Self-Assembly of Biopolymeric Structures below the Threshold of Random Cross-Link Percolation

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ABSTRACT Self-assembly of extended structures via cross-linking of individual biomolecules often occurs in solutions at concentrations well below the estimated threshold for random cross-link percolation. This requires solute-solute correlations. Here we study bovine serum albumin. Its unfolding causes the appearance of an instability region of the sol, not observed for native bovine serum albumin. As a consequence, spinodal demixing of the sol is observed. The thermodynamic phase transition corresponding to this demixing is the determinative symmetry-breaking step allowing the subsequent occurrence of (correlated) cross-linking and its progress up to the topological phase transition of gelation. The occurrence of this sequence is of marked interest to theories of spontaneous symmetry-breaking leading to morphogenesis, as well as to percolation theories. The present results extend the validity of conclusions drawn from our previous studies of other systems, by showing in one single case, system features that we have hitherto observed separately in different systems. Time-resolved experimental observations of the present type also bring kinetic and diffusional processes and solute-solvent interactions into the picture of cross-link percolation.

INTRODUCTION

The gel state of matter is reached through the topological phase transition of intermolecular cross-link percolation (Axelos and Kolb, 1990; Coniglio et al., 1982; de Gennes, 1976, 1979; Essam, 1980; Kolb and Axelos, 1990; Martin and Adolf, 1991; Stauffer, 1979, 1981; Stauffer et al., 1982). Physical gels with relatively weak and reversible cross-links are often encountered in nature, where their loose order provides the necessary blend of structural and functional stability and flexibility. In the classical theory of gelation, thermodynamically driven cross-linking is made possible by the existence of "functionality sites" (Flory, 1941; Stockmayer, 1943). In the simplest percolative description, cross-linking occurs at random (Essam, 1980; Flory, 1941; Stauffer, 1979, 1981; Stockmayer, 1943) and gelation corresponds to cross-link percolation (Essam, 1980; Stauffer, 1979, 1981). Self-assembly of biopolymeric gels, however, often occurs at low concentrations (Burchard and Ross-Murphy, 1988; Clark and Ross-Murphy, 1987; Mitchell and Ledward, 1985; Russo, 1987), well below the estimated threshold for random percolation. This requires the existence of correlations among solutes, that is, a break of symmetry in the initially homogeneous sol. It is of high theoretical, biological, and practical interest to sort out under which circumstances and thermodynamic drives symmetry is broken.

In the present work we report time- and temperature-resolved observations of the gelation behavior of bovine serum albumin (BSA) aqueous solutions, mainly at low concentrations. Our results endorse and extend the validity of conclusions drawn from our previous studies (Bulone and San Biagio, 1991; Bulone et al., 1993; Emanuele and Palma-Vittorelli, 1992, 1995a,b; Emanuele et al., 1991, 1993; Leone et al., 1987; Palma-Vittorelli, 1989; Palma-Vittorelli et al., 1993; San Biagio and Palma, 1991, 1992; San Biagio et al., 1986, 1990, 1993; Sciortino et al., 1993) by showing in one single case features that we have hitherto observed separately in different systems. More specifically, they show that gelation at low to moderate concentrations is the result of a sequence of up to three processes of different nature, occurring on different and sometimes competing time scales. This brings kinetic and diffusional processes measurably into the picture of cross-link percolation.

EXPERIMENTS AND DISCUSSION

Pure (99%) crystallized BSA from fraction V was purchased from Miles Diagnostics (code no. 81-001, lot no. 91) and used without further treatment. Solutions were prepared by dissolving the solid material at room temperature in Millipore water, buffered at pH 6.2 (0.1 M phosphate buffer). Samples were centrifuged at 15,000 × g for 10 min and filtered through 0.22 μm filters directly in the sample holder. Experimental apparatuses were as already described (Emanuele et al., 1991; San Biagio and Palma, 1991; San Biagio et al., 1986). Dynamic light scattering and static light scattering were measured using a Brookhaven BI-30AT 128-channel correlator with a 30-mW He-Ne Spectra Physics laser. We also performed measurements of optical rotation (Jasco DIP 370 polarimeter), differential scanning calorimetry (DSC) (Micro 7707, Hart Scientific), and low-shear viscosity and viscoelasticity (Contraves low-shear 30 viscometer, computer interfaced).

Received for publication 21 March 1995 and in final form 12 September 1995.

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0006-3495/96/01/494/06 $2.00

BSA gelation occurs upon heating (so-called inverse temperature, or entropy-driven, gelation). Available DSC data had already shown evidence that upon heating, protein unfolding comes first and gelation follows (Barone et al., 1992, and manuscript in preparation; Clark and Ross-Murphy, 1987; Mitchell and Ledward, 1985). A first set of experiments was aimed at a better understanding of this unfolding-gelation sequence. Aliquots of the same sample preparation were identically treated, used in different instruments, and subjected to identical heating, at a rate of 0.05°C/min. Several quantities were simultaneously measured in this way. In Fig. 1 we show for 1% w/v solutions of native BSA, DSC data confirming the already known behavior, along with present data of scattered light intensity (I/I_o), optical rotation ($-\Delta\alpha$), shear viscosity (η), and complex viscosity $\eta^* = (\eta'^2 + \eta''^2)^{1/2}$ (Ferry, 1980). It is evident that the endothermic DSC peak attributed to BSA unfolding (Barone et al., 1992, and manuscript in preparation; Clark and Ross-Murphy, 1987; Mitchell and Ledward, 1985) is associated to an optical rotation signal. Later in the scanning, the simultaneous sharp rises of η and η^* clearly reveal that the solution of the already unfolded protein undergoes gelation, in accord with the observation that when the sample has reached 77°C, it is already a clear, self-supporting gel.

The sharp rise of scattered light intensity in Fig. 1 actually occurs after BSA unfolding, and markedly precedes gelation. This suggests that interactions among BSA molecules responsible for the sharp rise of I/I_o must be a consequence of changes (due to unfolding) of the exposed parts of the protein. More precisely, the new system (that is, the solution of unfolded BSA) could be in a state close to its region of thermodynamic instability, and the I/I_o signal could be due either to diverging concentration fluctuations or to some rapid and remarkable aggregation (but not yet gelation). The flat behavior of η and η^* that accompanies the I/I_o rise in Fig. 1 does not support (even if does not need to contradict) the aggregation hypothesis. Instead, the fluctuation divergence hypothesis is in full accord with the behavior that we have observed in other systems (Bulone and San Biagio, 1991; Bulone et al., 1993; Emanuele and Palma-Vittorelli, 1992, 1995a,b; Emanuele et al., 1991; Leone et al., 1987; Palma-Vittorelli, 1989; Palma-Vittorelli et al., 1993; San Biagio and Palma, 1991, 1992; San Biagio et al., 1986, 1990, 1993; Sciortino et al., 1993). Indeed, unfolding (as revealed by DSC and optical rotation) alters solute-solute and solute-solvent interactions, as well as the protein shape and conformational flexibility. Consequently, the position and shape of the thermodynamic instability region and encompassing spinodal line will largely differ from those of solutions of native BSA, whose proximity is not even hinted at by our data.* Another set of experiments was then performed, to subject the spinodal line hypothesis to experimental test.

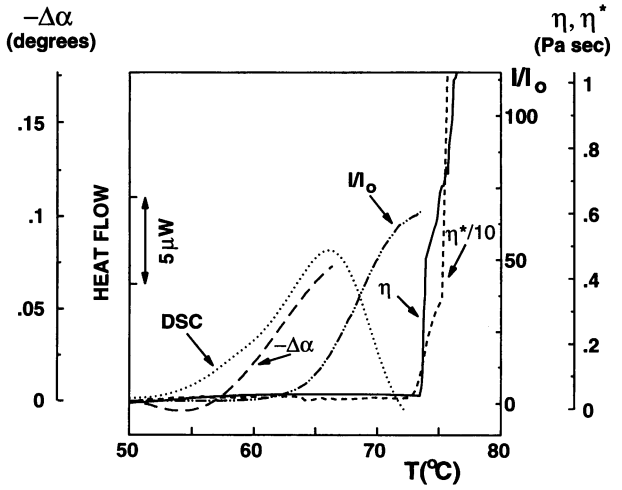

FIGURE 1 DSC, optical rotation ($\Delta\alpha$), scattered light intensity (I/I_o), low-shear viscosity (η), and complex viscosity ($\eta^* = (\eta'^2 + \eta''^2)^{1/2}$) relative to a 1% w/v ($\Phi$ = 0.73% v/v) solution of native BSA, measured in the course of heating at a rate of 0.05°C/min. Note that the DSC curve is attributed to protein unfolding so that the accompanying $\Delta\alpha$ signal also monitors unfolding, whereas processes monitored by the I/I_o, η, and η^* curves start when the specimen is a sol of already unfolded BSA.

Dilute (0.1% w/v) solutions of BSA, unfolded under the same conditions of the previous experiments to ensure the same final conformation of the protein, were preliminarily obtained by heating to 67°C, as suggested by DSC data in Fig. 1. At this concentration, unfolding could be monitored by measuring the protein hydrodynamic radius by dynamic light scattering (Madonia et al., 1983), and it was observed to be essentially complete in 25 min. Specimens were then recooled to 4°C. In these conditions and even at room temperature and above, the constancy of the hydrodynamic radius up to periods of 24 h or more suggested that a fortiori in the much shorter times needed for our experiments, no change in the conformation of unfolded BSA occurs. This conclusion and the overall correctness of our procedure are fully corroborated by additional data, to be presented and discussed with reference to Fig. 2 below. Higher concentrations were obtained using an AMICON ultrafiltration system at 4 °C with AMICON PM 10 membranes.

The hypothesis of the vicinity of a region of instability (and encompassing spinodal line) of solutions of unfolded BSA was then tested by the fluctuations divergence method (Scholte, 1971). The latter is based on the fact that when the temperature of thermodynamic instability T_{SP} at a given concentration is approached, the amplitude and size of concentration fluctuations are expected to diverge critically. When a mean-field approximation holds, the scattered light diverges as $[(T - T_{SP})/T_{SP}]^{-1}$, and the average geometric

*It must be remarked that in the present case there is no need to address the complex problem of protein unfolding (Chan and Dill, 1993; Dill and Shortle, 1991) or to know in detail the new conformation(s). What is relevant to the present discussion is the clear evidence for the occurrence of some kind of stable unfolded state (or population of states), entailing marked changes in protein interactions. Results to be discussed in the following will show the existence of unfolded conformation(s) that are not appreciably polydisperse.

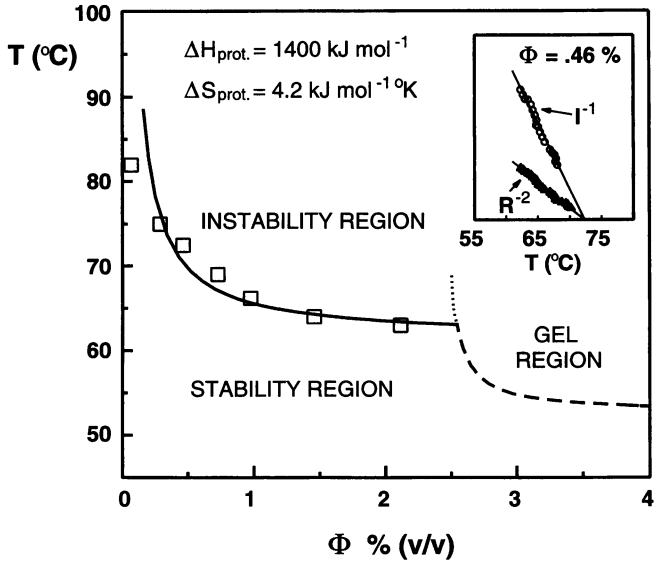

FIGURE 2 Phase diagram of solutions of BSA pre-unfolded as explained in the text. Data points are obtained by the fluctuation-divergence method (Scholte, 1971), and the quality of measurements is shown in the inset. The spinodal line was obtained by Flory-Huggins best-fitting. Corresponding best-fit values of Flory-Huggins ΔH and ΔS per (unfolded) molecule of BSA are also shown in figure.

size of fluctuations (measurable by dynamic light scattering) diverges as $[(T - T_{SP})/T_{SP}]^{-1/2}$ (de Gennes, 1979; Scholte, 1971). It follows that in this case plots of I^{-1} and R^{-2} versus temperature (where R is the fluctuations size) will be linear and will extrapolate to the same $T = T_{SP}$ value on the temperature axis (San Biagio and Palma, 1991, 1992; Sciortino et al., 1993). The inset of Fig. 2 shows that here, as in other cases (Bulone et al., 1993; San Biagio and Palma, 1991, 1992; Sciortino et al., 1993), critical divergences follow mean-field exponents. This allowed us to meaningfully use Scholte's method. In this way the spinodal temperature at any chosen concentration of the solution can be determined, and spinodal lines can be obtained by best fits of data points in terms of the Flory-Huggins expression (de Gennes, 1979; Kurata, 1982). Such fits also provide numerical values of Flory-Huggins χ parameter, which here contains an entropy and an enthalpy term (San Biagio and Palma, 1991, 1992; Sciortino et al., 1993) and quantitatively compares solute-solute and solute-solvent interactions. For unfolded BSA solutions the experimental points and best-fitting spinodal line are shown in Fig. 2. A value $p = 9$ was chosen for the Flory-Huggins parameter measuring the orientational correlation of individual segments in the proteic chain (de Gennes, 1979; Kurata, 1982; Sciortino et al., 1993). However, fittings based on different p values gave very close Flory-Huggins entropies and enthalpies referred to the entire protein, although, of course, they gave distinctly different entropies and enthalpies on a "per site" basis. Actual values referred to the entire protein were found to be $\Delta H = 1400$ kJ mol^{-1} and $\Delta S = 4.2$ kJ mol^{-1} °K^{-1}, within 10%. The direct gelation line also shown in the figure was determined by viscoelastic measurements and it is accurate to within a few degrees. This is fully adequate to the present purpose. Its more accurate determination will need further experiments.

From Figs. 1 and 2 the rationale of our preparation of solutions of unfolded BSA is now clear: i) We start with a low-concentration solution ($C = 0.1\%$ w/v, $\Phi = 0.073\%$ v/v) and let BSA unfold rapidly at 67°C. At this concentration, the solution of unfolded protein does not undergo any demixing because it remains in its stability region. ii) We subsequently decrease the temperature to 4°C. At this temperature, solutions of unfolded BSA are in their stability region at all concentrations that we use. The concentration may therefore be raised to any wanted value with no fear of demixing. In these conditions, the absence of complications due, for example, to re-folding or other changes is evidenced not only by the absence of changes of the hydrodynamic radius mentioned above, but also by the independence of the spinodal line in Fig. 2 and related Flory-Huggins best-fitting parameters of the unfolded BSA solutions for the storage time (up to 24 h) at 4°C.

This procedure allowed us to perform further quenching experiments to ascertain the actual sequence of steps leading to gelation of pre-unfolded BSA in the $0.1\% \leq C \leq 3\%$ w/v. A typical set of results, relative to $C = 1\%$ w/v and quenching temperature $T = 70$°C, is shown in Fig. 3. Here we see typical "signatures" of spinodal demixing (Cahn, 1965; Cahn and Hilliard, 1958; Emanuele et al., 1991) such as the initial exponential growth of scattered light (Fig. 3 *a*), the emergence of a maximum in the structure function that shifts in time toward lower q values (Fig. 3 *b*), and the linear Cahn plot of the rate of growth of scattered light (Fig. 3 *c*). From Fig. 3 *c* the characteristic length q_m can be derived (Cahn and Hilliard, 1958), and we obtain in this case $q_m \approx$ 192,000 cm^{-1}, in agreement with the position of the maximum in Fig. 3 *b*. Further support in favor of the occurrence and ripening of a spinodal demixing (which triggers the occurrence of gelation of unfolded BSA) comes from the inset of Fig. 3 *b*, which shows that all I/I_{Max} versus q/q_{Max} data fall on one and same master curve, as expected for demixing systems (Guenoun et al., 1987). Only after about 600 min from the start of spinodal demixing, gelation becomes observable, as shown by the large rise of η^* (Fig. 3 *d*). Consequently, our observations of spinodal demixing can conservatively be taken as meaningful at least up to 500 min (Fig. 3 *b*).

The following should be kept in mind for what concerns Fig. 3. Data in Fig. 3 *a* were obtained by continuous recording of the scattered intensity at constant angle. Data in Fig. 3 *b* were obtained by rapidly and automatically positioning the photomultiplier along a sequence of 13 different angles between 20 and 140° (this large angular span did not allow, of course, parallel recording by a videocamera). The photomultiplier was kept in each position for 2 s and the entire scan took 90 s. The length of these measurement did not distort to an appreciable extent the data in Fig. 3 *b* (except for the first two curves, which are not very rele-

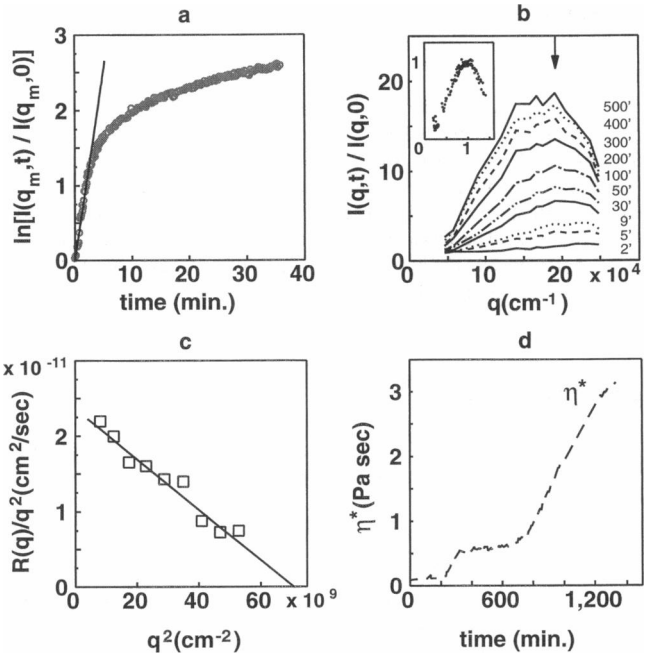

FIGURE 3 Typical results of quenchings of pre-unfolded BSA solutions in the region of thermodynamic instability of Fig. 2. In this specific case C = 1% w/v (Φ = 0.73% v/v), and the solution was quenched from 4°C to 70°C. Typical "signatures" of spinodal demixing: (*a*) initial exponential growth of light scattered at the $q = q_m$ value corresponding to the maximum value of $I(q,t)$; (*b*) emergence of a maximum in the structure function of the sol, shifting in time toward lower q values, as observed by scattering of laser light (= 632 nm). The inset shows the I/I_{max} versus q/q_{max} plot relative to all data points, which are seen to fall on one and the same "master curve." The scaled data in the inset for $q < q_{max}$ showed the expected $I \propto q^2$ scaling. The arrow marks the position of the maximum as computed from data in *c*; (*c*) linear Cahn plot of the rate of growth of scattered light; (*d*) time dependence of η^*, showing that only at times beyond about 600 min does gelation start.

vant), as shown by runs operated starting from either extreme of angular interval. Data in Fig. 3 *c* were derived from a set of data of the type of Fig. 3 *a*, obtained from aliquots of the same specimen, identically treated.

Quenchings of solutions to other points of the instability region in the T,c plane for $c < \sim 3.5\%$ w/v (corresponding to $\Phi < \sim 2.6\%$ v/v) cause the occurrence of the same sequence of events, that is: i) demixing of the sol as such; ii) self-assembly of the gel, presumably starting in protein-rich regions generated by demixing and ultimately enabling full percolation of cross-links (Bulone and San Biagio, 1991; Emanuele et al., 1991; Leone et al., 1987; San Biagio and Palma, 1991, 1992; San Biagio et al., 1990; Sciortino et al., 1993). At very low concentrations and appropriately high quenching temperature (see Fig. 2), the regions where polymers cluster as a consequence of the spinodal mechanism will be mutually disconnected (Hayward et al., 1987). This notwithstanding, gelation has been observed in similar low-concentration cases to initiate and proceed up to completion within mesoscopic regions, which remain freely drifting while their internal gelation proceeds to completion (Bulone and San Biagio, 1991). At concentrations that are not extremely low, these free-drifting properties in the course of internal gelation may favor (via collisions and sticking) the additive accretion of regions resulting from demixing, and the consequent occurrence of truly macroscopic gelation. This shows that the growth with time of high-concentration regions in cases (as the present one) where spinodal demixing prompts and is followed by cross-linking/gelation, does not need to follow exactly (beyond a certain time) the pattern relative to pure spinodal demixing.

Finally, it could be thought that because the diagram of Fig. 2 refers to unfolded BSA, it is of no use in understanding the gelation behavior of native BSA solutions. However, even if our experiments of gelation upon heating start with the native protein, the appropriate phase diagram above 62–67°C is always that of Fig. 2, because at those temperatures native BSA rapidly unfolds. Accordingly, and depending on kinetic competition between unfolding, demixing, and gelation (that is, upon the chosen temperature), processes very similar to those just described can be and have indeed been observed.

CONCLUSIONS

This work shows that BSA conformational changes and consequent changes of protein groups exposed to solvent have two distinct effects. One is to provide cross-linking sites, that is, the appropriate microscopically broken symmetry at the molecular surface. The existence of cross-linking sites is necessary for gel self-assembly. However, it is not sufficient for the occurrence of gelation at concentrations below the threshold for random cross-link percolation. The other effect (which is responsible for the process that precedes gelation and prompts nonrandom cross-linking) is a crucial thermodynamic contribution to the onset of solute-solute correlations, corresponding to a symmetry break at a coarser (mesoscopic) level. This contribution is explicitly and quantitatively measurable via the Flory-Huggins parameter, and it expresses the role of solute-solvent interactions in the sequence of processes leading to gelation. The mesoscopic symmetry break is due to concentration fluctuations showing either anomalously large amplitudes and lifetimes (diverging critically in the proximity of the instability region), or a self-sustained character leading to actual demixing (within the instability region). The two cases have been observed separately (Emanuele et al., 1991; San Biagio and Palma, 1991; San Biagio et al., 1986, 1990; Sciortino et al., 1993). In the present work we have observed in sequence both types of fluctuations, that is: i) those showing the critical mean-field divergence illustrated in the inset of Fig. 2 (which has allowed us to determine the spinodal line and related Flory-Huggins ΔH and ΔS) and ii) those going through a selective amplification, so causing spinodal demixing on the mesoscopic scale. The latter corresponds to a proper phase transition of the sol as such. At low concentrations this thermodynamic phase transition and related symmetry break of the system promote the topological

phase transition of cross-link percolation. In this case, cross-linking does not occur at random, being largely (if not strictly) channeled through the higher-concentration regions. It may be of interest to remark that many self-assembly and morphogenetic processes in nature occur at similarly low initial concentrations of involved molecules.

The observed double role of conformation and symmetry changes at the molecular level agrees with results of molecular dynamics simulations, which provide further evidence of the dependence of morphologies resulting from cross-linking upon the type and range of action of cross-linking sites (Brugé et al., 1991, 1992).

It must nevertheless be remembered that these and other interesting simulation results (Hayward et al., 1987) refer to situations where demixing and cross-linking are indistinguishable and correspond to one and the same process and kinetics. In the present work, instead, the two processes correspond to very different free energies and kinetics, as already observed in other cases (Bulone and San Biagio, 1991; Emanuele and Palma-Vittorelli, 1992, 1995a,b; Emanuele et al., 1991; Leone et al., 1987; San Biagio et al., 1986, 1990). Their sequence brings kinetic and diffusional processes measurably into the picture of gelation. Furthermore, it can be surmised that it is not strictly necessary for high-concentration regions resulting from spinodal demixing and providing channeled (nonrandom) percolation to constitute from the very beginning a macroscopic percolative pattern. This is because (as seen at the end of the preceding section) diffusional and kinetic processes may concur in establishing/reinforcing the macroscopic percolative pattern of cross-links bridging mesoscopic regions in the course of their individual gelation (Bulone and San Biagio, 1991). This covers an area of marked theoretical interest because such aspects, and the consequent time/temperature/concentration interplay evidenced here as well as in our previous work (Bulone and San Biagio, 1991; Emanuele and Palma-Vittorelli, 1992, 1995a,b; Emanuele et al., 1991, 1993; Leone et al., 1987; Palma-Vittorelli, 1989; Palma-Vittorelli et al., 1993; San Biagio et al., 1986, 1990, 1993), have still to be taken into account in current theories (Coniglio et al., 1982; Stauffer and Aharony, 1991; Stauffer et al., 1982). It also covers an extensive area of biological interest (Emanuele et al., 1991), because most biological self-assembly processes occur kinetically in the course of biosynthesis, rather than at fixed initial concentrations.

We thank Prof. N. Kurti and Mr. H. This-Benckhard for having stimulated our attention to the thermodynamics of albumin solutions, on the occasion of the First International Workshop on Molecular Gastronomy (Erice, Italy, 1992). Indeed, the present work is also relevant to our understanding of the statistical physics and thermodynamics underlying the making and the texture of so-called ricotta cheese, whose starting material is largely the solution of lactoalbumin remaining after the coagulation of casein. We wish to thank many local shepherds for gracious discussions on the temperature versus time profile used in the course of their work by traditional wisdom, for optimizing under various circumstances the yield and texture of the final product (to be published). Finally, we are indebted to Prof. M. B. Palma-Vittorelli and Prof. S. L. Fornili for discussions and a critical reading of the manuscript, to Prof. G. Barone for discussions and communication of unpublished data, and to Dr. D. Giacomazza for data elaboration.

The present work was done at IAIF-CNR and University Laboratories, with partial support from the Italian "Ministero per l'Università e per la Ricerca Scientifica e Tecnologica" MURST (local fundings) and from the Sicilian "Comitato Regionale Ricerche Nucleari e Struttura della Materia." The authors participate in the European Community Human Capital and Mobility Network "The Dynamics of Protein Structures," whose stimulus is also acknowledged. The present work is part of a research effort currently supported by INFM/MURST within the European Community Regional Development Program.

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