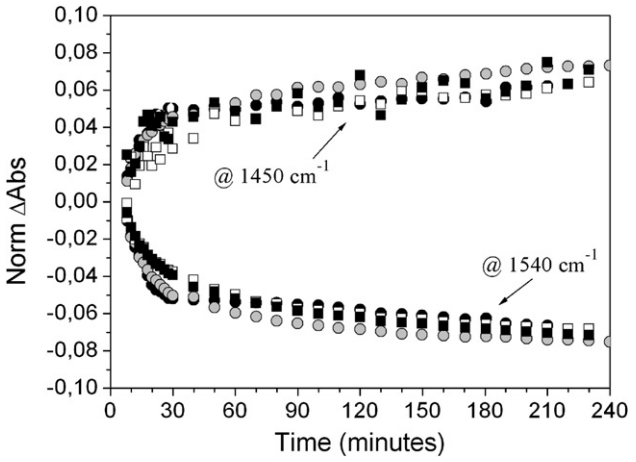

Fig. 6. Time evolution of normalized differential absorption intensity of Amide II at 1540 cm^{-1} and Amide II′ at 1450 cm^{-1} for BG0 (●), BG5 (◍), BG100 (□) and BG500 (■).

time evolution of these bands gives us information on the protein unfolding with consequent H–D exchange between protein and solvent [9]. The comparison of the time evolution of the normalized components at 1540 cm^{-1} (Amide II) and 1450 cm^{-1} (Amide II′) for different modified BSA (Fig. 6) shows that the partial unfolding, caused by heating, is not affected by the existence of AGE products. This result seems in contrast with those previously obtained by tryptophans fluorescence spectroscopy. Instead, it must be considered that the experimental conditions are similar but not identical: the concentration of protein for IR absorption measurements is very higher than that for fluorescence measurements. Besides with exchangeable hydrogens and the tryptophans for FTIR and fluorescence spectroscopy, respectively, the chromophores are different. Indeed, the fluorescence technique follows the modifications in the surrounding area around the tryptophans, while the FTIR spectroscopy, through the Amide II and II′ exchange, gives us information on the average of the hydrogen exchanged through partial unfolding of the whole protein.

4. Discussions

This study allowed achieving a better understanding of the importance of structural integrity of albumin on its biological properties. In particular, the aggregation study of glycated albumin represents a new way for investigating structural aspect of proteins glycation. Among numerous roles of albumin, binding and transport of several endogenous and exogenous molecules represent important biological functions which could be affected by glycation process. The changes of binding capacity for these metabolites depend on the tertiary structure of the binding sites which are distributed over the protein.

By using different techniques, we studied both structural modifications of bovine serum albumin induced by glycation process forming AGEs and changes in its susceptibility to thermal aggregation depending on the glucose content used for the glycation. By carrying out the changes of the protein conformation, we try to evidence the important role played by glucose, through non-enzymatic glycosylation pathway. In particular, an increasing amount of glucose used for the glycation leads to the formation of an increasing number of glycated protein molecules as also demonstrated in previous studies [19,30].

Regarding the glycation process, the increase of glucose concentration incubated with BSA solution at 37 °C for 7 weeks contributes to modify progressively the tertiary structures of the protein, as revealed by the modifications in the tryptophan fluorescence. Further, tertiary conformational changes could be a consequence of molecular rearrangements after the formation of AGE products. In particular, some of these AGEs forming covalent crosslinks with adjacent protein strands such as pentosidine, MOLD (methylglyoxal-lysine dimer imidazolium crosslink) or GOLD (glyoxal-lysine dimer imidazolium crosslink) [56,57] require conformational changes which produce more apolar and tightly molecules with respect to the native protein. Besides, we have also shown, by using ANSA probe, that the accessibility of the hydrophobic regions in the protein increases with the glycation.

The specific partial unfolding, due to the glycation, could explain partially the increase of copper binding capacity with glycated BSA, observed in a previous study [19], while aggregation of the native form of this protein reduces the affinity to this metal [12]. With the change of tertiary structure, a pronounced increase in the carbonyl content and a decrease in free thiol group content are also observed, as probes of an increased oxidative state of BSA. The increase of oxidative state in glycated albumin is observed because both phenomena of glycation and oxidation are completely intertwined [58]. In albumin, the only one reduced cystein is prone to oxidation. Surprisingly, an increase of hemolysis time of red blood cells, suggesting an increase of protective effect of albumin incubated with increasing concentration of glucose was observed; this result, related to different tertiary structures revealed in different glycated samples, may indicate that specific amino acids buried in the native conformation of BSA can now scavenge free radicals. Moreover, the Maillard products are well known for being responsible for an increase in antioxidant activity in food systems [59]. The increasing oxidative state and the new conformation of the glycated protein are associated with a better free radical scavenging property of glycated albumin.

From all these results about the characterization of the glycated protein, we can summarize:

i) The presence of increasing number of glucose molecules during the incubation induces an increasing number glucose units attached to protein molecules in solutions (higher glycophore emission intensity).
ii) The tertiary structure is the sole structure level sensitive to change with increasing of the glycated molecules (lower tryptophans emission intensity).
iii) With these conformational changes, the glycated proteins become more apolar molecules (blue-shift of glycophore emission and higher ANSA fluorescence emission intensity) characterized by a progressive increase in their new structures of carbonyls content and a decrease of free thiol groups content. Besides, as attested by the hemolysis test, free radical scavenging capacities are not altered in proteins modified by glycation, suggesting that antioxidative properties are not affected.

For the study of aggregation pathway of glycated BSA, several studies have reported that AGE products could induce amyloid-like aggregation or molten globule-like state [46,60,61]. No aggregation state is observed for glycated BSA at our experimental conditions. In particular, modifications in the time evolution of secondary and tertiary structures accompanying the aggregation process in all the glycated samples were observed. The aggregation proceeds by a conversion of the protein secondary structure from α-helices to β-sheets, but formation of β-aggregated structures progressively decreased when the glucose concentration used for the glycation was increased. The aggregates growth is progressively decreased when the number of AGEs is increased. Therefore, the aggregation process of BSA is markedly affected by the glycation.

A possible explanation could be suggested for this low susceptibility of aggregation for highly glycated albumin. Non-enzymatic glycation of proteins involves generally amino groups such as lysine and arginine [62] and N-terminal amino groups [16]. The thiol group

of cysteine residue is a well-known powerful nucleophile which may be likely glycated by glucose and give rise advanced glycation end products such as *S*-(carboxymethyl) cysteine (CMC) [63–65]. At our experimental conditions, in BSA modified in AGEs products, the adduction of glucose on the unique cysteine residue could occur. On the one hand, the decrease of thiol groups content with the extent of glycation assayed by Ellman's method may be attributed, not only to an increase of oxidative state of the protein but also to the involvement of the cysteine in the glycation process. On the other hand, it is well recognized that the unique free cysteine of BSA, analogously to HSA, has a noteworthy role contributing to the aggregates growth by the formation of intermolecular disulphide bridges and S–S exchange [6,66]. In conclusion, our results indicate that the free cysteine of modified BSA could be involved in the glycoxidation process directly by oxidation or formation of CMCs (*S*-(carboxylmethyl) cysteine) or, more simply, indirectly by the presence of a steric hindrance of near glucose molecules forming AGEs. As a consequence, it could not longer be available to take part in the formation of intermolecular bonds. The increase of the number of protein molecules involved in the formation of AGEs necessarily causes the decrease of the efficiency of the aggregation process up to inhibit it in the samples for which largest amount of glucose were used to induce glycation.

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