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Experimental evidence for a two-step process in the aggregation of β -lactoglobulin at pH 7

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RESUME

Un mécanisme en deux étapes pour l'agrégation de la β -lactoglobuline à pH7 a été proposé dans la littérature. La première étape réactionnelle est difficile à caractériser, dans la mesure où les particules formées sont de petite taille et s'agrègent rapidement. Cette étude fournit les preuves expérimentales de l'existence de deux étapes dans le processus d'agrégation, en se basant sur des expériences de diffusion du rayonnement et de chromatographie d'exclusion stérique. L'influence de la force ionique, de la concentration en protéine et de la température de chauffe est discutée. Nos résultats montrent que la première étape aboutit à la formation de petits agrégats globulaires, dont la taille est indépendante, ou seulement faiblement influencée par les paramètres pré-cités.

ABSTRACT

A two-step mechanism for β -lactoglobulin aggregation at pH7 has been reported in the literature. The first aggregation step is difficult to characterise, as small particles are formed with a strong tendency to further associate in bigger aggregates. Experimental evidence for a two-step aggregation process, using scattering techniques and size exclusion chromatography, is shown here. The effect of the ionic strength, the protein concentration and the heating temperature is discussed. The results show that the first step consists in the formation of small globular aggregates, whose size are only weakly, if at all, influenced by the parameters mentioned above.

KEYWORDS : globular protein ; β -lactoglobulin ; aggregation ; scattering techniques; size-exclusion chromatography.

INTRODUCTION

β -Lactoglobulin is a globular whey protein with a radius of about 2.0 nm and a molar mass of $18400 \text{ g}\cdot\text{mol}^{-1}$. The protein contains two disulphide bonds and one free sulphhydryl group [1]. In the native state it forms dimers in aqueous solution to an extent which depends on the pH, ionic strength, concentration and temperature. The effect of ionic strength and temperature on the monomer-dimer equilibrium has been reported elsewhere [2]. With increasing temperature, the equilibrium is displaced towards monomers. When the temperature is increased above about 50°C the proteins partially denature and aggregate [1-4]. The kinetics of the aggregation are strongly dependent on the temperature and concentration. We have reported elsewhere the results of a detailed static and dynamic light scattering study of the aggregates formed at pH7 with 0.1M added salt [5].

The idea of a two-step mechanism for β -Lactoglobulin at neutral pH has first been reported in the sixties : small aggregates, formed by interchange of disulphide bonds have been observed with sedimentation measurements [6-7]. These small particles can then associate in bigger aggregates by physical interactions. More recently, scattering techniques have been used to characterise the two-step model [5,8-12]. By studying the polydispersity index measured by dynamic light scattering for the onset of β -Lactoglobulin aggregation vs. pH, ionic strength and temperature, Griffin et al. [8-9] proposed the formation of small rod-like particles. Roefs and De Kruif [10] modelled the aggregation kinetics obtained in low salt conditions in terms of a radical polymerisation involving an initiation, a propagation and a termination step. The size of the protein polymer particles is predicted to be proportional to the square root of the initial β -lactoglobulin concentration.

We have studied the static structure factor of aggregates formed at pH7 over a wide range of scattering wave vectors q by the combination of small angle neutron scattering (SANS) and static light scattering (SLS) experiments [5,11,12]. Some of the results will be discussed here. The main discrepancies between our results and those mentioned above deal with the shape of the particles formed in the first step and with the influence of concentration, ionic strength and temperature on their size. Here, we present experimental evidence that the particle size is only weakly, if at all, influenced by these parameters.

MATERIALS AND METHODS

Sample preparation.

β -lactoglobulin has been obtained from Sigma (ref. L.0130, batch 98F8030) and is a mixture of the genetic variants A and B. The ionic strength was set by addition of sodium chloride. In all conditions 200 ppm NaN_3 has been added to avoid bacterial growth. The solutions were dialysed against the solvent and filtered through 0.1 μm pore size Anotop filters into glass vials. For the neutron scattering experiments, the solutions were prepared by directly dissolving salt-free protein and the required amount of salt in D_2O . Concentrations were measured by UV spectrometry at 280 nm and expressed in percentage weight per volume.

The samples were covered with paraffin oil to prevent evaporation and heated in a thermostated bath at temperatures between $70.0 \pm 0.2^\circ\text{C}$ and $90.0 \pm 0.2^\circ\text{C}$ for different periods of time. The aggregation is quenched by rapid cooling to room temperature. Except when otherwise indicated, light scattering experiments are performed on highly diluted samples so that effects of interaction and multiple scattering are suppressed. Raw scattering data were corrected by subtracting the contribution of non aggregated proteins. The amount of non aggregated monomers has been determined by SEC. The dilute samples remain stable at room temperature over a period of months without any detectable further aggregation or break-up of aggregates. Some measurements have been carried out on samples during the aggregation. These measurements are limited to the early stages of the aggregation process because the samples become opaque as the aggregation advances.

Experimental techniques.

SLS and SANS can be used to determine the static structure factor $S(q)$ which is proportional to the scattering intensity. At infinite dilution, the measured static structure factor can be used to deduce a number of structural parameters [13]. The weight average molar mass, M_w , (or the aggregation number, m_w) and the z-average radius of gyration, $\langle R_g \rangle_z$, can be derived from a so called Guinier analysis for $q \langle R_g \rangle_z < 1$. For not too polydisperse self-similar structures the fractal dimension df can be defined by : $M_w \propto \langle R_g \rangle_z^{df}$. In this case, $S(q) \propto (q \langle R_g \rangle_z)^{-df}$ for $q \langle R_g \rangle_z \gg 1 \gg qR_0$, where R_0 is the size of the elementary unit of the fractal structure.

Dynamic light scattering (DLS) on very dilute solutions can be used to determine the z-average diffusion coefficient, $\langle D \rangle_z$, of the particles if $q \langle R_g \rangle_z < 1$,

which in turn can be related to the z-average hydrodynamic radius, $\langle R_s \rangle_z$, via the so called Stokes-Einstein relation [14]:

$$\langle R_s \rangle_z = \frac{k_B T}{6\pi\eta \langle D \rangle_z} \quad (1)$$

LS measurements have been performed with a Spectra Physics argon ion laser operating with vertically polarised light with wave length $\lambda=488\text{nm}$. The range of wave vectors covered is $3.5 \times 10^{-2} > q > 3.0 \times 10^{-3} \text{ nm}^{-1}$ with $q=(4\pi n_s/\lambda)\sin(\theta/2)$, n_s and θ being the solvent refractive index and the angle of observation respectively. The temperature is controlled by a thermostated bath and was set at 20°C . Toluene has been used as a reference in the LS measurements with a value of $4.0 \cdot 10^{-5} \text{ cm}^{-1}$ for the Rayleigh factor at 488nm . 0.179 was taken as refractive index increment on the basis of previous results [2]. SANS experiments were performed in Saclay (France) and in the Rutherford Laboratory (UK). The range of wave vectors covered is $2 > q > 0.07 \text{ nm}^{-1}$. SEC measurements were carried out with columns adapted to low masses (Du Pont, Zorbax bio series GF 450) so that small aggregates are separated from the native proteins.

RESULTS :

Figure 1 shows the q-dependence of the scattering intensity of aggregates formed in D_2O at $\text{pD}7-0.1\text{M}$ at different concentrations and heating conditions. The scattering intensity of the aggregates is corrected by the scattering of residual monomers as described elsewhere [4]. The corrected scattering intensity is normalised by the intensity scattered by the native monomers, so that the extrapolation at $q \rightarrow 0$ directly provides for the weight average aggregation number m_w . The intensity scattered by the native proteins, which in these conditions are mainly dimers i.e. double spheres [2], is also shown for comparison.

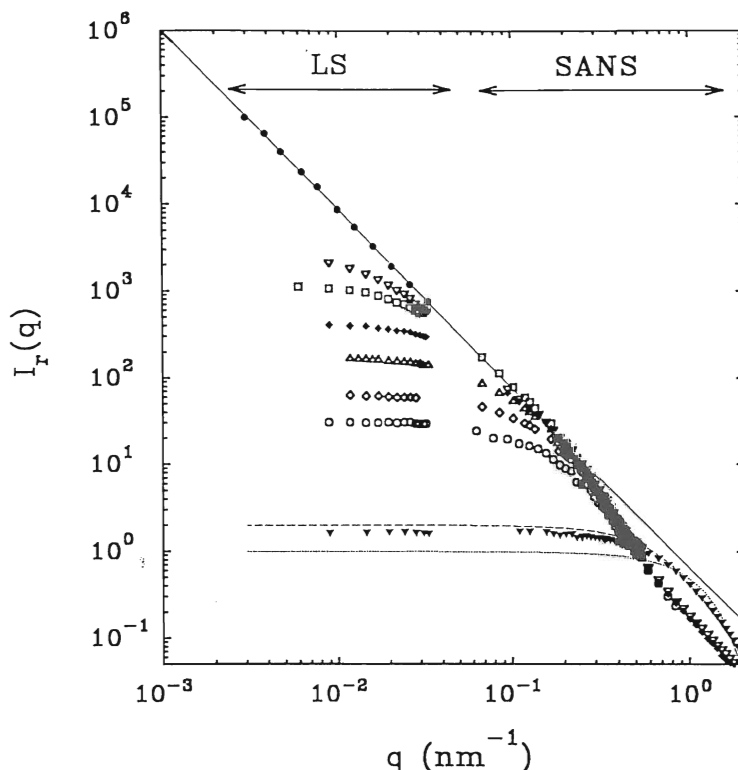


Figure 1 : Relative scattering intensity $I_r(q)$ as a function of the scattering vector for different concentrations of β -lactoglobulin and heating conditions in D_2O (pD7-0.1M) : (∇) 3% native solution; (\circ) 3%-70°C-0h10; (\diamond) 1%-70°C-0h40; (Δ) 1%-76°C-0h10; (\blacklozenge) 3%-70°C-2h; (\square) 1%-76°C-0h30; (∇) 3%-70°C-8h; (\bullet) 1%-70°C-61h. The solid line represents a linear least squares fit of the data at 1%-70°C-61h. The dotted line is the theoretical form factor of a homogeneous sphere of radius 1.85 nm and the dashed line the one of 2 tangent homogeneous spheres of radius 1.85 nm each.

The straight lines observed at $q < 0.1 \text{ nm}^{-1}$ imply that the aggregates have a fractal structure at distances larger than about 10 nm. The fractal structure seems independent of concentration and heating conditions in the range studied [5]. The fractal dimension obtained from the slope is equal to 2.05 ± 0.02 and the same slope has been measured in H_2O . The internal cut-off for the fractal regime ($q^{-1} = 10 \text{ nm}$) corresponds to $m_w \cong 60$. The larger elementary unit of the fractal structure suggests a two-step mechanism.

Further evidence comes from a study of the effect of electrostatic interactions. At low ionic strength (pD7-0.003M), only small globular aggregates are formed, see figure 2.

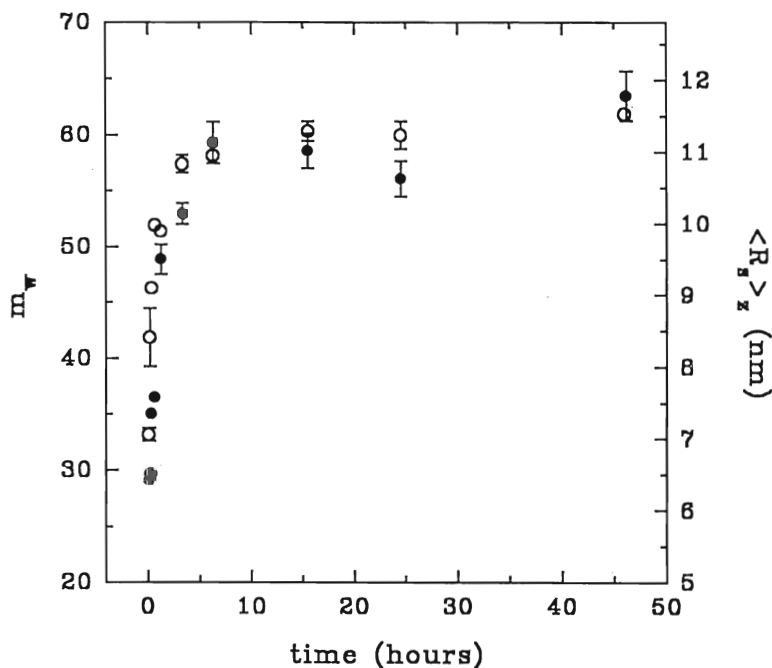


Figure 2 : Evolution of m_w , (●), and $\langle R_s \rangle_z$, (○), as a function of time of β -lactoglobulin aggregates (pD7-0.003M-4%-76°C). The experiments were performed on highly diluted samples in H₂O at 20°C.

$\langle R_s \rangle_z$ and m_w reach a plateau value with only a very slow increase with further heating: $\langle R_s \rangle_z = 11.5 \pm 0.5$ nm and $m_w = 60 \pm 5$. The plateau values for hydrodynamic radius and the weight average aggregation number are in agreement with the estimation made for pH7-0.1M aggregates (figure 1).

SANS experiments, carried out on samples corresponding to the plateau value (76°C-46h), clearly show that these aggregates have the same local structure as the larger ones formed with 0.1M added salt, see figure 3. At low ionic strength $S(q)$ shows a peak due to repulsive interactions. The relative light scattering intensity extrapolated to $q=0$ and at infinite dilution, is indicated in figure 3. It is clear that interactions remain important even at 1%. Unfortunately, it was not possible to

measure the neutron scattering intensity accurately at even lower concentrations. However, the structure factor at large values of q is not influenced by interactions. We previously showed that we can recover the fractal structure factor by dividing the structure factor of the large aggregates by that of the globules [12].

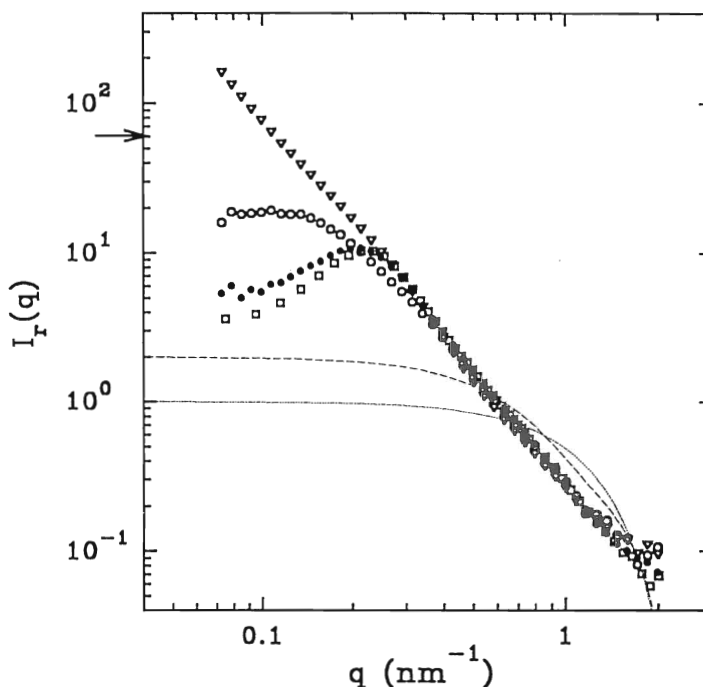


Figure 3 : $I_r(q)$ as a function of q for β -lactoglobulin aggregates in D_2O formed at various ionic strengths and heating conditions : (\square) pD7-0.003M-4%-76°C-46h; (\bullet) pD7-0.003M-4%-90°C-26h; (\circ) pD7-0.003M-4%-90°C-26h diluted at 1%; (\blacktriangledown) pD7-0.1M-4%-76°C-19h. The dotted line is the theoretical form factor of a homogeneous sphere of radius 1.85 nm and the dashed line the one of 2 tangent homogeneous spheres of radius 1.85 nm. The arrow on the left vertical axis indicates the value obtained by SLS measurements, extrapolated to $q=0$ and at infinite dilution (cf. fig. 2).

The globule is clearly seen as the elementary unit of the fractal aggregates. In screened conditions, the globules associate quickly to form bigger aggregates. In non screened conditions, due to repulsive electrostatic interactions, the reaction of the second step is much slower and the globules can be observed. It is worth noting that we do not observe any influence other than kinetic [11] of the heating temperature (from 70°C to 90°C) on the structures seen in figures 1 and 3.

Experimental evidence for a two-step mechanism is also given by SEC measurements : upon heating, the amount of native protein decreases due to aggregate formation. Two populations of aggregates are observed, suggesting that a physical separation of globules and bigger aggregates occurs in SEC columns. Electrostatic interactions do not strongly influence the globule size. Interchange of disulphide bonds have been observed in the early stages of aggregation and are probably involved in the globule formation [15]. This could explain that the same local structure is obtained whatever the ionic strength is. Moreover, the fact that a bigger proportion of globules is observed in the low ionic strength condition confirms our previous comments on the influence of interactions on the aggregation mechanism.

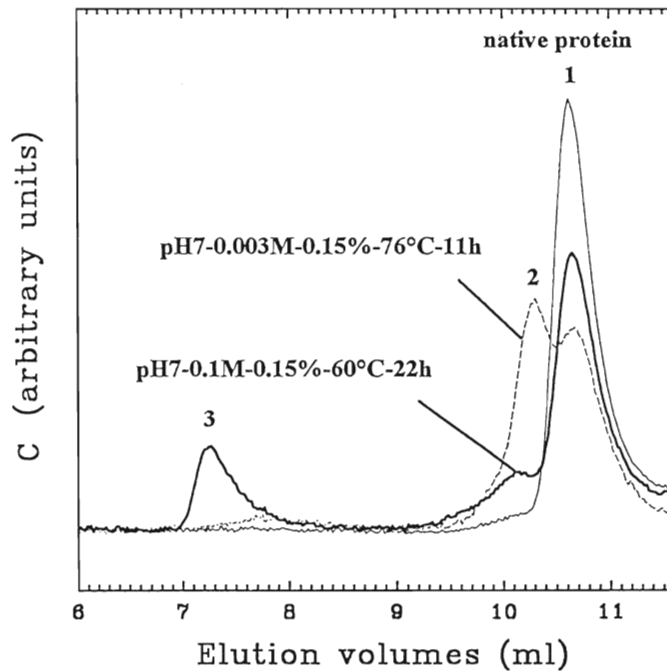


Figure 4 : SEC chromatograms obtained from various β -lactoglobulin solutions. Thin solid line : native protein; dashed line : pH7-0.003M-0.15%-76°C-11h; thick solid line pH7-0.1M-0.15%-60°C-22h. Numbers indicate the peak position of (3) the aggregates, (2) the globule, (1) the native protein.

In order to investigate the concentration effect on the size of the globules, the aggregation has been followed in situ by DLS at low ionic strength (0.003M) at concentrations from 0.15% to 2%. The inverse Laplace transform of the experimental

correlation functions shows two relaxation processes corresponding to the diffusion of the aggregates and the non-aggregated proteins. Diluting the sample at the end of the heating allowed us to correct for the interactions and to carry out SEC experiments reported in figure 4. The hydrodynamic radii were calculated using eq.1 and are shown in figure 5. The results of some measurements on samples quenched at different reaction times are also shown in figure 5. Although results obtained in situ are noisy, a clear increase in $\langle R_s \rangle_z$ of the globules is observed with reaction time, with a plateau for the two lowest concentrations (0.15% and 0.5%). For 2%, a true plateau is not observed. At this higher concentration the second step reaction is quicker and the weak increase of $\langle R_s \rangle_z$ is due to the increasing contribution of scattering by larger aggregates. The plateau value of 16 ± 1.5 nm is higher than the one obtained in D₂O solvent (11.5 ± 0.5) and the weight average aggregation number of the globule in H₂O is close to 95 ± 5 instead of 60 ± 5 in D₂O. The difference between the solvents could be due to differences in the physical properties, e.g., viscosity, polarity, pK_a of the thiol groups and the amount of dissolved oxygen [3]. It is possible that the globule size is related to the thiol reactivity, but further investigation is needed to clarify this point.

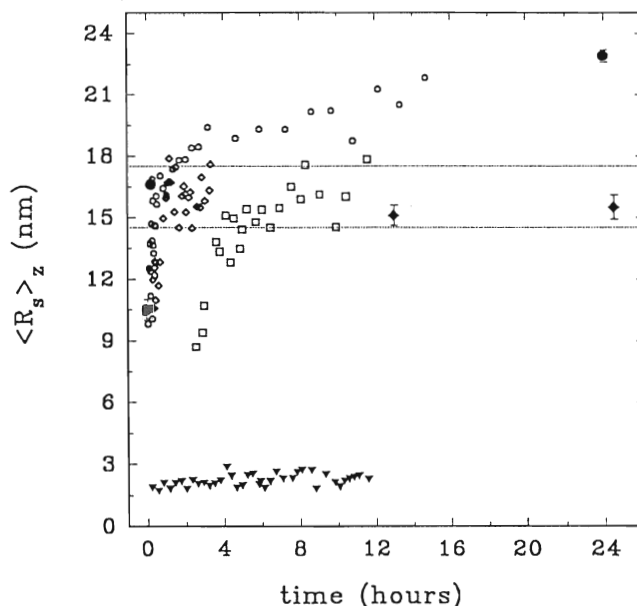


Figure 5 : Evolution of $\langle R_s \rangle_z$ of β -lactoglobulin aggregates as a function of heating time in pH7-0.003M solvent. (\circ) 2%-76°C. (\diamond) 0.5%-76°C. (\square) 0.15%-76°C. (\bullet) 2.2%-80°C. (\blacklozenge) 0.5%-80°C. (\blacktriangledown) residual monomers of β -lactoglobulin 0.15%-76°C.

DISCUSSION

We have shown that at pH7 the aggregation of β -lactoglobulin occurs in two steps. The first aggregation step consists in the formation of small particles (globules), whose size does not seem to depend on the initial protein concentration, the temperature and the ionic strength in the range tested. SANS experiments show that the shape of globules is not rod-like, in which case $I_r(q)$ would depend linearly on q . We have observed in all conditions tested (pH, ionic strength, concentration, temperature) that the decrease of the monomer concentration can be described by order 2 kinetics [4]. This implies that the monomers present at high temperatures first associate in dimers before forming globules. In a second step these globules aggregate to form fractal structures, see fig.6.

The existence of two steps in the aggregation process, as well as the importance of disulfide interchange in the first step is in agreement with the aggregation model proposed by Roefs and de Kruif [10] mentioned in the introduction. However, three experimental observations are incompatible with their model : 1) the size of the globule is insensitive to variations of the protein concentration, ionic strength and temperature in range tested; 2) the structure of the globule is compatible with the one of a small flexible linear chain but one would expect in this case a more rod like structure at small ionic strength due to electrostatic repulsions, which has not been observed; 3) the observed kinetics of monomer consumption are not of order 1.5 as it should be in their model.

We believe that the discrepancy is maybe in part due to the fact that their measurements were done at higher concentrations. However, they did not take into account the effect of electrostatic and excluded volume interactions which are especially important at low ionic strength conditions. In addition, they did not correct for the scattering due to residual non-reacted proteins. The authors use the cumulants methods to analyse the DLS data. This method is not suitable for the description of an aggregation mechanism as it does not allow the separation of the contributions of aggregated and non-aggregated species.

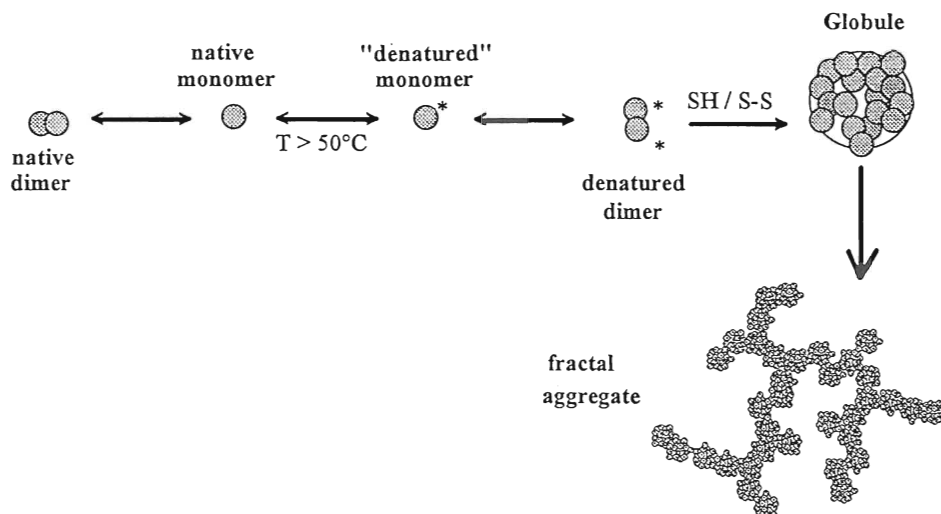


Figure 6 : Schematic representation of the two step aggregation process of β -lactoglobulin at pH7.

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