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Metal ions modulate thermal aggregation of beta-lactoglobulin: A joint chemical and physical characterization

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ABSTRACT

Molecular basis of the role played by Cu^{2+} and Zn^{2+} ions during the thermal aggregation processes of beta-lactoglobulin (BLG) was studied by using a joint application of different techniques. In particular, Raman spectroscopy was very useful in identifying the different effects caused by the two metals at molecular level (i.e. changes in His protonation state, disulfides bridge conformation, and micro-environment of aromatic residues), evidencing the primary importance of the protein charge distribution during the aggregation process. Both metal ions are able to act on this factor and favor the protein aggregation, but Zn^{2+} is able to alter the natural conformational state of BLG, causing a slight unfolding, whereas Cu^{2+} ions play a role only during the thermal treatment. Thus, Zn^{2+} ions favor the formation of bigger aggregates and branched fibril-like structures, whereas for Cu^{2+} ions a greater number of cross-beta structures during thermal incubation and finally, fibrillar structures. The aggregation process occurs in two phases, as suggested by the measurements on the time evolution of the BLG aggregates: the first one is characterized by a partial unfolding of the protein and aggregate growth, forming oligomers and protofibrils, whereas the second one is characterized by further supramolecular assembly, leading to the formation of fibrils.

1. Introduction

A large number of proteins may form under appropriate conditions a variety of aggregated structures whose morphology resembles those of fibrils that can be accumulated in biological environments under pathological conditions [1–4]. The fibril formation seems to be driven by an appropriate destabilization of the native state [1,5], and typical of the early phases of fibril formation is the conversion of α-helices into β-sheets. However, also proteins containing a large fraction of β-sheets can be transformed in vitro into fibrillar structures through a process generally involving destabilization and consequent auto-assembly of partially unfolded intermediates. Fibrils are the final state of aggregated and re-organized protofibrils. The formation of amorphous aggregates, fibrils or gels may be initiated by thermal or chemically induced protein unfolding [6,7].

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Bovine β-lactoglobulin (BLG), a small globular protein of bovine milk, is made of 162 residues forming two antiparallel β-sheets [8]. Its known three-dimensional structure (Fig. 1) contains one free thiol group and it is stabilized by two disulfide bonds, Cys66–Cys160 and Cys106–Cys119, of which the first one generates a conformational restraint that is reported to inhibit the formation of misfolded aggregation of BLG [9].

BLG is one of the most popular model proteins in the study of protein folding and conformation in vitro. Refolding of denaturated BLG proceeds through a non-native α-helical intermediate, making this protein a useful tool to investigate α–β transitions, important for example in the conformational transition of prion proteins. In addition, this protein can form either amorphous aggregates or amyloid fibrils upon changing the working parameter [10]. BLG, being the major whey protein in milk, is also important in food technological applications where the control of the protein denaturation/aggregation during heating is of outstanding importance for the acceptance of the final quality of the products and for avoiding allergenic problems [11]. Thus, we have selected BLG because of its double interest: it is a model beta-protein in aggregation processes and a thermal marker in industrial processes involved in milk treatment.

Although the mechanism of BLG heat-aggregation has been extensively studied, it is not still completely understood and controlled [7,12–14]. As a consequence of BLG aggregation, either amorphous aggregates or amyloid fibrils can be formed, depending on the experimental conditions [12,15].

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Metal ions can play a role in unfolding and/or aggregation processes thanks to their ability in acting as bridges of adjacent negatively charged groups, as well as by providing a shielding of negative charges of the neighboring protein molecules that, losing the repulsive forces, can get close enough to interact via non-covalent forces with a low potential energy [12,16,17]. The study of the interactions between metal ions and proteins is of increasing interest both in food processing, because of the capability of metal ions to induce cold gelation or aggregates [18,19], and in biomedical sciences since the presence of metal ions, together with the amyloid fibril formation, is one of the fundamental aspects in the etiology of different neurodegenerative pathologies (amyloidosis) [20–22]. Cu^{2+} and Zn^{2+} ions have a different physiological role, and have been observed to promote aggregation in amyloid fibrils and/or amorphous aggregates. Moreover, they are important essential trace elements for human nutrition.

To get deep insight into the molecular basis of the fibril formation, it is interesting to know the effect of both metal ions on BLG aggregation, since the propensity of a protein to aggregate is correlated to many physico-chemical parameters, some of which can be affected by the metal/protein ratio and the metal ion binding mode. Here, we describe how Cu^{2+} or Zn^{2+} ions modulate the changes in the BLG structure caused by thermal treatment. A joint combination of techniques was used 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 A) was purchased from Sigma-Aldrich (A0281). The protein was dissolved in a 20 mM MES (4-morpholineethanesulfonic acid) buffer solution prepared in H_2O or D_2O (99.9%, Aldrich) and titrated with KOH or KOD until pH or pD 7 (where pD is $pH_{meter\ reading} + 0.4$). The protein solution (3.3 mM) was centrifuged for 8 min and filtered with Sartorius filters (pore diameter 0.20 μm). The samples, freshly prepared in D_2O, were divided in two aliquots for IR and DLS measurements, in order to characterize the heating phase (at 60 °C) of the native protein solution. D_2O solutions were used to avoid the IR spectral overlaps between the Amide I band and the strong water absorption at 1650 cm^{-1}. $CuCl_2$ or $ZnCl_2$ (99.99%, Sigma-Aldrich) was prepared in Super Q Millipore water and added to freshly prepared BLG solutions, in order to obtain a 1 mM final concentration. 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.

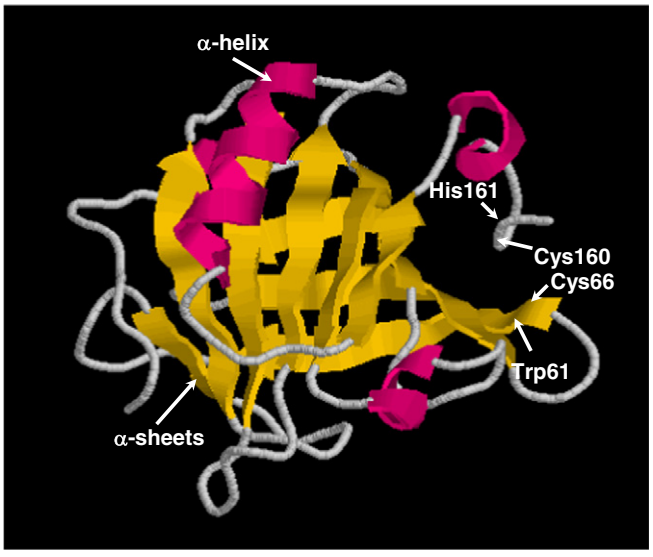

Fig. 1. Three-dimensional structure of BLG A that shows the relative positions of four amino acid residues reasonably involved in the BLG aggregation process (Cys66–Cys160, His 161, and Trp61). The diagram was drawn from PDB file 1BSQ using RASMOL Ver.2.7.

2.2. Raman spectroscopy

Raman spectra were obtained on lyophilised samples, before and after heating of the protein aqueous solution at 60 °C for 400 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. Lyophilization was performed on a 4 K freeze dryer module 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. In order to fit the vibrational bands, some parameters should be fixed or constrained within reasonable limits (number and position of all components). To obtain information regarding the number and position of the component peaks, smoothed fourth derivatives with a thirteen-point Savitsky–Golay function, whose resolution is superior with respect to the second-derivative spectra, were used. The S–S and secondary conformations, as well as the His tautomers, were estimated from the relative area of the individual components assigned to specific structural entities. The vibrational assignments of the band components were carried out on the basis of the literature. As regards the disulfide bridges, the components at about 505, 520 and 540 cm^{-1} were assigned to the three basic conformations of S–S bonds: gauche–gauche–gauche (*ggg*), gauche–gauche–trans (*ggt*) and trans–gauche–trans (*tgt*), respectively [23,24]. As regards the secondary structure, the α-helix content of BLG was calculated from the area of the component in the 1652–1657 cm^{-1} range, whereas the contribution from unordered conformations was reflected by the 1661–1663 cm^{-1} peak [25]. β-Turns mainly gave rise to the bands in the 1639–1645 and 1683–1695 cm^{-1} regions [26], whereas the components in the 1672–1678 cm^{-1} range were attributed to β-sheet conformation [24,27].

An important assumption implicit in such an approach is that the effective intrinsic intensities of the bands corresponding to different conformations or side-chains are very similar. As a consequence, the curve fitting procedure cannot give the absolute content of a structure. However, the curve fitting analysis may be of value in probing the nature and the extent of structural changes in proteins induced by external factors interacting with protein molecules, such as free radical attack or heating treatment [17,28].

2.3. IR spectroscopy

IR spectra were recorded by a Bruker Vertex 70 spectrometer, equipped with a mid-IR globar light source (a U-shaped silicon carbide piece). The spectral resolution was 2 cm^{-1} and the total number of scans for each spectrum was 100. All samples were placed between two CaF_2 windows, with a 0.05 mm Teflon spacer. The error on the wavenumber estimation was ±1 cm^{-1} and the absorbance accuracy was within 1%.

By using D_2O in the preparation of protein solutions, it was possible to monitor the H–D exchanges by means of the Amide II and the Amide II′ bands. Amide II (or II′) band (1400–1580 cm^{-1}) is predominantly associated with the N–H (or N–D) in-plane bending of the peptidic groups [17,29,30]. When H–D exchange occurs, a simultaneous Amide II′ increase and Amide II decrease are observed. In order to identify the evolution time of each spectral component under the broad amide bands, difference spectra were obtained by subtracting the