Heat-Induced Aggregation of β-Lactoglobulin as a Function of pH

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The effect of pH in the range 6.0−8.0 on the denaturation and aggregation of β-lactoglobulin (β-lg) was investigated. Results were interpreted in terms of the reaction scheme for the denaturation and aggregation of β-lg proposed by Roefs and De Kruif (*Eur. J. Biochem.* **1994**, *226*, 883−889). The rate of conversion of native β-lg increased strongly at higher pH values, whereas the molecular mass of the aggregates decreased strongly. In the pH range 6.4−8.0 aggregates were formed mainly by intermolecular disulfide bonds, but even at pH 6.0, thiol/disulfide exchange reactions were involved, although to a lesser extent. The time course of the exposure of the thiol group in native β-lg upon heating and the subsequent disappearance of this group through the formation of disulfide-linked aggregates was investigated by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) and varied strongly with pH. These observations could be used, in combination with the reaction steps of the reaction scheme, to describe qualitatively the strongly pH-dependent isothermal calorimetry curves, measured at 65 °C.

Keywords: *β-Lactoglobulin; denaturation; aggregation; pH; thiol reactivity*

INTRODUCTION

β-Lactoglobulin (β-lg) is the major protein in whey and therefore tends to dominate the thermal behavior of the total whey protein system. Because of this dominant role, a great deal of attention has been given to the properties of β-lg under various experimental conditions (Mulvihill and Donovan, 1987). Notwithstanding this, a complete picture is lacking. The objective of the present study was to systematically investigate the effect of pH (range pH 6.0−8.0) on the denaturation and aggregation of β-lg.

β-Lg is a globular protein and contains two disulfide bonds (C106−C119 and C66−C160) and one free cysteine (C121) (Papiz et al., 1986). At room temperature and at physiological pH β-lg exists mainly as a dimer, in which the monomers are noncovalently linked, but it dissociates into monomers (molecular mass = 18.3 kDa) at elevated temperatures (Georges et al., 1962; Dupont, 1965; Verheul et al., 1999). This dimer dissociation was shown to be a necessary step in the heat-induced aggregation mechanism (Cairoli et al., 1994; Iametti et al., 1996). Upon further heating (above 50 °C) the protein undergoes a conformational change, with increased exposure of previously buried hydrophobic groups and the thiol group. However, the nativelike backbone secondary structure is retained and this situation can be pictured as a "molten-globule state" (Ptitsyn, 1995). Hydrophobic interactions between the exposed groups can cause aggregation of the protein molecules while still in the molten-globule state. The thiol group in the modified monomer can induce thiol/disulfide exchange reactions, leading to the formation of disulfide-linked aggregates. The disulfide linkage involved in the intermolecular interchange reaction would most likely be the C66−C160 disulfide, which is found in one of the external loops of β-lg. The other disulfide is buried in the inner parts of the protein and is less available for reaction (McKenzie et al., 1972; Papiz et al., 1986).

The formation of aggregates via noncovalent interactions and via thiol/disulfide exchange reactions may occur simultaneously or sequentially, but it will be very difficult to distinguish between these steps experimentally. However, it is generally accepted that at neutral pH thiol/disulfide exchange reactions, leading to the formation of intermolecular disulfide bonds, are involved (Watanabe and Klostermeyer, 1976; Hillier et al., 1980; Shimada and Cheftel, 1989; Liu et al., 1994; McSwiney et al., 1994; Hoffmann and Van Mil, 1997).

In water at near-neutral pH the aggregation of β-lg can be described using the kinetic model proposed by Roefs and De Kruif (1994). In this kinetic model the denaturation and aggregation of β-lg is described by analogy with a radical-addition polymerization reaction. The reaction scheme contains an initiation, a propagation, and a termination step, and the free thiol group of β-lg plays the role of the radical. The initiation step consists of (a number of) reversible reactions, in which the β-lg dimer is split into two monomers, followed by an irreversible step, which is the real initiation reaction. This latter reaction is a first-order reaction in which the conformation of native β-lg (B) is transformed in such a way that the free thiol group becomes reactive (B*). In the propagation step the reactive thiol group of B* reacts via a thiol/disulfide exchange reaction with one of the two intramolecular disulfide bonds of a nonreactive β-lg molecule. An intermolecular disulfide bond is formed, and a new reactive thiol group becomes accessible, so the propagation step can be repeated many times, leading to the formation of (linearly linked) aggregates. The polymerization process stops when in the termination step two reactive intermediates react with each other, forming a polymer without a reactive thiol group.

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Assuming that the rate of initiation is much smaller than the propagation rate and using a steady-state principle, it follows that, at neutral pH, the decrease in concentration of native β-lg follows an overall reaction of order 1.5 (Roefs and De Kruif, 1994; Hoffmann et al., 1996). This assumption may not hold at other pH values, leading to different overall kinetics. However, although the kinetic equations may not hold at pH values other than neutral, it is still possible that the reaction scheme can be used in a broader pH range. The aim of this paper was to investigate to what extent the reaction scheme of Roefs and de Kruif can be used in the pH range 6.0−8.0.

In a previous paper we have shown that, by the use of isothermal calorimetry experiments, it was possible to model, at neutral pH, the rate of the individual steps in the reaction scheme (Hoffmann et al., 1997a). In these calculations a numerical integration of the reaction equations was used. In addition to the three reaction steps (initiation, propagation, and termination) of the model, the dissociation of the β-lg dimer into the monomers was treated as an extra reaction step.

In principle, all four reaction steps in the reaction scheme are pH dependent. This will be a combined effect of pH on the conformation of the molecule and the proportion of dissociated thiol groups. Although the exposed thiol group has been denoted the reactive species in the reaction scheme, it is in principle the thiolate anion. Because the pH values investigated are close to the p*K* value of the thiols, the proportion of thiolate anions will depend strongly on the pH of the reaction medium.

The effect of pH on the rate of aggregate formation, on the molecular mass of the aggregates formed, and on the role of the thiol group in aggregate formation was evaluated. The effect of pH on the molecular mass distribution of the aggregates was determined by the use of high-performance size-exclusion chromatography (HP-SEC) with multiangle laser-light scattering (MALLS) detection. The role of covalent disulfide bonds and noncovalent interactions in the formation of heat-induced aggregates was determined by treatment of heated solutions with 6 M urea or 6 M urea + 10 mM dithiothreitol (DTT) prior to analysis with the SEC-MALLS system.

Dissociation of noncovalently linked dimers and subsequent initiation reactions will lead to the exposure of reactive thiol groups. The propagation reaction does not affect the number of exposed, reactive thiol groups, whereas the formation of terminated polymers will lead to a decrease in exposed thiol groups. To understand in more detail the time course of these processes, the number of thiol groups accessible for reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in solutions that had been heated for different times was determined. Further insight into the effect of pH on the contribution of the different reaction steps to the overall reaction was obtained by isothermal calorimetry experiments at 65 °C. Results were interpreted in terms of the four steps of the reaction scheme.

MATERIALS AND METHODS

Materials. In all experiments a purified bovine β-lg sample, containing the genetic variants A and B (in a nearly 1:1 ratio) was used; it was prepared at the pilot plant of NIZO from whey, basically following the procedure of Maubois et al. (1987). The sample contained 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 (Hoffmann et al., 1996). It contained 4% moisture.

The protein was dissolved in double-distilled water in a concentration of 50 g of dry matter/L and stirred for 2 h. After the pH (range 6.0−8.0) had been adjusted with 0.1 M HCl or 0.1 M NaOH, the solution was stirred for 30 min. After preparation, all solutions were filtered (0.22 μm Millipore low-protein-binding filter). The solutions were stored at 4 °C and were used for further experiments within 24 h of preparation.

Heat Treatment. A series of test tubes each containing ∼5 mL of a β-lg solution was heated in a water bath at 65 °C. After various time periods, a tube was taken and cooled in ice−water and then stored at 4 °C.

Determination of Concentration of Native β-Lg. The concentration of native β-lg in the heated samples was determined by diluting the samples with double-distilled water to a concentration of ∼2.5 g of native β-lg/L. The pH was adjusted to 4.7 ± 0.1, and the aggregates of denatured proteins were separated by centrifugation at 20000*g* for 30 min. The native β-lg concentration present in the supernatant was determined by HP-SEC (Phenomenex column, type TSK G2000 SW_{XL}), with detection at 280 nm (Hoffmann et al., 1996; De Wit, 1990).

SEC-MALLS. The heated β-lg solutions were applied to a high-performance gel chromatography system, consisting of Phenomenex TSK G2000 SW_{XL} and TSK G4000 SW_{XL} silica gel columns (30 × 0.78 cm) in series (Hoffmann et al., 1997b). The exclusion limits of these two columns are 6×10^4 and 1×10^6 Da, respectively, for proteins. The columns were eluted with a phosphate buffer (6.956 g of KH_2PO_4, 6.956 g of K_2HPO_4, and 21.410 g of Na_2SO_4 in 1 L of double-distilled water, pH 6.5; buffer A) at a flow rate of 0.8 mL/min. Heated samples were diluted with double-distilled water to a β-lg concentration of 10 g of dry matter/L, and 0.01% (w/v) sodium azide was added to prevent microbial spoilage. These samples were further diluted with eluent to a final concentration of 1.0 g of dry matter/L. After filtration (0.22 μm Millipore low-protein-binding filter), 100 μL was injected into the chromatographic system. To determine the role of covalent and noncovalent interactions in the formation of aggregates, samples (concentration of 1.0 g of dry matter/L) were also incubated with buffer B (= buffer A containing 6 M urea; pH 6.8) or with buffer C (= buffer B containing 10 mM DTT; pH adjusted to 8.3 with 1 M NaOH). Samples were incubated at room temperature for 1 and 24 h in buffer B or C, respectively, and elution was done in buffer B.

For on-line light scattering detection a DAWN-F MALLS photometer (Wyatt Technology, Santa Barbara, CA) was used, equipped with a K5 flow cell and a linearly polarized He−Ne laser-light source (5 mW) with wavelength $\lambda = 632.8$ nm. The range of wave vectors (q) covered is $0.026 > q > 0.0066$ nm^{-1}, with $q = (4\pi n_s/\lambda)\sin(\theta/2)$, n_s being the solvent refractive index and θ being the scattering angle (Tanford, 1961). The mass of eluting material was determined on-line with a UV spectrophotometer (LKB Bromma 2140 Rapid Spectral Detector) at 220 nm (buffer A) or 280 nm (buffer B). The data were accumulated and processed using Astra for Windows, version 4.0, software. Prior to the measurements, the DAWN-F MALLS photometer was calibrated with filtered, HPLC-quality toluene and normalized using a bovine serum albumin solution. The molecular mass M_i and the mean square radius $\langle r^2\rangle_i$ of material eluting in each slice i were calculated with a first-order Debye fit, using a specific refractive index increment (dn/dc) of 0.161 cm^3/g for buffer A and 0.131 cm^3/g for buffer B and a second virial coefficient (A_2) of zero (Hoffmann et al., 1997b).

Isothermal Calorimetry Experiments. The microcalorimeter system used was the 2277 Thermal Activity Monitor (TAM), Thermometric AB (formerly LKB), Sweden (Suurkuusk and Wädso, 1982). The system consists of a 25 L thermostated water bath, which can hold up to four independently operated calorimeter units. At the beginning of an experiment 2.50 ± 0.01 g of protein solution (50 or 100 g of dry matter/L) was placed into a stainless steel vessel (4 mL) and sealed with a

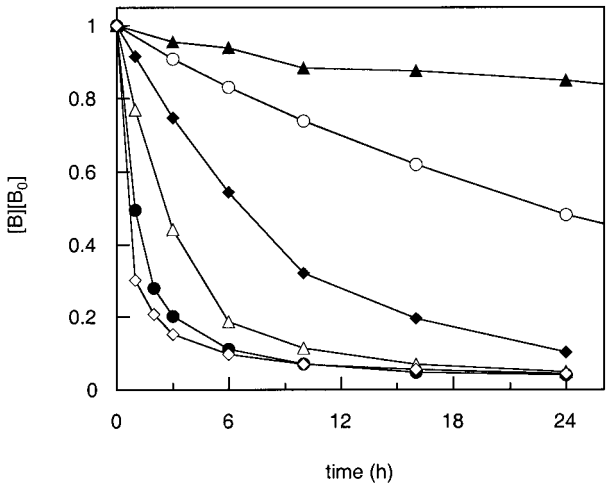

Figure 1. Influence of pH on the fractional decrease in concentration of native β-lg ([B]/[B_0]) as a function of time at 65 °C: (▲) pH 6.0; (○) pH 6.4; (◆) pH 6.8; (△) pH 7.2; (●) pH 7.6; (◇) pH 8.0. Initial concentration ([B_0]) was 50 g of dry matter/L).

Teflon washer and a stainless steel screw cap. The reference vessel contained 2.50 ± 0.01 g of double-distilled water. The vessels were allowed to equilibrate for 20 min in the temperature equilibration position of the TAM. After this time, the vessels were lowered into the measurement position and the computer program and the monitoring of the heat flow signal were started (Hoffmann et al., 1997a).

Determination of Number of Reactive Thiol Groups. The reactivity and the accessibility of the free thiol group in heated β-lg solutions (50 g of dry matter/L) was determined by reaction with DTNB′. DTNB was dissolved in a 0.05 M Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, at a concentration of 0.133 g/g of buffer. The heated β-lg solutions were, after cooling in ice−water, put at room temperature for 30 min prior to the analysis with DTNB (in a molar ratio DTNB/β-lg monomer of 5:1). DTNB solution (2340 μL) was placed in a 10 mm quartz cuvette, and after addition of 60 μL of β-lg solution, the absorbance at 412 nm was measured at room temperature as a function of time with a Perkin-Elmer Lambda 2 spectrophotometer. The cuvettes for the blank measurements contained 2340 μL of DTNB solution and 60 μL of 0.05 M Tris-HCl buffer.

RESULTS AND DISCUSSION

Decrease in Concentration of Native β-Lg. The concentration of native β-lg in the heated solutions was determined by HP-SEC analysis, and the rate of conversion of native β-lg was found to increase with increasing pH (Figure 1). At pH 6.8 the decrease in concentration can be fitted with a reaction of order 1.5 kinetics, as predicted by the model of Roefs and De Kruif (1994). At lower and higher pH values the determination of the reaction order is not unambiguous. At lower pH values there is a tendency toward order 1 kinetics, whereas for higher pH values the experimental results can be fitted best with a reaction of order 2 or higher.

In principle all four of the reaction steps in the reaction scheme will be pH dependent. High pH facilitates the dissociation and the subsequent unfolding. This may be related to the Tanford transition, a conformational change that is known to occur at room temperature at pH ∼7.5 (Tanford et al., 1959). In this conformational change the thiol group becomes more reactive (Dunnil and Green, 1965; Brownlow et al., 1997; Qin et al., 1998). With increasing pH the degree of dissociation of the thiol group increases. Because the thiolate anion is the reactive species in the thiol/disulfide exchange reactions, the rate of the propagation and termination step increases with increasing pH. The rate constants of the different reaction steps will be affected by pH in different ways, leading to different overall reaction kinetics.

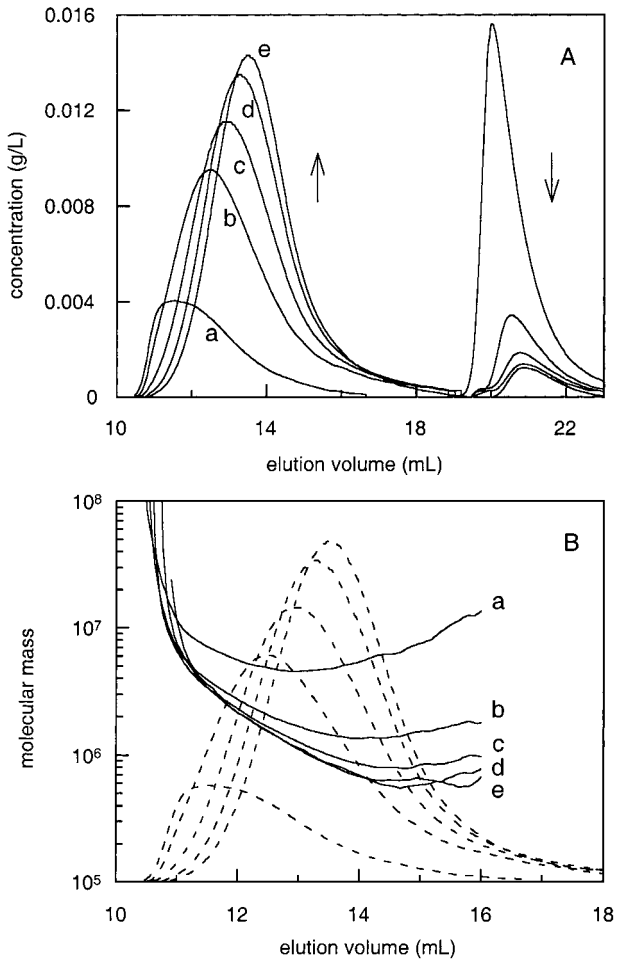

Figure 2. (A) SEC-UV elution profiles of 50 g/L β-lg solutions (pH range 6.4−8.0), heated for 24 h at 65 °C. As indicated by the arrows, the native β-lg peak decreases in the order pH 6.4 > pH 6.8 > pH 7.2 > pH 7.6 > pH 8.0, whereas the aggregate peak increases. (B) Molecular masses calculated by multiangle laser-light scattering for the aggregate peaks in (A) as a function of elution volume. These molecular masses (solid lines) have been laid over the SEC-UV elution profiles (dashed lines) of the aggregate peaks at various pH values: (a) pH 6.4; (b) pH 6.8; (c) pH 7.2; (d) pH 7.6; (e) pH 8.0.

SEC-MALLS Elution Profiles. The effect of pH on the molecular mass distributions of the aggregates formed was analyzed by SEC-MALLS, and the elution profiles for solutions heated for 24 h at 65 °C are shown in Figure 2A. In the chromatograms two peaks can be seen: a peak assignable to residual native β-lg (dimers and monomers) at a retention volume of ≈21 mL and an aggregate peak at lower elution volumes. The native β-lg peak in the 24 h heated solutions decreases with increasing pH, whereas the aggregate peak increases and shifts toward higher elution volumes, that is, lower molecular masses. At pH 6.0 no aggregate peak was observed (data not shown), probably due to the very slow reaction at this pH. From Figure 1 we can derive that at this pH after 24 h of heating, <15% of the initial β-lg concentration has been converted into aggregates.

The molecular masses calculated by the MALLS photometer for the material eluting in each slice of the

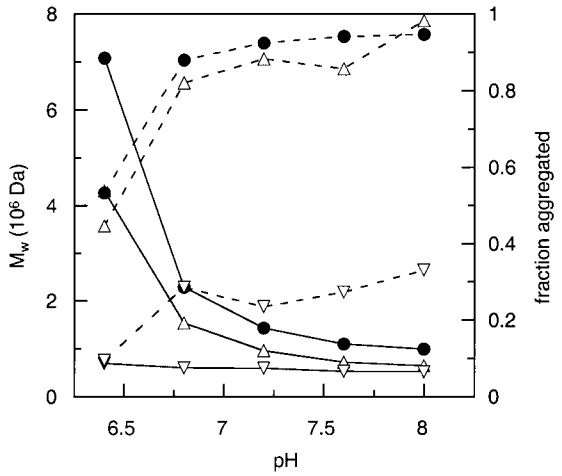

Figure 3. Effect of treatment in 6 M urea and in 6 M urea + 10 mM DTT (pH 8.3) prior to analysis of heated β-lg solutions (24 h, 65 °C). The fraction of aggregated β-lg (based on the peak area of the aggregate peak in the TSK G2000 SW_{XL}/TSK G4000 SW_{XL} chromatograms) (dashed lines) and the weight-averaged molecular masses (M_w) calculated for the aggregate peak (solid lines) are plotted as a function of pH for samples treated in three different ways: (●) no pretreatment, analysis in phosphate buffer, pH 6.5 (= buffer A); (△) incubated for 1 h in buffer A containing 6 M urea (= buffer B, pH 6.8), analysis in buffer B; (▽) incubated for 24 h in buffer B containing 10 mM DTT (pH 8.3), analysis in buffer B.

aggregate peak are shown in Figure 2B. In general, in the central part of all aggregate peaks a linear relationship between log *M* and elution volume was observed, which corroborates the separation power of the chromatographic system used. Although light scattering provides an absolute measurement of molecular masses and does not rely on a relation between molecular mass and retention time, the elution is not necessarily governed by differences in molecular mass only. The gross shape of the molecule (including the hydration shell) and its physicochemical characteristics, especially net charge and hydrophobicity, may also play a role in separation (Billingham, 1977). If separation were based on molecular mass only, one would expect the log *M* versus elution plots to overlap, as can be seen with pH 7.2, 7.6, and 8.0. However, the molecular mass calculated for material eluting at a particular retention time was found to increase at pH 6.8 and even more at pH 6.4. This indicates that the aggregates formed at pH 6.4 and 6.8 do not only have higher molecular masses but may also have a different conformation (i.e., more compact) or different physicochemical properties (i.e., less negatively charged) compared with the aggregates formed at higher pH values.

Interactions Involved in Aggregate Formation. To elucidate the role of covalent intermolecular disulfide bonds and noncovalent interactions in the aggregate formation at the different pH values, heated β-lg solutions were incubated with 6 M urea or 6 M urea + 10 mM DTT prior to elution in phosphate buffer, containing 6 M urea. The effects of these treatments on the fraction of aggregated material and the weight-averaged molecular mass (M_w) of the aggregates in β-lg solutions that had been heated for 24 h at 65 °C are reported in Figure 3.

Without treatment, M_w was found to decrease with increasing pH, whereas the fraction of aggregated material increased. Treatment with 6 M urea had little effect on the fraction of aggregated material (on the basis of the peak area of the aggregates relative to the total peak area). However, in the chromatograms of the solutions heated at pH values in the range 6.8−8.0 two or three small shoulders at the high molecular mass end of the native β-lg peak appeared (with molecular masses in the range $2 \times 10^4 - 3 \times 10^5$ Da). These oligomers of β-lg were not formed due to the dissociation of high molecular mass aggregates in 6 M urea but were formed from native β-lg (Hoffmann et al., 1997b). Upon treatment with 6 M urea, at all pH values a small decrease in M_w of the aggregate peak was observed, presumably due to the splitting of a fraction of high molecularr mass aggregates into smaller aggregates. This indicates that some (primary) disulfide-linked aggregates had reacted further to larger aggregates via noncovalent interactions.

After incubation with 6 M urea + 10 mM DTT at all pH values a considerable decrease in the amount of aggregated protein occurred, demonstrating that a large proportion of high molecularr mass aggregates were held by intermolecular disulfide bonds. However, no complete dissociation of the aggregates into monomers/dimers was observed, as also has been reported in an earlier SEC-MALLS study (Hoffmann et al., 1997b). The M_w values calculated for the aggregates obtained after incubation with 6 M urea + 10 mM DTT were independent of pH ($M_w \approx 7 \times 10^5$).

The results obtained illustrate that at all pH values in the range 6.4−8.0 intermolecular disulfide bridges play an important role in the formation of heat-induced β-lg aggregates. At pH 6.0 the conversion was too slow and the concentration of aggregates in the heated solutions too low for evaluating the effect of urea or DTT. However, with SDS−PAGE analysis under nonreducing conditions high molecular mass aggregates were observed on top of the gel, which disappeared under reducing conditions (results not shown), indicating that also at this pH intermolecular disulfide bonds are involved in aggregate formation.

Exposure and Disappearance of Reactive Thiol Groups. To understand in more detail how pH affects the aggregation of β-lg, we followed the exposure of the thiol group (dissociation and initiation step) and its subsequent disappearance upon the formation of terminated polymers (two thiol groups per terminated molecule). The propagation step does not affect the total number of free, reactive thiol groups, as one thiol group disappears in the formation of a disulfide bond and a new thiol group becomes reactive. The exposure and disappearance of free, reactive thiol groups in the heated solutions was determined by reaction with DTNB.

In the reaction of DTNB with the thiol groups of native β-lg two steps can be distinguished: (1) diffusion of DTNB into the β-lg molecule to the thiol group and (2) reaction of DTNB with the thiolate anion. At neutral pH the thiol group in native β-lg is shielded by other groups (Hoffmann and van Mil, 1997) and DTNB has to diffuse into the molecule before reaction can occur.

Control experiments showed that reaction of DTNB with generic thiols (cysteine and glutathione) at concentrations equivalent to that of β-lg was instantaneous on the experimental time scale. In unheated β-lg solutions no thiol groups were titrated within this time, independent of the pH. This shows that at all pH values investigated, the thiol group in unheated solutions is not exposed but shielded in the molecule. With increas-

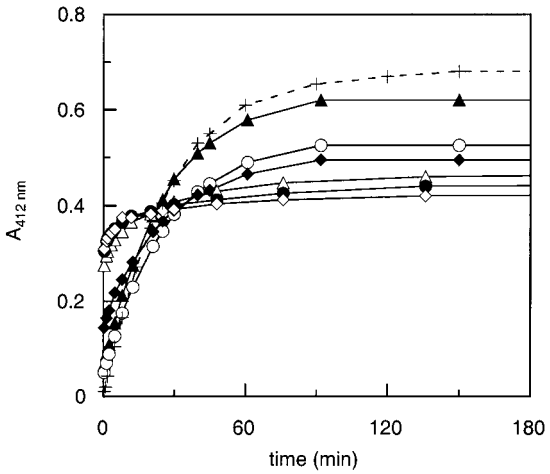

Figure 4. Time course of the increase in absorbance at 412 nm upon addition to DTNB solution in the cuvette of a β-lg solution that has been heated for 6 h at 65 °C: (▲) pH 6.0; (○) pH 6.4; (◆) pH 6.8; (△) pH 7.2; (●) pH 7.6; (◇) pH 8.0. Also, the increase in absorbance measured with an unheated solution (pH 6.8) is shown (+).

ing reaction time A_{412} was found to increase (see the results for the unheated solution at pH 6.8 in Figure 4), due to the slow diffusion of DTNB into the β-lg molecule. The results obtained with the unheated solutions at the other pH values were identical (results not shown). This demonstrates that even at pH 8 in the unheated solution the thiol group is shielded in the β-lg molecule. Furthermore, we can conclude from these results that step 1 (diffusion of DTNB in the β-lg molecule) is the rate-determining step. At all pH values within 2 h a plateau level ($A_{412,max}$) was reached, corresponding to titration of 0.85 thiol group/mol of β-lg monomer. Therefore, not all thiol groups could be titrated, and this is probably due to oxidation of thiol groups in the time needed to reach the maximum level.

Figure 4 also shows the effect of pH on the time course of the absorbance increase upon addition of a β-lg solution that has been heated for 6 h at 65 °C to the DTNB solution in the cuvette. Because the reaction of DTNB with generic thiols was shown to be very fast on the time scale of the experiments, we can assume that the thiol groups determined immediately after addition of heated β-lg solutions to the DTNB solution in the cuvette correspond to exposed, reactive thiol groups. Practically, this number was determined by extrapolation of the absorbance at 412 nm, measured at early times in the experiment, to time $t = 0$ ($A_{412,t=0}$). The number of directly accessible thiol groups in the 6 h heated solutions (i.e., $A_{412,t=0}$) increases with increasing pH, demonstrating that with increasing pH more reactive β-lg intermediates (B_i*, $i \geq 1$) with a reactive, free thiol group are present in the heated solution. The absorbance increases with time due to the reaction of DTNB with shielded thiol groups in the residual native β-lg molecules. The absorbance reaches a maximum level ($A_{412,max}$), corresponding to the total number of thiol groups in solution, that is, the total number of thiol groups in native β-lg minus the thiol groups that have been involved in the formation of a disulfide bond in a termination step. For all pH values $A_{412,max}$ of the 6 h heated solution was lower than the value obtained with the unheated solution and the value decreased with increasing pH, indicating that with increasing pH fewer thiol groups are available for reaction with DTNB

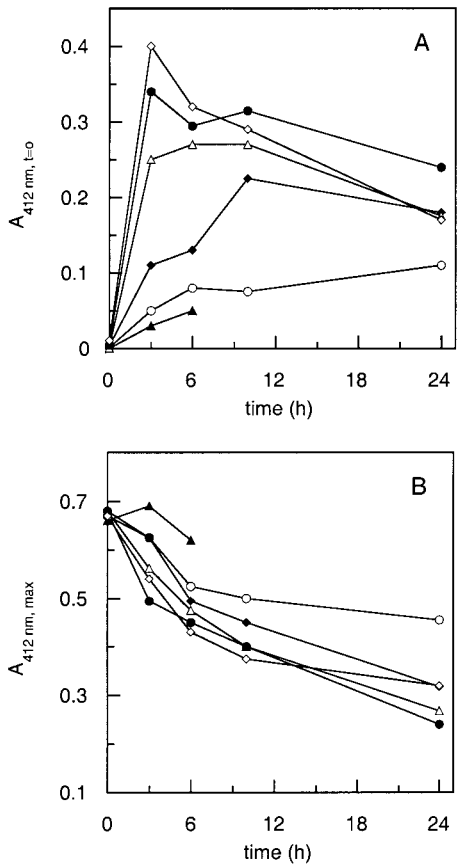

Figure 5. (A) Initial absorbance (e.g., absorbance extrapolated to $t = 0$; $A_{412nm,t=0}$) and (B) maximum absorbance at 412 nm ($A_{412nm,max}$) as a function of heating time at 65 °C for the various pH values: (▲) pH 6.0; (○) pH 6.4; (◆) pH 6.8; (△) pH 7.2; (●) pH 7.6; (◇) pH 8.0.

because more thiol groups have been involved in oxidation reactions leading to a terminated molecule.

The increase in the number of terminated molecules with increasing pH is in line with the above-discussed effect of pH on the rate of conversion of native β-lg (Figure 1). High pH facilitates the dissociation and the subsequent unfolding. Although the denaturation/aggregation of β-lg is accelerated with increasing pH; aggregate size was found to decrease with increasing pH (Figures 2 and 3). This can be ascribed to the fact that at early stages of the reaction already a large number of reactive intermediates, with a reactive, exposed thiol group, are formed. This increases the probability of termination reactions, resulting in the formation of more, but smaller, disulfide-linked, terminated aggregates and in a faster decrease in the number of thiol groups available for reaction with DTNB, compared with lower pH values (Figure 4). Figure 5 summarizes the $A_{412,t=0}$ and $A_{412,max}$ values obtained for the various pH values as a function of heating time at 65 °C. At pH 6.0 the solutions became turbid during heat treatment, due to the formation of a few but very large aggregates, which disturbed the absorbance measurement with DTNB. At pH 6.4 $A_{412,t=0}$ increases with increasing heating time (Figure 5A), due to the progressive formation of reactive intermediates, with an exposed thiol group. At the other pH values $A_{412,t=0}$ also increases in the initial part of the reaction but starts decreasing after a certain time. Although at this time reactive intermediates, with an exposed thiol group, will still be formed, the decrease in the number of free thiol

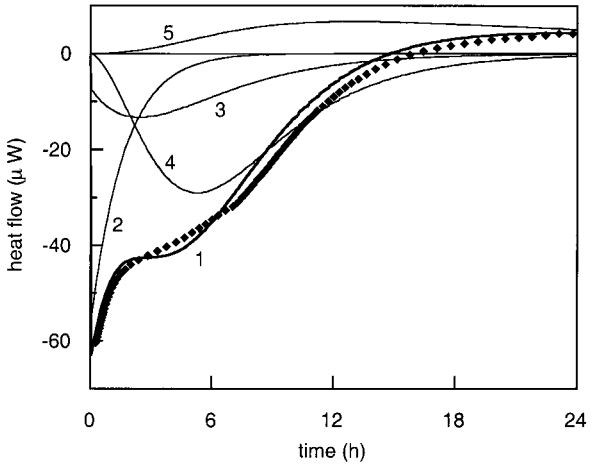

Figure 6. Experimental (◆) and calculated (line) heat flow versus time curves for 100 g/L β-lg at 65 °C (pH 6.8). The calculated curve (1) is calculated with the previously described set of reaction parameters at 65 °C (Hoffmann et al., 1997a). In addition to the total calculated heat flow versus time curve is shown the contribution of the different reaction steps is shown: (2) dissociation; (3) initiation; (4) propagation; (5) termination reaction.

groups due to the formation of terminated polymers has become dominant. The time at which $A_{412,t=0}$ reaches a maximum value decreases with increasing pH. At pH 7.6 and 8.0 the maximum level is reached within 3 h, and at this time <20% of native β-lg is left (see Figure 1), indicating that indeed only a limited further delivery of reactive β-lg intermediates can occur. For all pH values $A_{412,max}$ was found to decrease with increasing heating time (Figure 5B) due to the progressive formation of terminated molecules.

Isothermal Calorimetry Experiments. Further insight into the effect of pH on the contribution of the different reaction steps (dissociation, initiation, propagation, and termination reaction) to the overall reaction was obtained with isothermal calorimetry experiments. A previous study has shown that isothermal calorimetry is an adequate technique for unraveling these processes (Hoffmann et al., 1997a). By combining the strongly concentration-dependent isothermal calorimetry curves measured at 65 °C with experimental information on the rate of decrease in concentration of native β-lg at that temperature, we succeeded in deriving a "best" parameter set (i.e., values for the rate constants at 65 °C and the reaction heats of the dissociation, initiation, propagation, and termination reaction). Also, information on the temperature dependence of the rate constants was obtained.

On the baiss of the reaction parameters determined at neutral pH (Hoffmann et al., 1997a) the contribution of the different reaction steps to the overall reaction heat measured for 100 g/L can be visualized as shown in Figure 6. The dissociation reaction contributes much to the relatively large, endothermic heat flow measured at the early stage of the reaction and lasts for a relatively short time. The heat associated with the initiation step is relatively small, whereas the propagation step determines more or less the characteristic curvature of the thermograms. The exothermic heat effect of the termination reaction continues for a very long time and causes the total heat flow to become positive after longer reaction times, when the contribution of the initiation and propagation reaction has become very small.

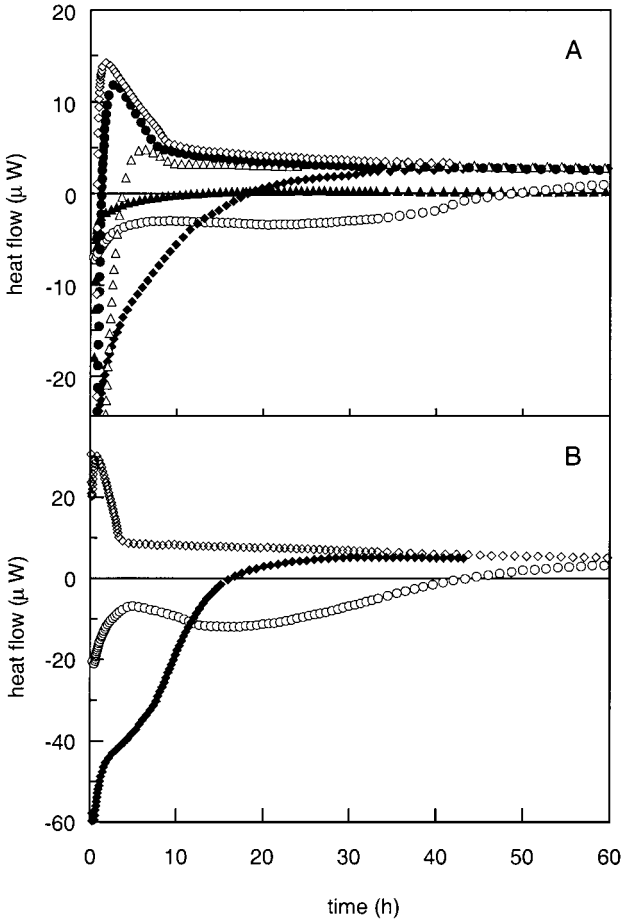

Figure 7. Heat flow versus time curves at 65 °C for (A) 50 g/L and (B) 100 g/L β-lg solutions of various pH values: (▲) pH 6.0; (○) pH 6.4; (◆) pH 6.8; (△) pH 7.2; (●) pH 7.6; (◇) pH 8.0.

Figure 7 shows the thermograms obtained at 65 °C with 50 and 100 g/L β-lg at various pH values, and as may be seen, the shape of the thermograms is strongly pH dependent. In principle all four reaction steps in the reaction scheme can be pH dependent, and therefore it will probably be difficult to derive the reaction constants and reaction heats at the different pH values from the thermograms obtained. However, with the help of the above-discussed effects of pH on the rate of conversion of native β-lg, the number of thiol groups accessible for reaction with DTNB, and the size of the formed aggregates, some trends can be derived that can explain the strongly pH-dependent appearance of the thermograms in a qualitative way. As most experiments were performed with 50 g/L β-lg, we start by discussing the thermograms obtained with this concentration (Figure 7A). However, with 100 g/L β-lg (Figure 7B) similar trends can be seen.

At pH 6.0 the conversion of native β-lg occurs very slowly and, although even at this pH value thiol groups seem to be involved in the formation of disulfide-linked aggregates, the very large aggregates formed at this pH are mainly physical aggregates. Physical aggregation will involve no, or only a very small, exothermic heat effect. At this pH the dissociation and subsequent unfolding of the molecule occur very slowly and, as a consequence, a very small heat effect is measured. Although the shape of the thermogram at pH 6.4 differs from that at pH 6.8, it can be reconstructed from the same four reaction steps, but the reactions are occurring

much more slowly. At pH 7.2, 7.6, and 8.0 initially a very large endothermic heat flow is measured. This endothermic heat flow decreases rapidly, and within a few hours the overall heat flow becomes exothermic. This can be ascribed to a fast dissociation and subsequent unfolding of the native β-lg molecules at elevated pH, and the rate of these processes increases with increasing pH. Before the isothermal calorimetry measurement was begun, the vessels had to be equilibrated for 20 min in the equilibration position of the TAM. In this time the reactions have already started and a substantial part of the overall reaction heat cannot be measured. After the heat flow signal has become positive, the overall reaction heat is caused mainly by the exothermic heat effect involved with the termination reaction. The time at which the heat flow signal becomes positive decreases with increasing pH. At all pH values (with the exception of pH 6.0) the fraction of native β-lg was below 20% at the moment the heat flow signal changed from endothermic to exothermic (Figure 1), so the contribution of dissociation, initiation, and propagation reactions has become very small. At pH 7.2, 7.6, and 8.0 after 10 h at 65 °C, a sudden change in the rate of heat production can be observed, and after that time only a very slow further decrease of the exothermic heat flow occurs. Ten hours corresponds with the time at which almost all native β-lg has been converted (Figure 1) and, as such, no further formation of B_1* will occur. This time corresponds also very roughly with the time at which the number of exposed thiol groups, which are directly available for reaction with DTNB, starts decreasing (i.e., the time at which the maximum in $A_{412,t=0}$ occurs). However, at this time reactive β-lg intermediates with an exposed thiol group are still available (Figure 5A), which can react to terminated molecules. However, we cannot exclude that this long-lasting exothermic heat flow is (partly) caused by other (secondary) reactions, such as, for example, intra- or intermolecular thiol/disulfide bond reshuffling reactions and exposure of a shielded thiol group in a terminated polymer, which can also initiate thiol/disulfide exchange reactions, leading to the formation of branched aggregates. The probability of the occurrence of such secondary reactions will increase with pH owing to the higher reactivity of the thiol group.

Conclusions. The aggregation of β-lg was shown to be very sensitive to the pH of the reaction medium. The rate of conversion increased strongly with pH, whereas the molecular mass of the aggregates, determined with size-exclusion chromatography in combination with laser-light scattering, decreased strongly. In the pH range 6.4−8.0 aggregates were mainly formed by intermolecular disulfide bonds, and even at pH 6.0, at which very large noncovalently linked aggregates were formed, also thiol/disulfide exchange reactions seem to be involved. The dissociation and initiation reactions are accelerated to a large extent with increasing pH, and due to the formation of many reactive intermediates in the initial stages of the reaction, the probability of termination reactions increases, leading to the formation of smaller aggregates. These effects could be used to describe qualitatively the strongly pH-dependent isothermal calorimetry curves, measured at 65 °C.

On the basis of the obtained results we think that the reaction scheme proposed by Roefs and De Kruif (1994) holds not only at neutral pH but in a broader pH range. However, the reaction rate constants, especially the rate constants of the dissociation and initiation reactions, depend strongly on pH, leading to different overall reaction kinetics.

ABBREVIATIONS USED

β-lg, β-lactoglobulin; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; HP-SEC, high-performance size-exclusion chromatography; M_n, number-averaged molecular mass: M_w, weight-averaged molecular mass; PAGE, polyacrylamide gel electrophoresis; MALLS, multiangle laser-light scattering; TAM, thermal activity monitor; UV, ultraviolet.

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