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Biophysical Chemistry 91 (2001) 61–69

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Effects of intermediates on aggregation of native bovine serum albumin

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Received 25 January 2001; received in revised form 19 March 2001; accepted 19 March 2001

Abstract

Protein aggregation has been recognized to be a pathological indicator for several fatal diseases, such as Alzheimer's disease, transmissible spongiform encephalopathies, Creutzfeldt–Jacob disease, etc. Aggregation usually involves conformational changes of proteins that have acquired an intermediate β-structure-rich conformation and can occur even at low protein concentration. Recent work in our laboratory has shown that bovine serum albumin (BSA), even at low-concentration, exhibits self-association properties related to conformational changes, so providing a very convenient model system to study this class of problems. Here we report data (obtained by different experimental techniques) on a mixture of BSA in native and intermediate (β-structure-rich) form. Results show that the interaction between the two species is responsible for a decrease in the thermodynamic stability of the solution. This occurs without requiring noticeable conformational changes of the native protein. Results presented here can provide new insight on the 'protein only' hypothesis proposed for the formation of plaques involved in several neurodegenerative diseases.

Keywords: Bovine serum albumin; Amyloid; Phase transition; Static light scattering; Dynamic light scattering

1. Introduction

Aggregation of proteins has become one of the major topics in biology and medicine in the last few years [1]. In fact, protein precipitates accompany many diseases such as Alzheimer's disease, Down's syndrome, Creutzfeldt–Jacob disease, bovine spongiform encephalopathy, and systemic AL amyloidosis, among others [2–4]. One common and defining feature of all these diseases is the formation and deposit (intracellular and extracellular) of proteins in plaques, amyloid fibrils and filaments [1–8]. Although amyloid deposits can be formed by different proteins, their structure and physical properties are very similar [7]. The established transmissibility of some of the above pathologies has suggested the existence of

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0301-4622/01/$ - see front matter
PII: S0301-4622(01)00155-7

a common infectious agent (prion), whose nature is still controversial. In fact, there is a lively debate in the current literature about the agent responsible for amyloidosis and related diseases [2]. The 'protein only' hypothesis [8], which states that prions do not contain any nucleic acid and consist of a modified form of a normal host-encoded protein, seems to be the most reliable, although no definitive data are currently available to support it against the 'virus agent' hypothesis [9]. Within the frame of the 'protein only' model, the prion replicates itself by acting as a template for the conversion of native protein into a priogenic conformational intermediate characterized by a higher content of β-structure and capable of self-assembly [8]. The existence of metastable intermediates with the structural characteristics of molten globule seems to be a common trait of all known human amyloidogenic proteins [10]. The central point to be elucidated concerns the thermodynamics of intermediate stabilization, the aggregation process, and its relation with the presence of already misfolded protein (or infective agent). A nucleation–polymerization mechanism catalyzed by the presence of an exogen prion [11] has been proposed to take into account the need of raising the local concentration of protein up to values required to start the aggregation process.

In this paper we report experimental results on a solution of bovine serum albumin (BSA) that can be taken as a convenient model system for studying the role of the conformational transition due to unfolding on the thermal aggregation of the protein and the underlying thermodynamic mechanism [12,13]. Previous work at our laboratory on aqueous solutions of BSA has shown that by incubating a BSA solution at 67°C for a short time and recooling the protein to 4°C, a partially unfolded metastable intermediate is obtained. Temperature scan experiments on a solution of this intermediate showed that the partial unfolding (so obtained) and the consequent exposure of buried groups to the solvent were responsible for the thermal aggregation of the protein through two different effects. The first one is to provide cross-linking sites at the molecular surface necessary for self-assembly. The second one is a thermodynamic contribution to the onset of solute–solute correlations due to the appearance of an instability region of the sol [12,13]. This last contribution was found to be responsible for the self-assembly of the protein at very low concentration. The same mechanism capable of leading the self-assembly at average concentration well below the threshold of random cross-links percolation has been observed in other proteins or polymers [12–20].

In the present work, we use static and dynamic light scattering, circular dichroism (CD) and differential scanning calorimetry (DSC) to characterize BSA intermediates and to study the interaction between native protein and intermediates. Results on a mixture of the two species show a decreased thermal stability in comparison with those of solutions containing only the native protein or the intermediate form.

2. Materials and methods

Pure (99%) crystallized BSA from fraction V was from Miles (81-001, Lot No. 94) and used without further purification. The samples were prepared in phosphate buffer 0.1 M (pH 6.2) as described in San Biagio et al. [12]. The partially unfolded BSA intermediates were obtained by heating a dilute 0.1% w/w solution of BSA at 67°C for 25 min and rapidly recooling the solution to 4°C. Higher concentrations were obtained using an AMICON ultrafiltration system with AMICON PM 10 membranes. The samples were prepared and stored in a cool room at 4°C. Static (SLS) and dynamic light scattering (DLS) measurements were done using a Brookhaven BI-30 AT 128-channel correlator with a 35-mW He-Ne Melles Griot laser and standard optical system. The intensity autocorrelation function measured in the case of a solution containing only native or intermediate BSA was analyzed using a single exponential [21] or cumulant analysis [22]. The two different methods gave the same diffusion coefficient (D_T). In the case of the mixture (native and intermediate BSA), data were fit to a double exponential [21], whose decay times allowed to obtain the diffusion coefficient of each

species in solution. Using the Stokes–Einstein relationship [21], an estimation of their molecular radii was obtained. The relative percent concentration in weight of the two species (native and intermediate) in the mixture was calculated from the relative ratio of the amplitudes of the two exponential functions. The shape of the BSA intermediate was determined by depolarized dynamic light scattering measurements analyzed as reported earlier [17]. Circular dichroism (CD) measurements were performed on a J-715 Jasco instrument. A differential scanning calorimeter (DSC) Micro 7707 (Harth Scientific) was used to measure the DSC curves.

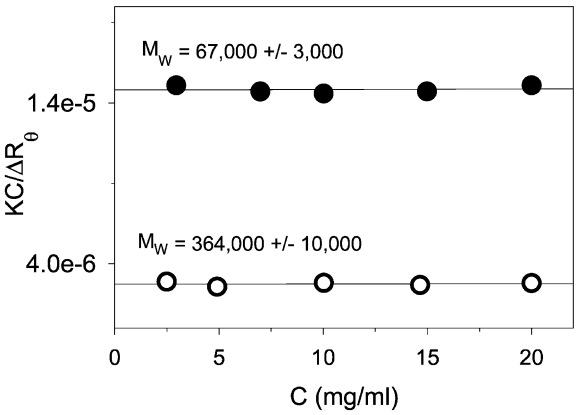

Fig. 1. The Debye plot of native (full circle) and intermediate (empty circle) BSA; $T = 25°C$; $\theta = 90°$.

3. Results and discussion

3.1. BSA intermediate

Static and dynamic light scattering and CD measurements were used to characterize the intermediate species. Its molecular weight was obtained by the dependence of the excess scattered light on protein concentration, through the following relationship based on Zimm's formalism of the Rayleigh–Debye–Gans light scattering model for dilute polymer solutions [21]:

$$\frac{K \cdot c}{\Delta R(\theta)} = \frac{1}{\mathrm{MW} \cdot P(\theta)} + 2 \cdot A_2 \cdot c \qquad (1)$$

where c is the sample concentration in g/ml, K is an optical parameter, $\Delta R(\theta)$ is the excess intensity of scattered light at angle θ, MW is the molecular weight of the macromolecule in solution, $P(\theta)$ is the particle scattering function and A_2 is the second virial coefficient. Since the molecular size of BSA, both in native or intermediate conformation, is small compared with the laser wavelength, $P(\theta) = 1$ for all angles. Fig. 1 shows the excess scattered light at 90° for native BSA (full circle) and intermediate (empty circle) solutions. The molecular weights of the two species derived from Eq. (1) are 67 ± 3 and 364 ± 10 kDa, respectively, suggesting that, following the partial unfolding of the protein, an oligomer of five or six molecules is formed.

An inspection of the species present in the solution was provided by DLS measurements. Data relative to an intermediates solution at 25°C and 2% concentration were analyzed using CONTIN [23] to obtain the size distribution of species. Results are shown in Fig. 2 together with those obtained for native protein at the same temperature and concentration. A cumulant analysis [22] of DLS data gives a polydispersity of 0.2 ± 0.05 and 0.08 ± 0.05 in the intermediate and native case, respectively. It has to be noted that a very monodisperse sample (like monodisperse latex spheres in solution) give consistently a polydispersity value less then 0.1. This appears to be the case of native protein solution. Instead, the polydispersity found in the case of the intermediate solution suggests the existence of a quite narrow distribution of small oligomers. Therefore, the molecular weight has to be taken as an average value. We recall that intermediate solutions at different concentrations are obtained by subsequent reconcentration at low temperature of a sample at 0.1% concentration quenched at 67°C for 25 minutes. As proved by our past work [20] the average size of intermediate is independent on final concentration, indicating that no further aggregation occurs. Solutions of intermediates are stable until they are brought close to or inside their thermodynamic instability region. Interest-

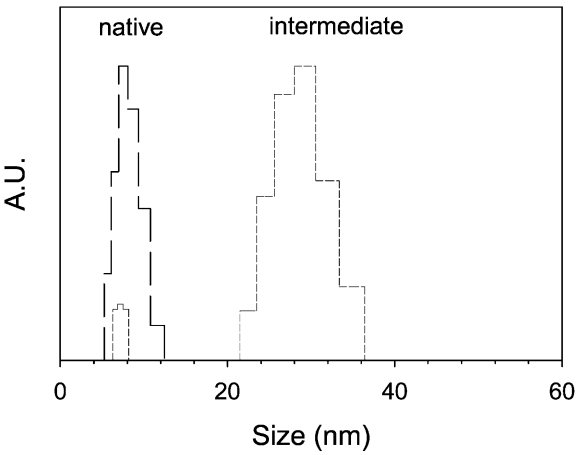

Fig. 2. CONTIN data analysis (intensity distribution) of dynamic light scattering data for native protein (long-dashed line) and intermediate (short-dashed line) at 2% w/w concentration; $T = 25°C$. $\theta = 90°$. In the intermediate case a little amount of native protein is also observed.

ingly, metastable oligomers have been observed in the case of β-lactoglobulin [24].

Depolarized dynamic light scattering experiments were also performed to obtain information on the molecular shape of intermediates. It is well known, in fact, that for optically asymmetric particles, the scattered light includes also a horizontally polarized component. When observing this horizontally polarized scattered light, the normalized field autocorrelation function can be written as [21,25]:

$$g^{(1)}_{VH}(q,t) = A \cdot e^{-\Gamma_{VH} \cdot t} \quad (2)$$

where $\Gamma_{VH} = 6D_R + D_T\ q^2$, D_T and D_R are the translational and rotational diffusion coefficients of the scatterer and $q = (4\pi n/\lambda) \sin(\theta/2)$ is the wave vector. In Fig. 3 we report the plot of Γ_{VH} vs. q^2 values. Values of D_T and D_R are determined from the slope and intercept ($q = 0$). Combined use of the two diffusion coefficients through Perrin's relations [25], enables us to determine that BSA intermediate has the shape of a prolate object with an axial ratio of approximately 0.8.

The structural properties of BSA intermediates were probed by CD measurements in the far UV range. CD spectra of the native protein at room temperature and after 25 min at 67°C are shown in Fig. 4. The observed change indicates a decrease of the ratio of α-helix/β-sheet components [26]. When the solution of intermediates is cooled to 20°C, the CD spectrum (not shown) does not change, confirming the stability of the intermediate conformation.

3.2. Mixture of intermediate and native form

Dynamic light scattering experiments were performed on a solution containing 1.5% w/w of native + 0.5% w/w of intermediate species. The 3:1 ratio was chosen to have comparable scattered light intensity from the two species under the hypothesis of no interaction between them. The size distribution of species in the mixture at 25°C is shown in Fig. 5 together with those of solution of native protein or intermediate alone (same data as in Fig. 1). The figure shows that, in the mixture, two different species are well resolved. The mean size of the small species corresponds to that observed for a solution of native protein, whereas that of the large species is

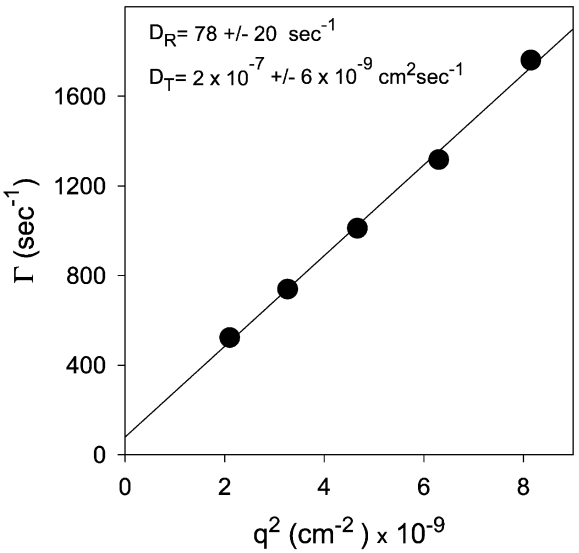

Fig. 3. Γ vs. q^2 for a depolarized dynamic light scattering experiment on a sample of intermediate BSA at 2% w/w concentration.

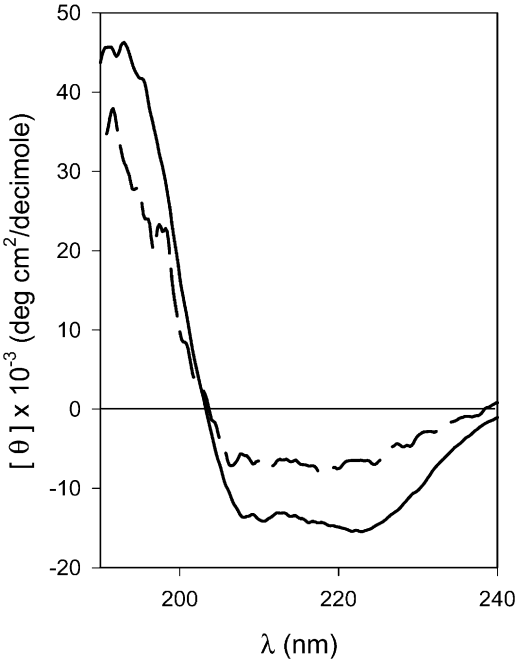

Fig. 4. Far-UV CD spectra of BSA at 0.01% w/w concentration. Continuous line: $T = 25$°C; broken line: $T = 67$°C.

larger than that of the intermediate alone in solution. This could be due to a conformational change of the intermediate or to its effective growth through aggregation with some molecule of native protein. (We note that the extent of size increase rules out the hypothesis of aggregation between intermediate molecules, which, however, are not seen to interact at room temperature in absence of native protein). To check the possible formation of higher oligomers by aggregation between intermediate and native protein, the autocorrelation function was fit to the double exponential form:

$$g_1(q,\tau) = A_S \exp(-D_S q^2 \tau) + A_L \exp(-D_L q^2 \tau) \quad (3)$$

where $A_{S,L}$ and $D_{S,L}$ are the amplitudes and diffusion coefficients of small and large species, respectively. The amplitude is proportional to the scattered intensity, which, in turn, is directly proportional to the weight-averaged molar mass multiplied by the weight concentration. The experimental value of the ratio A_L/A_S in the mixture was approximately 10% higher than that expected on the basis of the known concentration and

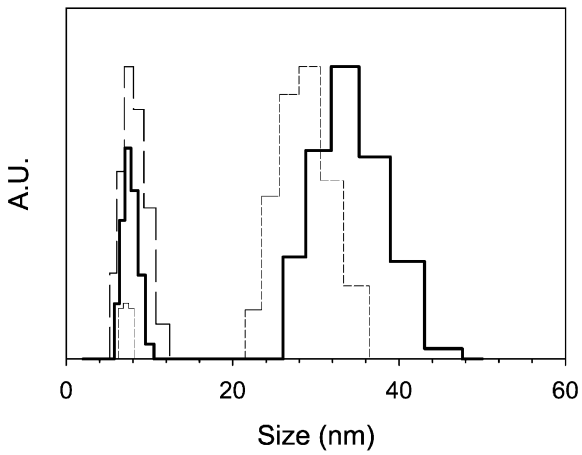

Fig. 5. CONTIN data analysis of dynamic light scattering data for a 3:1 mixture of native and intermediate BSA (continuous line). Concentrations of native and intermediate form are 1.5 and 0.5% w/w, respectively. The same data of Fig. 2 for native (long-dashed line) and intermediate (short-dashed line) are shown for comparison.

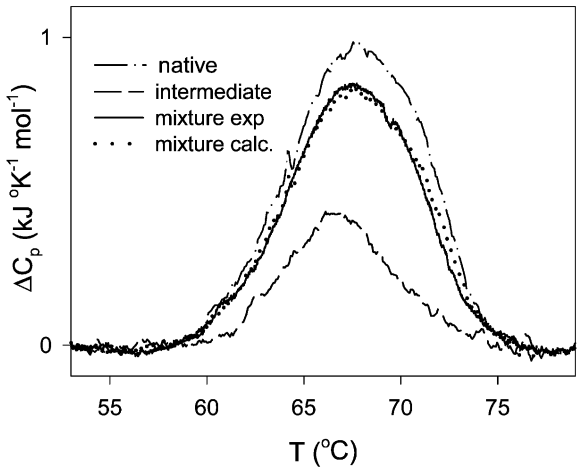

Fig. 6. Differential scanning calorimetry (background subtracted) signals for native (2.0% w/w long dashed), intermediate (2.0% w/w small-dashed) and mixture (1.5% w/w native + 0.5% w/w intermediate, continuous line). Dots represent theoretical values obtained combining the experimental native and intermediate curves in the 3:1 ratio.

molecular weight of each species. This difference could be accounted for by the aggregation of a single native protein molecule to each intermediate, but the effect is too small to be taken as concrete evidence that aggregation has occurred.

To investigate if interaction with the intermediate form can favor early unfolding of protein in native configuration, we performed some DSC experiments on solutions of native protein, intermediate, and a mixture of them. In Fig. 6 we report the DSC spectra obtained in the three cases. The short-dashed line is the DSC signal for a sample of 2% unfolded, the long-dashed line for 2% native and the continuous line is the signal for a mixture of 0.5% unfolded and 1.5% native BSA. The DSC signal from the unfolded solution is approximately a factor of 2.5 less than that from native BSA, indicating, in agreement with the CD signal, the partial unfolding of the intermediate protein. The dots in the figure represent the theoretical signal obtained by the *weighted-by-concentration* addition of the unfolded and native signal. We observe that the dots fit the continuous curve nearly perfectly, showing that the presence of unfolded BSA in solution does not favor the unfolding of native form.

An aliquot of the same sample was heated at a constant rate of 0.1°C/min from room temperature close to the denaturation temperature of the native protein (67–70°C [27] under our experimental conditions). The dynamic light scattering autocorrelation function was fit to a double exponential form and the molecular radii were obtained by the Stokes–Einstein relationship [21]. In the first trial all the four fitting parameters were left free to adjust. The radius of the small species (39 ± 2 Å) was found to correspond very closely to that measured for the protein in its native configuration (37 ± 2 Å) at all temperature values. The latter is in very good agreement with the value reported in the literature [28]. Furthermore, it remains constant with increasing temperature up to the denaturation temperature, just as observed in a solution of native protein [12,13] where thermal aggregation always follows denaturation [12]. Therefore, we chose to keep the ra-

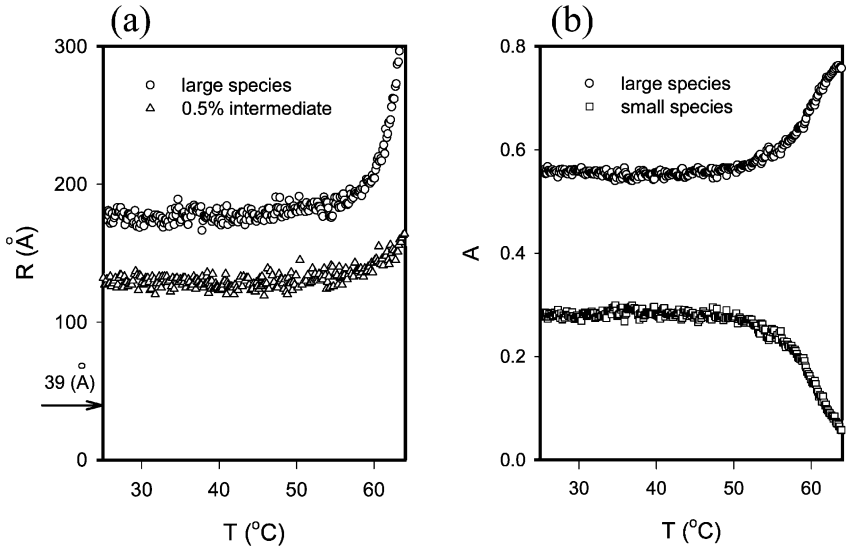

Fig. 7. Results of upward temperature scan (at 6°C/h) on a mixture of native and intermediate form of BSA. (a) Hydrodynamic radius measured by dynamic light scattering for the large species in the mixture (circles), as calculated from the autocorrelation function using double exponential analysis [21] and keeping constant (39 Å) the radius of the small species (see text). Data for intermediate alone in solution at $C = 0.5\%$ w/w (triangles) are shown for comparison. In this case the hydrodynamic radius was calculated by using a single exponential analysis. (b) Amplitudes of contributions of the large (circles) and small species (squares) to the autocorrelation function. The total amplitude is constant and equal to 0.84.

dius of the small species constant (39 Å) letting the diffusion coefficient of the small species change only for its dependence on temperature and solvent viscosity. In Fig. 7a the radius of the large species (circle) is reported as a function of temperature. In the same figure the radius of intermediate BSA (triangles) alone in solution at 0.5% concentration is also shown for comparison, while the radius of native BSA is indicated by the arrow. We observe that the size of the large species, starting from an initial higher value, begins to increase at a lower temperature. It has been shown in previous work [12,13] that the increase of the molecular radius observed in a solution of intermediates actually corresponds to the growth of lifetime of critical concentration fluctuations (also revealed by a diverging light scattered intensity) in approaching its thermodynamic instability region. Before discussing if in the present case the observed size increase should be attributed to concentration fluctuations or aggregation or both, it is appropriate to consider data relative to the contribution of the two species to the correlation function amplitude reported in Fig. 7b. We observe that, at temperature well below the unfolding threshold, the contribution of the large species starts to increase at the expense of the small one. To check if the ratio between the two populations is actually changing in favor of the larger one, we calculated the contribution of each species to the scattered light intensity. This contribution, in the hypothesis of observing the diffusion of two independent species of different molecular size can be derived from:

$$I_{i=1,2} = \frac{A_{i=1,2}}{A_1 + A_2} I \qquad (4)$$

In Fig. 8 the total light scattered intensity and the relative contributions of the two species are shown. We observe that the contribution of the small species remains constant up to the unfolding temperature, instead of decreasing as expected from a change in the balance of the two populations. This inconsistence can be recovered by considering that the total amplitude of the correlation function is normalized by dividing the total light scattered intensity. If the latter increases as a consequence of a contribution due only to one component of the solution, the amplitude of the other component is apparently reduced. Similarly to what is observed in the case of intermediate solution (also in the mixture), increasing concentration fluctuations at increasing temperature give rise to an increasing scattered intensity and their diverging lifetime displays in an increasing correlation time. The thermal stability of the large species in the mixture can be compared with that of solution of the intermediate alone, by looking at the temperature dependence of the contribution of the large species to the scattered intensity. This is obtained by subtracting from the total intensity the contribution of the native form (data of Fig. 8). Results are shown in Fig. 9, where the plots of the inverse of scattered light intensity evidence a lower critical temperature in the case of the mixture. It has to be remarked that the difference cannot be ascribed to an increase of intermediate concentration, because this should happen only at the expense of the native form. The latter, as seen above, does not come to a premature unfolding

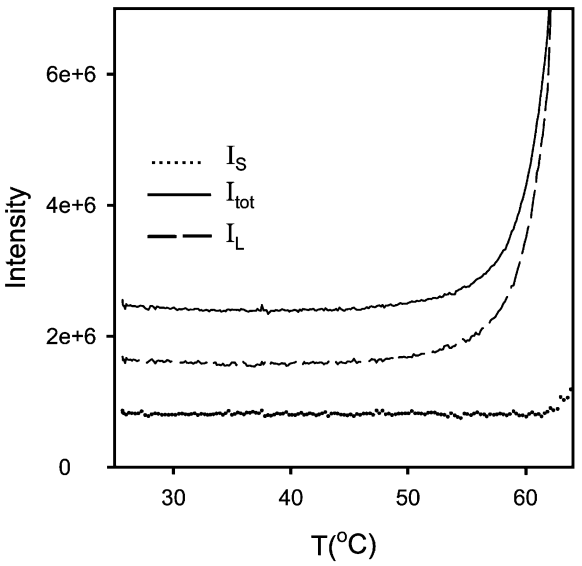

Fig. 8. Light scattered intensity in the course of temperature scan on the mixture. The total light scattered (solid line) is decomposed in the contributions of the large (dashed line) and the small species (dotted line) by using Eq. (4) in the text.

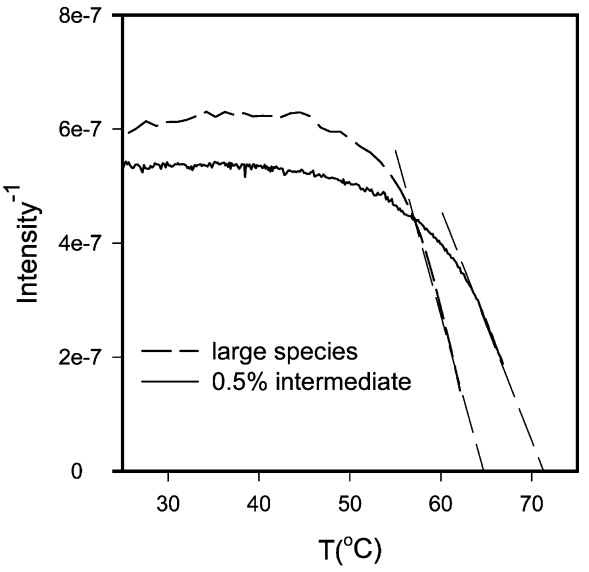

Fig. 9. Temperature dependence of the inverse of the scattered light intensity for the large species in solution (dashed line) as compared with that observed for a solution containing only intermediates (continuous line). In the frame of the mean field theory, the temperature value at which the intensity diverges is the spinodal temperature at the given concentration [30].

and, at the most, it aggregates on intermediates at a little percent, as could be suggested by the larger size of intermediate observed in the presence of the native form. Further work on isolated large species is in order to clarify if this size increase is due instead to a conformational change induced by the presence of the native form. Nevertheless the present results show that interaction between native protein and intermediate (either direct or solvent mediated) is responsible for a decreased thermal stability of the solution.

4. Conclusions

Results presented here suggest that protein aggregates in solution can favor the aggregation of the protein in its native form. Data are in agreement with the hypothesis of a 'protein only' mechanism being responsible for fiber deposit and consequent amyloidoses. The presence of an instability region (spinodal) can be taken into account for aggregation and growth of protein clusters. Interestingly, recent work by Urbanc et al. [29] has shown that the plaques formed in the brain of Alzheimer patients appear clustered in aggregates of different sizes as would be expected if a spinodal mechanism was responsible for their formation. The present results, although obtained in a simple and easily controlled system, can help to understand the role of unfolded intermediates in protein aggregation with implications in medicine, biotechnology and food production.

Acknowledgements

We thank Prof. J. Newman and Dr M. Manno for helpful discussions. We thank Prof. G. Paradossi for helping in the interpretation of CD spectra. We thank Dr D. Giacomazza for expert technical assistance. The technical assistance of Mr F. Giordano, Mr A. La Gattuta, Mr G. Lapis, Mr M. Lapis and Mr R. Megna is also acknowledged. Partial support comes from the 'Progetto Strategico Biosensori' of the National Research Council (CNR).

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