Kinetics of Aggregation and Gelation of Globular Proteins after Heat-Induced Denaturation

Christel Le Bon, Taco Nicolai, and Dominique Durand*

Chimie-Physique des Matériaux Polymères, UMR-CNRS 6515, Université du Maine Avenue Olivier Messiaen, 72085 Le Mans Cedex 9, France

Received April 12, 1999; Revised Manuscript Received June 23, 1999

ABSTRACT: The rate of native protein consumption upon heating solutions of β-lactoglobulin at pH 7 and 0.1 M salt is characterized by a single activated process for temperatures up to 85 °C. The activation energy is only weakly dependent on the protein concentration and is about 390 kJ/mol. The effective order of the reaction responsible for the decrease of native proteins is 1.5, independent of concentration and temperature in the range investigated (2.5−115 g/L; 52−76 °C). Gel times were measured over a wide range of concentrations (9−180 g/L) and temperatures (55−87 °C). The temperature dependence of the gel time is characterized by the same activation energy as the consumption of the native proteins. The concentration dependence of the gel time diverges when the concentration approaches 7 g/L independent of the heating temperature. The divergence occurs when all native proteins have aggregated before the gel is formed. The growth of the aggregates was measured using light scattering. Below 7 g/L the growth of the aggregates stagnates.

Introduction

It is well-known that heating globular proteins in aqueous solutions induces aggregation which, if the concentration is sufficiently large, can lead to gel formation.[1] One of the most intensively studied globular proteins is β-lactoglobulin (β-lg), which is the major whey protein. β-lg has a molar mass of 18.6 kg/mol and a radius of about 2 nm.[2−4] At room temperature β-lg dimerizes with an association constant that depends on the ionic strength and the pH.[5−7] If the solution is heated, the equilibrium is displaced toward the monomer.[5,8] However, if the temperature is raised above about 50 °C, the monomers denature[9] and aggregate.[2,10−12] The aggregation process is irreversible and can be quenched by rapidly reducing the temperature. The aggregates are stable at room temperature, and the structural properties of the aggregates can be studied in highly diluted solutions. We have reported a detailed study of the structure of β-lg aggregates formed at pH 7 and ionic strength 0.1 M.[10] One of the conclusions of this study and subsequent work is that the structure of the aggregates is independent of the concentration (C) and the temperature (T) over the range investigated (0.4−30 g/L; 70−76 °C). However, the rate at which the aggregates grow increases strongly with increasing C and T.

At neutral pH, the aggregation occurs in two distinct stages,[13] see Scheme 1. In the first step, small well-defined clusters are formed from about 90 proteins in H_2O and about 60 proteins in D_2O. In the second step these so-called globules aggregate themselves into large scale clusters with a self-similar fractal structure which eventually leads to gel formation. The second step is inhibited at low ionic strength due to repulsive electrostatic interactions. The first step involves exchange of sulfide bridges.[14,15] The large clusters are stable upon dilution, but can be partially broken up using surfactants such as SDS. As far as we are aware it is not known what the mechanism of the aggregation of the globules is.

In the past the kinetics of the aggregation process of β-lg has been mostly studied by measuring the decrease of the native protein concentration as a function of time.[11,16−29] It was found that the time dependence of the concentration of nonaggregated protein (C_{na}) is well described by

$$\frac{dC_{na}}{dt} = -KC_{na}{}^{n} \tag{1a}$$

and after integration

$$C_{na}^{1-n} = C^{1-n} - (1-n)Kt \quad n \neq 1 \tag{1b}$$

with n the order of the reaction and K the rate constant. In the literature reaction orders 1,[18,26] 1.5,[11,16,17,20,28] and 2[19,21−23,25] have been reported.

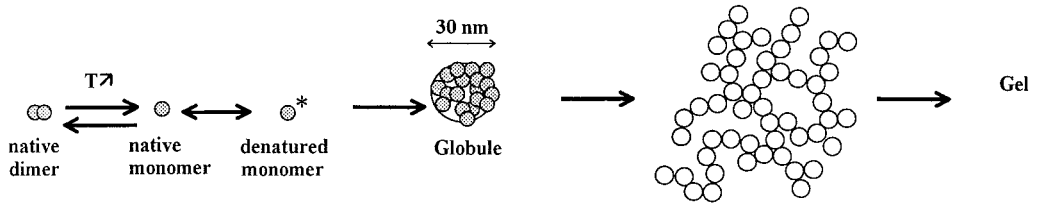
Another characteristic measure of the aggregation kinetics is the gel time (t_g). A systematic study of the concentration and temperature dependence of the gel time of the globular protein bovine serum albumin (BSA) has been reported by Tobitani and Ross-Murphy.[30,31] They found approximately linear behavior in plots of $\log(t_g)$ against C and $\log(t_g)$ against T. They propose a model for the gelation which leads to

$$\log(t_g) = a_1 + a_2 \log(C) + a_3 T + a_4 T \log(C) \tag{2}$$

where a_1−a_4 are system-dependent coefficients. The temperature dependence in eq 2 is based on the assumption that the Arrhenius model is valid for the gelation process. Of course, the Arrhenius model predicts $\log(t_g) \propto 1/T$, but over the small temperature range investigated the difference with $\log(t_g) \propto T$ is small. Equation 2 implies that for a given temperature t_g has a power law concentration dependence: $t_g \propto C^{\alpha}$. They introduced an ad hoc cross term to allow for a temperature-dependent exponent α and a concentration-dependent activation energy. The experimental results were consistent with eq 2, but no attempt was made to interpret the coefficients.

10.1021/ma9905775 CCC: $18.00
Published on Web 09/03/1999

Scheme 1. Aggregation and Gelation Process of β-Lactoglobulin in Aqueous Solutions at pH 7



In this paper we present an investigation of the kinetics of the aggregation process of β-lg at pH 7 and 0.1 M ammonium acetate. We have studied both the rate of protein consumption and the gel time over a wide range of concentrations and temperatures. One of the objectives of this work is to correlate t_g with the rate of protein consumption. We will show that for a proper understanding of the gelation kinetics it is essential to measure the rate of protein consumption. We have also studied the growth of the aggregates at different concentrations and temperatures using light scattering.

Experimental Section

Sample Preparation. β-lg used in this study was kindly provided by Besnier (batch no. 754). High performance liquid chromatography shows that the sample consists of equal fractions of genetic variants A and B and that the fraction of α-lactalbumin is negligible. The purity is better than 95%. Solutions were prepared by dialyzing against water at pH 7 containing 0.1 M CH_3COONH_4. A total of 200 ppm NaN_3 was added to avoid bacterial growth. The solutions were filtered through 0.2 or 0.45 μm pore size Anatope filters depending on the concentration. The protein concentrations were determined after filtration by UV absorption at 278 nm using extinction coefficient 0.96 L g^{-1} cm^{-1}.[32]

Size Exclusion Chromatography. SEC experiments were carried out at room temperature with a TSK PW 5000 + PW 6000 column set (30 cm + 60 cm) in series and a differential refractive index detector SHODEX RI 71. The columns were eluted with a 0.1 M $NaNO_3$ solution at a flow rate of 1 mL/min; 200 ppm of NaN_3 was added as a bacteriostatic agent. The injected volume was 300 μL and the injected concentration was approximately 0.1%. The analysis lasted for 45 min. The refractive index increment dn/dC is taken as 0.189 mL/g.[33,34]

Light Scattering. Static and dynamic light scattering measurements were made using an ALV-5000 multi-bit multi-τ correlator in combination with a Malvern goniometer and a Spectra Physics laser operating with vertically polarized light with wavelength $\lambda = 532$ nm. The range of scattering wave vectors covered was $3.0 \times 10^{-3} < q < 3.5 \times 10^{-2}$ nm^{-1} with $q = (4\pi n/\lambda) \sin(\theta/2)$, θ being the angle of observation and n the refractive index. The temperature was controlled by a thermostat bath and was set at 20 °C.

The aggregation process was quenched by quickly reducing the temperature to 20 °C. At room-temperature dilute solutions of aggregates are stable for a period of months. The weight-average number of proteins per aggregate (m_w) was determined in highly diluted solutions by a combination of static and dynamic light scattering and SEC as detailed elsewhere.[10,35]

Rheology. Rheological measurements were made with a Low Shear 40 rheometer using a Couette geometry (gap 0.5 mm). The measuring cup was preheated at the desired temperature and filled with 2.5 mL of the β-lg solution. The sample was recovered with paraffin oil to prevent evaporation. The storage and loss moduli were measured at 0.6 Hz applying a strain of 0.2%.

Results and Discussion

Native Protein Consumption. We have measured the fraction of nonaggregated protein ($F = C_{na}/C$) as a function of heating time using two methods. One method consists of precipitating the aggregates at pH 4.6[36] and measuring the protein concentration of the supernatant. We verified using dynamic light scattering that the supernatant contained only native protein. The second method utilizes size exclusion chromatography (SEC). The two methods give the same results within experimental error.

Figure 1 shows an example of the chromatographs of a solution of 10 g/L β-lg heated for different periods at 70 °C. The aggregates are well separated from the native proteins, and the fraction of residual proteins can be determined from the variation of the peak area. Aggregate separation is limited by the total exclusion volume, so that for large aggregates ($t = 100$ min and $t = 600$ min) the elution profile is not representative of the size distribution of the aggregates.

Figure 2a shows the fraction of nonaggregated protein as a function of heating time at 67 °C for three concentrations. If we normalize the time axis by the time needed to consume half the proteins (t_h), all data superimpose within experimental error; see Figure 2b. For clarity we show in Figure 2 only three concentrations, but in fact the data at all concentrations and temperatures investigated superimpose within the experimental error. In Figure 3 we compare our results with literature data. Figure 3 contains data on β-lg aggregation in whole milk, sweet milk, distilled water, or different buffers. In the literature, F is usually represented in a linearized form assuming a particular order for the reaction. Within the considerable scatter of the data, no systematic temperature, concentration, or ionic strength dependence is observed. The data can be described by an apparent aggregation rate with order

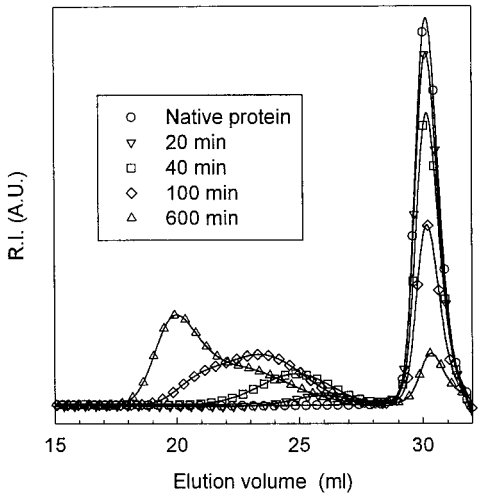


Figure 1. Chromatographs of β-lg solutions heated for different periods (0 min [native protein] 20 min, 40 min, 100 min, and 600 min) at 70 °C ($C = 10$ g/L, pH 7, 0.1 M CH_3COONH_4).

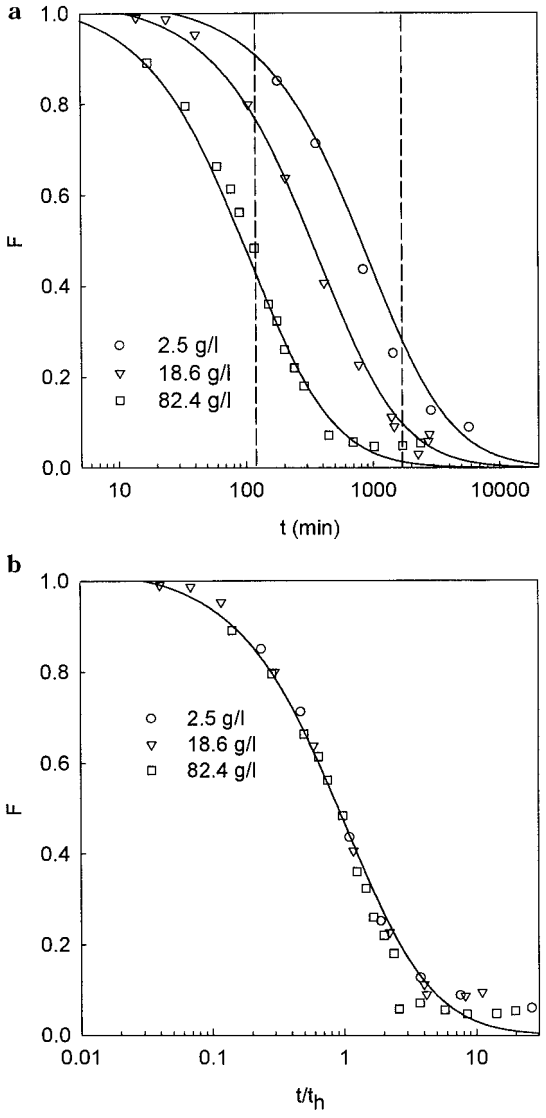


Figure 2. (a) Fraction of nonaggregated proteins F as a function of heating time at 67 °C for different concentrations indicated in the figure. The dashed lines indicate the gel time as determined with the insolubility criterion for the two higher concentrations (82.4 and 18.6 g/L). The continuous lines represent fits to eq 1 with $n = 1.5$. (b) Same data as in part a plotted as a function of the heating time normalized by the time needed to consume half of the native proteins, t_h. The continuous line represents a fit to eq 1 with $n = 1.5$.

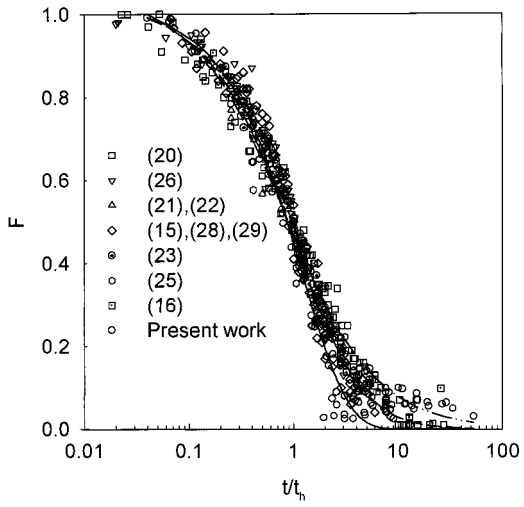


Figure 3. Fraction of nonaggregated proteins as a function of the heating time normalized by the time needed to consume half the proteins. The figure contains results at different temperatures, concentrations and external conditions taken from the present study and the literature. The lines represent fits to eq 1 with $n = 1$ (solid line), 1.5 (dashed line), and 2 (dash−dotted line).

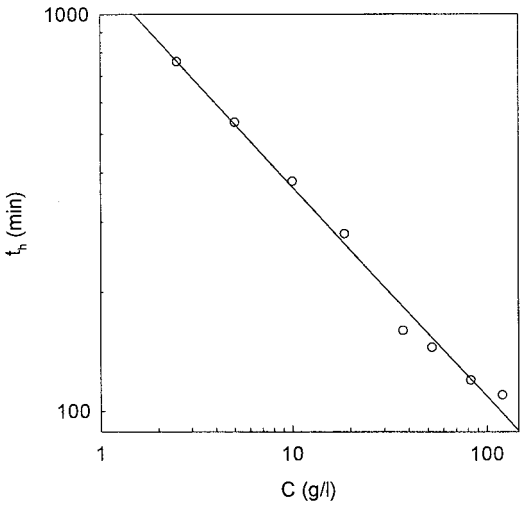


Figure 4. Concentration dependence of the time needed to consume half of the proteins t_h at 67 °C (pH 7, 0.1 M CH_3COONH_4). The solid line has a slope of 0.5.

intermediate between 1 and 2; see Figures 2 and 3. In view of the large scatter of the experimental results the difference between the reaction orders reported in the literature as 1, 1.5, and 2 is not significant except perhaps for $F < 0.1$. A more accurate method to obtain the effective order of the aggregation process is to measure the concentration dependence of t_h over a broad range of concentrations. Such measurements are more demanding and have rarely been done.[28] Figure 4 shows the concentration dependence of t_h over a range of concentrations covering almost two decades. The solid line in Figure 4 represents the result of a linear least-squares fit to the data in a double logarithmic representation the data and has slope -0.53 ± 0.3 (95% confidence). This means that within the experimental error $t_h \propto \sqrt{C}$ implying an effective order $n = 1.5$. This fractional effective order is probably due to a combination of successive reactions. Roefs and de Kruif[17] have proposed a model of the first step of the aggregation process that leads to an effective order 1.5. This model is based on that of free radical polymerization and predicts that the molar mass decreases with reaction time and increases with the concentration. In fact, the size of the "globules" is independent of the concentration. The local structure of the fractal aggregates obtained at 0.1 M salt is concentration independent.[10,37] At low ionic strength where the "globules" do not further aggregate, the size of the "globules" initially increases with time and stabilizes at a value independent of the concentration.[13,37]

We have studied the temperature dependence of t_h in the range 52.5−76 °C at different concentrations (2.5−115 g/L). In Figure 5, we have plotted $t_h\sqrt{C}$ in an Arrhenius representation, which collapses the data onto a single straight line showing that the activation energy is not strongly concentration dependent:

$$t_h\sqrt{C} = A \exp\left(\frac{E_a}{RT}\right) \qquad (3)$$

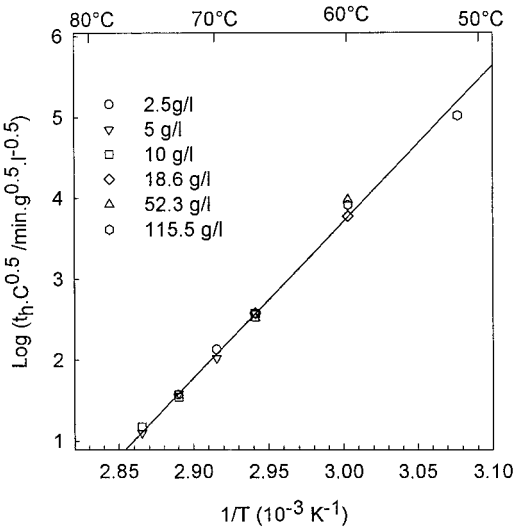


Figure 5. Arrhenius representation of the temperature dependence of $t_h\sqrt{C}$ at different protein concentrations. The solid line represents the results of a linear least-squares fit.

Here R is the gas constant, T the absolute temperature, and E_a the activation energy. In this temperature range, the aggregation process is characterized by a single activation energy. A linear least-squares fit gives E_a = 390 kJ/mol and $\log(A) = -56.8$ min^{-1} g$^{-0.5}$ L$^{0.5}$. For all concentrations and temperatures tested, the reaction order is 1.5, and so it follows from eq 1b that

$$F = (1 + 0.5K\sqrt{C}t)^{-2} \tag{4}$$

with the rate constant K given by

$$K = K_0 \exp(-E_a/RT) \tag{5}$$

K_0 follows directly from A using $F = 0.5$ at $t = t_h$: $A = (\sqrt{2}-1)/(0.5K_0)$, which yields $K_0 = 5.5 \times 10^{56}$ min^{-1} g$^{-0.5}$ L$^{0.5}$.

The value of E_a is consistent with activation energies reported in the literature,[11,16,19−23,25,26,38] which vary between 270 and 430 kJ/mol for temperatures below 90 °C. The large activation energy suggests that the temperature dependence of the aggregation rate is dominated by the highly cooperative denaturing process of the proteins. At temperatures above 85 °C a lower activation energy is reported.[20] The free energy (ΔG^*), enthalpy (ΔH^*), and entropy (ΔS^*) of activation can be calculated from the Eyring equation, $K = (k_b T/h) \exp(-\Delta G^*/RT)$, with the following relationships $\Delta H^* = E_a - RT$ and $\Delta G^* = \Delta H^* - T\Delta S^*$. Here k_b is Boltzmann's constant and h is Planck's constant. In the temperature range used, we obtain ΔH^* close to 387 kJ/mol and $\Delta S^* \approx 0.8$ kJ/(mol/K). The free energy of activation is close to the value (100 kJ/mol) reported for the thermal denaturation of eighteen proteins.[39]

Gel Time. At sufficiently high concentrations, heated β-lg solutions form an insoluble gel characterized by a storage shear modulus independent of the frequency in the range (10^{-2}−10^2 Hz) and much larger than the loss modulus.[40] The sol−gel transition is characterized by the divergence of the viscosity and the appearance of a frequency independent storage shear modulus. The percolation model predicts at the gel point a power law frequency dependence of the loss (G'') and storage (G') shear modulus at low frequencies.[41] For many covalent gels such a power law frequency dependence is indeed observed.[42] Therefore, an unambiguous method to determine t_g would be to measure the frequency dependence of G'' and G' as a function of heating time. Unfortunately, this method is not feasible for globular proteins because the shear modulus is too weak at the gel point to measure accurately. For this reason Tobitani and Ross-Murphy[30] have determined the gel point by measuring G' as a function of heating time at a single frequency and defining t_g arbitrarily as the time needed to reach 1−2 Pa. Verheul et al.[43] and Renard et al.[44] defined the gel point as the moment when the solution no longer flows on tilting the tube. We have compared three different criteria to determine t_g: (1) the rise of G'; (2) the divergence of size of the aggregates; (3) the solubility of the system. For the third criterion, aliquots were put in an excess of solvent at different heating times, and t_g was defined as the time where we first observed a precipitate. Figure 6 shows a comparison of the three methods for one system. It is clear that the three methods lead to a value of t_g that is the same within 20%. More importantly, we verified that the concentration and temperature dependence of t_g is the same independent of the method used.

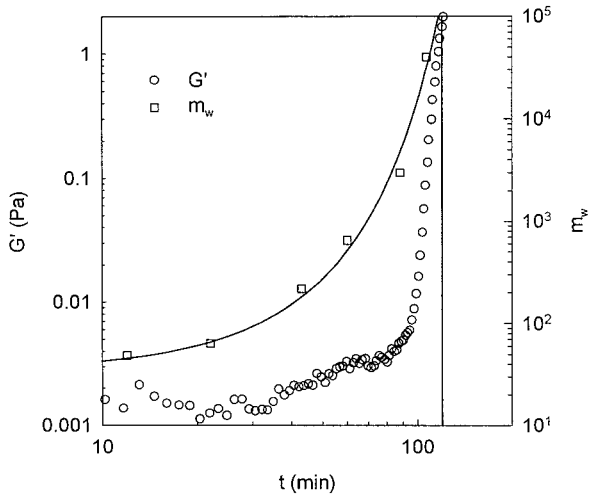


Figure 6. Comparison of the time evolution of m_w and G' of a solution containing 82.3 g/L β-lg at pH 7 and 0.1 M salt heated at 67 °C. The constant value of G' at times up to 80 min is due to the limitation of the apparatus used. The vertical line indicates the time when we first observe an insoluble gel fraction.

We have determined t_g over a broad range of concentrations (9−180 g/L) and temperatures (55−87 °C) using the insolubility criterion, which is by far the easiest. Figure 7 shows the temperature dependence of t_g at different concentrations in an Arrhenius representation. Clearly t_g is strongly temperature dependent and can be determined only over a small range of temperatures. For temperatures up to 85 °C the temperature dependence of t_g can be characterized by a single activation energy that is only weakly concentration dependent; see Figure 8. At 87 °C t_g is smaller than expected for a simple Arrhenius dependence. The value of E_a averaged over all concentrations is about 350 kJ/mol, which is close to that found for the native protein consumption. It appears that both the initial aggregate formation which leads to native protein consumption and the macroscopic gel formation are controlled by the same large activation energy. Most likely, the temperature dependence is in both cases determined by denaturation of the proteins.

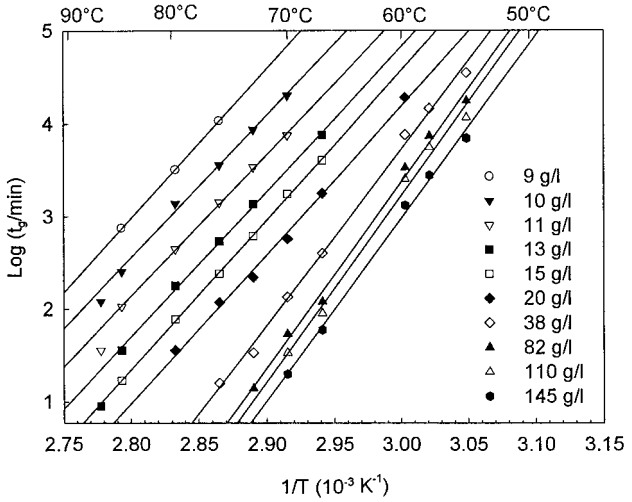


Figure 7. Arrhenius representation of the temperature dependence of the gel time of β-lg solutions at a number of concentrations (pH 7, 0.1 M CH_3COONH_4). The solid lines represent the results of linear least-squares fits.

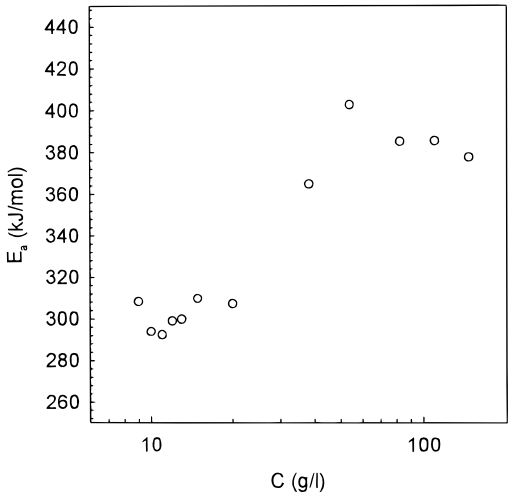


Figure 8. Concentration dependence of the activation energy characterizing β-lg gelation at pH 7 and 0.1 M CH_3COONH_4.

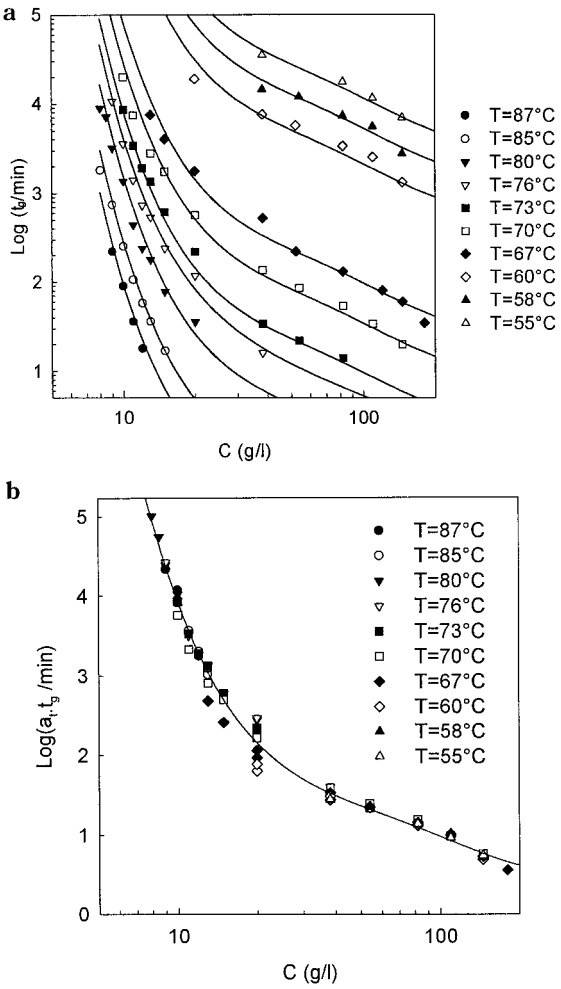


Figure 9. (a) Double logarithmic representation of the concentration dependence of the gel time at different temperatures indicated in the figure. The solid lines are obtained by vertical shifts of the line through the master curve shown in part b. (b) Master curve obtained by superposition of the data shown in part a using vertical shifts with the reference temperature being 73 °C. The line through the data is a guide to the eye.

Figure 9a shows a double logarithmic representation of the concentration dependence of t_g for a range of temperatures. In this representation it is clear that t_g diverges at $C \approx 7$ g/L. It is possible to superimpose the data by simple vertical shifts; see Figure 9b, because the activation energy is only weakly concentration dependent. The temperature dependence of the shift factors is determined by the average activation energy: $\ln(a_T) \propto E_a/T$. The superposition is certainly not perfect, but shows that the shape of the concentration dependence is not strongly temperature dependent. It also allows visualization of the concentration dependence of t_g over a wider range of concentrations. The concentration dependence decreases with increasing concentration except at the very highest concentrations. However, at these highest concentrations the contribution of counterions to the total ionic strength is no longer negligible. At pH 7 the net charge of β-lg is -6 so that the contribution of counterions to the total ionic strength is $3.3 \times 10^{-4}C$ M, with C in g/L.

The critical slowing down of the aggregation process was confirmed by measuring the growth of the aggregates at low concentrations, see Figure 10. Details of these measurements are given elsewhere.[37] For $C >$ 7 g/L the average aggregation number m_w of the aggregates increases exponentially and diverges at the gel point. For $C < 10$ g/L the growth stagnates at values of m_w that decrease with decreasing concentration.

In Figure 11 we compare the rate of protein consumption characterized by t_h with the gel time. The concentration dependence of t_h and t_g is very different, which implies that the fraction of residual nonaggregated proteins at the gel point (F_g) varies with the concentration. The concentration dependence of F_g is shown in Figure 12 and compared to the concentration dependence of t_g. The concentration dependence of F_g can be calculated utilizing eqs 4 and 5. The solid line in Figure 12 shows that the calculated concentration dependence of F_g describes the data within the experimental error.

At low concentrations all proteins aggregate before a gel is formed, but for $C > 10$ g/L an increasing fraction of proteins aggregates after the gel point. This observation was made recently by Verheul et al.[43] to explain the structural properties of the gels formed at different concentrations and ionic strengths. They found that the structural properties of the aggregates are mainly related to the concentration of aggregates at the gel point (C_g) and not the total protein concentration. In

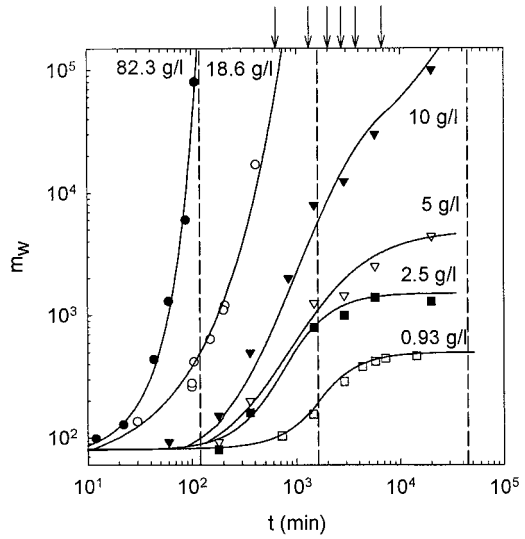


Figure 10. Growth of the weight-average aggregation number of β-lg solutions heated at 67 °C for different concentrations indicated in the figure (pH 7, 0.1 M CH_3COONH_4). The vertical lines indicate the gel point, while the arrows indicate the time when 90% of the native proteins is aggregated.

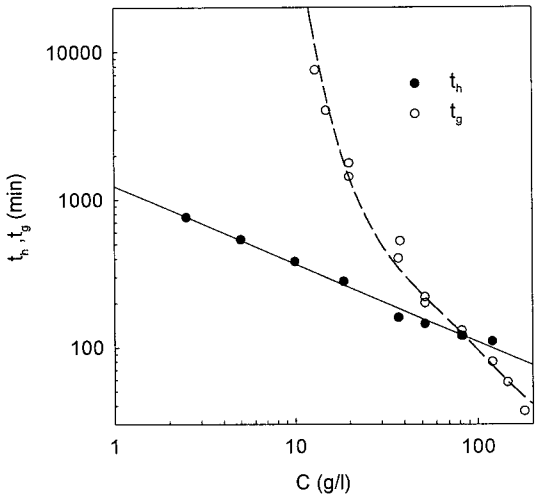


Figure 11. Comparison of the concentration dependence of the time needed to consume half of the native proteins and the gel time for β-lg heated at 67 °C (pH 7, 0.1 M CH_3COONH_4). The straight line through the filled circles has slope 0.5. The dashed line through the open circles is the same as the guide to the eye used in Figure 9b.

Figure 13 we plot C_g as a function of the total protein concentration. The value of C_g at a given protein concentration is independent of the temperature as changing the temperature only influences the rate of growth of the aggregates, but not their structure. Again C_g can be calculated utilizing eqs 4 and 5 using the concentration dependence of t_g as indicated by the dashed line in Figure 12. The experimental results are well described by the calculated values; see the solid line in Figure 13. For comparison, we also show the results obtained by Verheul et al.[43] on a whey isolate which shows the same qualitative behavior.

Discussion

It appears that the temperature dependence of the aggregation and the gel formation of β-lg at pH 7 and 0.1 M salt is controlled by a single activated process, at least in the temperature range 50−85 °C. The activation

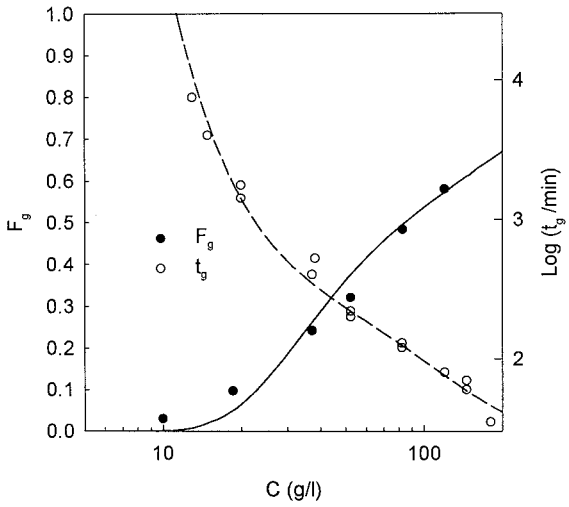


Figure 12. Comparison of the concentration dependence of the fraction nonaggregated proteins at the gel point with the gel time for β-lg solutions heated at 67 °C (pH 7, 0.1 M CH_3COONH_4). The dashed line is the same as the guide to the eye used in Figure 9b. The solid line results from a calculation of F_g based on the concentration dependence of t_g as indicated by the dashed line; see text.

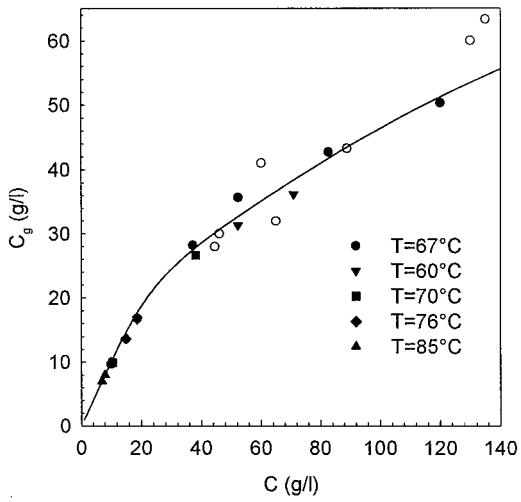


Figure 13. Aggregate concentration at the gel point as a function of the total β-lg concentration for various temperatures indicated in the text (pH 7, 0.1 M CH_3COONH_4). The open symbols represent results obtained by Verheul et al.[43] for a whey isolate at 68.5 °C (pH 6.9, 0.1 M NaCl). The solid line results from a calculation based on the concentration dependence of t_g; see text.

energy is only weakly dependent on the concentration, but it is systematically larger at higher concentrations. There is no indication for the existence of a minimal temperature of gelation (T_m). In fact, aggregation is clearly seen even at temperatures as low as 40 °C. We have kept a solution containing 120 g/L β-lg in an oven at about 43 °C which gelled after 2 months.

On the other hand there does appear to be a minimum protein concentration necessary for aggregation (C_m), which is the same for all temperatures investigated. t_g clearly diverges at $C_m \approx 7$ g/L, and the light scattering measurements show a qualitative difference of the aggregation kinetics at concentrations above and below C_m even though the structure of the aggregates is the same. The light scattering measurements are important because they allow investigation of the aggregate growth even when gel times become prohibitively long. It is not straightforward to establish whether there exists a true

minimum temperature or concentration of gelation or simply a strong slowing down so that it is no longer possible to obtain gels in a reasonable time. In practice, often state diagrams are determined in which the temperature[30,31] or ionic strength[44] needed to form a gel in a fixed heating time is plotted as a function of the concentration. Such diagrams are useful for a characterization of the system but cannot give C_m or T_m. We note, however, that at high temperatures the concentration dependence of t_g is very steep so that the value of C_m obtained by Renard and Lefevre after heating for 1 h at 100 °C is close to the value estimated from the divergence of t_g shown in Figure 9 for the case pH 7 and 0.1 M salt.

The present results are in clear disagreement with the model proposed by Tobitani and Ross-Murphy as expressed by eq 2. In a first approximation there is no need to invoke a cross-term because the concentration dependence of t_g is only weakly temperature dependent and vice versa. Tobitani and Ross-Murphy do not observe for BSA a minimum concentration needed for gelation but found a power law concentration dependence of t_g. However, they limited their investigation to concentrations above 60 g/L. Most likely, a divergence of t_g will occur also for BSA at lower concentrations. The authors make the interesting remark that "there *must* be a critical temperature for each concentration and vice versa" (italic type is our emphasis) although neither their data nor their model shows the need for introducing a critical concentration or temperature. From the present results, it is evident that the temperature dependence is controlled by an activation energy, and there is no indication for a critical temperature for gelation other than the practical constraint that gelation will take more than a week at temperatures below 55 °C. We do indeed observe a minimum concentration for gelation, but it is important to realize that there is no a priori reason for the existence of such a concentration.

In the past the existence of C_m has been inferred from a particular model of gelation. In this model it is assumed that the aggregation is reversible and characterized by an association constant.[45] The effect of ionic strength on C_m was interpreted by Renard and Lefevre[44] in terms of an ionic strength dependent association constant. However, the aggregation of globular proteins is clearly irreversible. Aggregates in very dilute solution are stable over periods of months and are stable even at the temperature at which they are formed. Another model which leads to a minimum concentration for gelation supposes that the monomers contain a small number of functional groups that form the cross-links in the aggregates and the gels.[45,46] When the aggregates grow, there is a competition between intra- and interparticle cross-linking. In more dilute solutions, intraparticle cross-linking (cyclization) is more likely so that the overall functionality of the aggregates decreases with decreasing concentration. Below a critical concentration, the functionality of the aggregates becomes zero, and the aggregation process stops before a gel is formed. However, there is no indication that globular proteins contain well-defined functional sites which is even less likely for the "globules". In addition, increasing intraparticle cross-linking would lead to denser structures at lower concentrations, which is not observed.

A different picture appears if we assume that the "cross-links" are formed by nonspecific attractive forces such as van der Waals forces, hydrogen bonding, and hydrophobic or electrostatic interactions. This model is more useful for colloidal gels. The simplest version of such a system is the aggregation and gelation of spheres in solution that stick as soon as they encounter each other. Monte Carlo simulations have been used to model this so-called diffusion-limited cluster aggregation (DLCA).[47,48] The aggregates that are formed have a fractal structure and a broad size distribution. When the volume fraction of the aggregates becomes important, the initial flocculation process crosses over to the percolation process that leads to the formation of a gel.[48] There is no critical concentration of gelation for such an aggregation process although in practice a gel cannot be observed if C is too small because it is too weak. Nevertheless, even if at very low concentrations the macroscopic gel is not stable to thermal fluctuations, one would still expect that very large aggregates are formed. The same is true for reaction limited aggregation (RLCA), but with longer gel times.

DLCA and RLCA were shown to be good models for the flocculation of spherical colloids,[47] but are they good models for the present system? The protein aggregates have a fractal structure with a fractal dimension and polydispersity close to that predicted by RLCA. In addition, there is no indication for specific functional sites involved in the cross-linking of the "globules" which are the elementary units of the fractal structure. The connection between the "globules" is more likely to be due to nonspecific physical interactions as mentioned above. So we would expect β-lg gels and globular protein gels in general to be more like colloidal gels than covalent polymer gels. Nevertheless, we do observe a clear divergence of the gel time at a particular concentration, and at lower concentrations only small aggregates are formed. The stagnation of the aggregate growth has to be related to the details of the aggregation process.

An important difference between the aggregation of gold or polystyrene spheres and globular proteins is that in the latter case not all particles start aggregating at the same time. The fraction of native protein decreases with an effective reaction order of 1.5 independent of the temperature. A striking feature of the comparison between the concentration dependence of F_g and t_g is that t_g increases strongly as F_g approaches zero; see Figure 12. This correlation suggests that the growth of the aggregates slows down dramatically if the native proteins become scarce. Apparently, the presence of native proteins or the "globules" formed in the first step is important for the association of the larger clusters. Note that cluster−cluster aggregation is necessary for the formation of a gel. We have no explanation for this observation and it would be interesting to see if this correlation holds for other globular proteins and other conditions of pH and ionic strength.

Conclusions

The decrease of native protein when heating solutions of β-lg at pH 7 and with 0.1 M salt can be described by eqs 4 and 5 over the whole concentration range investigated (2−115 g/L) and for temperatures below 85 °C. The temperature dependence is controlled by an activation energy that is only weakly concentration dependent. The concentration dependence implies an effective reaction order of 1.5, independent of the temperature. The free energy of activation is close to that found for the denaturation process of many globular proteins.

Similar gel times are found from the rise of the shear modulus, the divergence of the aggregate size and the solubility criterion. An Arrhenius temperature dependence of the gel time is found with a weakly concentration dependent activation energy which is the same as found for the decrease of native protein. The concentration dependence of t_g diverges at $C \approx 7$ g/L at all temperatures investigated (55−85 °C). At lower concentrations, the aggregation stagnates. The divergence of t_g occurs when the residual fraction of native protein at the gel point becomes very small.

References and Notes

(1) Clark, A. H. In *Functional properties of food macromolecules*; Mitchell, J. R., Ed.; Elsevier Applied Science; London and New York, 1998; p 77.
(2) McKenzie, H. A. In *Milk proteins: chemistry and molecular biology*; McKenzie, H. A., Ed.; Academic Press: New York and London, 1971; Vol. II, p 257.
(3) Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. *Nature* **1986**, *324*, 383.
(4) Pessen, H.; Kumosinski, T. F.; Farrell, H. M. J. *J. Ind. Microbiol.* **1988**, *3*, 89.
(5) Aymard, P.; Durand, D.; Nicolai, T. *Int. J. Biol. Macromol.* **1996**, *19*, 213.
(6) Verheul, M.; Pedersen, J. S.; Roefs, S. P. F. M.; de Kruif, K. G. *Biopolymers* **1999**, *49*, 11.
(7) Pessen, H.; Purcell, J. M.; Farrell, H. M. J. *Biochim. Biophys. Acta* **1985**, *828*, 1.
(8) Dupont, M. *Biochim. Biophys. Acta* **1965**, *102*, 500−513.
(9) Casal, H. L.; Köhler, U.; Mantsch, H. H. *Biochim. Biophys. Acta* **1988**, *957*, 11.
(10) Gimel, J. C.; Durand, D.; Nicolai, T. *Macromolecules* **1994**, *27*, 583.
(11) Hoffmann, M. A. M.; Roefs, S. P. F. M.; Verheul, M.; van Mil, P. J. J. M.; de Kruif, K. G. *J. Dairy Res.* **1996**, *63*, 423.
(12) Griffin, W. G.; Griffin, M. C. A.; Martin, S. R.; Price, J. *Faraday Trans.* **1993**, *89*, 3395.
(13) Aymard, P.; Gimel, J. C.; Nicolai, T.; Durand, D. *J. Chim. Phys.* **1996**, *93*, 987.
(14) Shimada, K.; Cheftel, J. C. *J. Agric. Food Chem.* **1989**, *37*, 161.
(15) Hoffmann, M. A. M.; van Mil, P. J. J. M. *J. Agric. Food Chem.* **1997**, *45*, 2942.
(16) Anema, S. G.; McKenna, A. B. *J. Agric. Food Chem.* **1996**, *44*, 422.
(17) Roefs, S. P. F. M.; de Kruif, K. G. *Eur. J. Biochem.* **1994**, *226*, 883.
(18) Larson, B. L.; Jenness, R. *J. Am. Chem. Soc.* **1952**, *74*, 3090.
(19) El-Shazly, A.; Mahran, G. A.; Hofi, A. A. *Milchenw.* **1978**, *33*, 166.
(20) Dannenberg, F.; Kessler, H. G. *J. Food Sci.* **1988**, *53*, 258−263.
(21) Hillier, R. M.; Lyster, R. L. J. *J. Dairy Res.* **1979**, *46*, 95−102.
(22) Lyster, R. L. J. *J. Dairy Res.* **1970**, *37*, 233.
(23) Manji, B.; Kakuda, Y. *Can. Inst. Food Sci. Technol. J.* **1986**, *19*, 163.
(24) Gimel, J. C. Thesis, Université du Maine, Le Mans, France, 1993.
(25) Dupont, M.; Yon, J. *J. Chim. Phys.* **1961**, *58*, 682.
(26) Gough, P.; Jenness, R. *J. Dairy Sci.* **1962**, *45*, 1033.
(27) Cairoli, S.; Iametti, S.; Bonomi, F. *J. Protein Chem.* **1994**, *13*, 347.
(28) Verheul, M.; Roefs, S. P. F. M.; de Kruif, K. G. *FEBS Lett.* **1998**, *421*, 273.
(29) Verheul, M.; Roefs, S. P. F. M.; de Kruif, K. G. *J. Agric. Food Chem.* **1998**, *46*, 896.
(30) Tobitani, A.; Ross-Murphy, S. B. *Macromolecules* **1997**, *30*, 4845.
(31) Tobitani, A.; Ross-Murphy, S. B. *Macromolecules* **1997**, *30*, 4855.
(32) Townend, R.; Winterbottom, R. J.; Timasheff, S. N. *J. Am. Chem. Soc.* **1960**, *82*, 3161.
(33) Halwer, M.; Nutting, G. C.; Brice, B. A. *J. Am. Chem. Soc.* **1951**, *73*, 2786.
(34) Perlmann, G. E.; Longsworth, L. G. *J. Am. Chem. Soc.* **1948**, *70*, 2719.
(35) Aymard, P.; Durand, D.; Nicolai, T. *Macromolecules* **1999**, *32*, 2542.
(36) de Witt, J. N. *J. Dairy Sci.* **1990**, *73*, 3602.
(37) Le Bon, C.; Nicolai, T.; Durand, D. *Int. J. Food Sci. Technol.*, in press.
(38) Park, K. H.; Lund, D. B. *J. Dairy Sci.***1984**, *67*, 1699.
(39) Labuza, T. P. *Food Technol.* **1980**, *67*, 67.
(40) Paulson, M.; Dejmek, P.; Van Vliet, T. *J. Dairy Sci.* **1990**, *73*, 45.
(41) Rubinstein, M.; Colby, R. H.; Gillmor, *J. R. Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* **1989**, *30*, 81.
(42) see for examples: Winter, H. H.; Chambon, F. *J. Rheol.* **1986**, *30*, 367; Adolf, D. B.; Martin, J. E.; Wilcoxon, J. P. *Macromolecules* **1990**, *23*, 527; Prochazka, F.; Nicolai, T.; Durand, D. *Macromolecules* **1996**, *29*, 2260.
(43) Verheul, M.; Roefs, S. P. F. M.; Mellema, J.; de Kruif, K. G. *Langmuir* **1998**, *14*, 2263.
(44) Renard, D.; Lefebvre, J. *Int. J. Biol. Macromol.* **1992**, *14*, 287.
(45) Clark, A. H.; Ross-Murphy, S. B. *Adv. Polym. Sci.* **1987**, *83*, 57.
(46) Suematsu, K.; *Eur. Phys. J. B* **1998**, *6*, 93; Clark, A. H. *Polym. Gels Networks* **1993**, *1*, 139.
(47) Meakin, P. *Phys. Scr.* **1992**, *46*, 295.
(48) Gimel, J. C.; Durand, D.; Nicolai, T. *J. Sol−Gel Sci. Technol.* **1999**, *15*, 129.

MA9905775