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ORIGINAL PAPER

Thermally induced denaturation and aggregation of BLG-A: effect of the Cu^{2+} and Zn^{2+} metal ions

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Abstract There is growing evidence that metal ions can accelerate the aggregation process of several proteins. This process, associated with several neuro-degenerative diseases, has been reported also for non-pathological proteins. In the present work, the effects of copper and zinc ions on the denaturation and aggregation processes of β-lactoglobulin A (BLG-A) are investigated by differential scanning calorimetry (DSC), fluorescence, electron paramagnetic resonance (EPR) and optical density. The DSC profiles reveal that the thermal behaviour of BLG-A is a complex process, strongly dependent on the protein concentration. For concentrations $\leq$0.13 mM, the thermogram shows an endothermic peak at 84.3°C, corresponding to denaturation; for concentrations >0.13 mM an exothermic peak also appears, above 90°C, related to the aggregation of the denaturated BLG-A molecules. The thioflavin T fluorescence indicates that the thermally induced aggregates show fibrillar features. The presence of either equimolar Cu^{2+} or Zn^{2+} ions in the protein solution has different effects. In particular, copper binds to the protein in the native state, as evidenced by EPR experiments, and destabilizes BLG-A by decreasing the denaturation temperature by about 10°C, whereas zinc ions probably perturb the partially denaturated state of the protein. The kinetics of BLG-A aggregation shows that both metal ions abolish the lag phase before the aggregation starts. Moreover, the rate of the process is 4.6-fold higher in the presence of copper, whereas the effect of zinc is negligible. The increase of the aggregation rate, induced by copper, may be due to a site-specific binding of the metal ion on the protein.

Keywords β-Lactoglobulin · Thermal denaturation · Aggregation · Copper · Zinc

Abbreviations

BLG-A	β-Lactoglobulin A
DSC	Differential scanning calorimetry
EPR	Electron paramagnetic resonance
PBS	Phosphate buffer solution
ThT	Thioflavin T
Trp	Tryptophan

Introduction

Protein aggregation has been the subject of several biophysical and biochemical researches since it is related to the pathogenesis of many neurodegenerative (such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and prion diseases), cardiovascular and tumoural diseases (Ross and Poirier 2004; Frokjaer and Otzen 2005). In the aggregated state, the proteins inactive are abounding in anti-parallel β-sheets and form amorphous aggregates or regular fibrils (Selkoe 2003).

Recently, it has been evidenced that aggregate formation is not specific of some amino acidic sequences, but it is a

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general property of the polypeptide chains (Dobson 1999; Selkoe 2003; Uversky and Fink 2004). In fact, under suitable conditions, even proteins not involved in pathological states, such as SH domain in PI3 kinase, myoglobin and C_{552} cytochrome can form fibrillar aggregates with features similar to those found in the pathological states (Uversky and Fink 2004).

Aggregation is a complex process not yet completely understood: its activation requires that the interacting protein molecules are in a partially or totally unfolded conformation. Such a state can be experimentally induced in vitro by variation of a great number of chemico-physical parameters such as temperature, pressure, protein concentration, ionic strength and pH value of the dispersion media (Militello et al. 2004; Uversky and Fink 2004). In particular, experimental results point out that the change of some of these parameters would lead to the partial solvent exposure of apolar amino acids, which in the native state are buried in the protein matrix, and would give rise to new protein–protein interactions, that cannot take place when the protein molecules are in their native state (Zhang and Liu 2003; Fedurkina et al. 2006). Moreover, shift of the equilibrium between the correctly folded molecules and the partially unfolded ones, genetic mutations of the primary protein structure, and reduced protein stability are factors also involved in triggering protein aggregation in vitro. Recent reports highlight that the hydrophobicity, the net charge and α or β structure propensity influence the rate of aggregation of non-native proteins (Chiti et al. 2003).

It has been observed that the senile plaques, typical of the Alzheimer's disease, contain a great amount of metal ions such as Cu^{2+}, Fe^{3+} and Zn^{2+}. So far, their role is not very well clarified. The formation of amyloid fibrils from the β-amyloid peptide, which is the main constituent of amyloid plaques in the brain of Alzheimer's disease patients, becames faster in the presence of copper and zinc ions (Mantyh et al. 1993; Bush et al. 1994). A similar behaviour has been noticed in α-synuclein, a protein involved in Parkinson's disease, in the prion protein entailed in Creutzfeld-Jacob disease and in β_2-microglobulin (Hashimoto et al. 1999; Morgan et al. 2001), as well as in proteins not involved in pathological states, e.g. acylphosphatase (AcP) (Capanni et al. 2004). Therefore, the metal ions could play a role on the unfolding and/or aggregation processes, as already reported (Suzuki et al. 2001; Capanni et al. 2004). It is therefore of interest to study the aggregation process under well defined experimental conditions, aiming to correlate the observed effects with the variation of the chemico-physical parameters.

In this work we focus on the heat- and concentration-induced denaturation and aggregation mechanisms of β-lactoglobulin A (BLG-A) at pH 6.2, and on the influence of copper and zinc metal ions on both processes.

β-Lactoglobulin A is a small globular protein (MW = 18,300 Da) that is the major component of the whey proteins in the milk of ruminants and many other mammals (Kontopidis et al. 2004). Although it is known that it can bind in vitro to a variety of hydrophobic substrates, mainly retinol and long-chain fatty acids, its physiological function is still unknown. At neutral pH, it exists in a native conformation constituted by a monomer $\Leftrightarrow$ dimer equilibrium. BLG-A has a core structural pattern formed by one α-helix and eight strands forming antiparallel β-sheets and its tertiary structure is stabilized by two disulfide bonds. Moreover, there are also one free, highly reactive –SH group of Cys-121, buried in the hydrophobic core of the protein, and two tryptophan (Trp) residues at 19 and 61 position (Brownlow et al. 1997).

Several studies report on the heat-induced aggregation of BLG-A (Capron et al. 1999; Hamada and Dobson 2002; Fitzsimons et al. 2007). The data show that the aggregation of BLG-A strongly depends on the experimental conditions. In particular, this protein can form either amorphous aggregates or amyloid fibrils upon changing the working parameters (Carrotta et al. 2001; Bromley et al. 2005). Thus, it is of interest to analyse the metal ions effects on both denaturation and aggregation processes.

In the present work we investigate, by means of differential scanning calorimetry (DSC), intrinsic Trp and extrinsic thioflavin T (ThT) fluorescence, electron paramagnetic resonance (EPR) and optical density measurements, the denaturation and the aggregation mechanisms induced in BLG-A by temperature and protein concentration. The influence of the addition of equimolar Cu^{2+} and Zn^{2+} metal ions on both processes are also studied.

The results evidence that BLG-A, under particular thermal conditions, forms amyloid fibrils. Cu^{2+} ions, compared to Zn^{2+}, destabilize the native conformational state of the protein and speed up the aggregation process.

Materials and methods

Materials

Genetic variant A of β-lactoglobulin (product type L-7880, lot no. 026K7000) and thioflavin T (T-3516) were purchased from Sigma, while the salts of reagent grade $CuCl_2$ and $ZnCl_2$ dihydrate and those for the 100 mM phosphate buffer solution (PBS) at pH 6.2 were from Merck. All materials were used as purchased with no further purification. Distilled water was used throughout.

Sample preparation

All the samples used in each experiment were aqueous protein solution prepared in PBS (100 mM, pH = 6.2). The protein concentration was determined by spectrophotometric measurements, using ε = 17,600 M^{-1} cm^{-1} at λ = 280 nm. For the experiments in the presence of copper and zinc, the ions were dissolved in the buffer solution and the protein was added to the aqueous-metal solutions in 1:1 molar ratio. Each sample was freshly prepared and filtered with Sartorius 17597-K MINISART 0.2 μm filters before the measurements, to avoid aggregation due to water-suspended particles.

Differential scanning calorimetry

Differential scanning calorimetry experiments were performed on a VP-DSC MicroCalorimeter (MicroCal, Inc.), with cell volumes of 0.52 ml. The temperature resolution is ΔT = ±0.1°C. Protein samples were extensively degassed before measurements. The scans ran from 20 to 120°C at the scan rate of 90°C h^{-1}. The experiments were performed at protein concentration ranging from 0.08 to 1.00 mM.

To obtain a reproducible baseline, at least four buffer vs. buffer scans were performed. After the reference measurements, the sample cell was emptied, reloaded with the protein solution and equilibrated for 50 min at the starting temperature. The reversibility of the transition was checked by a second scan of a previously scanned sample, cooled down to 20°C in about 30 min. In addition, the same procedure was applied to a sample for which the temperature of the first scan was interrupted at the end of the endothermic peak. All the apparent heat capacity (Cp) curves were obtained from the calorimetric profiles, corrected by baseline subtraction and normalized with respect to the protein concentration. The data were analysed by using the Origin software (Microcal Software, Inc., USA).

Fluorescence spectroscopy

Fluorescence spectra were obtained with a Perkin-Elmer LS 50B spectrofluorimeter (accuracy $\Delta\lambda$ = ±0.5 nm) equipped with a Peltier Temperature Programmer PTP-1 (accuracy ΔT = ±0.5°C). Protein samples, at concentration of 0.02 mM, were monitored for both Trp and ThT fluorescence. The excitation wavelength for the Trp fluorescence was 280 nm and the emission spectra were recorded between 290 and 430 nm. Fluorescence measurements vs. temperature were performed by scanning the temperature from 20 to 86°C at the scan rate of 60°C h^{-1}. Temperature was measured directly by an YSI thermistor dipped into the cuvette.

The ThT fluorescence was measured in the range 460–600 nm using an excitation wavelength of 442 nm. In the presence of ThT the following sample preparation procedure has been adopted: after an incubation treatment at 80°C of different time length, ranging from 1 to 3 h, protein samples were mixed with ThT to obtain a final concentration of the dye of 0.05 mM. The spectra were then recorded at RT.

All the emission spectra were recorded at the rate of 100 nm min^{-1}.

EPR spectroscopy

The EPR experiments on BLG-A (protein concentration 5 mM) in the presence of equimolar copper were carried out with a Bruker ESP 300 X band spectrometer, equipped with the ESP 1600 data acquisition system. The experimental conditions were as follows: 100 kHz magnetic field modulation, 10 mW microwave power and 5 G peak-to-peak magnetic field modulation amplitude. The Cu^{2+} EPR spectra were recorded at −196°C; for the temperature dependent studies, the protein samples in the presence of copper were first incubated for about 5 min at 70°C and then rapidly frozen by plunging the EPR tube into a finger dewar containing liquid nitrogen, inserted into the TE_{102} (ER4201, Bruker) standard rectangular cavity.

Turbidity measurements

Optical thermal profiles of buffered aqueous solution of 0.13 mM BLG-A with or without equimolar metal ions were acquired with a JASCO 7850 spectrophotometer equipped with a Peltier thermostated cell, model TPU-436 (accuracy ΔT = ±0.5°C) and an EHC-441 temperature programmer. Quartz cuvettes with 1-cm optical path were used throughout.

The changes in the turbidity of the solution resulting from protein aggregation were recorded vs. temperature or time (at fixed temperature), measuring the optical density at 400 nm (OD_{400}), at which the native protein did not show any absorption. Data analysis on the aggregation kinetics was performed using the program Origin.

Results and discussion

Thermal behaviour of BLG-A

DSC experiments

It is known that the aggregation process is favoured when the interacting proteins are in a partial or total unfolded conformation. This state can be induced in different ways

(Clark 1998; Fink 1998; Holm et al. 2007). In the present study we use the temperature as perturbing agent of the protein native state.

Figure 1a (solid line) shows the calorimetric profile of a BLG-A in PBS, recorded at 90°C h^{-1} in the 20–120°C temperature range at 0.13 mM concentration. Similarly to samples at lower protein concentration (data not shown), a large endothermic peak is observed with maximum heat absorption at T_{max} = 84.3°C, defined as the transition temperature from the native to the denaturated state. The rescan of the sample (Fig. 1a, dotted line) does not show any absorption peak, indicating that the thermal unfolding of the protein is irreversible. In general, different processes, such as deamidation of asparagine/glutamine residues and reaction of the molecular oxygen dissolved in solution with cysteines, as well as protein aggregation, can be considered responsible of the irreversibility of the thermal denaturation (Zale and Klibanov 1986; Jacob et al. 2004).

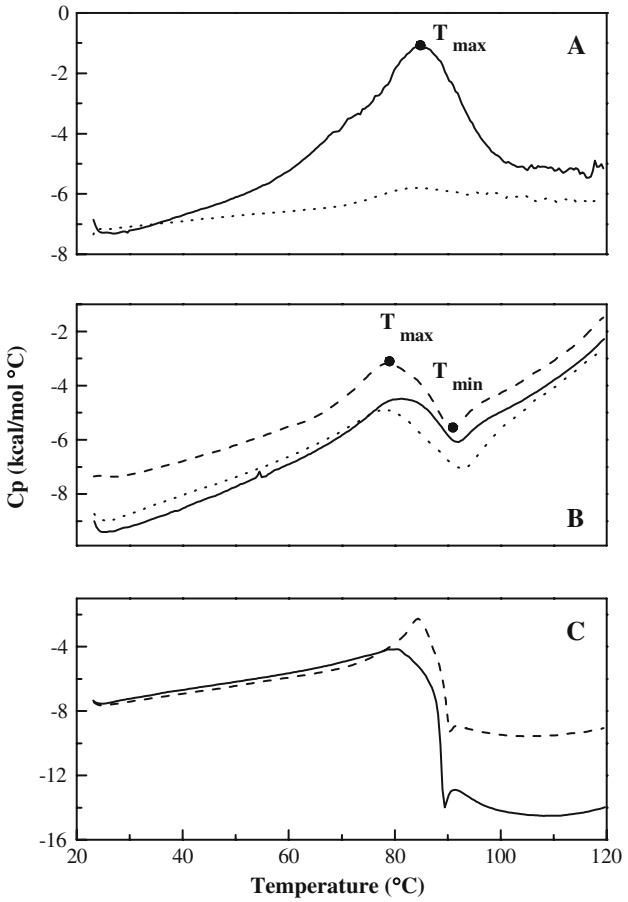

Fig. 1 Differential scanning calorimetry scans (scan rate: 90°C h^{-1}) showing the apparent heat capacity curves of BLG-A in PBS (100 mM, pH = 6.2), at different concentrations (mM): **a** (*solid line*) 0.13; *dotted line* indicates the rescan of the protein sample; **b** (*solid line*) 0.14, (*dashed line*) 0.16 and (*dotted line*) 0.26; **c** (*solid line*) 0.52 and (*dashed line*) 1.00. *Dots* correspond to the maxima and minima of the apparent heat capacity. Errors are within the symbol size

One experimental parameter that plays an important role on the denaturation and aggregation mechanisms is the protein concentration (Qi et al. 1995; De la Fuente et al. 2002; Fitzsimons et al. 2007). In Fig. 1b, the calorimetric profiles of BLG-A at different concentrations are reported. The DSC curves show a strong dependence of the apparent heat capacity on the BLG-A concentration: at concentrations higher than 0.13 mM the broad endothermic peak at T_{max} is followed by an exothermic one at higher temperature, T_{min}.

The presence of two well distinct peaks in the thermograms suggests that, in contrast to what is observed for protein concentration $\leq$0.13 mM, at these concentrations the denaturation and the aggregation processes are sequential (Berkowitz et al. 1980; Dzwolak et al. 2003). In addition, the results indicate that both processes are influenced by the protein concentration. In fact, as regards the denaturation process, a progressive down shift of T_{max} from 84.3 to 78.0°C is registered by increasing the concentration from 0.13 to 0.26 mM (Fig. 1a, b), indicating a thermal destabilization of BLG-A. Furthermore, comparing the results reported in Fig. 1a, b, it can be stated that 0.13 mM is a threshold concentration, above which BLG-A aggregation is evidenced in the DSC scan. This phenomenon was found also in other proteins (Finke et al. 2000; Dzwolak et al. 2003; Wang and Kurganov 2003), and was explained with a nucleation model, i.e. the formation of a stable nucleus that, as soon as a critical size is achieved, incorporates additional monomeric protein units into a growing aggregate (Harper and Lansbury 1997). Nucleation-initiated aggregation dramatically occurs immediately above the critical concentration, i.e. the concentration at which a substantial number of nuclei have formed. By increasing the concentration of BLG-A from 0.14 to 0.26 mM (Fig. 1b), besides a decrease of T_{max}, both modification of the exothermic peak shape and progressive increase of $\Delta T = T_{min} - T_{max}$ (i.e. the difference between the temperature corresponding to the two calorimetric peaks) are observed. Such an effect has also been evidenced on insulin (Dzwolak et al. 2004). Thus, in the examined range, the concentration increment shifts the aggregation process to slightly higher temperatures even if the denaturation is favoured. In addition, as the scan rate is equal for all the measurements, time elapsing between the denaturation and aggregation processes increases with the protein concentration. The whole thermal process is irreversible even in this case, as observed by the rescan of the sample (data not shown).

By further increasing the protein concentration to 0.52 mM and then to 1 mM (Fig. 1c), it can be noted that T_{max} reverses its trend, restoring the initial value of about 84°C. This result could be explained by assuming a higher protein dimers concentration in solution. Moreover, for these protein concentrations, the aggregation process immediately

follows denaturation. This result is in agreement with the nucleation-growth mechanism (Dzwolak et al. 2004). On the other hand, one can expect that increasing the protein concentration will favour the formation of a stable nucleus and, consequently, will decrease the duration of the lag period between the denaturation and aggregation processes.

On the overall, the DSC results describe how the unfolding and aggregation processes of BLG-A depend both on temperature and concentration. In particular, a threshold concentration of 0.13 mM was identified, for which the denaturation temperature is 84.3°C. Accordingly, to trigger the protein aggregation, in the following the measurements were performed under conditions close to denaturation, with an incubation temperature of 80°C and a protein concentration of 0.13 mM, or lower.

Fluorescence

In BLG-A there are two Trp residues: Trp-61 is completely exposed to the solvent, while Trp-19 is located in a hydrophobic cavity in the protein core (Brownlow et al. 1997). In Fig. 2 the Trp fluorescence spectra of BLG-A recorded at different temperatures are reported. By increasing the temperature from RT to 86°C, it is observed a red-shift of the maximum emission wavelength, λ_{max}, and a fluorescence intensity reduction. In fact, the λ_{max} value passes from 335.5 nm, in the spectrum recorded at RT, to 349.5 nm for the curve at 86°C: this red-shift is attributable to an increased solvent exposure of the Trp in the denaturated state. The reduction of the fluorescence intensity, indeed, can be due to a fluorescence quenching from both water molecules and charged amino acid residues close to the Trp residues.

To highlight the presence of aggregates in solution and, in particular, of amyloid fibrillar structures, we have also measured the ThT fluorescence in the presence of BLG-A after different incubation times. For comparison the BLG-A and ThT alone have been also measured and do not show fluorescence in the 460–600 nm range (data not shown).

Figure 3 shows the intensity of the emission at λ_{max} of the BLG-A in presence of ThT measured for the various samples. The fluorescence of native BLG-A containing ThT shows a weak fluorescence signal at λ_{max} = 494 nm (Fig. 3, time = 0 min). To induce the aggregation process, the protein solution has been incubated at 80°C for different time intervals and the spectra were then recorded at RT. After 60 min of incubation of the protein at 80°C followed by addition of ThT, we observe an emission signal at λ_{max} = 496.5 nm with an increased intensity with respect to the previous one. By increasing the incubation time to 120 min, a strong increment of the emission intensity with respect to the native BLG-A is observed. A further increases of the emission of ThT is registered after 180 min of incubation, while λ_{max} is almost unchanged.

For the samples incubated 120 and 180 min, the RT fluorescence has also been measured at different times (Fig. 3). The results indicate that the emission intensity of the fluorescence spectra, recorded at RT, increases with time. In all cases, the fluorescence progressively increases until a saturation value is reached.

In particular, the fluorescence of the dye in the protein sample incubated 180 min at 80°C was measured at 25°C for the following 120 min, showing that the aggregation process slowly continues with time. This result suggests that, under these experimental conditions, the BLG-A aggregation is an irreversible process.

The ThT dye is widely used to evidence the presence in solution of molecular aggregates with fibrillar structures

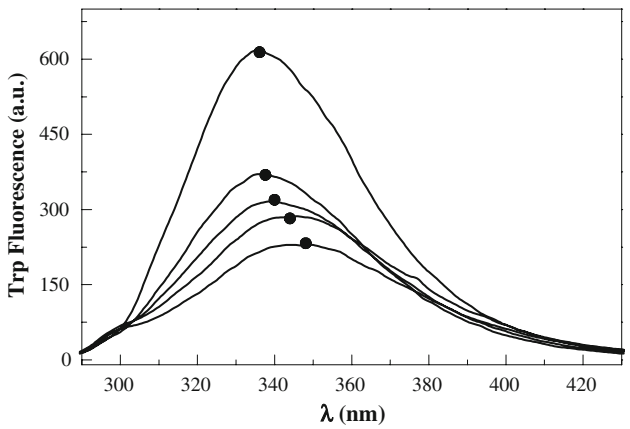

Fig. 2 Tryptophan fluorescence spectra of BLG-A in PBS (100 mM, pH = 6.2) at λ_{exc} = 280 nm for different temperatures (°C); from *top* to *bottom*: RT, 50, 70, 80 and 86. *Dots* correspond to the maximum emission. Errors are within the symbol size

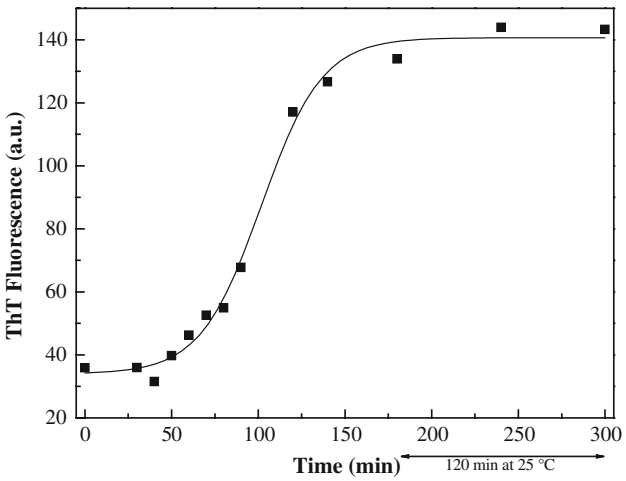

Fig. 3 Changes in ThT fluorescence intensity at λ_{max} as a function of the incubation time of BLG-A in PBS (100 mM, pH = 6.2) at 80°C. The spectra are recorded at RT, λ_{exc} = 442 nm. Errors are within the symbol size

(LeVine 1999). Binding with such structures leads both to an increment of the fluorescence intensity of ThT and to a red-shift of the emission wavelength (Rezaei et al. 2002). Therefore, the ThT fluorescence suggests the presence of BLG-A fibrillar aggregates in solution: their concentration increases with the incubation time. To determine the kinetic parameters describing the aggregation process at 80°C, the experimental data have been fitted with a sigmoidal Boltzmann curve (solid line in Fig. 3). Such a fit allows us to obtain the rate constant of approach to the final state, $k = 0.057 \pm 0.006$ min^{-1}, and the half-time of aggregation, $t_0 = 102 \pm 2$ min. The latter value agrees very well with that obtained by Carrotta et al. (2001), $t_0 = 105$ min, by incubating at 67.5°C a solution of BLG-A at 1.1 mM concentration. At this concentration the protein is in a non-native state, below the denaturation temperature, as it can be also seen from our experiments in Fig. 1c.

One of the mechanisms proposed to explain the thermally induced aggregation of BLG-A is that, upon heating to approximately 80°C, BLG-A dimers dissociate into monomers. The thiol group and hydrophobic residues become solvent accessible. Subsequently, aggregates are formed via intermolecular thiol-disulphide exchange and, to a lesser extent, thiol–thiol oxidation (McSwiney et al. 1994; Hoffmann and van Mil 1997). The sigmoid behaviour of the fluorescence results in Fig. 3 are consistent with a two step cooperative process in which the dimer-to-monomer dissociation in BLG-A is followed by aggregation.

Metal ions effects on BLG-A

DSC experiments

Copper and zinc are essential elements for life, widely distributed in plants and animals. Their presence, especially at high concentration, is believed to trigger or promote protein aggregation.

The effect of equimolar concentration of either Cu^{2+} or Zn^{2+} on the thermal denaturation of BLG-A, at the concentration of 0.13 mM, is shown in Fig. 4.

In the presence of Zn^{2+}, the calorimetric profile of BLG-A (Fig. 4, dotted line) shows an endothermic peak at higher temperature ($T_{max} = 87.2$°C) and, moreover, an exothermic peak at 98°C appears. Exothermic peaks are usually related to aggregation processes (Dzwolak et al. 2003); therefore, this result suggests that Zn^{2+} affects the denaturation and promotes the aggregation of BLG-A.

In particular, the increase of T_{max} could be explained by assuming a metal binding to the partially denaturated state, which favours the formation of intermolecular links and can be considered as the starting point of the aggregation process. A similar mechanism has also been proposed to interpret the zinc interaction with several β-amyloid peptides (Stellato et al. 2006).

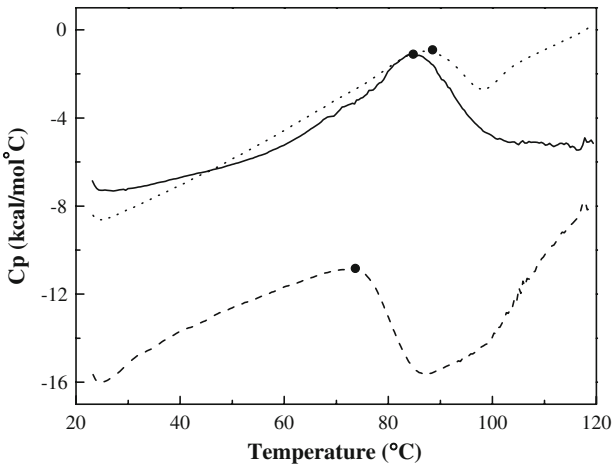

Fig. 4 Differential scanning calorimetry thermogram of BLG-A (*solid line*) and of BLG-A in presence of (*dotted line*) Zn^{2+} and (*dashed line*) Cu^{2+}. The protein concentration is 0.13 mM and the protein/metal ions molar ratio is 1:1. *Dots* correspond to the maxima of the endothermic peaks and errors are within the symbol size

A different behaviour is observed when Cu^{2+} is added to the protein solution (Fig. 4, dashed line). The DSC scan shows a dramatic reduction of the protein stability. In fact, the denaturation temperature decreases of about 10°C ($T_{max} = 73.6$°C) and, even in this case, an exothermic peak appears. The reduction of T_{max} may be also ascribed, at least in part, to the overlap of the endothermic peak with the exothermic one due to aggregation, causing a possible cancellation of negative and positive heat flow. Destabilization in the presence of Cu^{2+} is also evident in the denaturation curves of β_2–microglobulin (Villanueva et al. 2004). The result for BLG-A could be due to the fact that Cu^{2+} is preferentially bound to the protein native state. Binding could occur by means of the two His (His-146 and His-161) of BLG-A, similarly to what is observed in β_2–microglobulin (Verdone et al. 2002). In fact, both proteins possess two histidine residues exposed to the solvent. Moreover, in the presence of metal ions in solution, a negative apparent ΔCp value of about -7 kcal mol^{-1} °C^{-1} is estimated as the difference between the heat capacity of the protein at 20°C and the completely aggregated one at 120°C. This value must be interpreted with caution since it includes both the difference in amino acid hydration between the aggregated and the native protein and, probably, a macroscopic effect related to precipitation of the aggregates and global reduction in solvent-solute interface (Dzwolak et al. 2003, 2004). It is interesting to note that the starting of the apparent Cp in the DSC thermogram shifts to more negative values in the presence of Cu^{2+}. This result is a further indication of the interaction of the metal ion with the native state of the protein. A similar thermal response

has been observed in the DSC profile of β_2-microglobulin, under conditions where amyloid fibrils are formed (Sasahara et al. 2006, 2007).

EPR experiments

The EPR spectrum of Cu^{2+} in interaction with native BLG-A is shown in Fig. 5, line a. In the parallel region of the spectrum, at low magnetic field, three hyperfine lines are observed: the fourth is hidden by a large resonance line in the perpendicular region. The EPR parameters, $g_{\|} = 2.450$ and $A_{\|} = 159.06$ G, are typical of type-2 copper species and indicate a distorted tetragonal coordination. This magnetic signal clearly evidences the binding of Cu^{2+} to the protein in the native state. In fact, it is significantly different from the EPR spectrum of a frozen aqueous solution of hydrated copper (Peisach and Blumberg 1974).

The EPR spectrum of Cu^{2+}-BLG-A does not display any significant change (Fig. 5, line b) after the protein sample is incubated for 5 min at 70°C, a temperature slightly lower than the denaturation temperature of BLG-A in the presence of copper ions, according to the DSC measurements (see Fig. 4). This result suggests a negligible effect of the temperature on the geometry and coordination atoms of the copper sites of the protein.

Since Zn^{2+} ion is diamagnetic, no magnetic information can be obtained for this sample.

In conclusion, the DSC and EPR results indicate that the effects observed with the two different metals agree with data reported by Stellato et al. (2006) on Cu^{2+} and Zn^{2+} interaction with several β-amyloid fragments investigated by X-ray absorption technique. In contrast, in α-synuclein Cu^{2+} promotes nucleation of fibrillar aggregates, even if it has no effect on the structural features inherent to the spontaneous aggregation of the protein, i.e. copper-induced fibrils have the same morphology as those formed in the absence of the metal ions (Rasia et al. 2005).

Turbidity experiments

Fluorescence spectroscopy does not allow an effective analysis of the copper and zinc effects on the aggregation process of BLG-A, since transition metal ions are fluorescence quenchers (Lakowicz 1983). Such an analysis can be carried out by means of optical absorption spectroscopy to investigate the protein-metal interactions and the changes in the aggregation kinetics.

Information on the associative behaviour of BLG-A with the temperature can be obtained by performing turbidity measurements at 400 nm on the protein without and with metal ions, at the same protein concentration used in the DSC experiments, 0.13 mM (Fig. 6).

The plot in Fig. 6 (solid line) shows that for BLG-A in PBS the optical density at $\lambda = 400$ nm (OD_{400}) is constant at low temperatures and abruptly increases after 85°C. Such an increase suggests the starting of formation of protein aggregates in solution. This temperature shifts downwards to 79°C, when zinc (dotted line) is present in the protein solution. In the presence of copper (dashed line), there is a progressive increase of OD_{400} starting at about 70°C that becomes steeper at 80°C.

The kinetics of the thermal aggregation of BLG-A was measured by following the OD_{400} at 80°C as a function of the time: the results are reported in Fig. 7. The typical behaviour of kinetic curves can be observed (Kurganov 2002; Fedurkina et al. 2006), consisting in an increment

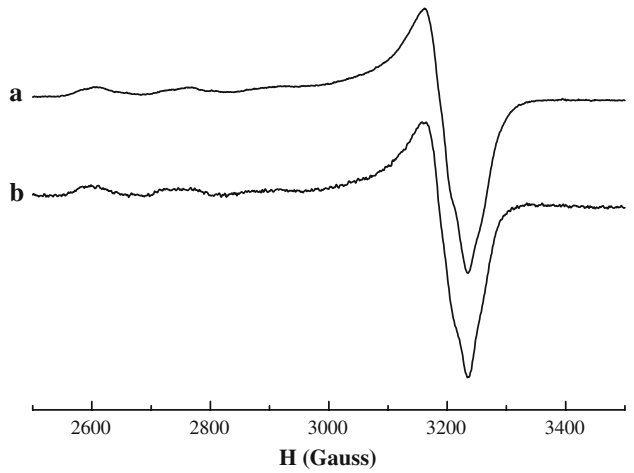

Fig. 5 Electron paramagnetic resonance spectra at −196°C of Cu^{2+} interacting with BLG-A in **a** the native state and **b** after incubation for 5 min at 70°C. The protein concentration is 5 mM and the protein/metal ions molar ratio is 1:1

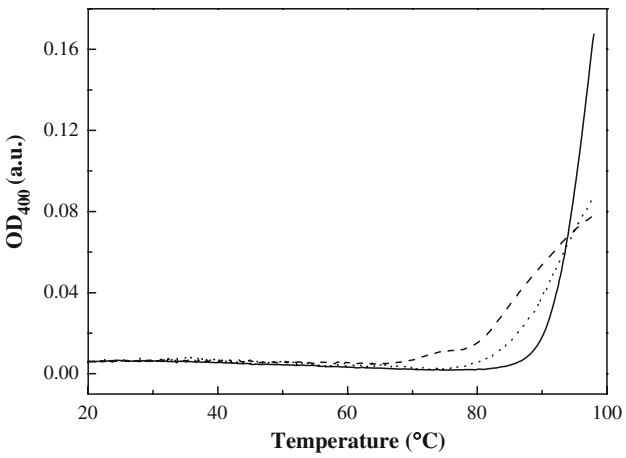

Fig. 6 Optical density at 400 nm, OD_{400}, as a function of temperature for BLG-A alone (*solid line*) and in the presence of (*dashed line*) Cu^{2+} and (*dotted line*) Zn^{2+}. The protein concentration is 0.13 mM and the protein/metal ions molar ratio is 1:1

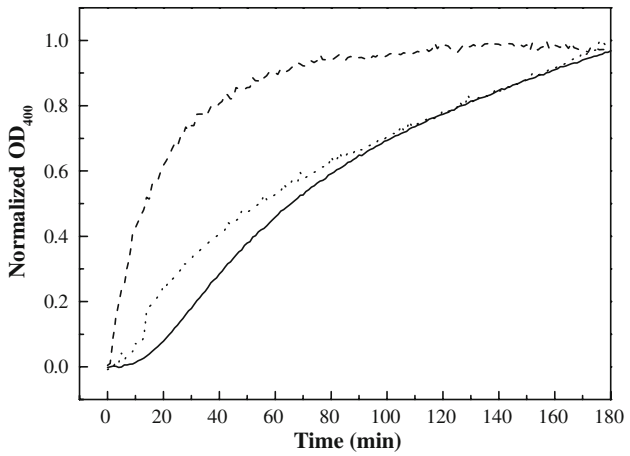

Fig. 7 Kinetics of aggregation at T = 80°C of BLG-A alone (*solid line*) and in the presence of (*dashed line*) Cu^{2+} and (*dotted line*) Zn^{2+}. The protein concentration is 0.13 mM and the protein/metal ions molar ratio is 1:1

with time of the optical density at 400 nm. BLG-A (solid line) shows an initial lag phase in which OD_{400} is almost constant; afterwards, a significant increase of the absorption is observed. The lag phase indicates the time required by the protein to pass from the native to the partially unfolded state necessary to trigger the aggregation.

The rise in the optical density is appreciable only after the appearance of sufficiently large aggregates in solution (Kurganov 2002). Such behaviour has also been observed in other proteins, such as creatine kinase (Fedurkina et al. 2006). In the presence of Zn^{2+} in solution (Fig. 7, dotted line) the curve is slightly different at the beginning, while after 100 min it has the same trend of BLG-A alone. More marked effects are obtained in the presence of Cu^{2+} (dashed line). In fact, the lag phase is completely absent and the OD_{400} increase is faster in comparison with the other cases. To quantify these effects the curves in Fig. 7 have been fitted with the following equation, under the hypothesis that the protein aggregation process is a reaction of first order (Fedurkina et al. 2006):

$$OD_{400}(t) = 1 - exp(-kt)$$

where OD_{400} (t) is the OD_{400} value at the time t and k the kinetic constant of the reaction.

Table 1 Aggregation rate constants of BLG-A aqueous solution in PBS (100 mM, pH = 6.2) at T = 80°C in the absence (k) and in the presence (k_{met}) of metal ions

Sample	k (min^{-1})	k_{met} (min^{-1})	k_{met}/k
BLG-A	0.011 ± 0.001	–	–
$+ZnCl_2$	–	0.013 ± 0.001	1.2 ± 0.2
$+CuCl_2$	–	0.051 ± 0.001	4.6 ± 0.5

The protein concentration is 0.13 mM and the protein/metal ions molar ratio is 1:1

The data were fitted excluding the lag phase (12 min) and the k values obtained are reported in Table 1. These values suggest that the zinc effect on k is almost negligible, whereas the copper ion increases the rate of protein aggregation of about 4.6-fold. By analyzing the kinetics of the aggregation process of the AcP, Capanni et al. (2004) have observed that an excess of $CuCl_2$ (about 400:1 molar ratio with AcP) induces only a 2.6-fold acceleration of AcP aggregation. Therefore, the effects observed in BLG-A are more marked, notwithstanding a much lower Cu^{2+} concentration. In our case, at the highest copper concentrations, we observe protein precipitation in solution, ascribable to the combined effect of metal ions and temperature. The difference in the observed effects supports the hypothesis on the role of hystidine residues in the Cu^{2+} binding, since there is no histidine in AcP. The same mechanism has also been proposed to explain the results obtained with α-synuclein (Rasia et al. 2005), β2-microglobulin (Morgan et al. 2001) and β-amyloid protein (Atwood et al. 1998).

Conclusion

Combining the results obtained with DSC, fluorescence, EPR and OD techniques, it has been highlighted that the aggregation of BLG-A, at pH = 6.2, is compatible with a nucleation model with a threshold concentration of 0.13 mM. The protein aggregation is characterized by a two step cooperative mechanism in which the dimer-to-monomer dissociation is followed by aggregation. The nature of the aggregates is fibrillar, as evidenced by ThT fluorescence.

Transition metal ions influence both the denaturation and aggregation processes. BLG-A–Cu^{2+} interaction is specific and binding with the protein in the native state is favoured. On the contrary, the interaction between the protein and the Zn^{2+} ion is less specific than Cu^{2+} and, probably, favours the aggregation by means of formation of intermolecular bonds among the denaturated polypeptide chains. Moreover, the Cu^{2+} metal ion is more effective than Zn^{2+} ion in modifying the kinetics of protein aggregation, which is about 4.6-fold faster compared to the control experiment in the absence of metals.

Copper is known to be a more dangerous metal in vivo than zinc. Our results, together with those obtained for other proteins, suggest that an excess of copper in the organism could represent a high risk factor for aggregation processes. These dangerous effects could explain why some proteins that are able to chelate metals in vivo, such as albumin, are necessary in living organisms.

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