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ORIGINAL PAPER

Thermal aggregation of bovine serum albumin at different pH: comparison with human serum albumin

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Abstract We report here a study on thermal aggregation of BSA at two different pH values selected to be close to the isoelectric point (pI) of this protein. Our aim is to better understand the several steps and mechanisms accompanying the aggregation process. For this purpose we have performed kinetics of integrated intensity emission of intrinsic and extrinsic dyes, tryptophans and ANS respectively, kinetics of Rayleigh scattering and of turbidity. The results confirm the important role played by conformational changes in the tertiary structure, especially in the exposure of internal hydrophobic regions that promote intermolecular interactions. We also confirm that the absence of electrostatic repulsion favours the disordered non-specific interactions between molecules and consequently affects the aggregation rate. Finally, the comparison between BSA and another relative protein, HSA, allows us to clarify the role of different domains involved in the aggregation process.

Introduction

During the last years, protein aggregation processes have been extensively studied for their relevance in many fields of biotechnological, pharmaceutical and medical research (Chiti et al. 2002; Calamai et al. 2005). Nevertheless, a global comprehension of the phenomenon and of the underlying mechanisms is still far from being accomplished. Protein aggregation is characterized by multiple interactions and cross-feedback among different processes, in particular conformational changes and intermolecular interactions, which strongly affect each other. In addition, a remarkable role may be played by solvent induced/guided interactions. The hierarchy of all these mechanisms and their extent depend on the environment and on several physical and chemical parameters such as temperature, pH, ionic strength, denaturant addition, etc. (Durbin and Feher 1996; Guijaro et al. 1998; Vaiana et al. 2001; Foguel et al. 2003; Munishkina et al. 2004; Uversky and Fink 2004; Dzwolak et al. 2005).

In general, it is commonly accepted that the aggregation process takes place from misfolded and partially unfolded states acting in competition with the normal folding pathway (Uversky and Fink 2004; Calamai et al. 2005; Guijaro et al. 1998; Dobson 2004; Kelly 1998; Morel et al. 2006). We remind that, for proteins, the lost of structural stability may trigger partial unfolding, which, in turn, may result in the formation of supramolecular aggregates. Conformational changes play a fundamental role in the aggregation pathways and have their onset under particular external conditions (San Biagio et al. 1999; Bellotti et al. 2000; Militello et al. 2003, 2004; Vetri et al. 2007). For example, for a wide class of proteins under specific conditions, conformational changes at the secondary structure may promote the formation of intermolecular β-strands, leading to formation of strongly ordered aggregates, known as amyloid fibrils (Arai et al. 1999; Bellotti et al. 2000; Stefani et al. 2003; Uversky and Fink 2004; Vetri et al. 2007). This particular self-association of proteins in amyloidal aggregation is involved in a wide range of pathologies, such as

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Alzheimer’s and Parkinson’s disease, in which deposition of large amounts of aggregated proteins is observed (Foguel et al. 2003; Calamai et al. 2005; Dobson 2004; Walsh et al. 2004).

Thermal induced aggregation has to be considered as a process beginning from partially denatured conformations: after the temperature increase, the native and compact form of the protein becomes more flexible and as a consequence, some molecular regions of the protein molecule, such as hydrophobic regions or free SH-groups, become accessible to new intermolecular interactions, thus bringing about the formation of large aggregates through non-covalent and/or disulphide bonds (Militello et al. 2003). In addition, intermolecular association can be favoured by the formation of intermolecular β-sheets. This short and general description is necessary, in our opinion, to acquaint the readers with the idea that a very large variety of mechanisms are involved in aggregation process and that these mechanisms are difficult to separate and are strongly affected even by small differences in the external parameters. At the moment, it is necessary to collect a lot of data using different techniques and proteins, in way to make much more clear the following in the steps and pathways that constitute the whole aggregation process.

Bovine serum albumin (BSA) is a well-known protein, which under a variety of conditions displays tendency to self-assembly in large macromolecular aggregates. It is composed of a single polypeptide chain, organized into three domains (I, II and III), each one formed by six helices. At room temperature, its secondary structure is predominantly α-helical. The 17 disulphide bridges ensure some rigidity within each subdomain, but allow significant modifications in the shape and size of serum albumin in response to changes in pH and other influences (Foster 1977; Carter and Ho 1994). BSA undergoes reversible conformational isomerization as a function of pH, going from the *N* form, at neutral pH, to the less compact F form, close to pH 4 (Katchalski 1957; Sadler et al. 1993; Nakamura et al. 1997; Brooks et al. 2000). It is worth noting that the secondary structure of BSA displays few changes in a range of pH values between 6.5 and 5 (El Kadi 2006), the latter value being close to the isoelectric point of the protein and reported to be around 4.6-4.9 (Putnam 1975; Peters 1996; Taboada et al. 2004; Brewer et al. 2005).

We have already performed studies on thermal aggregation of BSA and we have shown that, at low concentration regime (μM) and at pH 6.2, the aggregation process involves conformational changes at the secondary and tertiary level. Moreover, a relevant role is played by the free cysteine 34 together with intermolecular hydrophobic interactions (Militello et al. 2003). In the high concentration regime (mM), the aggregation pathway is strongly affected by pH, as previously described (Militello et al. 2004). At pH 7.4, far from the pI (i.e. with a net charge in the protein surface), the aggregation proceeds through the formation of relatively small ordered aggregates (oligomers) that are characterized by a sizeable amount of intermolecular β-sheets, as revealed by FTIR spectroscopy. At lower pH values, 6.8 and 6.2, more close to the pI (i.e. with a minor net charge in the protein surface), a large fraction of bigger disordered aggregates was observed. All these previous studies clearly indicate that the mechanism of BSA aggregation can proceed via two different pathways: either producing smaller ordered aggregates through the conversion from α-helix to β-aggregated structures addressed by specific intermolecular interactions (as electrostatic interactions) or producing aggregates of larger dimensions via non-specific and more disordered interactions (as hydrophobic interactions).

For continuity and for better understanding of the several mechanisms and steps involved in the aggregation of BSA, here we report a study on BSA thermal aggregation at lower pH values, 4.7 and 5.7, and in low concentration regime in such a way as to neglect the role of thermodynamic instability of BSA solutions against liquid–liquid demixing (San Biagio et al. 1999; Militello et al. 2003). The experimental approach consists of a kinetic study of the emission of tryptophans present in the protein and of ANS (anilino naphthalene sulphonate) used as an extrinsic dye. In particular, we remind that BSA has two tryptophans, embedded in two different domains: Trp134, located in domain I, in a hydrophobic pocket near the surface of the protein molecule; Trp214, located in an internal part of domain II (Moriyama et al. 1996; Militello et al. 2003). The quantum yield of tryptophans depends on their solvent exposure that, in our conditions, is caused by conformational changes in tertiary structure due to high temperature. For these reasons, following the aggregation process through the kinetics of tryptophans emission, we are able to provide information on the conformational changes in domain I and II. At the same time, the progress of the aggregation process can be monitored by means of Rayleigh scattering of the excitation peak at 90°.

Moreover, we have also studied, through suitable turbidity measurements, how the rate of the aggregation processes depends on the concentration at the two pH values. Finally, additional information on the conformational changes in the tertiary structure was obtained by using ANS, whose emission vanishes in water solution, while is increased when the probe is buried in hydrophobic regions. For this reason, ANS is a valid tool for studying the properties of intermediate states in folding/unfolding pathways and the conformational changes involved in the aggregation processes (Semisotnov et al. 1991; D’Alfonso et al. 1999; Lindgren et al. 2005; Marcon et al. 2005). In general, BSA has two specific binding sites for ANS, located respectively in domain II and III of the protein, with different affinities (Zolese et al. 2000). At high ANS concentration, both sites are populated, whereas at low concentration the probe interacts essentially with the site buried in domain III. In particular, in this study we have selected an ANS concentration suitable to obtain information on the role played by domain III.

The experimental approach is also applied, for comparison, to human serum albumin (HSA) samples. In fact, the aim of this study is to better understand the mechanisms related to the aggregation of BSA, also comparing itself with the aggregation process of a relative protein, the HSA. In particular, HSA and BSA present closely homologous structures stabilized by a repeating pattern of the 17 disulphide bridges, both at the tertiary and secondary structure level (Carter and Ho 1994) and they have the same isoelectric point (Putnam 1975; Peters 1996; Taboada et al. 2004; Breweret al. 2005; El Kadi 2006; Oliva et al. 2002). HSA has only one tryptophan (Trp214), localized in domain II (Moryiama et al. 1996; Gelamo et al. 2002), and it is known to undergo different pH-dependent structural transitions and conformational changes with different involvement of domains (Dokal et al. 2000) that can bring to amyloid fibril formation (Taboada et al. 2006).

The results show that aggregation processes in human and bovine serum albumin, for the same conditions, follow different aggregation pathways strictly affected by hydrophobic interactions modulated by pH.

Materials and methods

Sample preparation

BSA (A-0281), HSA (A-3782) and ANS (A-1028) were purchased from Sigma. All the measurements on BSA and HSA were performed in phosphate buffer 0.1 M at pH 4.7 and 5.7. Every solution was freshly prepared and filtered just before the measurements and the protein concentration was spectrophotometrically determined. Protein concentration was approximately 3.5 μM. ANS/protein molar ratio was 0.4 to ensuring that the dye was linked to subdomain III.

Spectral measurements

Fluorescence spectra were carried out on Jasco FP-6500 equipped with a Jasco ETC-273T peltier-thermostat. Samples were positioned in a cuvette of 1 cm and, after thermal equilibration; all emission spectra were recorded at 0.5 nm wavelength intervals, with emission and excitation bandwidth of 3 nm, scan-speed of 100 nm/min and integration time of 1s.

Excitation spectra at 25°C, before and after the kinetics, were measured to control variations of the excitation band profile. The spectral distributions were corrected for spectral response of the detection system (Lackovicz 1983; Leone et al. 1999) and normalized with respect to sample absorption at the exciting wavelength. Spectra were analysed by calculating the integrated intensity of the spectral distributions, after having subtracted the tangent to the minima of each band. Contemporary Rayleigh scattering at 90° was also measured as the maximum of the elastic peaks of excitation light. The experimental errors were about 2 and 5% for fluorescence and scattering data, respectively.

The tryptophans emission spectra in the range 280–430 nm were obtained under excitation at 270 nm, a good compromise between spectral quality and the requirement of avoiding significant contributions to the tyrosine emission (Militello et al. 2003;Vetri and Militello 2005). ANS-protein complexes spectra were detected using an excitation wavelength $\lambda_{exc} = 380$ nm.

Absorption measurements were carried out on a Jasco V-570 spectrophotometer. Turbidity experiments were performed following the absorbance at $\lambda = 320$ nm during the progress of the aggregation process.

Results and discussions

BSA aggregation

In Fig. 1, we report the kinetics of Rayleigh scattering intensity at 60°C for two BSA samples, at pH 4.7 and 5.7.

As can be seen, during the aggregation process, the Rayleigh scattering intensity increases with increasing time and dimensions of scattering objects in solution. The data show also a marked dependence on the pH values: the growth of

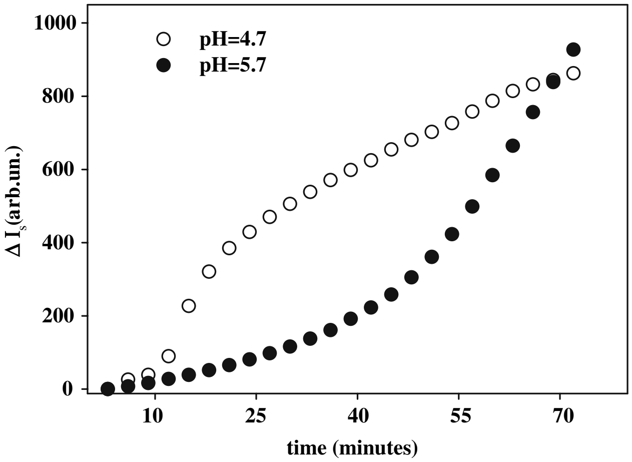

Fig. 1 Kinetics of elastic scattering peak measured at $\lambda = 270$ nm and $T = 60$°C for BSA at pH 4.7 (*white circles*) and 5.7 (*black circles*)

macromolecular aggregates at pH 4.7 is faster than that at pH 5.7. In particular, at pH 4.7, i.e. very close to the isoelectric point, the signal shows a sudden growth of macromolecular aggregates in the first 20 min and a subsequent slower phase. At pH 5.7, the behaviour appears inverted, with a slower and more regular growth phase at the beginning and a final faster phase. It is worth noting that the initial points are coincident (within 10 min) and that after about 65 min the two behaviours cross themselves. This last statement could mean that we are observing the growth of macromolecular aggregates, which can be roughly considered to be of the same dimensions, but they are produced via different pathways of aggregation dependent on pH. Indeed, in the first 10 min the protein, at both the pH values, probably proceeds towards a thermal partial unfolding before aggregating (Militello et al. 2003, 2004).

To have more information on the nature of the observed aggregates, we investigated the behaviour of the aggregation rate as a function of protein concentration through the turbidity method. In fact, concentration is a key parameter in aggregation and the dependence of the process evolution on this parameter could give interesting insights into understanding the involved mechanisms.

Figure 2a shows a typical kinetic of the absorbance measured at 320 nm and at 60°C for a BSA sample at pH 5.7. Moreover, an indication of the velocity of the process can be obtained considering the inverse of the time necessary to reach 1/2 of the maximum value of the sample turbidity, as shown in Fig. 2b, in which we put in relation the dependence of this parameter from the protein concentration at the two pH values. In both cases, the rate of aggregation depends linearly on the concentration with different slopes. Such a linear dependence, which is reasonable for an associative process (Chandrasekhar 1943), suggests that the aggregation is not affected by particular nucleation steps, which would give higher concentration dependencies (see e.g. Ferrone 1999). Even if the explored concentration range is restricted, this behaviour is not surprising also in view of the fact that the observed phenomena occur far from the instability region, so liquid–liquid demixing mechanisms can be excluded. It should be noted that this kind of observation allows us to evidence that the process, at lower pH, is characterized by a steeper concentration dependence and by the existence of a threshold concentration below which no aggregation process occurs. This seems to indicate the existence of a critical concentration of monomers below which aggregate cannot form. In fact, threshold phenomena in general occur in the presence of two equilibrium states coexisting in a certain range of parameters (i.e. concentration), represented here by monomeric and aggregate states. Non-specific interactions may lead to steeper concentration dependence and also to the existence of the observed threshold concentration, which is probably brought about by a non-negligible dissociation rate between monomers and aggregates, equal to the relative association rate (see e.g. Naiki and Gejyo 1999).

It is worth marking that, as a function of pH, BSA presents different aggregation pathways that seem to be characterized by different mechanisms: while temperature in this concentration regime is a “knob” that makes the process faster or slower (through partial unfolding of the native state), pH induces variation in the nature of aggregation pathways since it varies/diversifies in both the initial three-dimensional structure (as isomeric forms) and in the rate at which the different interactions are involved.

To investigate if and how changes at the secondary structure level are involved, we have performed CD measurements that are not reported here because, for both samples, the aggregation pathways proceed without relevant changes in secondary structures. This fact allows us to exclude the intermolecular association favoured by intermolecular *β*-sheets, found previously with the same protein at higher pH. Under these conditions, on the contrary, the aggregation process seems to evolve through conformational changes at the tertiary structure level, as the kinetics of tryptophans and ANS fluorescence emissions show.

Figure 3 shows the kinetics of tryptophans emission of the BSA at pH 4.7 and 5.7. We remind that the emission band of BSA at about 330 nm is attributed to the two tryptophans: Trp134, located in a hydrophobic pocket near the surface of the domain I and that gives the major contribution to emission intensity (Militello et al. 2003); Trp 214, located in an internal part of domain II. As evident, both samples at 60°C exhibit quenching of tryptophans emission,

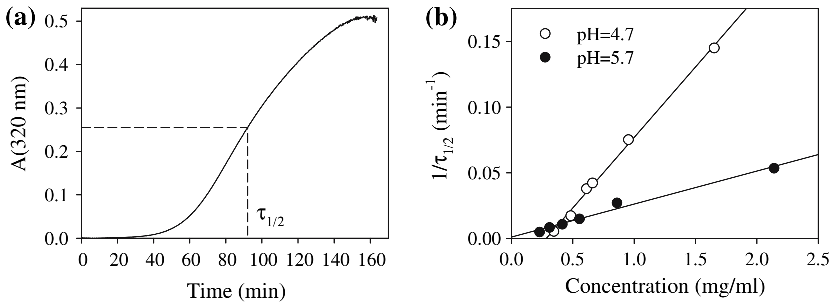

Fig. 2 **a** Kinetics of absorbance measured at (λ = 320 nm and T = 60°C, for BSA at pH 5.7. **b** Values of inverse of $\tau_{1/2}$ (the time necessary to reach 1/2 of the maximum value of the turbidity) as a function of BSA concentration at two pH. As in Fig. 1, symbols

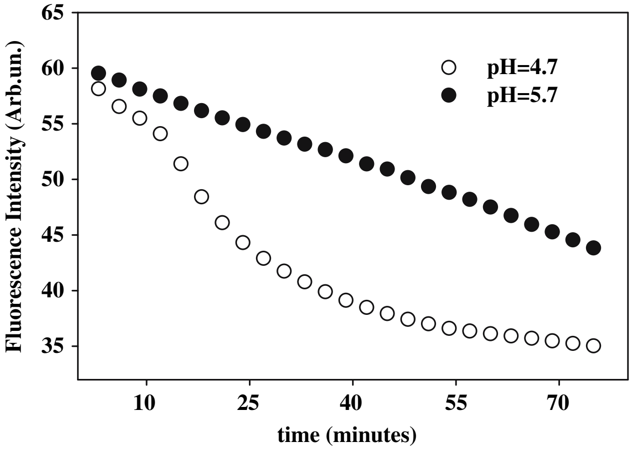

Fig. 3 Kinetics of the integrated intensity of tryptophans emission at $T = 60°C$ for BSA at two pH. As in Fig. 1, symbols

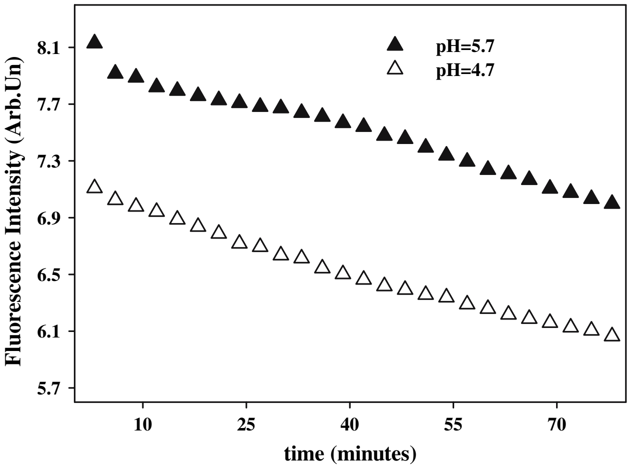

Fig. 4 Kinetics of the integrated intensity of ANS emission at $T = 60°C$ for ANS-BSA at pH 4.7 (*white triangles*) and 5.7 (*black triangles*)

whose temporal evolution is in strict relation with the scattering increase reported in Fig. 1. Such quenching has been attributed to tertiary structure changes, which cause the exposure of tryptophans to the solvent. Furthermore, the decrease of fluorescence emission appears monotonic and slower for the sample at pH 5.7, while it seems to be biphasic at a lower pH. In Fig. 1, during the first 10 min no change is observed, while up to the beginning, and especially at pH 4.7, a slow partial unfolding of the protein, i.e. a change in tertiary structure due to the temperature is evident. We outline, however, that the first point showed in all the kinetics is already at thermal equilibrium (60°C). The differences in the observed decrease of tryptophans emission can be rationalized by assuming that the different pH affect the rate of the exposure of tryptophans, i.e. it is reasonable to think that the different ratio between the two tryptophanyl contributions at the emission band is also pH dependent. Notwithstanding, BSA at these two pH is reported to exist prevalently in its *N* conformation, so we suggest that the main contribution is still attributable to Trp 134 in domain I. This could indicate that the overall effects observed until now are related to local rearrangement of the surrounding of this chromophor, leading to the partial opening of the native conformation. As a consequence of the observed opening, there is the exposure of new reactive regions prone to aggregation.

To further explore the nature of the exposed sites, we studied the emission kinetic of ANS dye. In particular, ANS binding site in BSA are of two types: one with larger affinity identified on subdomain IIIA and another with a lower affinity identified on subdomain IIA, where Trp 214 is located (Zolese et al. 2000). The ANS concentration studied here enables us to obtain information on the conformational changes involving principally domain III. Figure 4 shows the kinetics of the integrated emission intensity of ANS at the two pH. The selected ANS concentration enables us to obtain information on the conformational changes involving principally domain III. Moreover, it has been verified that, in these conditions, the aggregation process is not slackened by ANS presence.

These data show at pH 4.7, a lower emission intensity of ANS, in analogy with the tryptophans emission in Fig. 3, which indicates a major exposure to the solvent of ANS binding site in domain III with respect to that at pH 5.7. Both the behaviours at the two pH studied are parallel, thus indicating that we are observing the same process. Indeed, the different intensity values suggest a different "affinity" of BSA for this dye, which depends on pH; in fact, it is plausible to attribute these differences to the subtle changes in BSA conformation due to different pH.

Our results up to now suggest that domains I and III are certainly involved in the conformational changes that address the protein towards partial unfolding, and their conformation is influenced by pH.

HSA aggregation

To better understand the mechanisms of aggregation of BSA, we compared the data shown until now with analogous data regarding the aggregation process of HSA, a relative protein with homologous tertiary and secondary structures and same isoelectric point. HSA has only one tryptophan (Trp 214) localized in domain II (Moryiama et al. 1996; Gelamo et al. 2002; Viallet et al. 2000) and is known to undergo different pH-dependent structural transitions and conformational changes with different involvement of domains (Dokal et al. 2000). With this protein, we tried to separate the contributions of domain II to the aggregation process.

In particular, we followed the thermal aggregation of HSA under the same experimental conditions as that used for BSA. In Fig. 5, we report the kinetics of Rayleigh scattering intensity at 60°C for two HSA samples, at pH 4.7 and 5.7, in the same time interval observed before for BSA, in Fig. 1.

As can be seen, this protein shows an analogous slow growth of Rayleigh scattering intensities in both the samples at different pH up to 40 min from the beginning of the process. Afterwards, the behaviour becomes faster at pH 4.7 than at pH 5.7, as shown in BSA. Again, in the first 10 min, the behaviours are the same for both the samples. With respect to BSA, the aggregation of HSA at pH 5.7 seems much faster than BSA at the same pH, while at pH 4.7 it seems slower from the beginning. These observations suggest different aggregation pathways explored by the two proteins depending on pH. From the other measurements performed but not reported, in the same experimental conditions, as observed for BSA, the aggregation pathways for HSA proceed without relevant changes either with respect to protein concentration or with respect to secondary structures.

Figure 6 shows the kinetics of tryptophan emission for HSA at pH 4.7 and 5.7. As can be seen, for both samples the intensity decreases in correlation with the observed scattering intensities showed in Fig. 5, thus indicating that, also in this protein, conformational changes in tertiary structure occur and that also domain II is involved in the partial opening to the solvent, but shows less dependence on pH. The Trp 214 localized in domain II, in fact, is situated in an internal site.

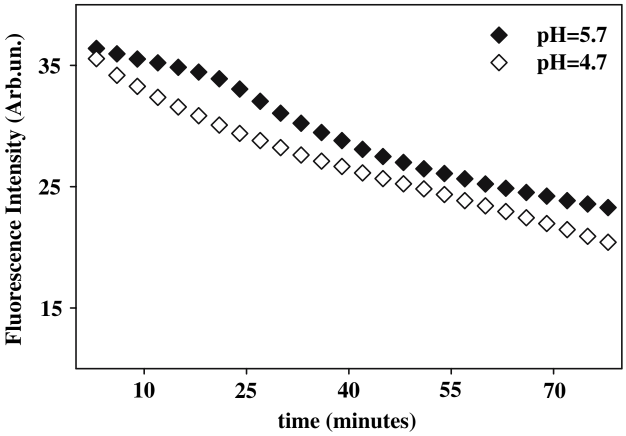

Fig. 6 Kinetics of the integrated intensity of tryptophans emission at $T = 60$°C, for HSA at two pH. As in Fig. 5, symbols

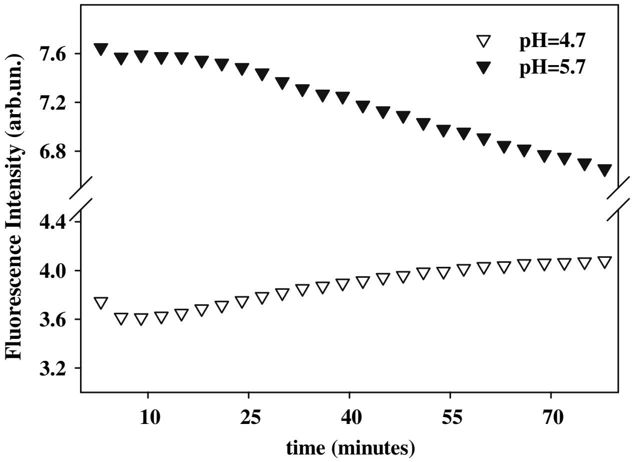

Fig. 7 Kinetics of the integrated intensity of ANS emission at $T = 60$°C for ANS-HSA at pH 4.7 (*white triangles*) and 5.7 (*black triangles*)

In Fig. 7, the kinetics of ANS emission are reported; the experiments were rigorously performed under the same conditions used for BSA (ANS/HSA = 0.4). Due to the close structural homology of the two proteins, the binding sites (and the relative affinities) for ANS are considered to be the same as that of BSA.

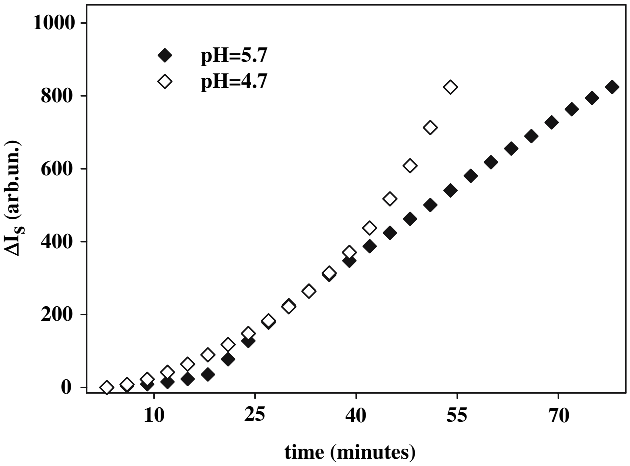

Fig. 5 Kinetics of elastic scattering peak measured at $\lambda = 270$ nm and $T = 60$°C for HSA at pH 4.7 (*white diamonds*), and 5.7 (*black diamonds*)

From Fig. 7, it is evident that, as also for HSA, the ANS affinity appears to be different depending on pH; as in BSA, at pH 4.7 the emission intensity of ANS is lower, thus indicating a major exposure to the solvent of ANS binding site of domain III, with respect to that at pH 5.7. But the behaviour shown by this hydrophobic dye is opposite with respect to BSA: a major quenching at pH 5.7, while at pH 4.7 the initial lower quenching is very short (only in the first 10 min), then increases but the whole variation in the emission signal is very small. In general, we can discuss the results as follows:

1. at pH 5.7, ANS emission remains quite constant in the first step (during about 20 min) indicating not immediate and appreciable variations of domain III, and then a

subsequent quenching appears and it could be attributed to a major exposure to the solvent of ANS binding site in domain III; in BSA, the partial opening of the ANS pocket occurs immediately, as shown in Fig. 4;

2. at pH 4.7, ANS emission shows a very small initial quenching (in the first 10 min), which can be attributed to a preliminary exposure of the dye to the solvent, due to the partial opening of tertiary structure caused by temperature; afterwards, the signal shows a small increase of emission intensity, which could be attributed to the formation of new intermolecular interactions that produce new hydrophobic sites for the dye. This last behaviour is not observed in BSA.

Conclusions

The data shown, consistently, seem to confirm the role played by the net charge of the protein in the aggregation process. The absence of electrostatic repulsion favours the disordered interaction between proteins and consequently affects the aggregation rate. The intermolecular interactions between the hydrophobic regions exposed to the solvent are initially dominant, as shown by ANS behaviour, and then other mechanisms add themselves.

We already had suggested that when the net charge of the BSA, at higher concentrations with respect to those shown in this study, is increased (i.e. when pH of solution is far from the pI), the aggregation mechanism proceeds in an ordered way to form β-aggregates that prevalently come from the α-helix changes and produce aggregates of small dimensions; on the contrary, when the net charge is decreased, (i.e. when pH of solution is close to pI), the aggregation mechanism is more disordered and other aggregates of larger dimensions add to the smaller ones. In other words, BSA can aggregates via smaller specie (β-aggregates) when the electrostatic repulsions are increased and this mechanism prevalently contributes to form oligomers, whereas, other type of aggregates structures add when the net charge of the protein decreases (Militello et al. 2003).

Coherently, in this study at lower concentrations, the minor molecular crowd shows, for both the proteins, similar dependence on pH, in the sense that the changes in the net charge favours more or less the electrostatic repulsion between neighbours and drives towards one or another aggregation pathway. We remind that here we are studying a pH range closer to the pI than that of previous papers.

We have also found that, in the experimental conditions studied here, the “incipit” of BSA aggregation is not guided by nucleation mechanisms. In addition at the lower pH, close to the isoelectric point, the poorly specific interactions result in the presence of a threshold concentration below which no one aggregation can occur. We can also notice the absence or the presence of the threshold concentration for the same protein in different conditions. The aggregation rate linearly depends on concentration; instead, the dissociation rate of the first small aggregates most likely does not, probably being more related to intrinsic properties of protein–solvent interaction.

Moreover, we can infer that different domains are involved in different conformational changes depending on pH; in fact, as monitored by tryptophans and ANS fluorescence, aggregation is favoured from progressive exposure of internal hydrophobic groups in the three protein domains. This exposure appears different at the two pH and in the two proteins and it is probably due to the slight differences in the initial native structure, like different isomeric forms, as also reported in a paper by Dockal et al. (2000).

The nature of aggregation pathways appears to be composed of two different and inverted phases at the two pH. In particular, at these two acid pH, closest to the pI, conformational changes involve only tertiary structure and the aggregation proceeds via disordered and non-specific interactions without conformational changes at the secondary structure for both the relative proteins. Although a marked homology in protein structure exists, data concerning HSA provide information on different aggregation pathways with respect to those of BSA.

These results confirm that the process is strictly dependent on external conditions and, in particular, assessment of the role of the solvent in aggregation processes is crucial. We can guess that the conformational changes inducing partial exposure of hydrophobic residues have a common feature in being the step for initiating intermolecular cross-linking interactions also without changes in secondary structures. Other type of interactions, especially the electrostatic ones, seem to drive the aggregation process toward more or less disordered aggregation that needs changes also in secondary structures.

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References

Arai S, Hirai M (1999) Reversibility and hierarchy of thermal transition of hen egg-white lysozyme studied by small-angle X-ray scattering. Biophys J 76:2192–2197

Bellotti V, Mangione P, Merlini G (2000) Immunoglobulin light chain amyloidoses: the archetype of structural and pathogenic variability. J Struct Biol 130:280–289

Brewer SH, Glomm WR, Johnson MC, Knag MK, Franzen S (2005) Probing BSA binding to citrate-coated gold nanoparticles and surfaces. Langmuir 21(20):9303–9307

Brooks DE, Haynes CA, Hritcu D, Steels BM, Müller W (2000) Size exclusion chromatography does not require pores. PNAS 97:7064–7067

Calamai M, Chiti F, Dobson CM (2005) Amyloid fibril formation can proceed from different conformations of a partially unfolded protein. Biophys J 89:4201–4210

Carter DC, Ho JX (1994) Structure of serum albumin. Adv Protein Chem 45:153–203

Chandrasekhar S (1943) Stochastic problems in physics and astronomy. Rev Mod Phys 15:1–89

Chiti F, Taddei N, Baroni F, Capanni C, Stefani M, Ramponi G, Dobson CM (2002) Kinetic partitioning of protein folding and aggregation. Nature Struct Biol 9:137–43

D'Alfonso L, Collini M, Baldini G (1999) Evidence of heterogeneous 1-anilinonaphthalene-8-sulfonate binding to β-lactolgobulin from fluorescence spectroscopy. Biochimica et Biophysica Acta 1432:194–202

Dobson CM (2003) Protein folding and misfolding. Nature 426:884–890

Dockal M, Carter DC, Rüker F (2000) Conformational transitions of the three recombinant domains of human serum albumin depending on pH. J Biol Chem 275:3042–3050

Durbin SD, Feher G (1996) Protein crystallization. Annu Rev Phys Chem 47:171–204

Dzwolak W, Grudzielanek S, Smirnovas V, Ravindra R, Nicolini C, Jansen R, Loksztejn A, Porowski S, Winter R (2005) Ethanol-perturbed amyloidogenic self-assembly of insulin looking for origins of amyloid. Biochemistry 44(25):8948–8958

El Kadi N, Taulier N, Le Huerou JY, Gindre M, Urbach W, Nwigwe I, Kahn PC, Waks M (2006) Unfolding and refolding of bovine serum albumin at acid pH: ultrasound and structural studies. Biophys J 91:3397–3404

Ferrone F (1999) Analysis of protein aggregation kinetics. Methods Enzymol 309:256–274

Foguel D, Suarez MC, Ferrão-Gonzales AD, Porto TCR, Palmieri L, Einsiedler CM, Andrade LR, Lashuel HA, Lansbury PT, Kelly JW, Silva JL (2003) Dissociation of amyloid fibrils of α-synuclein and transthyretin by pressure reveals their reversible nature and the formation of water-excluded cavities. Proc Natl Acad Sci 19:9831–9836

Foster JF (1977) In: Rosenoer VM, Oratz M, Rothschield MA (eds) Albumin structure, function and uses. Pregamon Press, Oxford, pp 53–84

Gelamo EL, Silva CH, Imasato H, Tabak M (2002) Interaction of bovine (BSA) and human (HSA) serum albumins with ionic surfactants: spectroscopy and modelling. Biochim Biophys Acta 1594(1):84–99

Guijarro JI, Sunde M, Jones JA, Campbell ID, Dobson CM (1998) Amyloid fibril formation by an SH3 domain. Proc Natl Acad Sci U S A 95(8):4224–4228

Katchalski E, Benjamin GS, Gross V (1957) The availability of the disulfide bonds of human and bovine serum albumin and of bovine gamma-globulin to reduction by thioglycolic acid. J Am Chem Soc 79:4096–4099

Kelly JW (1998) The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. Curr Opin Struc Biol 8:101–106

Lakovicz JR (1983) Principles of fluorescence spectroscopy. Plenum Press, New York

Leone M, Agnello S, Boscaino R, Cannas M, Gelardi FM (1999) Conformational disorder in vitreous systems probed by photoluminescence activity in SiO_2. Phys Rev B 60:11475–11481

Lindgren M, Sorgjerd K, Hammarstrom P (2005) Detection and characterization of aggregates, prefibrillar amyloidogenic oligomers, and protofibrils using fluorescence spectroscopy. Biophys J 88(6):4200–12

Marcon G, Plakoutsi G, Canale C, Relini A, Taddei N, Dobson CM, Ramponi G, Chiti F (2005) Amyloid formation from HypF-N under conditions in which the protein is initially in its native state. J Mol Biol 347(2):323–335

Militello V, Casarino C, Emanuele A, Giostra A, Pullara F, Leone M (2004) Aggregation kinetics of bovine serum albumin studied by FTIR spectroscopy and light scattering. Biophys Chem 107:175–187

Militello V, Vetri V, Leone M (2003) Conformational changes involved in thermal aggregation processes of bovine serum albumin. Biophys Chem 105:133–141

Morel B, Casares S, Conejero-Lara FA (2006) Single mutation induces amyloid aggregation in the alpha-spectrin SH3 domain: analysis of the early stages of fibril formation. J Mol Biol 356(2):453–68

Moriyama Y, Ohta D, Hachiya K, Mitsui Y, Takeda K (1996) Fluorescence behaviour of tryptophan residues of bovine and human serum albumins in ionic surfactant solutions: a comparative study of the two and one tryptophan(s) of bovine and human albumins. J Prot Chem 15:265–271

Munishkina LA, Henriques J, Uversky VN, Fink AL (2004) Role of protein–water interactions and electrostatics in (α-synuclein fibril formation. Biochemistry 43:3289–3300

Naiki H, Gejyo F (1999) Kinetic analysis of amyloid fibrils formation. Methods Enzym 309:305–318

Nakamura K, Era S, Ozaki Y, Sogami M, Hayashi T, Murakami M (1997) Conformational changes in seventeen cysteine disulfide bridges of bovine serum albumin proved by Raman spectroscopy. FEBS Lett 417(3):375–8

Oliva FY, Avalle LB, Camara OR (2002) Electrochemical behaviour of human serum albumin-TiO2 nanocrystalline electrodes studied as a function of pH, part 1: voltammetric response. J Electroanal Chem 534(1):19–29

Peters TJ (1996) All about albumin biochemistry, genetics, and medical applications. Academic Press, San Diego

Putnam FW (1975) The plasma protein structure, functions and genetic control. Academic Press, New York, pp 133–172

Sadler PJ, Tucker A (1993) pH-induced structural transitions of bovine serum albumin: histidine pKa values and unfolding of the N-terminus during the N to F transition. Eur J Biochem 212:811–817

San Biagio PL, Martorana V, Emanuele A, Vaiana SM, Manno M, Bulone D, Palma-Vittorelli MB, Palma MU (1999) Interacting processes in protein coagulation. Proteins 37:116–120

Semisotnov GV, Rodionova NA, Razgulyaev OI, Uversky VN, Gripas AF, Gilmanshin RI (1991) Study of the "molten globule" intermediate state in protein folding by a hydrophobic fluorescent probe. Biopolymers 31:119–128

Stefani M, Dobson CM (2003) Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. J Mol Med 81(11):678–699

Taboada P, Barbosa S, Castro E, Mosquera V (2006) Amyloid fibril formation and other aggregate species formed by human serum albumin association. J Phys Chem B 110(42):20733–20736

Taboada P, Gutierrez-Pichel M, Mosquera V (2004) Effects of the molecular structure of two amphiphilic antidepressant drugs on the formation of complexes with human serum albumin. Biomacromolecules 5(3):1116–1123

Uversky VN, Fink AL (2004) Conformational constraints for amyloid fibrillation: the importance of being unfolded. Biochim Biophys Acta 1698(2):131–53

Vaiana SM, Manno M, Emanuele A, Palma-Vittorelli MB, Palma MU (2001) The role of solvent in protein folding and in aggregation. J Biol Phys 27:133–145

Vetri V, Militello V (2005) Thermal induced conformational changes involved in the aggregation pathways of beta-lactoglobulin. Biophys Chem 13:83–91

Vetri V, Canale C, Relini A, Librizzi F, Militello V, Gliozzi A, Leone M (2007) Amyloid fibrils formation and amorphous aggregation in concanavalin. A Biophys Chem 125(1):184–90

Viallet M, Vo-Dinh PT, Ribou AC, Vigo J, Salmon JM (2000) Native fluorescence and mag-indo-1-protein interaction as tools for probing unfolding and refolding sequences of the bovine serum albumin sub-domain in the presence of guanidine hydrochloride. J Prot Chem 19:431–438

Walsh DM, Selkoe DJ (2004) Oligomers on the brain: the emerging role of soluble protein aggregates in neurodegeneration. Protein Pept Lett 11(3):213–228

Zolese G, Falcioni G, Bertoli E, Galeazzi R, Wozniak M, Wypych Z, Gratton E, Ambrosini A (2000) Steady-state and time resolved fluorescence of albumins interacting with *N*-oleylethanolamine, a component of the endogenous *N*-acylethanolamines. Proteins 40(1):39–48