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Conformational changes involved in thermal aggregation processes of bovine serum albumin

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Abstract

We report a kinetic study on thermal aggregation process of the model protein bovine serum albumin (BSA) in low concentration regime. Aim of this study is to provide information on relationship between conformational changes and initial step of aggregation. The experimental approach is based on steady-state fluorescence spectra of the two tryptophans located in two different domains, in way to study conformational changes in the surrounding of these residues. We also follow emission spectra of Fluorescein-5-Maleimide dye bound to the single free cysteine of BSA. Complementary information on the extent of aggregation and on the structural changes is obtained by Rayleigh scattering and circular dichroism measurements. These data contribute to clarify the connection between conformational changes at tertiary and secondary structure level during the aggregation and how the different domains are involved. We also discuss the relevant role played by cysteine 34 in the aggregation pathways.

Keywords: Bovine serum albumin; Protein aggregation; Steady-state fluorescence; Conformational changes; Circular dichroism

1. Introduction

It is well known that the unique native fold of a protein is crucial to its efficiency even in the complex environment of a living cell. Nevertheless, under some conditions, proteins can fail to fold correctly and this failure results in a wide range of diseases, such as amyloidoses, in which deposition of aggregated proteins in a variety of tissues is involved [1–3]. Neurodegenerative pathologies, such as Alzheimer's and Parkinson's diseases and transmissible spongiform encephalopathies, belong to this category.

In general, proteins aggregation process acts in competition with the normal folding pathway [4,5] and it takes place from misfolded and partially unfolded states [6,7]. In particular, a reduced folding stability appears to be a unifying property of amyloidogenic proteins [8]. In other words, the native protein and its aggregates can be seen as originating from a common population of partially unfolded, inter-converting molecules that, due to interactions with the solvent and with the neighbor molecules, proceed towards different pathways. For this reason, once undertaken the aggregation

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process in particular conditions of temperature and solvent composition the new macromolecules can coagulate to form different kind of gels [7,9–11].

Bovine serum albumin (BSA) is a well-known globular protein that has the tendency to aggregate in macromolecular assemblies [12]. Its three-dimensional structure is composed of three domains, each one formed by six helices, and its secondary structure is essentially α-helix [13,14]. At room temperature, tertiary structure is well defined and stabilized. As temperature increases, some molecular regions become accessible to new intermolecular interactions, producing soluble aggregates through disulphide and non-covalent bonds [15,16]. It has also been reported that, in overall aggregation process of BSA, an equally important role is also played by conformational changes of protein and by thermodynamic instability of the solution against liquid–liquid-demixing (LLD) [7]. Specifically, BSA aggregation appears to be the result of no less than three interconnected mechanisms: critically diverging concentration fluctuations associated with LLD, conformational changes and proteins cross-linking [7,10]. However, the hierarchy of all these mechanisms is strictly dependent on the experimental conditions, as put in evidence by temperature scan measurements of static and dynamic light scattering and circular dichroism (CD) [7].

In this paper, we focus our attention to the initial steps of BSA aggregation process consisting substantially in conformational changes of different regions of the protein. To this aim, we have chosen a BSA concentration low enough to be far from LLD instability region, in a way to obtain slow aggregation kinetics. This allows us to single out information on tertiary and secondary structural changes that go together with the initial intermolecular cross-linking processes, well separated from subsequent gelation processes.

The experimental approach consists in a kinetic study of steady-state fluorescence of intrinsic probes of BSA, the tryptophans, at three significant temperatures. BSA has two tryptophans, embedded in two different domains: Trp-134, located in proximity of the protein surface, but buried in a hydrophobic pocket of domain I, and Trp-214, located in an internal part of domain II [17]. Since their quantum yield depends on interaction with the neighbor environment [18–22] and on solvent exposure [23,24], time evolution of triptophan's emission spectra allows us to obtain information on conformational changes in both these domains. To explore separately the different environments, we also report a study on the emission kinetics of Fluorescein-5-Maleimide, that is a molecular probe covalently bound to the single free cysteine (Cys-34) of BSA, located within the domain I. Moreover, the presence of this dye in the proximity of Trp-134 causes the almost total quenching of its fluorescence, so, in these samples, only Trp-214 is optically active. In this way, we can obtain directly information on conformational changes of domain II separately by those of domain I. The extent of the aggregation process is contemporary followed by Rayleigh scattering of the excitation light and complementary information on secondary structure changes are obtained by CD measurements.

2. Materials and methods

2.1. Sample preparation

BSA (type A-0281) and human serum albumin (HSA) (type A-3782) were purchased from Sigma. BSA–Fluorescein-5-Maleimide conjugate (BSA–FM) was produced by Helix Research (Springfield, Oregon); the fluorescein dyes are in a ratio 1:1 with BSA, no free dyes being present in these samples. All measurements were performed in phosphate buffer 0.1 M at pH 6.2. Each sample was freshly prepared and filtered just before the measurements. For all samples, final protein concentration, determined by spectrophotometric measurements, was approximately 7 μM.

2.2. Spectral measurements

Emission spectra, excitation profiles and Rayleigh scattering measurements were carried out on Jasco FP-570 spectrofluorometer equipped with a xenon lamp (150 W). All samples were positioned in a thermostatized 1-cm path cuvette and, at a required temperature, the time evolution of fluo-

rescence is followed after 6 min for thermal equilibration. For all samples, fluorescence excitation spectra at 25 °C, before and after each kinetic experiment, are measured to monitor significant variations of the excitation band profile. For all samples, the emission spectra were obtained with emission and excitation bandwidth of 3 nm, scan-speed of 50 nm/min and integration time of 2 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. For further improvement of experimental data, we have repeated the Rayleigh scattering measurements in analogous samples in the same experimental conditions, using emission and excitation bandwidth of 1.5 nm. The experimental errors were approximately 2 and 5% for fluorescence and scattering data, respectively.

The energy dependence of the detection system was preliminary determined by placing an MgO scatterer in the sample position and measuring a synchronous spectrum of both monochromators [23,25]. Moreover, the spectral efficiency of the exciting light was corrected by using a Rhodamine B sample in glycerol as a reference [23]. The tryptophan 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 BSA and BSA–FM samples. The choice of 270 nm as excitation wavelength allows us to avoid significant tyrosine contributions to the tryptophan emission and to avoid partial overlap between the Rayleigh scattering peak and fluorescence signal that can alter the analysis of the emission band, maintaining good spectral quality. For BSA–FM samples, the fluorescein emission spectra, in the range 490–600 nm, were obtained under excitation at 490 nm, i.e. about the maximum of the absorption band. Finally, fluorescence spectra so obtained were analyzed by calculating the integrated fluorescence intensity of the spectral distributions, after subtracting the tangent to the minima of each band. Moreover, for all samples and in the same experimental conditions, the 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).

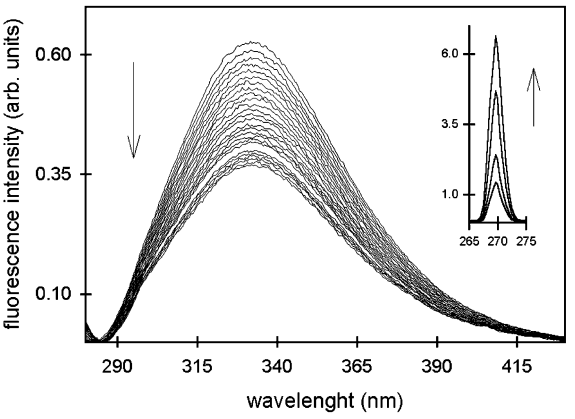

Fig. 1. Kinetic of BSA emission at 70 °C. The excitation wavelength was 270 nm. The inset shows the Rayleigh peak evolution measured at 270 nm. The arrows indicate the changes of the signals as a function of time.

To ensure that the effects on emission intensity of the increasing turbidity at 270 nm were negligible, we have measured the emission of BSA samples at 25 °C in presence of increasing concentration of polystyrene microspheres as scatterers (500 Å size); this allow us to reach values of Rayleigh scattering comparable with those observed in kinetic measurements. In these conditions, no significant changes on fluorescence quantum yield of BSA tryptophans were found.

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

3. Results and discussion

Fig. 1 shows the time evolution, in the first 120 min, of emission spectra of BSA at 70 °C. In the inset is also reported the simultaneous behavior of Rayleigh scattering for the same sample. A first inspection of raw data shows that Rayleigh scattering increases as a function of time, because of the increasing dimensions of scattering objects due to the proceeding of aggregation process [26,27] and, almost in the same time interval, the emission intensity of tryptophans decreases.

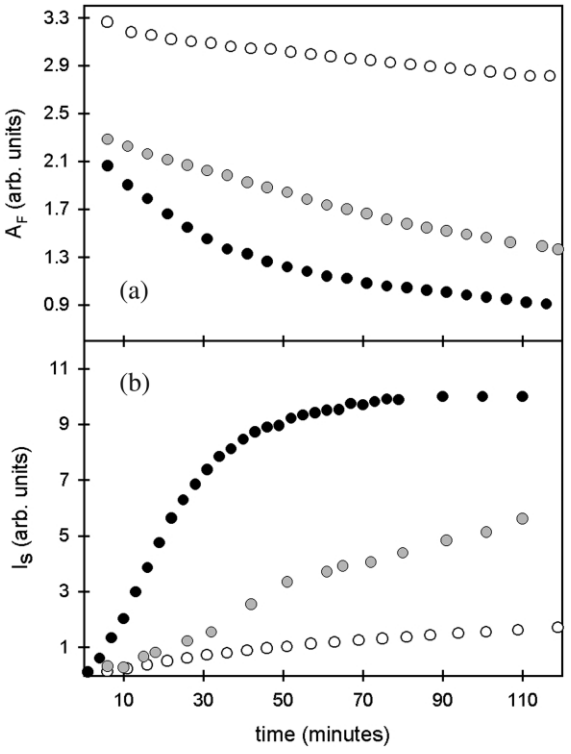

Fig. 2. (a) Integrated intensity of emission spectra (A_F) of BSA, as a function of time, at 65 °C (white circles), 70 °C (grey circles) and 75 °C (black circles). (b) Values of Rayleigh scattering maxima (I_S) as a function of time, for the same samples in the same experimental conditions.

Fig. 2a shows the time evolution of integrated intensity of tryptophans emission band, at three different temperatures. Fig. 2b shows the maxima of Rayleigh peaks, as a function of time, at the same temperatures.[1] These results confirm what observed before, i.e. a strict correlation exists between the decrease of tryptophans emission and the extent of the aggregation, as monitored by scattering measurements. In particular, we note that: (i) the growth rate of aggregation and of fluorescence quenching increases by increasing temperature; (ii) after an initial rapid trend within the first 50 min, the processes increase in a slower way, as more evident at 75 °C; (iii) in the time interval observed, no evidence exists of macroscopic aggregates like visible floccula formation, that is instead observed after several hours.

It is well known that the fluorescence quantum yield of tryptophan decreases as its exposure to solvent increases [28,29]. According to this, we take into account the progressive fluorescence quenching in terms of local rearrangements of the tryptophans surroundings. Therefore, we can infer that, at these temperatures and in this concentration regime, tertiary conformational changes take place and concur to the initial step of aggregation. We wish to remind that BSA emission band is attributed to two spectral contributions, the larger assigned to Trp-134 emission and the smaller to Trp-214 emission [20]. This suggests that the effects observed until now are prevalently related to the Trp-134 surrounding in the external part of domain I, more accessible to the solvent.

To see if and how secondary structure changes are involved in the initial step of aggregation processes, we report in Fig. 3a CD measurements in the UV region at 70 °C in the same samples and in the same time scales. As can be seen, native BSA spectrum shows a double peak shape, typical of high α-helical content [30], that, as a function of time, evolves towards a shape more similar to that typical of β-rich structures. The time evolution of CD signal at 225 nm, as shown in Fig. 3b, put in evidence that the most prominent changes occur in the first 70 min, after this time no changes being present. CD data suggest that not only conformational changes at tertiary structure level, as seen by fluorescence data, but also structural changes at secondary structure level are involved in the aggregation process. This agrees with results based on kinetics of static and dynamic light scattering and near–far UV CD on BSA (Vaiana and Palma, in preparation and Eur. Biophys. J. 29 (2000) 2D13). Furthermore, our data show that, after the first 70 min, the Rayleigh

[1] We note here that, before the analysis in terms of spectral moments, fluorescence bands were corrected according to the procedure outlined in Section 2. In this respect, we also outline that no significant changes in the absorption band profile are observed, as monitored by parallel absorption measurements performed on the same samples as a function of time, but for increasing turbidity, due to proceeding of the aggregation. Therefore, the reported fluorescence spectra are not affected by significant shifts of the exciting wavelength within the absorption band, as also confirmed by fluorescence excitation spectra.

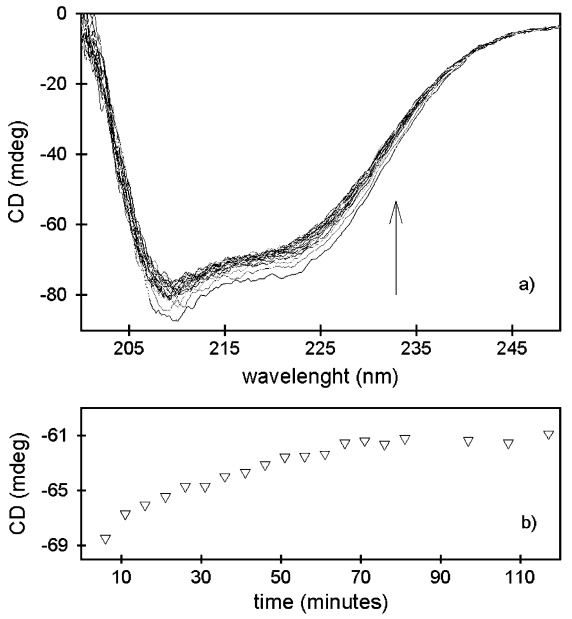

Fig. 3. (a) Kinetic of CD spectra of BSA sample at 70 °C. (b) Time evolution of CD signal measured at 225 nm at 70 °C. The arrow indicates the changes of the signal as a function of time.

scattering continues to grow and the fluorescence quantum yield of tryptophans continues to diminish, while secondary structural changes are exhausted. This suggests that aggregation is mainly driven by conformational changes at tertiary structure level.

To assign the above inferred conformational changes to a specific region of the protein, we have also measured the emission kinetics of Fluorescein-5-Malemide covalently bound to single free cysteine (Cys-34) of BSA. This dye shows an emission band in a spectral region (490–600 nm) well distinguished from that relative to tryptophanil residues.

Surprisingly, the presence of the dye strongly influences the emission band of tryptophans, as showed in Fig. 4a. We hypothesize that, in BSA–FM samples, a selective quenching of Trp-134 emission is induced by the fluorescein dye, due to its closeness to this tryptophanil residue. To confirm this hypothesis, we have compared BSA–FM emission spectrum with those of BSA and HSA, obtained in the same experimental conditions. BSA and HSA are homologous proteins, which have similar sequence and conformation but differ in the number of tryptophans, HSA having only one tryptophan located in a microenvironment similar to that of Trp-214 of BSA. Fig. 4a shows that BSA–FM emission spectrum is very similar to the HSA one, confirming that the presence of the dye quenches almost totally the spectral contribution of Trp-134.

In Fig. 4b, we report the absorption spectrum of Fluorescein-5-Maleimide dye. As can be seen, this dye presents several absorption bands, two of those fall in the same spectral region of the tryptophan emission, suggesting that, for this chro-

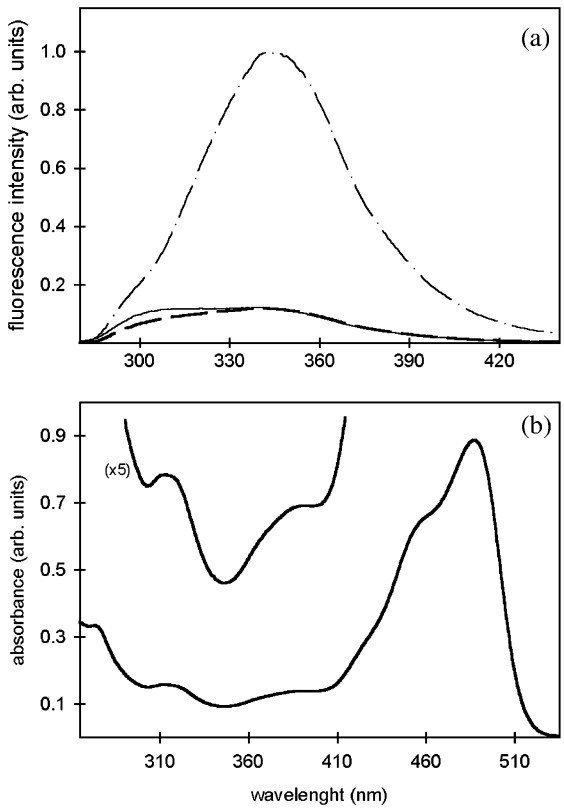

Fig. 4. (a) Normalized emission spectra of BSA (dash-dot line), HSA (dashed line) and BSA–FM (solid line) samples, obtained at 25 °C under excitation at 270 nm. The spectra were corrected for the corresponding absorbance value at 270 nm. (b) Absorption spectrum of free Fluorescein-5-Maleimide, at room temperature.

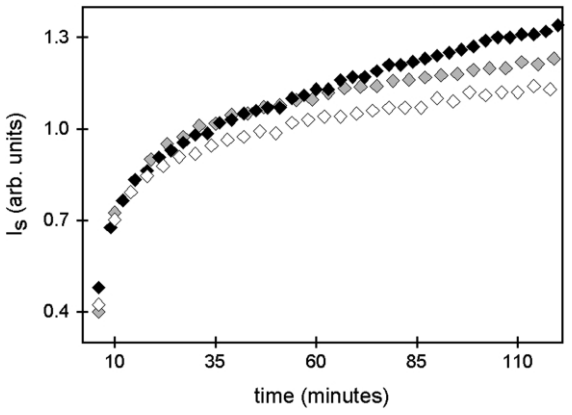

Fig. 5. Rayleigh scattering maxima (I_s), as a function of time, of BSA–FM sample at 70 °C (white diamonds), 75 °C (grey diamonds) and 80 °C (black diamonds).

mophore, a de-excitation pathway involving electronic states of the dye can take place in competition with radiative process. Nevertheless, we cannot exclude that the presence of the dye induces conformational changes in the surroundings of Trp-134 causing a larger exposure to the solvent and, in turn, a fluorescence quenching of this tryptophan.

In view of the above reported considerations, we believe that the comparison between measurements on BSA–FM and BSA samples gives us clear and separate information on conformational changes involving the inner part of domain II, monitored by Trp-214 and the external part of domain I, monitored by the dye.

In Fig. 5 are reported the maxima of Rayleigh peaks in BSA–FM as a function of time and at three different temperatures. Data show a trend similar to that obtained for BSA sample in the same experimental conditions (70 and 75 °C) but, unpredictably, the Rayleigh peak intensity appear to be lower (note the different scales in Figs. 2 and 5), indicating that the introduction of the dye in BSA molecule slows down the thermal aggregation processes. Moreover, in the first 50 min, the Rayleigh scattering intensity is independent on temperature, i.e. follows the same initial pathways. We have also made CD measurements on the same BSA–FM samples (data not reported); CD spectra did not show, within the time interval observed (120 min), any significant changes on secondary structure.

These data indicate that the presence of the dye brings two main effects: (1) it stabilizes the BSA native structure preventing conformational changes at secondary structure level; (2) it slows down the aggregation process.

Fig. 6 shows the time evolution of integrated intensity of Trp-214 emission for BSA–FM. Data show a fluorescence quenching similar to that found for BSA samples, suggesting that conformational changes involved in aggregation processes also interest the Trp-214 surrounding in the inner part of domain II. However, in agreement with Rayleigh scattering data and with the fact that only internal tryptophan contributes to emission spectra, the variation of fluorescence intensity is smaller.

In Fig. 7, we report the time evolution of integrated intensity of fluorescein dye in BSA–FM. As can be seen, the extension of the variation is comparable to that found for BSA. The faster initial emission quenching can be attributed to a larger exposure of the dye to the solvent, for a partial opening of tertiary structure, and seems to go in parallel with rapid increase of Rayleigh scattering. The subsequent behavior, consisting in an increase of emission intensity, is more or less

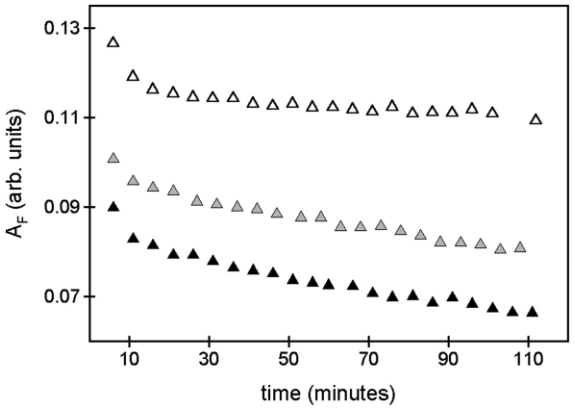

Fig. 6. Integrated intensity of emission spectra (A_F), as a function of time, for Trp-214 in BSA–FM at 70 °C (white diamonds), 75 °C (grey diamonds) and 80 °C (black diamonds).

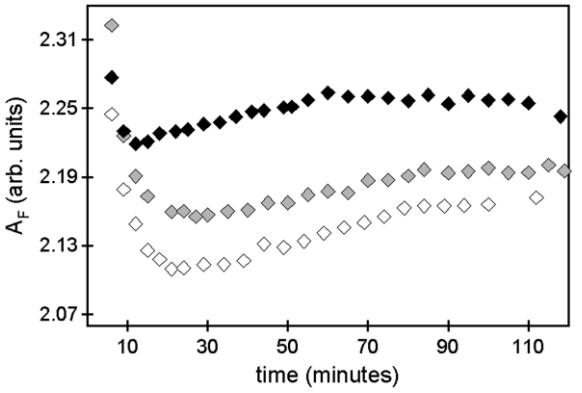

Fig. 7. Integrated intensity of emission spectra (A_F), as a function of time, for fluorescein in BSA–FM at 70 °C (white diamonds), 75 °C (grey diamonds) and 80 °C (black diamonds).

fast as a function of temperature and can be attributed to a progressive covering of the dye due to initial protein cross-linking formation. After this, an essentially time-independent behavior is observed, while the scattering intensity continues to grow slowly. Therefore, we have to note that, also in this sample, the surrounding of dye (domain I) undergoes conformational changes. Once again, data show the relevant role of tertiary structural changes, in the initial steps of aggregation, also without secondary structural changes.

4. Conclusions

Overall analysis of emission kinetics of BSA and BSA–FM provides information on structural changes in these two samples when placed at high temperatures. Data reported in this paper focus on conformational changes of the environments of the intrinsic and extrinsic probes in BSA, the tryptophans and the fluorescein, involved in the first steps (120 min) of aggregation pathway. As seen by CD measurements, conformational changes in secondary structure (from α- to β-rich structures) are present only in BSA while, in BSA–FM, these changes are not observed, in the same time interval considered. The aggregation of BSA–FM slows down with respect to that of BSA, as seen by Rayleigh scattering data of two samples at the same temperature. These results suggest that the presence of fluorescein inhibits, in the time interval considered, conformational changes at secondary structure level and slows down the initial aggregation process. We wish to highlight that domain I, where the fluorescein is bound, seems to have a predominant role during the aggregation of BSA.

From these results, we also suggest a noteworthy role of the unique free cysteine of BSA, the Cys-34 that, in BSA–FM samples, is bound covalently with fluorescein dye and cannot be available to form intermolecular bonds; in other words, this kind of ‘capping’ of Cys-34 elicits its role in intermolecular bonding, as also seen previously on HSA samples [31].

The comparison between these BSA and BSA–FM allows us to conclude that, in these experimental conditions of temperature and concentration, conformational changes at secondary and tertiary structure level are the driving step for a rapid initial intermolecular cross-linking process while, where secondary structural changes are not present and the free Cys-34 cannot do intermolecular bonds, the aggregation is slower and proceeds only through conformational changes at tertiary structure level. These conformational changes in domain I lead to a larger and rapid exposure of the probes to the solvent, due to a partial opening of native structure, while the accessibility of the solvent to more buried regions, like that in which is located the Trp-214, is smaller. We wish to underline that protein flexibility reveals once again its fundamental role not only on proteins function [32–35] but also on this kind of macroscopic processes.

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