Kinetics of Heat-Induced Aggregation of β-Lactoglobulin

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Heat-induced aggregation of β-lactoglobulin was investigated as a function of pH, heating temperature, and NaCl concentration by measurements of reaction kinetics, differential scanning calorimetry, and light scattering. The aggregation can be well interpreted using a reaction scheme consisting of two steps: a denaturation equilibrium, with a first-order unfolding reaction, followed by second-order aggregation reactions. Denaturation becomes rate limiting at high heating temperature, pH values close to the isoelectric point of the protein, and high NaCl concentration. At neutral pH a maximum is seen in the overall reaction rate as a function of NaCl concentration, which is explained by a stabilizing, salting-out effect of the salt in combination with an increase in the rate of successive aggregation reactions. At high NaCl concentrations physical bonding becomes increasingly important; large aggregates that continue to grow in time are formed, and two phases are distinguished in the aggregation step. The onset time of the secondary aggregation is related to a critical concentration of primary (denatured or small, aggregated) particles.

Keywords: *Whey; β-lactoglobulin; aggregation; kinetics; light scattering*

INTRODUCTION

The functional properties of whey proteins are changed by heat treatment, and this is essential for their application in foods (de Wit, 1984; Mulvihill and Donovan, 1987). The major whey protein is β-lactoglobulin (β-lg), which constitutes >50% of total whey protein in bovine milk (concentration ≈3 g/L; Walstra and Jenness, 1984). β-Lg is a globular protein and has a molecular mass of 18.3 kDa. It contains two intramolecular disulfide bonds and one thiol group (Papiz et al., 1986; Mulvihill and Donovan, 1987). The native molecule can form dimers and oligomeric structures by noncovalent, reversible association. At room temperature and in concentrations as in bovine milk the protein is predominantly present as a dimer in aqueous solutions at a pH between 5.5 and 7.5 (Georges et al., 1962; McKenzie and Sawyer, 1967; Verheul et al., 1998).

Upon heating, β-lg undergoes intramolecular and intermolecular changes. Raising the temperature shifts the β-lg monomer−dimer equilibrium at β-lg concentrations below 10 g/L toward monomers (Georges et al., 1962; Verheul et al., 1998). Upon heating above ≈60 °C the molecule undergoes conformational changes and partially unfolds (i.e. it denatures); nonpolar groups and the thiol group are exposed (Iametti et al., 1996). Denaturation can be followed by an irreversible aggregation reaction so that the whole heat-induced process becomes irreversible (Sawyer, 1968). The mechanism by which the processes involved take place is complex and is influenced by many factors, such as electrostatic and hydrophobic interactions, hydrogen bonding, and disulfide cross-linking (Mulvihill et al., 1990).

Recently in our laboratory a kinetic aggregation model was developed for the aggregation of β-lg at neutral pH and low ionic strength in the temperature range 60−70 °C (Roefs and de Kruif, 1994; Hoffmann et al., 1996). Under such conditions transparent dispersions are formed, containing relatively small polymeric protein particles (size <100 nm). These particles are formed by thiol group−disulfide bond exchange (propagation) reactions between reactive, unfolded intermediates and native β-lg molecules and by thiol group−thiol group (termination) reactions of reactive intermediates (Roefs and de Kruif, 1994; Hoffmann, 1997). When a steady-state principle is applied, the model predicts that β-lg is converted via a reaction of order 1.5. The question arises as to what mechanism can be attributed outside the specified range of conditions. For instance, adding NaCl to the β-lg solution prior to heating or lowering the pH to values closer to the isoelectric point of the protein (pI = 5.1) results in the formation of turbid dispersions or gels, which means that much larger aggregates are present. The formation of these larger aggregates occurs via a complex mechanism in which both physical aggregation (by noncovalent interactions, i.e. electrostatic and hydrophobic interactions and hydrogen bonding) and chemical aggregation (by disulfide exchange reactions) are involved (Mulvihill and Donovan, 1987).

In this paper a systematic study of the influence of pH, temperature, and NaCl concentration on the heat-induced aggregation of β-lg is presented. The kinetics of the process were followed by measuring the concentration decrease of native β-lg as a function of heating time. The formation of the protein aggregates was monitored in situ by dynamic light scattering (Hoffmann et al., 1996; Elofsson et al., 1996). We will introduce a simplified model for the aggregation of β-lg. Using this model we can indicate the conditions (pH, salt concentration, temperature) at which either the unfolding or aggregation reactions prevail.

Model for Heat-Induced Denaturation and Aggregation. For the heat-induced denaturation and aggregation of β-lg under a much wider range of

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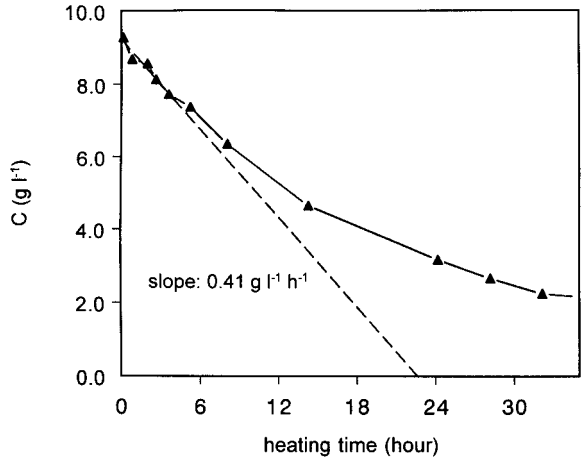

Figure 1. Concentration of nonaggregated β-lg versus heating time at 68.5 °C for an initial protein concentration of 9 g/L at pH 6.5 and 0 M NaCl; the initial reaction rate is defined as the initial slope of the concentration decrease.

conditions than before (Roefs and de Kruif, 1994), we now propose a less detailed reaction scheme consisting of two successive steps: a denaturation step and an aggregation step (de Jong et al., 1992):

step 1: denaturation

$$B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B^*$$

step 2: aggregation

$$B \text{ or } B^* + B_x^* \xrightarrow{k_2} B_{x+1}^* \rightarrow \ldots \rightarrow B_m^*$$

In the denaturation step in principle an equilibrium is established between the native state (B) and the partially unfolded state of the protein (B*). The unfolding and refolding are considered to be first-order reactions, which may involve a number of consecutive changes in the molecule. Upon unfolding the thiol group is exposed and becomes reactive (Hoffmann and van Mil, 1997), and subsequently several irreversible aggregation reactions (step 2) can take place. B_x^*, B_{x+1}^*, and B_m^* are β-lg aggregates consisting of x, $(x + 1)$, and m monomeric units, respectively (x, $m \geq 2$). These aggregates result from (several subsequent) irreversible aggregation reactions. All aggregation steps are bimolecular and are therefore second-order reactions. In step 2 the formation of protein particles takes place via (a) exchange reactions between free reactive thiol groups and disulfide bonds (chemical aggregation), (b) physical aggregation of unfolded protein molecules or chemically formed aggregates, or (c) an even more complex combination of chemical and physical aggregation.

The reaction order, n, of the overall aggregation reaction of β-lg is expected to be somewhere between 1 and 2 depending on the ratio of the reaction rates of the different steps. The general rate equation for a reaction with order n is

$$-dC_t/dt = k_n C_t^{\,n} \quad (1)$$

where t (s) is the reaction time, C_t (g/L) is the concentration of the reactant after a given reaction time, and k_n (g^{1-n} L^{n-1} s^{-1}) is the reaction rate constant. In the initial stage of the reaction (at $t \cong 0$; see Figure 1)

$$-dC_t/dt \cong k_n C_0^{\,n} \quad (2)$$

where C_0 (g/L) is the initial concentration of the reactant, and thus, initially,

$$C_t \cong C_0 - k_n C_0^{\,n} t \quad (3)$$

Experimental conditions determine the rate of the reactions in steps 1 and 2. The results of the present study are explained in terms of the above-described reaction scheme.

MATERIALS AND METHODS

Materials. Chemicals were of analytical grade. Purified β-lg was prepared at NIZO from cheese whey, basically following the procedure of Maubois et al. (1987). It contained the genetic variants A and B in a nearly 1:1 ratio (Hoffmann et al., 1996). The sample contained about 92% β-lg, 2% α-lactalbumin, 2% nonprotein nitrogen material, and 2.1% ash (including 0.73% Na^+, 0.02% K^+, 0.12% Ca^{2+}, and 0.008% Mg^{2+}) on a dry mass basis. It contained 4% moisture (Hoffmann et al., 1996).

Methods. *Sample Preparation.* β-Lg dispersions (2−46 g/L) were prepared by dissolving the β-lg powder in 0−1.0 M NaCl solutions that were made using double-distilled water. The protein dispersions were stirred for at least 2 h, and the pH was adjusted to the desired value using HCl or NaOH. The dispersions were then filtered using a 0.22 μm nonprotein-adsorbing filter for the kinetic experiments and double filtered using 0.1 μm nonprotein-adsorbing filters for the light-scattering experiments to remove insoluble materials and dust.

Kinetics of Heat-Induced Aggregation. β-Lg dispersions were heated in test tubes at 65−80 °C (predominantly at 68.5 °C) for various time periods. The tubes were cooled in ice water for 5 min, and the pH was adjusted to 4.7 ± 0.1, at which denatured/aggregated protein precipitates (de Wit, 1990). The denatured/aggregated proteins were sedimented by centrifugation for 30 min at 20000*g*. The concentration of native β-lg in the supernatant was determined by high-performance gel permeation chromatography (HP-GPC) as described by Hoffmann et al. (1996). Note that β-lg that is denatured (step 1) but not yet aggregated at a certain heating time is considered as native protein by this heat−quench method, since the equilibrium of step 1 shifts to the left upon cooling. Figure 1 shows a typical plot of the concentration of native (nonaggregated) β-lg as a function of heating time (9 g/L β-lg, 0 M NaCl, pH 6.5, 68.5 °C). Initial reaction rates of the aggregation process (dC/dt) were determined as the slope of the initial concentration decrease, as indicated in Figure 1.

Differential Scanning Calorimetry (DSC). The thermal behavior of β-lg solutions (44.5 g/L; pH 2−8) was studied using DSC with a Perkin-Elmer DSC7 within the temperature interval from 35 to 120 °C at a scan rate of 5 °C/min. Approximately 20 mg of sample solution was weighed into coated aluminum pans (TA Instruments). As reference, the same amount of already denatured/aggregated β-lg solution was used. The peak temperature, T_p (i.e. the temperature corresponding to maximum excess heat capacity), was determined from three to five replicate runs and varied by not more than 0.5 °C (Hoffmann et al., 1995).

Light Scattering. Dynamic light-scattering experiments were made using a Malvern Autosizer IIC Submicron particle size distribution analyzer. The system consisted of a Malvern PCS41 optics unit with a 5 mW He−Ne laser and a Malvern K7032-ES correlator used in serial configuration. The Autosizer IIC worked at a fixed scattering angle of 90°, and the wavelength of the laser beam was 632.8 nm, resulting in a wave vector of 0.0187 nm^{-1}. β-Lg dispersions (2−46 g/L; pH 6.5−7.5; 0−1.0 M NaCl) were mounted in 10 mm quartz cuvettes and put in the Autosizer IIC. Heat-induced aggregation measurements were carried out in situ in the apparatus (Hoffmann et al., 1996). Both the scattering intensity, $I_s(q)$,

and the apparent diameter, d_h, were evaluated. The quartz cuvette containing the sample was thermostated to within ±0.1 °C by a Joule-Peltier thermostat. The temperature was raised from 20 to 68.5 °C, and within 4 min the Peltier heater reached the desired temperature. At that time the recording of the autocorrelation function was started and the growth of aggregates with heating time was followed. Depending on the sample conditions, measurements were made every 0.5−3 min. The Malvern software was used in "Easy Mode", which means that the interval time was automatically adjusted to the "optimal" value. The apparent diameter of the particles in solution was calculated from a cumulant fit of the intensity autocorrelation function (Hoffmann et al., 1996). The scattered intensity of a particle dispersion in first-order approximation (small particles, low particle concentration, small wave vector) is given by (van de Hulst, 1981; Lyklema, 1991)

$$I_s \propto C_p M_p \quad (4)$$

where C_p is the particle weight concentration (g/L) and M_p is the weight-averaged molar mass of the particles (g/mol).

RESULTS AND DISCUSSION

We studied the sensitivity of β-lg to pH and temperature in a broad pH range with no extra salt added and studied the effect of NaCl more systematically at pH 6.5−7.5. Results on aggregation kinetics and in situ light scattering are presented subsequently.

Kinetics of β-Lg Aggregation. *pH.* In Figure 2A the initial reaction rate of the denaturation/aggregation process (logarithmic axis) is shown as a function of pH for various heating temperatures and an initial β-lg concentration of 9 g/L in water (low ionic strength; apart from pH adjustment no extra salt was added). Two main features are observed, i.e. a pronounced minimum in reaction rate between pH 3 and 4 at 75 and 80 °C and a strong increase at 65 and 68.5 °C between pH 6 and 8. These features can be coupled to the peak temperature of DSC thermograms as a function of pH (see Figure 2B). The enthalpy peak in the DSC thermogram arises mainly from conformational changes of the β-lg molecule (de Wit and Klarenbeek, 1981; Paulsson et al., 1985; Harwalker and Ma, 1989; Gotham et al., 1992). Since DSC thermograms are not recorded isothermally but at a given (relatively high) heating rate, they mainly probe the unfolding reaction of step 1, as shown by Hoffmann (1997) at neutral pH. The peak temperature, T_p, is strongly coupled to the temperature dependence of this unfolding reaction; above T_p the unfolding rate, k_1, in step 1 is very high. The minimum in reaction rate at pH 3−4 coincides with the maximum in T_p (≈85 °C), which is higher than the experimental temperatures (65−80 °C). Consequently, β-lg is mainly present in its native form ($k_1/k_{-1} \ll 1$), and the unfolding reaction of step 1 is slow and rate limiting, leading to low overall reaction rates (see Figure 2A). In step 2 mainly physical aggregation will occur under these conditions. At temperatures above T_p denaturation (step 1) occurs quickly and only a minor influence of pH on the overall reaction rate is observed.

The conformation of a protein is most stable near its isoelectric point, because the electrostatic repulsion between the charges of the protein molecule is at a minimum. The maximum stability of β-lg is found at a pH below the isoelectric point, which is probably related to the titration of carboxyl groups leading to extra internal hydrogen bonding and/or loss of localized unfavorable electrostatic interactions (Kella and Kinsella, 1988). At pH values further from the isoelectric point, the conformational stability decreases as a result of intramolecular charge repulsion (Harwalker and Ma, 1989). The decreased stability at low and high pH leads to a decreasing T_p (Figure 2B) and increasing reaction rates (Figure 2A).

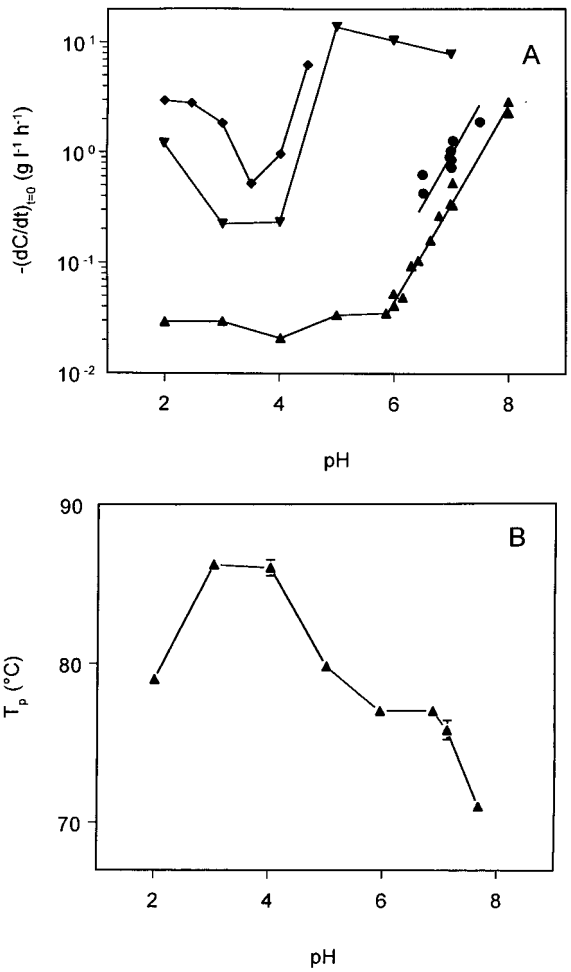

Figure 2. (A) Initial reaction rate of the denaturation/aggregation of β-lg in water versus pH for an initial protein concentration of 9 g/L. Heating temperatures: ▲, 65 °C; ●, 68.5 °C; ▼, 75 °C; ◆, 80 °C. (B) Peak temperature in DSC thermograms (T_p) versus pH: β-lg concentration, 46 g/L; heating rate, 5 °C/min (data kindly provided by M. A. M. Hoffmann).

The sharp increase in reaction rate above pH 6 at temperatures (65 and 68.5 °C) below T_p is caused not only by the decreasing stability, in which conformational changes of β-lg around pH 7 are involved, which are commonly referred to as the Tanford transition (Tanford et al., 1959), but also by an increasing contribution of chemical reactions in step 2. The latter can be attributed (probably quantitatively) to the increased reactivity (i.e. dissociation) of the thiol groups (Shimada and Cheftel, 1989; Hoffmann, 1997). Physical aggregation, on the other hand, decreases with increasing pH above pH 6, as a result of increasing intermolecular charge repulsion (Xiong et al., 1993). Above pH 8 accurate experiments were not possible, since the β-lg concentration decreased significantly in the heating-up time (even at 65 °C), and actually reactions occurred already at room temperature (results not shown). This has also been observed by others, and explained by the formation of intermolecular disulfide bonds (Akroyd, 1965; McKenzie and Sawyer, 1966, 1967).

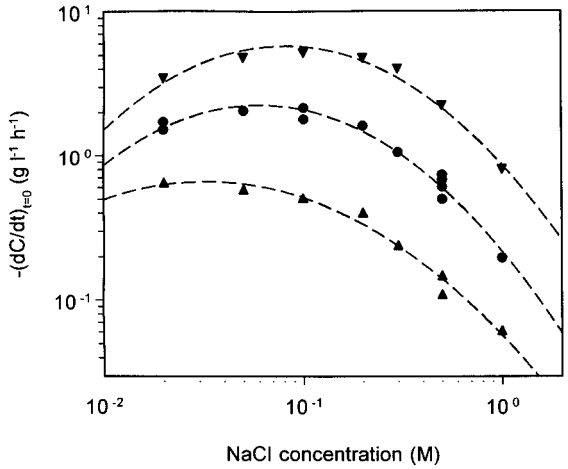

Figure 3. Initial reaction rate of the denaturation/aggregation of β-lg versus NaCl concentration at 68.5 °C for an initial protein concentration of 9 g/L: ▲, pH 6.5; ●, pH 7.0; ▼, pH 7.5.

NaCl Concentration at pH 6.5−7.5. In Figure 3 the initial reaction rate is plotted as a function of NaCl concentration (logarithmic axis) for pH 6.5, 7.0, and 7.5 and initial protein concentration of 9 g/L. An increase in pH from 6.5 to 7.5 causes an acceleration of the denaturation/aggregation process at each NaCl concentration, which is consistent with Figure 2 and is related to a shift toward the unfolded, denatured molecules in step 1 and an increasing contribution of chemical aggregation in step 2 (Shimada and Cheftel, 1989; Hoffmann and van Mil, 1997). As a function of the NaCl concentration the reaction rate shows a maximum, which shifts to higher NaCl concentration with increasing pH.

The NaCl concentration will affect both steps in the reaction scheme, and the maximum is caused by the combined effect of a reduced denaturation rate and increased aggregation rates at increasing salt concentrations. The equilibrium of step 1 is directly related to the conformational stability of β-lg, which is strongly coupled to the solubility of the protein; under solvent conditions at which proteins are highly soluble, their conformational stability is poor and vice versa (von Hippel, 1975; Arakawa and Timasheff, 1984; Kristjánsson and Kinsella, 1991). NaCl belongs to the salting-out class of salts and thus stabilizes the native protein conformation and decreases denaturation rate at high salt concentrations (von Hippel and Schleich, 1969). The rate of the aggregation reactions in step 2 will increase with NaCl concentration over the whole salt range investigated. At low salt concentrations (<0.1 M) the charge of the protein particles is screened and chemical and physical aggregation reactions are enhanced with increasing NaCl concentration. In addition, at high salt concentrations the solubility of protein particles is reduced and physical aggregation is further enhanced, leading to large aggregates [see for example Xiong et al. (1993)]. So, the maximum in overall reaction rate with increasing NaCl concentration, observed in Figure 3, is related to a simultaneous decrease in the rate of the denaturation reaction (step 1) and an increase in the rate of the aggregation reactions (step 2). The shift in the maximum to higher NaCl concentration with increasing pH (Figure 3) indicates that the screening by salt ions becomes more important at high pH. By measuring the differential change in optical density (dA_{320}/dT) as a function of temperature, Xiong (1992) observed that the transition temperature (defined as the temperature at which dA_{320}/dT has a maximum) first decreases and then increases with NaCl concentration. At pH 6.0 he found a minimum in the transition temperature at ~0.02 M NaCl, consistent with our findings.

Measurements by Arakawa and Timasheff (1987) indicate that the salting-in behavior of β-lg at low NaCl concentration will also contribute to the initial increase in reaction rate (Figure 3). They have reported that β-lg binds NaCl at neutral pH, caused by a unique charge distribution on the surface of the protein, giving the protein a large dipole moment (Cohn and Ferry, 1943). This binding of NaCl leads to an abnormal solubility behavior: the solubility (and thus denaturation rate) increases at low salt concentration, at which the binding of NaCl dominates, and decreases at high salt concentration. This is confirmed by DSC experiments, in which around 0.02 M NaCl a small minimum in thermostability as a function of NaCl concentration is observed (Hoffmann et al., 1995).

Order of Reaction. Figure 4 shows the fractional concentration of nonaggregated β-lg as a function of heating time for various initial protein concentrations at 0 M NaCl, pH 6.5 (Figure 4A) and pH 7.0 (Figure 4B), and 0.5 M NaCl, pH 6.5 (Figure 4C) and pH 7.0 (Figure 4D). At each initial protein concentration an increase in reaction rate is seen when the pH is increased from 6.5 to 7.0, for both 0 and 0.5 M NaCl. The insets in Figure 4 show the initial reaction rate as a function of the initial protein concentration on a double-logarithmic scale. The slope of these plots gives the initial reaction order, n, of the total denaturation/aggregation reaction (see eq 2). At 0 M NaCl n is 1.5 at pH 6.5 (Figure 4A) and 1.7 at pH 7.0 (Figure 4B), and at 0.5 M NaCl n is 1.0 at pH 6.5 (Figure 4C) and 1.4 at pH 7.0 (Figure 4D). The initial reaction order increases with pH and decreases with NaCl concentration. The increase with pH is caused by a decrease in stability of β-lg with pH (see Figure 2) and an increase in rate of the chemical reactions. The rate of the first-order unfolding reaction in step 1 will increase and the second-order (physical and chemical) aggregation reactions of step 2 will become rate limiting. The decrease in n with NaCl concentration (from 0 to 0.5 M) is caused by an increase in stability of β-lg at high salt levels, which decreases the rate of the first-order unfolding reaction in step 1 and makes it rate limiting at pH 6.5.

Aggregation of β-Lg Studied by *in Situ* Light Scattering. Light-scattering experiments were done at 68.5 °C as a function of pH (pH 6.5−7.5) and NaCl concentration (0−0.5 M). In Figure 5 the hydrodynamic diameter, d_h, and the scattered intensity, I_s, are shown as a function of heating time for 9 g/L β-lg solutions at pH 7.0 and various NaCl concentrations. The results allow an additional analysis of the aggregation reactions of step 2 at temperatures below T_p. Without added salt the size of the protein particles initially grows rapidly and after a short time reaches a more or less constant value of ~25 nm. Under these conditions, particles are formed via chemical aggregation (Roefs and de Kruif, 1994). Comparison with Figure 4B indicates that they do grow in number concentration rather than size upon prolonged heating (see eq 4). If NaCl is added prior to heating, the hydrodynamic diameter of the protein aggregates no longer remains constant but grows in

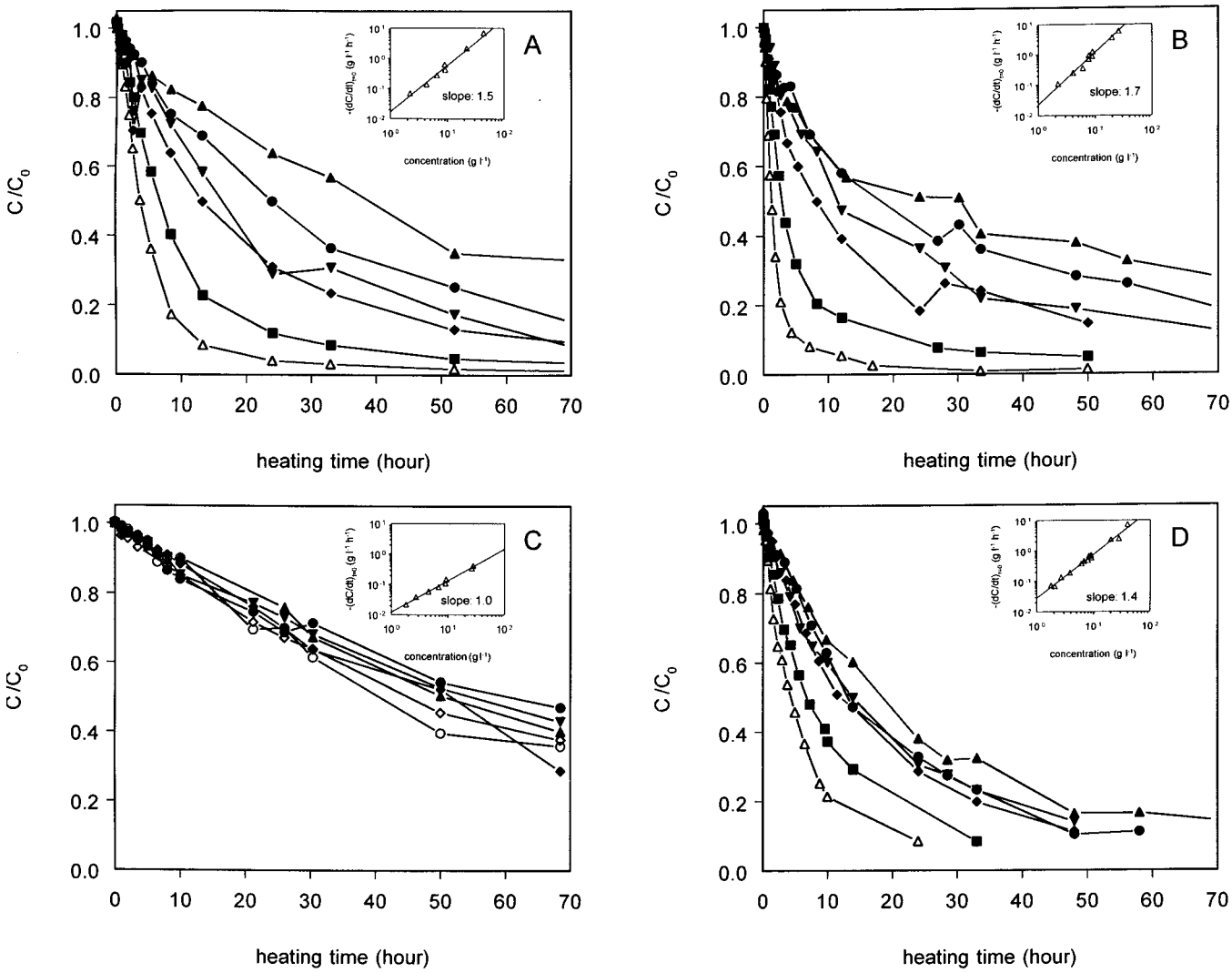

Figure 4. Fractional concentration of nonaggregated β-lg versus heating time at 68.5 °C for various initial protein concentrations: (A) 0 M NaCl, pH 6.5; (B) 0 M NaCl, pH 7.0; (C) 0.5 M, pH 6.5; (D) 0.5 M, pH 7.0. Nominal initial protein concentrations: ▲, 2 g/L; ○, 3 g/L; ●, 5 g/L; ▼, 7 g/L; ◆, 9 g/L; ■, 23 g/L; ◇, 27 g/L; △, 46 g/L. (Inset) Initial reaction rate versus initial β-lg concentration; the slope is the order of the total denaturation/aggregation reaction.

time. The particle size strongly increases with NaCl concentration. This is related to a decreased intermolecular repulsion, which promotes both chemical and physical aggregation, and a decreased solubility of the protein particles ("salting-out" effect), which promotes physical aggregation in step 2 (Kristjánsson and Kinsella, 1991).

In Figure 5 at salt concentrations of ≥0.1 M a lag phase is observed, followed by a second phase with clear growth of particle size and scattered intensity. This lag phase was reported earlier (Verheul et al., 1995; Elofsson et al., 1996) and is dependent on NaCl concentration, pH, heating temperature, and protein concentration. The NaCl concentration at which the particles in the second phase start to grow at pH 7.0 (0.1 M NaCl) corresponds to the position of the maximum in Figure 3. Above this NaCl concentration the lag time decreases with increasing NaCl concentration. In the lag phase only small particles are formed (i.e. denatured protein molecules or small aggregates such as those formed in the absence of salt). The concentration of these primary particles grows as the amount of nonaggregated protein decreases in time: native β-lg acts as a source that produces primary particles (see Figure 4). Above a certain concentration of primary particles, C^* (after the lag phase), a secondary (Schmoluchowski-type) aggregation mechanism becomes predominant. This is confirmed by Figure 6, in which I_s is plotted against the degree of conversion of nonaggregated β-lg (i.e. the relative amount of denatured/aggregated protein) at corresponding heating times. At higher NaCl concentrations an "upswing" in I_s is observed and the concentration of aggregated primary particles (C^*) at which the upswing starts is relatively low ($C^* \cong 0.6$ g/L at pH 7.0 and 0.5 M NaCl). Furthermore, C^* is independent of the initial β-lg concentration but decreases with increasing NaCl concentration and decreasing pH (Figure 6). At high NaCl concentration and low pH values very turbid systems are formed.

The kinetics of particle formation (Figure 7), including secondary aggregation, correlate well with the kinetics of the concentration decrease (Figure 4). Substituting $C^* = C_0 - C_{t^*}$ in eq 3 leads for the onset time of secondary aggregation, t^* (see Figure 7 for graphical definition), to

$$t^* \cong C^*/k_n C_0^{\,n} \qquad (5)$$

A double-logarithmic plot of t^* versus C_0 gives straight lines with a slope of $-n$. In Figure 8 the onset time is

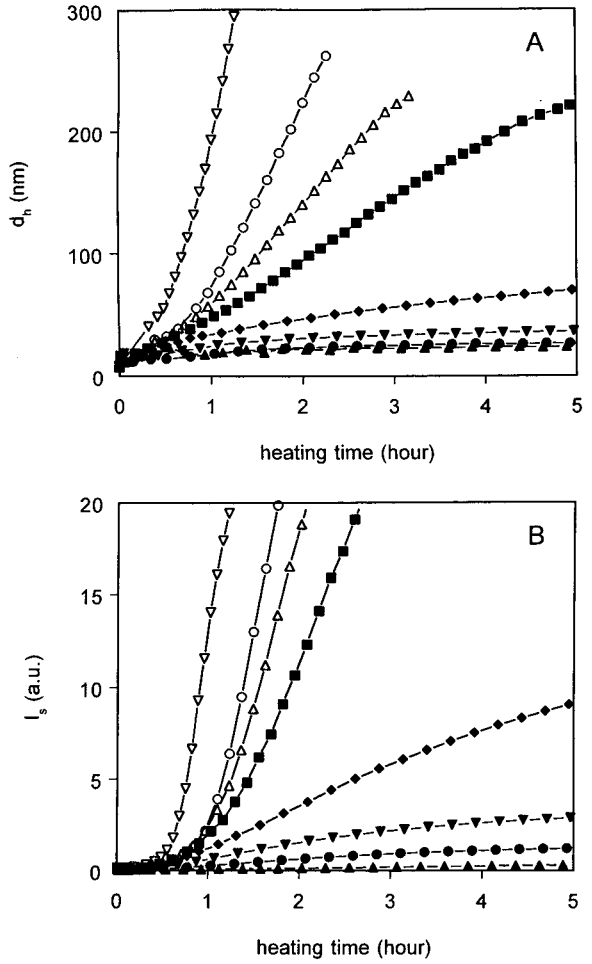

Figure 5. Hydrodynamic diameter (A) and scattering intensity (B) against heating time at 68.5 °C for β-lg solutions of 9 g/L at pH 7.0. NaCl concentrations: ▲, 0 M; ●, 0.02 M; ▼, 0.05 M; ◆, 0.1 M; ■, 0.2 M; △, 0.3 M; ○: 0.5 M; ▽, 1.0 M.

plotted against the initial protein concentration (2−46 g/L β-lg) at 0.5 M NaCl and pH 6.5, 7.0, and 7.5 on a double-logarithmic plot. t^* increases with decreasing initial protein concentration and decreases with increasing pH for all β-lg concentrations, and straight lines are obtained for the different pH values. The reaction order increases with pH, consistent with Figure 4, and the values of the slopes (−1.1 for pH 6.5, −1.3 for pH 7.0, and −1.5 for pH 7.5) agree quite well with the reaction orders of 1.0 for pH 6.5 and 0.5 M NaCl and 1.4 for pH 7.0 and 0.5 M NaCl in Figure 4.

General Discussion. The heat-induced aggregation of β-lg can be interpreted using a reaction scheme consisting of two steps: a denaturation equilibrium (with a first-order unfolding reaction) followed by second-order aggregation reactions. In the aggregation reactions either or both chemical and physical bonds are formed between the molecules. The experimental conditions determine the rate and nature of the reactions occurring. Two limiting cases concerning the overall reaction kinetics can be distinguished. These are (1) conditions under which the unfolding reaction is rate limiting, leading to an overall reaction order of 1, and (2) conditions under which the aggregation reactions are rate limiting, with an overall reaction order of 2. Situation 1 will occur at low heating temperature, at pH values closer to the isoelectric point of the protein and at high NaCl concentrations (because the unfolding reaction is delayed), and situation 2 at high heating

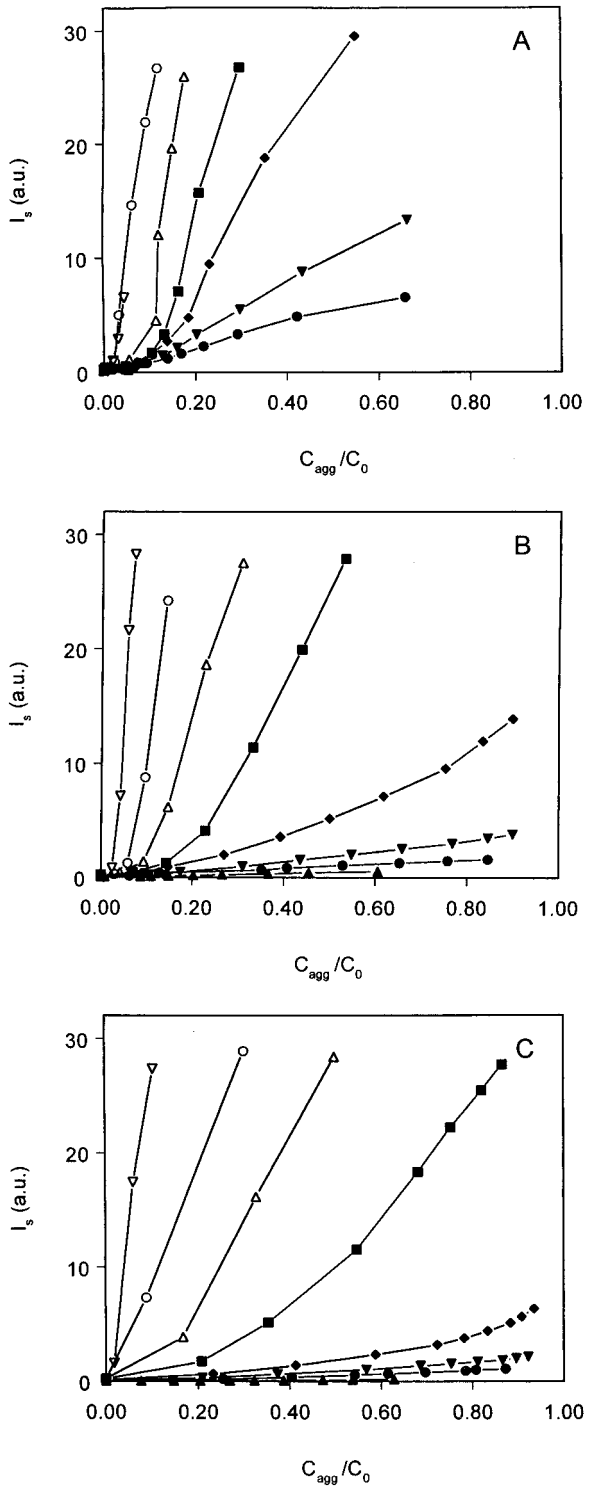

Figure 6. Scattering intensity versus fraction of denatured/aggregated protein in a 9 g/L β-lg solution heated at 68.5 °C (C_{agg} is the concentration of β-lg that is denatured/aggregated at a certain heating time) at pH 6.5 (A), pH 7.0 (B), and pH 7.5 (C). NaCl concentrations: ▲, 0 M; ●, 0.02 M; ▼, 0.05 M; ◆, 0.1 M; ■, 0.2 M; △, 0.3 M; ○, 0.5 M; ▽, 1.0 M.

temperature, pH values further from the isoelectric point, and low ionic strength (Figures 2−4). Furthermore, aggregation via chemical reactions is promoted at high pH values, at which the reactivity of thiol groups is increased (Shimada and Cheftel, 1989; Hoffmann and van Mil, 1997). Formation of physical bonds between proteins, on the other hand, is enhanced at pH values close to the isoelectric point and at high ionic strength,

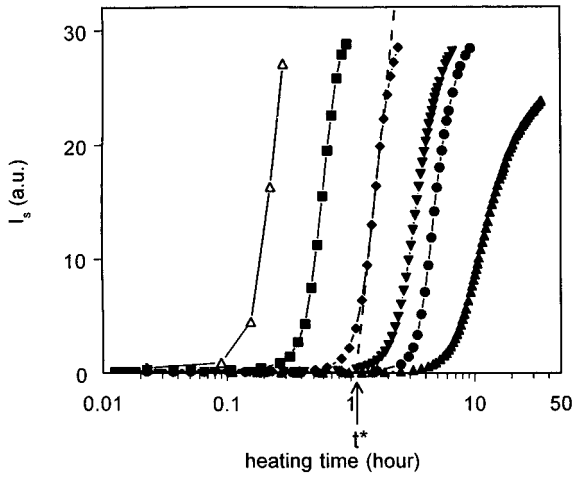

Figure 7. Scattering intensity versus heating time at 68.5 °C for β-lg solutions at pH 7.0 and 0.5 M NaCl. Nominal initial protein concentrations: ▲, 2 g/L; ●, 5 g/L; ▼, 7 g/L; ◆, 9 g/L; ■, 23 g/L; △, 46 g/L. Onset time of secondary aggregation, t^*, is the intersection of the slope of the secondary phase of I_s versus time with the time-axis as indicated for 9 g/L β-lg.

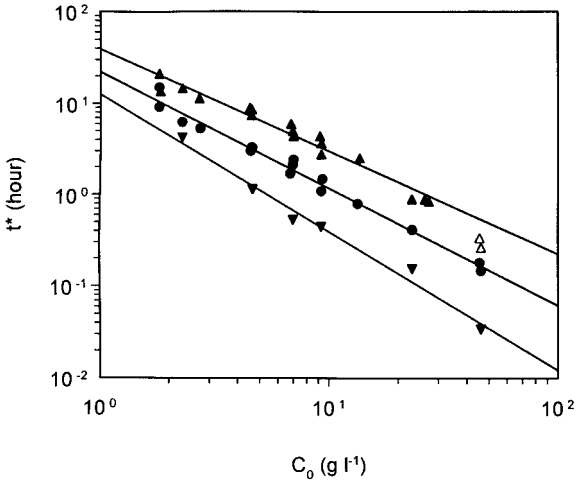

Figure 8. Onset time of secondary aggregation, t^*, versus initial protein concentration for β-lg solutions at 0.5 M NaCl and pH values of (▲, △) pH 6.5, (●) pH 7.0, and (▼) pH 7.5; (△) not used in linear fit.

because of decreased intermolecular repulsion and decreased solubility of the protein (Figure 5). The reaction rate shows a maximum as a function of NaCl concentration, caused by the opposing effects of an increasing aggregation rate and a decreasing unfolding rate (Figure 3). This observation is consistent with findings from other studies. For example, the position of the optimum coincides with the shift from a transparent to a very turbid system (Xiong, 1992; Xiong et al., 1993) and with the shift from a fine-stranded to a particulate gel (Langton and Hermansson, 1992) on heating a β-lg dispersion. Furthermore, exactly at these salt concentrations optima are found in gel hardness and gel stiffness of β-lg gels (Mulvihill et al., 1990; Matsudomi et al., 1991; Tang et al., 1995) and in the shear stress (at failure) of whey protein isolate gels (Kuhn and Foegeding, 1991).

At high NaCl concentrations physical bonding becomes increasingly important and large aggregates that continue to grow in time are formed (Figure 5). Under these conditions, two phases are observed in the aggregation step. The secondary aggregation starts at a certain critical concentration of primary particles, C^* (Figures 6−8). C^* is lower at higher salt concentrations and at pH values closer to the isoelectric point (Figure 6). Furthermore, the onset time of the secondary aggregation is dependent via a power law on the initial protein concentration (Figure 8), which is related to the order of the overall denaturation/aggregation reaction.

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