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Thermal induced conformational changes involved in the aggregation pathways of beta-lactoglobulin

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Abstract

Aggregation of proteins appears to be associated most often with conformational and structural changes that lead to exposure of some apolar residues. Depending on the native structure of the protein in exam, aggregation is a process that involves different mechanisms, whose time of occurrence and interplay can depend upon temperature.

To single out information about the multistages of the aggregation pathway, here we investigate the thermally induced conformational and structural changes of the beta-lactoglobulin (BLG). The experimental approach consists in studying steady-state fluorescence spectra of intrinsic chromophores, two tryptophans, and Anylino-Naphthalene-Sulfonate dye (ANS) molecular probe. This technique revealed to be particularly suitable in investigating samples in the low concentration range and at the initial steps of the aggregation process. The Rayleigh scattering of the exciting light follows the growth of the intermolecular interactions at the same time. Complementary information is also obtained by circular dichroism (CD) measurements on samples in the same experimental conditions.

The obtained data indicate a well-defined interconversion between quaternary, ternary and secondary structures, together with conformational rearrangements driven by hydrophobic interactions and intermolecular bonds. The results are also discussed in comparison with similar studies on BSA aggregation.

Keywords: Beta-lactoglobulin; Protein aggregation; Conformational changes; Fluorescence; ANS emission; Rayleigh scattering; Circular dichroism

1. Introduction

Protein aggregation is the focus of several studies in biophysical and biochemical research since it is associated with a large amount of neurodegenerative diseases, such as the amyloidoses, prion diseases and cataracts, that are caused by protein association [1–3]. This complex process is not yet totally understood as it depends from a great number of physical and chemical parameters (temperature, pressure, concentration, ionic strength, pH, denaturant addition) [4,5].

The aggregation process usually involves conformational changes of the whole protein or of a specific domain and it is often attributed to the association of partially unfolded molecules [2,6–9]. In fact, small changes in local configuration could allow a great stereo-reorganization of macromolecules. For this reason, aggregation process interests proteins both on a microscopic scale, involving structural and conformational changes that lead to the exposure of apolar residues, and on macroscopic scale, as result of intramolecular interactions.

In this respect, the native protein and its aggregates can be seen as originating from a common population of partially unfolded, interconverting molecules that, due to interactions with the solvent and with the neighbour molecules, proceed towards different pathways. The aggre-

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gation process is today considered as process acting in competition with the normal folding pathway and it is common property of any polypeptide chain [10]. In particular, under specific conditions globular proteins with no homology to each other or to the disease-associated proteins have the ability to form fibrils that resemble those extracted from diseased tissue. These proteins are prone to fibril formation under conditions that stabilize a partially folded structure of the protein [11].

However, is not yet fully understood how the conformational changes, at secondary and tertiary structure level, take part in the aggregation pathways. It widely diffused the idea that the aggregation could occur via structural changes leading to formation of β richer structures. This is in agreement with the observation that the core structure of all amyloid consists of β-sheets (with the strands perpendicular to the long axis of the fibre) and that many short peptide sequences containing several hydrophobic residues and high tendency to β-sheet formation have a strong bent to form aggregates [2,12].

In this paper, we report an experimental study aimed to single out information about the multistages of aggregation pathway and we put in evidence the role played by the native structure of the protein in this process. To this purpose, we perform kinetic data on thermal aggregation of β-Lactoglobulin (BLG), at pH 6.2 and at different temperatures. The experimental approach consists of steady-state fluorescence measurements by Rayleigh scattering kinetics and circular dichroism measurements. This protein, like other globular proteins, when heated tends to aggregate, but a clear picture of the sequence of the molecular events occurring during the aggregation and the gelation process [13] has not yet emerged.

BLG is a small globular protein (MW=18,300 Da) contained in the whey of ruminant milk. At neutral pH, it assumes a dimeric native conformation. Its secondary structure is composed of 15% of α-helices, 50% of β-strands and ~20% of reverse turn. It has a core structural pattern formed by an α-helix and eight strands of antiparallel β-sheets and its tertiary structure is stabilized from two disulfide bonds; moreover, there is also one free, highly reactive –SH group, corresponding to Cys-121, buried in the hydrophobic core of the protein [14–18].

Fluorescence measurements in low concentration region permit to observe conformational changes in the first phases of aggregation pathway; in fact, thanks to the high efficiency of the native chromophores, we are able to do measurements on very dilute solutions. It is well known that fluorescence of tryptophanil residues in protein can provide valuable information concerning their environments and their relative conformational changes [19–26]. To explore different domains of the protein eventually involved in the process, we have also performed kinetic fluorescence spectra of the Anylino-Naphthalene-Sulfonate dye (ANS). This probe has been largely used to study the properties of intermediate states at folding and unfolding equilibrium of globular proteins [27–30]. Fluorescence measurements on BLG–ANS complex give information on changes of protein–probe interactions and on the variations of the accessible hydrophobic areas. It has also been reported that BLG possess two different binding sites for ANS: an external site, in proximity of a hydrophobic patch on the protein surface, and an internal site, located in the hydrophobic core of BLG. The external site has a nonspecific interaction with ANS, contrarily to that of the internal one where one of the two S–S bridges of BLG (Cys-106/Cys-119) is located [30]. The primary cause of initiation of heat aggregation of BLG is thought to involve disulfide bridge formation by exchange between the free cystein and one of these two disulfide bridges [3].

We have already applied the same experimental approach to Bovine Serum Albumin, whose structure is almost totally α-helical, and we found that conformational changes at secondary structure level from α to β were involved in the aggregation pathway [5,19]. Here, to extend our comprehension on relevance of conformational changes in this process, we have chosen BLG because its structure is essentially formed by β structures. In this way, it is interesting to make a comparison with the results we have already obtained on BSA to lead to some general considerations.

2. Materials and methods

2.1. Sample preparation

BLG genetic variant A (typeL7880) and ANS (S-1028) were purchased from Sigma. All measurements were performed in phosphate buffer 0.1 M at pH 6.2. For all samples, final protein concentration, determined by spectrophotometric measurements, was approximately 2 μM.

The ANS concentration was 50 μM. We note that the low concentration used allow to avoid the distortion of the tryptophanil emission due to the absorption of the dye in the spectral region between 250 and 320 nm. Each sample was freshly prepared and filtered just before the measurements.

2.2. Spectral measurements

Fluorescence spectra, excitation profiles and Rayleigh scattering measurements were carried out on Jasco FP-6500 spectrofluorimeter equipped with a Jasco ETC-273T peltier as temperature controller. All samples were positioned in a 1 cm path cuvette and, at a required temperature, the time evolution of the emission spectra was followed after 3 min for thermal equilibration. For all samples, excitation spectra at 25 °C, before and after each kinetic, are measured to monitor significant variations of the excitation band profile. The emission spectra of the intrinsic and extrinsic chromophores were obtained with emission and excitation band-

width of 3 nm, scan-speed of 100 nm/min and integration time of 1 s, and recorded at 0.5-nm intervals. Contemporary, Rayleigh scattering at 90° was also measured as the maximum of the elastic peaks of excitation light at 270 nm. The experimental errors were about 2% and 5% for fluorescence and scattering data, respectively. The spectral distributions $A(E)$ were corrected for spectral response of the detection system [25,31].

The tryptophans emission spectra in the range 280–430 nm were obtained under excitation at 270 nm, i.e. at about the maximum of the absorption band, both for BLG and BLG–ANS samples. The choice of 270 nm as excitation wavelength was a good compromise between the requirements, avoiding significant tyrosine contributions to the emission and spectral quality [19,31].

For BLG–ANS samples, the ANS emission spectra, in the range 380–420 nm, were obtained under excitation at 380 nm, i.e. about the maximum of the absorption band of the dye.

Circular dichroism (CD) measurements in the Far and Near UV region were carried out on a Jasco J-715 spectropolarimeter, equipped with a Jasco PCT 348WI temperature controller. Sample cell paths were 0.1 mm, spectral resolution of 0.2 nm and averaging time of 1 s.

For all samples and in the same experimental conditions, time evolution of the absorption spectra was also followed by using a Jasco V-570 Spectrophotometer (scan speed 40 nm/s, integration time 2 s, bandwidth 1 nm).

2.3. Data analysis

For each fluorescence spectrum, the zeroth (M_0), first (M_1) and second (M_2) moments of the spectral distribution $A(E)$ were calculated from the experimental data, after subtracting the tangent to the minima of each band, according to the following definitions [32].

$$M_0 = \int_{-\infty}^{\infty} A(E)\mathrm{d}E$$

$$M_1 = \frac{\int_{-\infty}^{\infty} EA(E)\mathrm{d}E}{M_0}$$

$$M_2 = \frac{\int_{-\infty}^{\infty} E^2A(E)\mathrm{d}E}{M_0} - M_1^2$$

M_0 measures the integrated intensity, M_1 is the mean value of the emission energy and measures the position of the band and M_2 is the mean square deviation of the spectral distribution and measures its width.

3. Results and discussion

Fig. 1 shows the thermal kinetic evolution of the Rayleigh scattering peak intensity of BLG at four different temperatures, 70, 75, 77 and 80 °C, in the time interval of 200 min. At the beginning of the kinetics, all the samples show constant values. Then, the behaviors show that the objects in the solution begin to increase their dimension as a function of time and temperature. At 70 °C, the process is too slow to be observed in the time interval considered and the objects in the solution appear approximately of the same size, while at 80 °C we can better distinguish different

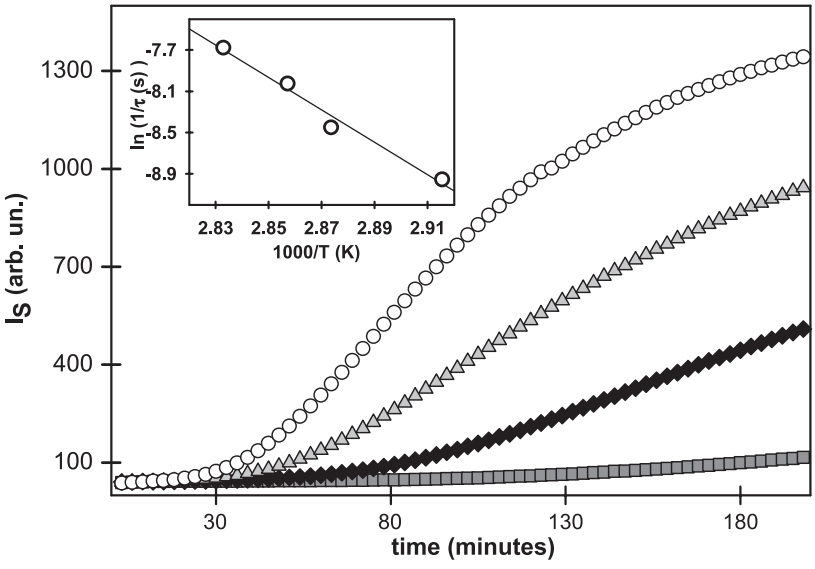

Fig. 1. Values of Rayleigh scattering maxima (I_S) of BLG at 70 °C (dark grey squares), 75 °C (black diamonds), 77 °C (grey triangles) and 80 °C (white circles), as a function of time. The inset shows the Arrhenius plot of τ [s] at the four temperatures (see text).

regimes on the whole aggregation process. As can be seen, after the first 35 min the aggregation has not begun and this delay changes as a function of temperature; then, from 35 to 120 min, the Rayleigh scattering intensity rapidly increases with a quasi-linear trend, suggesting a linear polymerization [33]; at last, up to 200 min, a less rapid increase is evident.

To quantify the temperature dependence of the delay in the beginning of aggregation, we have calculated the derivative of the intensity of the scattering as a function of time. In particular, for each temperature, we can define a different characteristic time (τ) given by that time corresponding to the minimum in the first derivate of the data reported in Fig. 1.

These values, reported in an Arrhenius plot in the inset of Fig. 1, indicate the end of the first step and the beginning of the aggregation. We analyze these data according to the law $\ln 1/\tau = A + \Delta E/K_B T$, where with ΔE we indicate an activation energy of the process. At our condition, we draw a value of ΔE of about 130 kJ mol.

Fig. 2 shows the time evolution of the intrinsic fluorescence of BLG at T=80 °C during the first 200 min. The Rayleigh scattering peaks as a function of time are also reported in the inset. As can be seen, the intensity of the emission increases as a function of time. Moreover, a significant red shift is observed in the initial steps of the kinetic, whereas the position of the band remains constant in the second part of the process. Contemporarily, the scattering peaks remain almost constant at the beginning, i.e. in the same initial time intervals corresponding to the shift of the peak positions; then, they begin to increase only when the maxima have completed their red shift.

In this respect, we recall that emission spectra of tryptophans are widely used to monitor the solvent exposure degree of the chromophores and a red shift of the bands indicates a more polar environment; moreover, their emission quantum yields may either decrease or increase upon the conformational changes in the surroundings [23–26].

To obtain more quantitative information, we report in Fig. 3 the behavior of M_0, M_1 and M_2 relative to the tryptophanil emission at four different temperatures. These data clearly show that the emission kinetic is strictly dependent from temperature. In particular, at 80 °C we observe the faster kinetic and an initial M_0 increase that, after about 120 min, changes its slope leading to a slow decay. M_1 shows an initial decrease leading to a constant value. The behavior of M_2 is characterized by a first increase, followed by a decrease that reaches a constant value. We outline a strict correlation between the temporal evolution of tryptophanil emission and the aggregation process, well showed by the behaviors at 80 °C reported in Figs. 1 and 3.

At 70 °C, the slowest kinetic, we observe a monotonic growth of M_0, accompanied by a decrease of M_1. The behaviors at 75 and 77 °C are coherent with those described above. All these results confirm the temperature dependence of the process. We wish to note the similar slopes of M_1 in the first data and at the three higher temperatures; it seems to indicate that the red shift extent is temperature independent. At the same time, the increase of M_2 at the same temperatures seems to indicate an increase in heterogeneity of the samples.

We have to underline that the emission band of BLG is the result of two different spectral contributions relative to the two tryptophans present in different parts of the

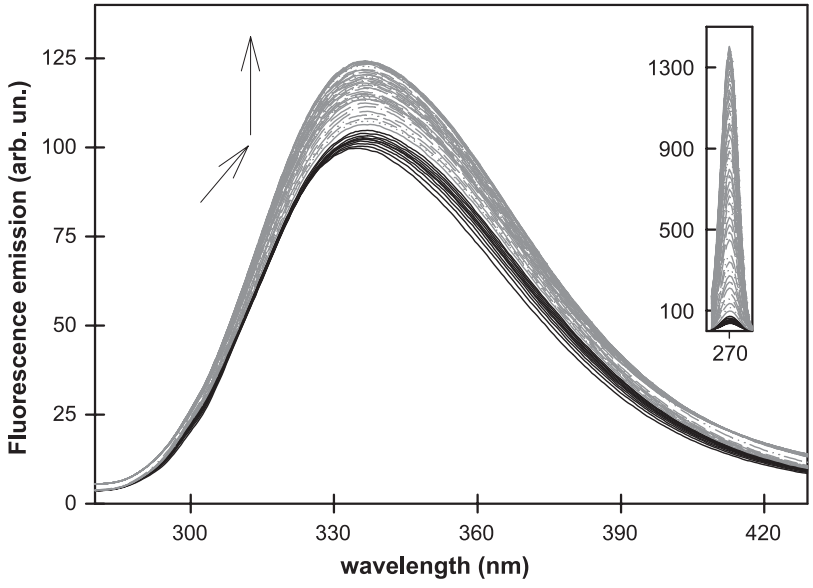

Fig. 2. Kinetics of BLG emission at 80 °C. The excitation wavelength was 270 nm. The inset shows the Rayleigh peak evolution measured at 270 nm. The arrows indicate the changes of the band as a function of time.

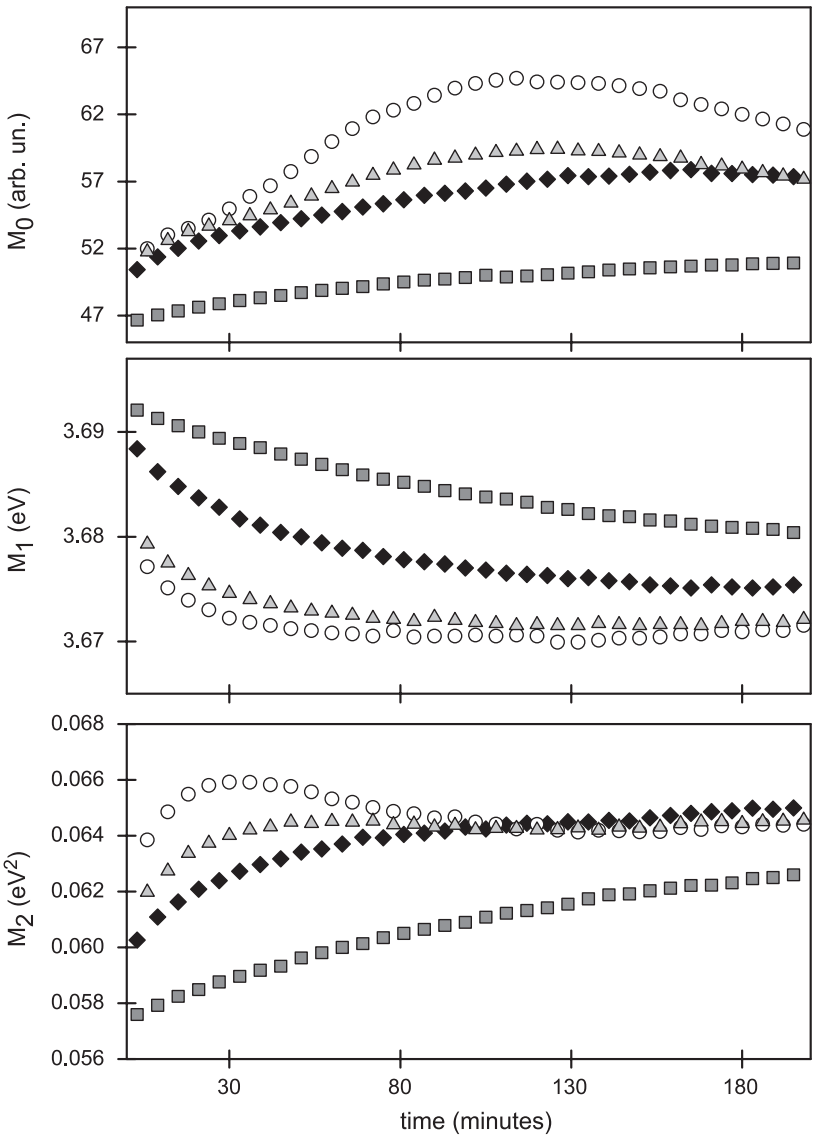

Fig. 3. M_0, M_1, M_2 of emission spectra of BLG, at 70 °C (dark grey squares), 75 °C (black diamonds), 77 °C (grey triangles) and 80 °C (white circles), as a function of time.

protein, as showed in Fig. 4. However, at the beginning of the kinetic, the main role is played by the internal Trp-19 because the other one, Trp-61, is exposed to the solvent and consequently its fluorescence is almost totally quenched. By proceedings of aggregation process, the Trp-61 emission becomes significant suggesting that this residue undergoes a partial covering.

The above reported results suggest that, at the first phase of the aggregation process, we can therefore attribute emission spectra to the single contribution of Trp-19, whose surrounding performs conformational changes. In particular, the increase of the fluorescence intensity can be attributed to the dissociation of the native dimer into monomers, whereas the red shift of the peak position

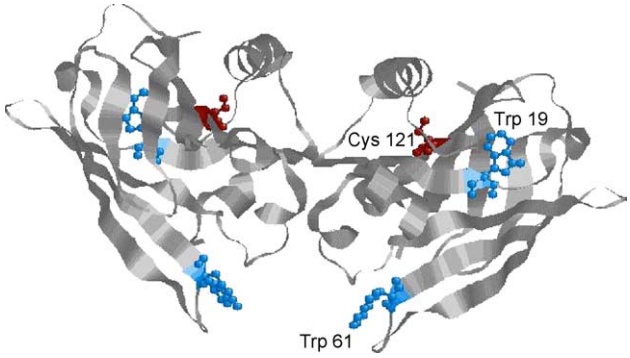

Fig. 4. The crystal structure of BLG dimer. The position of the free cystein and of the two tryptophans are indicated in the structure. The diagram was created by Rasmol with the Protein Data Bank code 1BEB [35].

corresponds to conformational changes involving the protein [25,33]. The activation energy value found in the Arrhenius plot (in Fig. 1) could be the energy necessary to perform the transition from quaternary to ternary structures [34].

Moreover, the single free cystein, Cys-121, is buried in the hydrophobic core of the dimer as Trp-19, and the partial opening of the protein structure and the conversion from dimer to monomer leads to the exposure of this residue. Once exposed, Cys-121 binds itself in forming new intermolecular bonds. In fact, it is already reported that, when heated, globular proteins tend to aggregate, thanks to the contribution of intermolecular disulfide bonds [6,19,36,37].

To best explore the role of hydrophobic intra- and intermolecular interaction, we performed kinetic emission of ANS, that is a well-known hydrophobic probe. In Fig. 5, the kinetics of M_0 and M_1 of ANS emission band at 75 °C are reported. M_2 is not reported because it does not show significant variations. In the first 30 min, no changes occur and this suggests that there are no changes in the hydrophobic environment seen by the dye; this means that the preliminary dimer–monomer transition does not influence the emission of the dye, as already reported in literature [28]. After, it is possible to observe a rapid increase of the moments that indicates progressive changes in the internal hydrophobic zones. The fluorescence intensity increases while the spectrum shifts towards higher energies (blue shift). This behavior indicates the existence of new accessible hydrophobic sites to the dye [27,30] and remind to the behavior showed by the scattering data in Fig. 1.

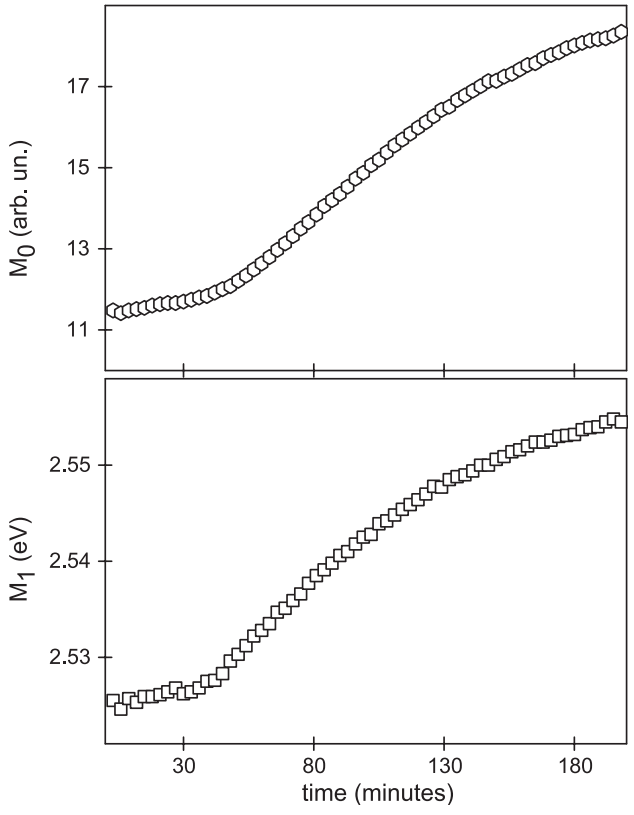

Fig. 5. M_0 (white hexagons) and M_1 (white squares) of emission spectra of ANS dye at 80 °C as a function of time.

To observe the secondary structural changes that, as already reported [19], are the driving steps toward the aggregation process together with conformational ones, we have performed CD measurements. In Fig. 6, we report CD spectra in the far-UV region at 80 °C, together with the time evolution of CD signal at 215 nm. These data show that the native β-structures of this protein change in favor of other different type of structures and show again that, after 100 min, the kinetic proceeds with a different slope.

FTIR absorption measurements (data not reported here) in the Amides region of a BLG sample in native (room temperature) and aggregated forms (after 3 h of incubation at 80 °C) have shown a decrease of the band assigned to native antiparallel β-sheet (1635 cm^{-1}) and a little increase of the band assigned to the β-turn aggregated structures (1685 cm^{-1}). The appearance of the two typical shoulders at 1620 and 1680 cm^{-1} attributed to the intermolecular β-sheet aggregates is not observed [5,38–40].

To sum up, we observe the absence of intermolecular association by β-sheet aggregates, seen by FTIR measurements, the decrease in the native β-structure, seen by CD and FTIR measurements, and the increase in the hydrophobic surroundings, seen by fluorescence of ANS. All these information seems to indicate the growth of macromolecular aggregates principally through hydrophobic interactions.

In the thermal kinetics of BSA, we have found that the β-aggregates, originating by α−β conversion prevalently contribute to form oligomers and that the ways in which the tertiary and secondary structure partially unfold is an important factor in determining the type of macromolecular network performed.

In BLG, the conformational changes observed are attributable to the transition dimer–monomer and to rearrangement of the monomer structure itself with consequent polymerization due principally to the hydrophobic interactions.

All data discussed here suggest a global picture that could be summarized in the following:

1. at the beginning of the kinetics, while the scattering data show that the aggregation has not begun (and is clearly dependent by temperature), the spectral data show a clear change in the tryptophan emission (spectral moments of the emission band), not found in ANS emission that shows analogous behavior to scattering data;
2. up to 120 min, the scattering data increase their values rapidly; the same happens for the ANS emission, while the tryptophan emission decreases;

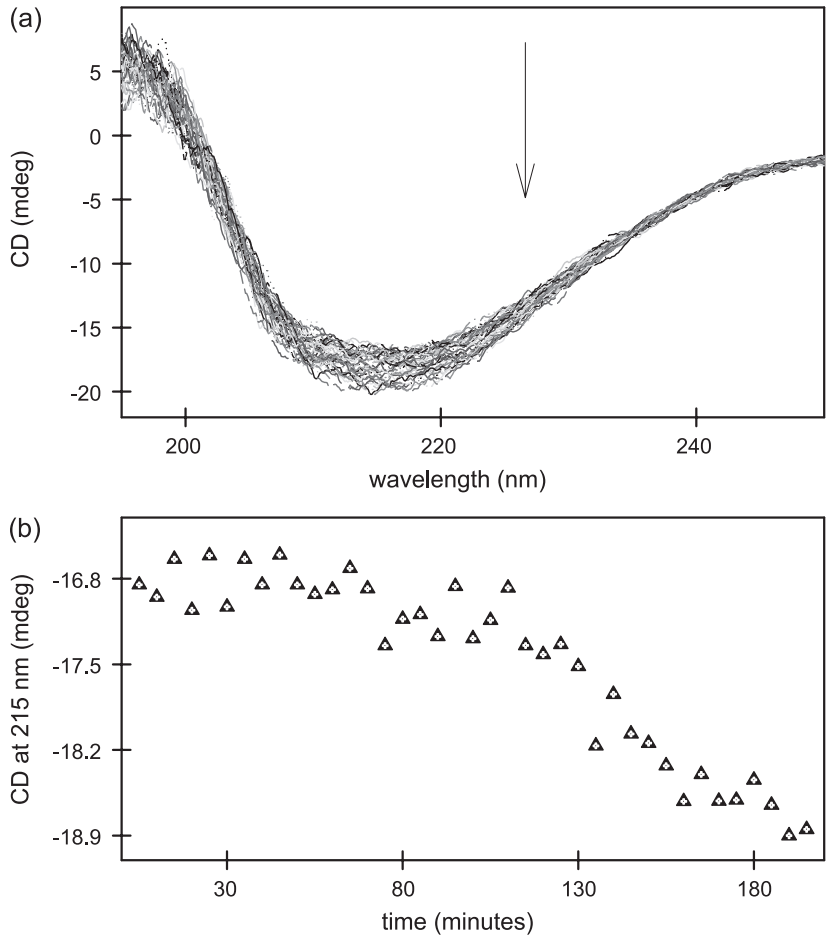

Fig. 6. (a) Kinetic of CD spectra of BLG sample at 80 °C, the arrow indicates the change of the signal as a function of time. (b) Time evolution of CD signal measured at 215 nm at 80 °C.

3. from 120 to 200 min, the scattering data and the ANS emission increase less rapidly, the tryptophan emission remains quite constant and the CD data now show a rapid decrease.

4. Conclusions

The results give evidence that different native structures lead to different mechanisms in the aggregation process. The thermally induced aggregation pathway of BLG seems to proceed by a sequence of different steps in terms of changes in quaternary, tertiary and secondary structures. We identify a first time, of about 40 min, in which we suppose a change in quaternary structure with subsequent rearrangement, a successive linear polymerization and a final rearrangement in macromolecular aggregates. All the conformational changes drive to the exposure and/or cover of specific residues and hydrophobic regions that play a fundamental role in the whole process. These changes do not stop after the exposure of hydrophobic zones of the protein but continues, giving the "aggregates" a very different structure even at secondary and tertiary level.

The BLG is also a little stable protein at extreme pH and this characteristic make its monomer structure very compact with the consequence that the aggregation process is well defined in different phases in comparison to the interconnected phases observed during BSA thermal aggregation pathway.

Aggregation process appears strictly dependent on temperature and it starts only after rearrangement of the protein structure [34,41–43]. Aggregate formation seems to proceed via a linear polymerization mechanism by monomer addiction [43]. The rate of this polymerization increases more steeply with increasing temperature.

As showed in Fig. 4, we remind that the BLG has a unique free cystein (Cys-121). This residue could contribute to the new intermolecular bonds, as already proposed for BSA, whose conformational changes lead to the exposure of this residue.

In other words, a global view of the reported results shows a multistep aggregation pathway that once undertaken proceeds through even hydrophobic and disulfide interactions.

Acknowledgments

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