Biomedical Spectroscopy and Imaging 5 (2016) 197–205
DOI 10.3233/BSI-160145
IOS Press

Chemical and physical characterization of thermal aggregation of model proteins modulated by zinc(II) and copper(II) ions

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Abstract.
BACKGROUND: Metal ions are implicated in protein aggregation processes of several neurodegenerative pathologies, where the protein deposition occurs, and in the biotechnology field like the food technology where many processes in food manufacturing are based on thermal treatments.
OBJECTIVE: The influence of Cu^{2+} or Zn^{2+} ions on the thermal aggregation process of Bovine β-lactoglobulin (BLG) and Bovine Serum Albumin (BSA), two protein models, was studied with the aim of delineating the role of these ions in the protein aggregation kinetics and to clarify the related molecular mechanisms.
METHODS: The protein structure changes were monitored by Raman spectroscopy, whereas the aggregate growth was followed by Dynamic Light Scattering measurements.
RESULTS: Both metal ions are able to favour the BLG aggregation, whereas only Zn^{2+} ions have a promoter effect on the thermal aggregation of BSA. The reason of this different behaviour is that the BLG aggregation evolution is manly affected by the redistribution of charges, whereas that of BSA by the metal coordination binding which depends on metal.
CONCLUSIONS: Raman spectroscopy, combined with dynamic light scattering experiments, was very useful in identifying the role played by Cu^{2+} and Zn^{2+} on the aggregation pathways of BLG and BSA. The results provide evidence for the role of histidine residues both in the redistribution of charges and in the two modes of metal binding that take place in BLG- and BSA-containing systems, respectively.

Keywords: Beta-lactoglobulin, Bovine Serum Albumin (BSA), copper and zinc ions, Raman spectroscopy, dynamic light scattering

1. Introduction

Protein activity depends on native folded structure, thus protein misfolding can lead to dysfunctions or results in a wide range of diseases. Degenerative disorders such as Alzheimer's, Parkinson's and type II diabetes are believed to derive from misfolded protein aggregates, known as amyloid fibrils, which build up in organs and tissues [3,4]. These diseases are particularly intriguing because evidence is accumulating that the formation of the highly organized amyloid aggregates is a generic property of polypeptides, and not simply a feature of the few proteins associated with recognized pathological

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conditions. The fibril formation seems to be driven by an appropriate destabilization of the native state and typical of the early phases of fibril formation is the conversion of α-helices into β-sheets [22]. Raman spectroscopy is exceptionally sensitive to protein secondary structure, and of consequence, combined with other techniques such as dynamic light scattering (DLS), can be succesfully used to study protein aggregates and fibrils.

The idea that metal ions are implicated in these protein aggregation processes arises from the finding that increased metal concentrations (mainly copper, iron and zinc) are present in the brains of Alzheimer's disease patients [2]. Understanding the nature of such aggregation mechanisms is a crucial step in the development of strategies to inhibite fibril formation and so to prevent and treat debilitating diseases.

Metal ions may act as inhibitors or promoters in aggregation processes depending on the metal/protein ratio and the metal ion binding mode; thus, they can differently affect the time evolution of the aggregates and their morphology [6,17]. The study of the interactions between metal ions and proteins is of increasing interest both in biomedical sciences since the presence of metal ions, together with the amyloid fibrils formation, is one of the fundamental aspects in the aetiology of different neurodegenerative pathologies (amyloidosis) [16], and in food processing, because of the capability of metal ions to induce cold gelation or aggregates [9].

Bovine β-lactoglobulin (BLG), a small globular protein of bovine milk, and Bovine Serum Albumin (BSA), the most abundant protein of plasma, are two of the most popular model proteins in the studies of protein folding, conformation *in vitro*, and aggregation for biotechnology applications (i.e. BLG is a thermal marker in industrial processes involved in milk treatment).

Although the mechanism of BLG and BSA heat-aggregation has been extensively studied, it is not still completely understood and controlled [5,12,13]. So, here we describe how Cu^{2+} or Zn^{2+} ions modulate the changes in the BLG and BSA structures caused by thermal treatment. We focus on the effects of these metal ions since they may have important medical applications being involved in aetiology and therapy of some illness, such as Menkes and Wilson diseases.

A joint combination of techniques allowed to obtain a more complete picture of the aggregation process mechanism at molecular level.

2. Materials and methods

2.1. Sample preparation

Bovine β-Lactoglobulin A (BLG), essentially fatty acid free BSA, $CuCl_2$ and $ZnCl_2$ were purchased from Sigma-Aldrich. The proteins were dissolved in a 20 mM MES (4-Morpholineethanesulfonic acid) buffer solution prepared in H_2O (99.9%, Aldrich) and titred with KOH until pH 7. The final concentration for BLG solution was 3 mM, whereas for BSA 1 mM. $CuCl_2$ or $ZnCl_2$ solutions (1 mM) were prepared in Super Q Millipore water and added to freshly prepared BLG and BSA solutions, in order to obtain a BLG/Metal(M) and BSA/M ratio of 3:1 and 1:1 ratio. The chloride salts of Cu^{2+} and Zn^{2+} were chosen for a spectroscopic reason, since they do not display any IR or Raman bands that can interfere with the protein spectrum.The freshly prepared samples in H_2O were incubated at 60°C for 400 min.

2.2. Raman spectroscopy

Raman spectra were obtained on lyophilised samples, before and after heating of the BLG aqueous solution at 60°C for 400 min and BSA at 58°C for 120 min, by a Bruker IFS 66 spectrometer equipped

with a FRA-106 Raman module and a cooled Ge-diode detector. The excitation source was a Nd^{3+}-YAG laser (1064 nm), the spectral resolution was 4 cm^{-1} and the total number of scans for each spectrum was 6000. The laser power on the sample was about 100 mW. Lyophilisation was performed on a Modulo 4K Freeze Dryer equipped with a RV8 Rotary Vane Pump (Edwards). The lyophilized product was kept at −80°C until use.

The curve fitting analysis was implemented using the OPUS/IR v 5.0 program, which uses the Levenberg–Marquardt Algorithm.

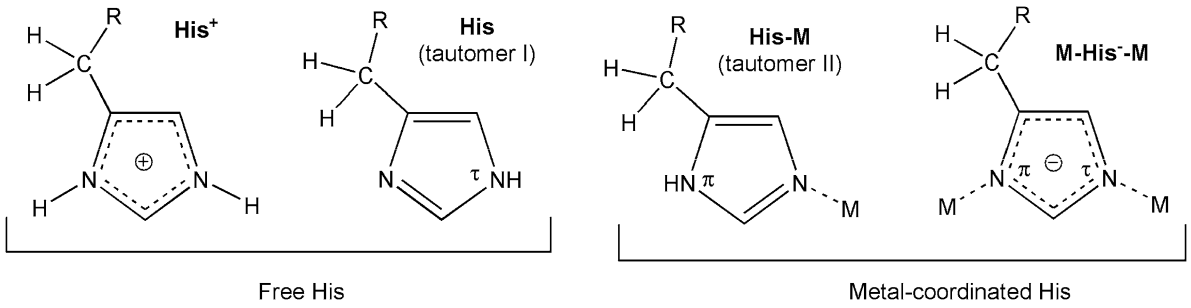
2.3. Dynamic light scattering (DLS)

DLS measurements were performed by using a Nano-S Zetasizer (Malvern Instruments) equipped with a He–Ne laser source tuned at 633 nm. Data were collected at 58°C for BSA and 60°C for BLG (at neutral pH the BLG unfolding starts at about 54°C and the aggregation kinetics are too fast to be followed over 60°C). The sample compartment was completely self enclosed and the sample temperature was automatically controlled within 0.1°C. The intensity scattering data were collected after a thermal equilibration time of 7 min. The evolution of the BLG and BSA thermal aggregation in the absence and in the presence of metal ions was followed using a Malvern particle sizer, based on the light scattering theory. The UV/Vis absorption measurements, realized before and after thermal incubation, probed the absence of precipitate during the kinetics in all the investigated samples.

3. Results and discussion

3.1. Heat-induced changes in specific protein moieties

His residues are present in both BLG and BSA sequence (BLG: 2 His; BSA: 17 His) and can act as potential donors for transition metal ions. In fact, the imidazole group of His side chain possesses two nitrogen atoms, which can be protonated or deprotonated, giving rise to four different protonation forms: two neutral tautomers, fully protonated imidazolium cation (His^+), and imidazolate anion (His^-) deprotonated at both the nitrogen sites (Scheme 1). Raman spectroscopy is one of the most powerful methods to study the protonation state of His in proteins since some bands are sensitive to the His tautomeric and ionic state [19,21]. Generally, three Raman bands (at 1570, 1290 and 990 cm^{-1}) can be useful, which appear at different wavenumbers depending on the His tautomeric form (tautomer I or II, also referred as Nτ-H or Nπ-H) and its involvement in metal coordination. The analysis of the free and



Scheme 1. Free and metal-coordinated states of His residue: protonated and neutral tautomeric I forms and two different Metal-coordinated forms (tautomer II and His^-).

metal-coordinated forms of His from Raman spectra is usually quite simple for proteins containing few aromatic residues, but this is not the case of BSA and BLG, which contains many aromatic amino acid residues.

Thus, a curve fitting analysis of the His bands is necessary because of their overlapping with some Tyr and Phe bands. In the case of BLG the band at ~990 cm^{-1} (=C–H in-plane bending vibration) was used [14]; it is generally assumed as a marker of His tautomer I, whereas a higher wavenumber band (~1004 cm^{-1}) has been assigned to the other neutral tautomer [1]. If the histidyl moiety is in its cationic form, this band has been observed at ~996 cm^{-1} [7], whereas it is shifted towards lower wavenumbers by metal coordination [7,20].

In the native BLG the presence of the His band at ~990 cm^{-1} indicated that the two His residues are both in the tautomeric I form (100%), and the thermal treatment induced only a small change in the protonation state of His (~10% from His^+) (Fig. 1(A) and (B)).

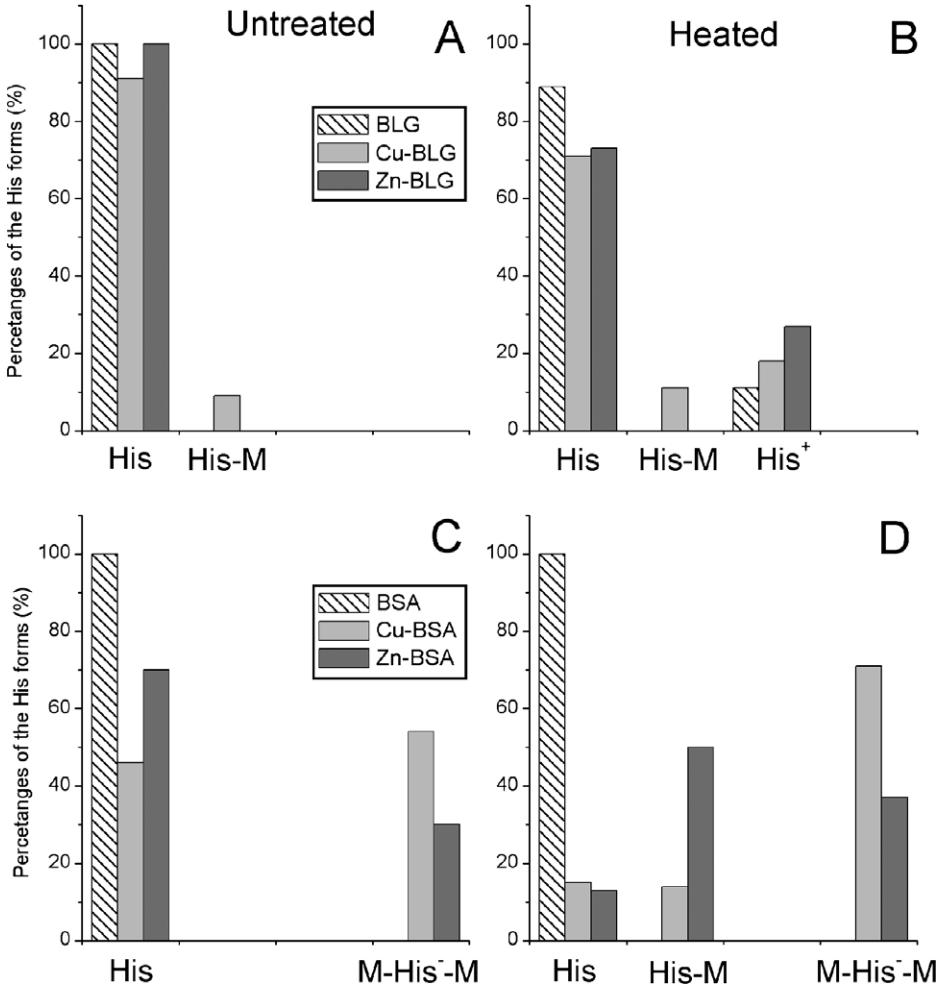


Figure 1. Percentages of His forms in BLG and BSA before (A and C) and after thermal treatment (B and D) in the absence and presence of metal ions.

The presence of metal ions induced a more evident change towards the cationic form of His (His^+) (Fig. 1(B)) and, in the case of Cu^{2+}, also a partial involvement of His in the metal coordination (~10% His-M) (Fig. 1(A) and (B)). Conversely, in the case of BSA, the His involvement in the metal coordination was found to be relevant both before and after the thermal treatment (Fig. 1(C) and (D)), as suggested by the appearance of a new Raman band component at ~1554 cm^{-1}, besides to the ~1565 cm^{-1} one due to free His ($\nu C_4{=}C_5$), characteristic of the His^- form bridging two metal ions through N_π- and N_τ-ligation (Fig. 2(A)) [10,15,18].

It's worth noting that after heating, His residues in BSA resulted to be almost completely involved in the Cu^{2+} and Zn^{2+} coordination (~85%) (Fig. 1(D)). However, the predominant mode of metal binding is greatly affected by the kind of metal ions present in the system: Cu^{2+} ions are mainly coordinated to His^- by both N_π and N_τ (~70%) (Scheme 1), whereas Zn^{2+} mainly binds to the neutral His (50%).

Other spectral changes further support the different coordination mode of Zn^{2+} ions in respect to Cu^{2+} in BSA, favoured by the temperature increase. In particular, a strong intensity increase of the shoulder at ~1420 cm^{-1} was visible in Cu-BSA (Fig. 2(A)), due to the deprotonation of some amide groups ($C{=}O/C{-}N^-$), as well as a rearrangement in the distribution of the charges (the COO^- vibrations contribute in this region). In fact, Cu^{2+} ion has been found to bind main-chain amide nitrogen in many protein or peptide-containing systems (i.e. soluble Cu(II)-amyloid β-peptide complex), since it is able to promote ionization of amide groups if a His residue is available as binding site [11,18].

The presence of Zn^{2+} ions induced a similar rearrangement in the distribution of the charges both inside BSA and BLG during heating. In fact only slight changes in 1400–1430 cm^{-1} region ($\nu_s COO^-$) were visible in the Zn-BSA spectra (Fig. 2(B)) and in the difference spectrum of Zn–BLG system (Fig. 3).

Other spectral differences between the Zn-BSA and Cu-BSA systems were visible in the bands due to Tyr residues. In particular, the Tyr intensity ratio (I_{860}/I_{840}), sensitive to the environment of Tyr residues, showed a different behavior in the two systems (Fig. 2). An I_{860}/I_{840} decrease of ~10% took place in Zn-BSA, whereas the doublet completely reversed in Cu-BSA. This result suggests that in Zn-BSA a

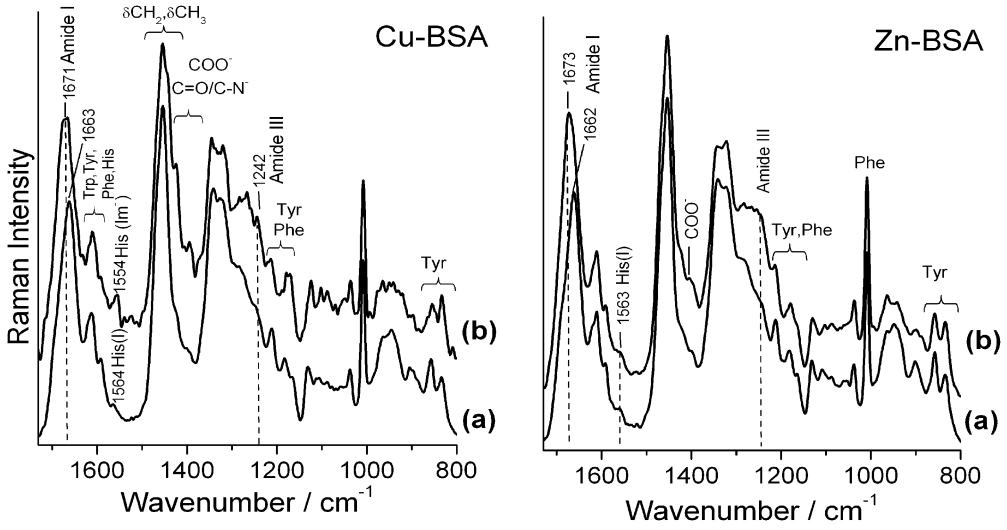


Figure 2. 1730–800 cm^{-1} Raman range of Cu–BSA and Zn–BSA systems, before (a) and after thermal treatment (b). The main band assignments are also indicated.

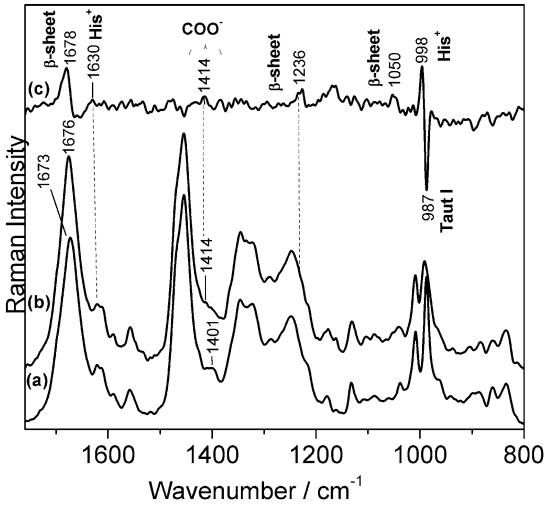


Figure 3. Raman spectra of Cu–BLG system: (a) before thermal treatment; (b) after thermal treatment; (c) difference spectrum obtained by subtracting spectrum (a) from spectrum (b).

higher number of Tyr residues are exposed to the surface of the protein; this can be due to a larger partial unfolding undergone by the protein in the presence of Zn(II).

Also in BLG-containing systems the decrease in the ratio of the tyrosyl doublet at ∼860 and 835 cm^{-1} (I_{860}/I_{835}), occurred, indicating that some of the four Tyr residues of BLG are more strongly hydrogen bonded to a negative acceptor after heating (this ratio decreases when the phenolic hydroxyl oxygen is ionized or strongly hydrogen bonded to a negatively charged acceptor, such as a carboxylate ion in Asp and Glu residues).

In conclusion, in the presence of metal ions the His residues of the two proteins show a different behaviour, enhanced by the thermal treatment: in BLG the change in the His protonation state is the prevalent result (more evident in the presence of Zn^{2+}), whereas in BSA they are strongly involved in metal binding, which is sensitive to the nature of the metal ion (Fig. 1). In particular, in BSA two modes of metal binding mainly take place depending on metal [15]. Since Cu^{2+} is able to bind to main-chain amide nitrogens, an intra-molecular Cu^{2+} coordination can take place, whereas the Zn^{2+} binding to N_τ-imidazole nitrogen can give rise to the formation of inter-molecular His(N_τ)–Zn(II)–His (N_τ) bridges.

3.2. Heat-induced changes in backbone conformations

The amide I band, due to the stretch vibration of the peptide C=O group (1640–1700 cm^{-1}), and the amide III band (1240–1300 cm^{-1}), resulting from coupled Raman active C–N stretching and N–H bending motions, are the most used bands for the secondary structure determination.

Both amide I and amide III bands of the BLG, Cu–BLG and Zn–BLG spectra, being at ∼1672 and ∼1242 cm^{-1}, respectively, indicated that all the systems mainly contain β-sheets. After the heating treatment a significant increase in the β-sheet content (more than 20%) was detected for all samples. In Fig. 3 the Raman spectra of Cu–BLG before and after heating are reported as example. By subtracting the Raman spectrum of the unheated sample from the heated ones, some positive bands marker of β-sheet conformation (i.e. at ∼1235 cm^{-1} and ∼1050 cm^{-1}) were well visible. It is worth pointing out that

the curve fitting analysis of Amide I revealed that the addition of Zn^{2+} ions to native BLG, differently from Cu^{2+} ions, slightly alters the protein conformation just before the thermal treatment [14].

Analogously, the BSA-containing samples, mainly α-helix before the treatment, showed a significant increase in the β-sheet content as a consequence of the thermal treatment (Fig. 2). Indeed, the Amide I band showed a significant shift towards higher wavenumbers (from 1663 to 1672 cm^{-1}), more pronounced in the presence of Zn(II) ions (Fig. 2(B)), and the Amide III band, visible in the typical β-sheets region, strongly increased in intensity (about 20%), reflecting the higher contribution from β-sheets.

3.3. Effects of metal ions on aggregate growth

The time evolution of the aggregation process of the two proteins and their metal-containing samples was followed by dynamic light scattering kinetic studies. As regards BLG, it resulted to assemble into supramolecular structures at neutral pH after about 60–90 min and the mean diameter of the aggregates was about 25 nm after 400 min (Fig. 4(A)). The presence of Cu^{2+} ions during the aggregation process was able to activate, immediately after the incubation and with a higher rate, the formation of a more numerous aggregated species, although the latter had a mean diameter similar to that of those formed in the solution of BLG alone, in agreement with the data reported in the literature [8]. As regards Zn^{2+} ions, their presence led to the formation of larger supramolecular structures with diameter of about 65 nm. The latter result agrees well with Raman data, suggesting that Zn^{2+} ions are more efficient than Cu^{2+} in affecting the re-distribution of charges in the protein and, as a consequence, in promoting protein aggregation.

As regards BSA, only Zn^{2+} ions slightly promoted the heat-induced aggregation of the protein and favoured the formation bigger aggregates (from 50 to 100 nm), as clearly shown by following the kinetics of the mean dimension of molecular species in solution (Fig. 4(B)) [15]. Conversely, the aggregates induced in BSA and Cu-BSA samples followed a similar evolution in the heat-induced aggregation pathway.

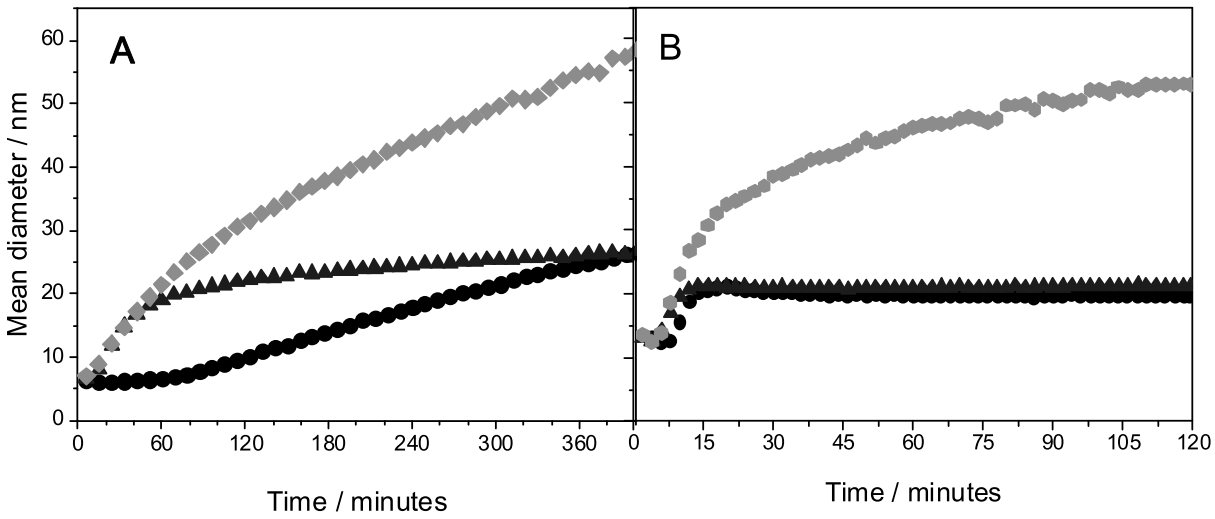


Figure 4. Time evolution of the mean diameter of the particles in solution in (A) BLG and (B) BSA at pH 7 and respectively heated at 60 and 58°C: protein (black circle), Cu–Protein (dark gray triangle), or Zn–Protein systems (gray rhombus).

4. Conclusions

Metal ions can play an important role in unfolding and/or aggregation processes by many molecular mechanisms i.e. by providing a shielding of negative charges of the neighbouring protein molecules that, losing the repulsive forces, can get close enough to interact via non-covalent forces with a low potential energy, or by acting as bridges of adjacent negatively charged groups.

Raman spectroscopy, combined with dynamic light scattering experiments, was very useful in identifying the role played by Cu^{2+} and Zn^{2+} on the aggregation pathways of BLG and BSA, i.e. evidencing the differences in the His protonation state and the metal coordination binding.

Although capable to affect in a different manner the aggregate dimension, both metal ions are able to favour the BLG aggregation, whereas only Zn^{2+} ions have a promoter effect on the thermal aggregation of BSA. The formation of bigger aggregates in BLG solutions in the presence of Zn^{2+} is probably due to the capability of Zn^{2+} ions to slightly modify the BLG native secondary structure and to induce a more relevant redistribution of charges; this gives rise to a different screening of electrostatic interactions between charged BLG molecules.

Conversely, in the case of BSA, Cu^{2+} and Zn^{2+} ions play a different role in the evolution of the aggregation process of this protein and of consequence they can differently affect the mean dimension of the molecular species formed in solution since the two modes of metal binding takes place depending on metal. Zn^{2+} ions promote the aggregate growth and aggregates of greater mean dimension (about 50 and 100 nm), since they can create inter-molecular bridges, whereas Cu^{2+} ions do not affect the evolution of BSA aggregation process since it is coordinated intra-molecularly, giving rise to little oligomers (about 20 nm) such as those formed in their absence.

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