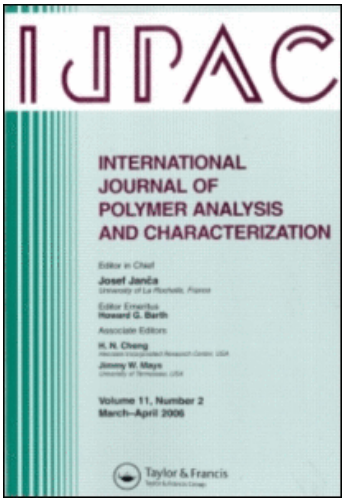

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A Comparison of the Structure of β-Lactoglobulin Aggregates Formed at pH7 and pH2

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In order to investigate the influence of electrostatic interactions on the thermal aggregation of β-lactoglobulin, different conditions of pH and ionic strength were used. Some structural features of the aggregates over a broad spatial range were determined with the use of scattering techniques. In the case of pH7, a first step of the aggregation process, leading to particles of about 60 monomers (which we refer to as "globules"), occurs whatever the ionic strength. Depending on the degree of screening, further aggregation can take place, leading to the formation of self-similar aggregates with fractal dimension 2.0. At pH7, both hydrophobic interactions and interchange of disulphide bonds could be involved. At pH2, the very low reactivity of the sulfhydric group may inhibit the formation of the globules. The aggregates are locally more or less rod-like and exhibit again fractal behaviour at larger distances, with a fractal dimension depending on the degree of screening. The influence of the ionic strength on the branching of the aggregates is also discussed.

KEY WORDS Globular protein; β-lactoglobulin; aggregation; light scattering; neutron scattering; fractal structure.

INTRODUCTION

β-Lactoglobulin is a globular whey protein with radius 2.0 nm and molar mass 18400 g mol^{-1} and attracts much attention for its potential in the food industry. The proteins are globally positively charged at pH > 5.2 and are negatively charged at lower pH [1]. The protein contains two disulphide bonds and one free sulfhydric group. In the native state it dimerizes in aqueous solution, to an extent which depends on the pH, ionic strength, concentration and temperature. β-lactoglobulin exhibits a rather unusual solubility at pH2, where it is strongly ionized (21 positive charges for 162 amino acids).

When the temperature is increased above 70°C the proteins partially denature and aggregate. In certain circumstances this aggregation leads to the formation of a gel. The kinetics of the aggregation are strongly dependent on the temperature and concentration. The ionic strength and pH do not only influence the kinetics, but also the structure of the aggregates and gels, e.g. at neutral pH the solution becomes opaque upon heating, while at pH2 it stays clear [2]. We have reported elsewhere the results of a detailed

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static and dynamic light scattering study of the aggregates formed at pH7 with 0.1M added salt [3].

Here we present the main results of a comparative study of the structure of the aggregates formed at pH2 and pH7 with various ionic strengths. A more complete study will be reported later. We have used light scattering (LS) and small-angle neutron scattering (SANS) to determine the static structure factor $S(q)$ which is proportional to the scattering intensity. From the static structure factor it is possible to deduce a number of structural parameters. For $q\langle R_g\rangle_z << 1$ and in the dilute state (Guinier regime), the weight-average molar mass M_w and the z-average radius of gyration $\langle R_g\rangle_z$ can be determined. For $q\langle R_g\rangle_z >> 1$, the static structure factor provides information on the internal structure of the object. For self-similar structures, the fractal dimension df is related to the degree of space filling and 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 $q\langle R_g\rangle_z >> 1 >> qr_0$, where r_0 is the size of the elementary unit of the fractal structure. If the system has a sufficiently low polydispersity, df is the true fractal dimension [5].

MATERIALS AND METHODS

β-lactoglobulin has been obtained from Sigma (ref. L.0130, batch 98F8030) and is a mixture of the genetic variants A and B. The pH2 solutions have been obtained by addition of HCl, whose contribution to the ionic strength has been taken into account. The ionic strength was set by addition of either sodium chloride or ammonium acetate. No difference in structure was observed with the nature of salt. 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. Concentrations were then measured by UV spectrometry at 280 nm.

The samples were covered with paraffin oil to prevent evaporation and heated in a thermostated bath at temperatures between 70.0 ± 0.2 and 90.0 ± 0.2°C for different periods of time. Temperatures and heating times were chosen on the basis of a preliminary study of the kinetics of the aggregation process that showed that concentration and temperature changed the kinetics but had no influence on the structures formed [3]. The use of D_2O instead of H_2O for SANS experiments again only influences the kinetics. The aggregation is quenched by rapid cooling to room temperature. 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 for pH7 and by UV spectrometry (after precipitation of the aggregates) at pH2. The dilute samples remain stable at room temperature over a period of months without any detectable further aggregation or break-up of aggregates.

LS measurements have been performed with a Spectra Physics argon ion laser operating with vertically polarised light with wave length $\lambda = 488$nm. The range of wave vectors covered is $3.5 \times 10^{-2} < q < 3.0 \times 10^{-3}$ 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 10^{-5} cm^{-1} for the Rayleigh factor at 488nm.

SANS experiments were performed in Saclay (France) and in the Rutherford Laboratory (UK).

RESULTS AND DISCUSSION

In Figure 1 we compare the wave vector (q) dependence of the scattering intensity from large aggregates (>1 μm) formed at pH2 with those formed at pH7, both with 0.1M added salt. The scattering intensity of the aggregates is normalized by the intensity of the solutions before heating (reduced intensity I_r). The intensity scattered by the native proteins (which can be considered here as dimers i.e. double spheres) at pH7-0.1M is also shown for comparison.

The straight lines observed at $q < 0.1$ nm^{-1} imply that the aggregates have a fractal structure at distances larger than about 10 nm. We have observed that the fractal structure is independent of concentration, temperature and heating time conditions in the range studied [3]. The fractal dimension obtained from the slope are equal to 2.0 at pH7 and 1.8 at pH2. Locally, however, at distances smaller than about 10 nm, the structure is different. At pH2 the reduced scattering intensity has a linear q-dependence over a small q-range ($0.1 < q < 0.2$ nm^{-1}) which indicates a local rod-like structure. At pH7 the local structure is a particle of bigger size than in the case of pH2. This globular particle, whose size is estimated at about 60 monomers can be considered as the elementary unit of the fractal aggregates.

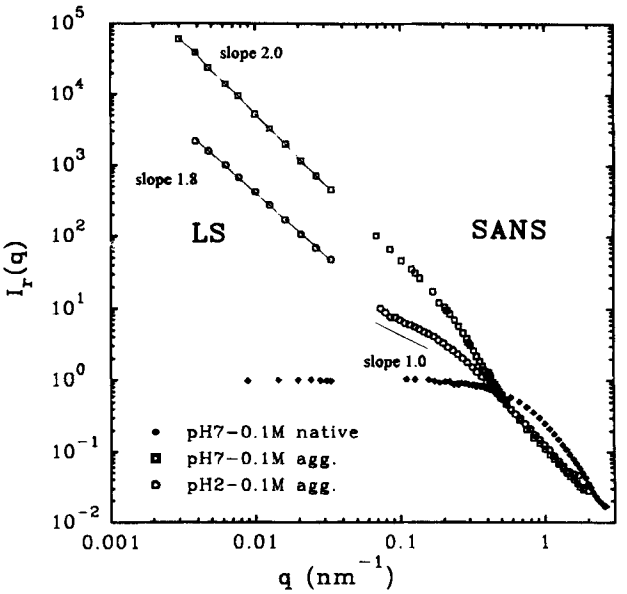

FIGURE 1 q-dependence of the normalised scattering intensities of pH7-0.1M native protein (♦), pH7-0.1M aggregates (□) and pH2-0.1M aggregates (○). β-lactoglobulin solutions of concentration c_0 have been heated at the temperature Θ during the time t.

□ pH7-0.1M agg: LS: c_0 = 1%, Θ = 70°C, t = 61h SANS: c_0 = 1%, Θ = 76°C, t = 30 min
○ pH2-0.1M agg: LS: c_0 = 2%, Θ = 80°C, t = 2h SANS: c_0 = 2%, Θ = 90°C, t = 26h
♦ pH7 0.1M native solution: c_0 = 1%

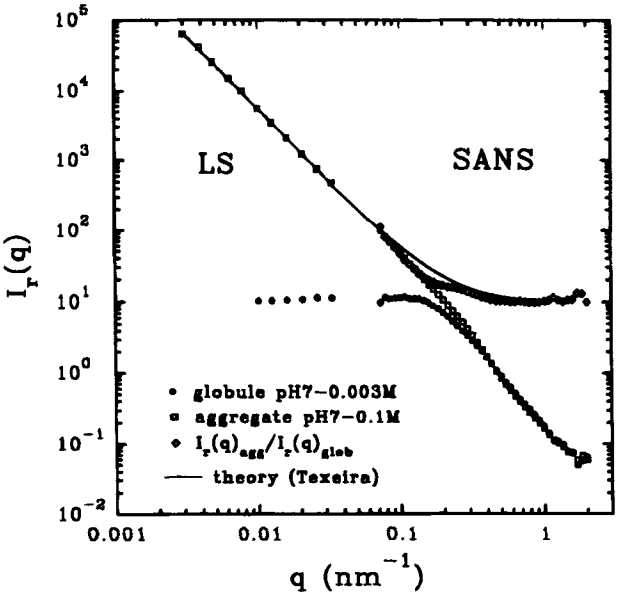

FIGURE 2 q-dependence of the normalised scattering intensities of pH7-0.1M aggregates (□) and pH7-0.003M aggregates, called here globule (●). The result of a division of the q-dependence of the large aggregates by that of the globule (◇) is compared to the theoretical structure factor given by Texeira (solid line). β-lactoglobulin solutions of concentration c_0 have been heated at the temperature Θ during the time t.
□ pH7-0.1M agg: LS: c_0 = 1%, Θ = 70°C, t = 61h SANS: c_0 = 1%, Θ = 76°C, t = 30 min
● pH7-0.003M agg: LS: c_0 = 4%, Θ = 76°C, t = 48h SANS: c_0 = 4%, Θ = 76°C, t = 48h

The difference between the aggregation at pH7 and pH2 is even clearer if we reduce the ionic strength of the solutions. At pH7-0.003M (200 ppm NaN_3) only small globular aggregates are formed and are refered to as "globules". In Figure 2 we have compared the reduced scattering intensity of these small aggregates with the large aggregates formed with 0.1M added salt. It is clear that the local structure of the large aggregates is the same as that of the globules formed at 0.003M. We believe that the large aggregates are fractal structures with as elementary unit the globule. We can recover the fractal structure factor by dividing the structure factor of the large aggregates by that of the globules. In Figure 2 we have compared the result with the theoretical structure factor of fractals given by Texeira [4]. The deviations around $q^{-1} = 0.1$ are probably due to the local excluded volume effects which are ignored in the derivation of the theoretical structure factor.

At pH2 the aggregates formed at low ionic strength have a more elongated structure. At the lowest ionic strength investigated (0.013M i.e. HCl and NaN_3 only), the reduced scattering intensity has an almost linear q dependence (slope = 1.1, see Figure 3). This means that the aggregates at low ionic strength are almost rodlike and contain very few branching points. With increasing ionic strength both the flexibility of the aggregates and the number of branching points increases leading to an increasing fractal dimension. For pH2-0.2M, electrostatic interactions are screened and the fractal dimension is the same as for pH7-0.1M.

Based on these experiments and other observations reported in the literature we propose the following model for the aggregation at pH7 and pH2.

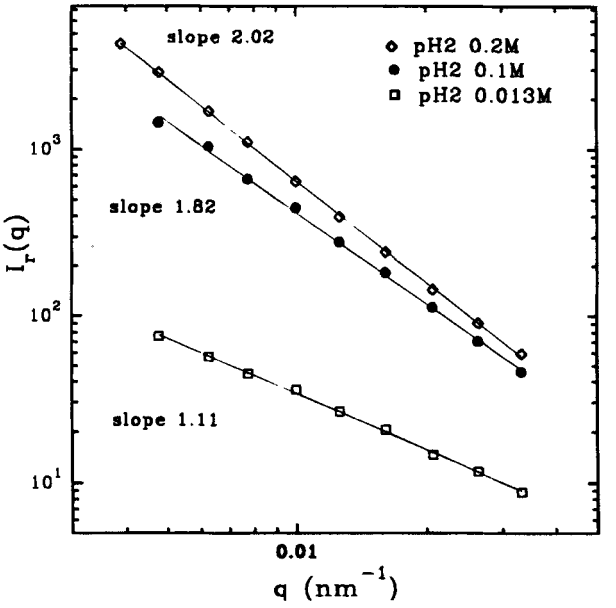

FIGURE 3 q-dependence of the normalised scattering intensities of aggregates formed at pH2 at different ionic strengths. β-lactoglobulin solutions of concentration c_0 have been heated at the temperature Θ during the time t.
□ pH2-0.013M agg: LS: $c_0 = 0.5\%$, $\Theta = 80°C$, $t = 11h$
● pH2-0.1M agg. LS: $c_0 = 2\%$, $\Theta = 80°C$, $t = 2h$
◇ pH2-0.2M agg. LS: $c_0 = 0.5\%$, $\Theta = 80°C$, $t = 3h$

At pH7 the aggregation occurs in two steps. First small globular aggregates are formed which subsequently aggregate to form fractal structures. The reaction rate of the second step is much reduced at low ionic strength due to repulsive electrostatic interactions. Interchange of disulphide bonds, leading to intermolecular bonding is probably involved in the formation of the globule.

At pH2 the reactivity of the sulfhydric groups is very small which inhibits the formation of the globule. However, the diameter of the rod-like local structure at pH2 is larger than the native protein. In both cases of pH, the step leading to the formation of fractal aggregates involves mainly an end to end aggregation, with occasional branching. The amount of branching and the persistence length of the aggregates are determined by the concentration of added salt which screens the repulsive electrostatic interactions.

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