Heat-Induced Aggregation of β-Lactoglobulin: Role of the Free Thiol Group and Disulfide Bonds

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The heat-induced aggregation of bovine β-lactoglobulin, dispersed in water at neutral pH and in different concentrations (10, 30, or 50 g of dry matter/L), was studied at 65 °C, and the results are related to a kinetic model. Native PAGE and SDS−PAGE analysis under nonreducing and reducing conditions showed that on heating disulfide-linked aggregates were formed and that the average size of these aggregates increased with increasing initial β-lactoglobulin concentration. In the presence of the thiol-blocking agent *N*-ethylmaleimide (NEM), at a molar ratio of NEM/β-lactoglobulin monomer of 1, all thiol groups were blocked and no disulfide-linked aggregates were formed, although with native PAGE high molecular mass noncovalently linked aggregates were observed. The formation of these aggregates accelerated with increasing NEM concentration until a molar ratio of NEM/β-lactoglobulin monomer of 1 was reached. In separate experiments we studied the effect of pH (in the range pH 6.0−8.0) on the aggregation of β-lactoglobulin and related this to the pH dependent reactivity of the thiol group.

Keywords: *β-Lactoglobulin; thermal treatment; denaturation; aggregation; thiol reactivity*

INTRODUCTION

Whey proteins are functional ingredients in foods, and the effect of heat treatment on their functional properties is essential to a variety of applications of these proteins (Mulvihill and Donovan, 1987). The major whey protein is β-lactoglobulin (β-lg) (McKenzie, 1971). This globular protein contains two disulfide bonds (C106−C119 and C66−C160) and one free cysteine (C121) (McKenzie et al., 1972; Papiz et al., 1986). At room temperature and at physiological pH, β-lg exists mainly as a dimer, but it dissociates into monomers (M_r = 18 300) at elevated temperatures. At 40 °C the protein undergoes some small, reversible conformational changes, whereas on further heating the protein (partially) unfolds, resulting in a molten-globule-like structure (Iametti et al., 1996), with increased exposure of the previously buried inner hydrophobic groups and the thiol group. 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).

It is generally accepted that these thiol/disulfide exchange reactions, leading to the formation of intermolecular disulfide bonds, play a role in the heat-induced aggregation and gelation of β-lg (Sawyer, 1968; Watanabe and Klostermeyer, 1976; Hillier et al., 1980; Shimada and Cheftel, 1989; Liu et al., 1994; McSwiney et al., 1994a,b). In addition to this chemical aggregation by covalent intermolecular disulfide bonds, also noncovalent interactions (ionic, van der Waals, hydrophobic) may be involved. The extent of their relative contribution to the overall aggregation and gelation process is unclear (Mulvihill and Donovan, 1987; Shimada and Cheftel, 1989; McSwiney et al., 1994a,b) and depends on experimental conditions such as pH and salt concentration. The contribution of noncovalent interactions will become of increasing importance with pH values closer to the isoelectric point and/or with higher salt concentrations.

Recently in our laboratory a kinetic aggregation model was developed. The reaction scheme contains several consecutive steps and is based on thiol/disulfide exchange reactions leading to the formation of polydisperse aggregates of disulfide-linked β-lg monomers (Roefs and De Kruif, 1994). The monomers are linearly linked, but the polymeric aggregates are not stiff rods and may even have a spherical shape. In the temperature range 60−75 °C at neutral pH, this kinetic model gives a quantitatively correct description of the concentration decrease of native β-lg on heating and of the dependence of the scattering intensity, as measured by *in situ* light-scattering experiments, on the initial β-lg concentration (Roefs and De Kruif, 1994; Hoffmann et al., 1996).

The role of the thiol group of β-lg in heat-induced aggregation has been extensively investigated. However, the aggregation of β-lg and the underlying mechanism vary with temperature, pH, and salt and protein concentrations. Most studies were performed in buffers of varying pH and salt concentration, whereas our model holds for β-lg dissolved in water or low salt concentrations and at near-neutral pH. Given the differences in experimental conditions, a comparison of the literature data is not easy and an extrapolation of these data to our system seems impossible. Therefore, the aim of this study was to investigate the aggregation of β-lg under well-defined conditions, where the kinetic predictions following from the model hold. We investigated whether in water at neutral pH, in addition to disulfide aggregation, also aggregation by noncovalent interactions is involved. Furthermore, we focused on the influence of

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pH (range 6.0−8.0) on the denaturation and aggregation of β-lg and on the role of the thiol group and disulfide bonds in aggregate formation.

In this paper all experiments were performed at 65 °C. This temperature is relatively low compared with temperatures used in industrial processes. However, we chose a lower temperature because reactions proceed at a much lower rate, which enables a better investigation of the processes involved in the denaturation and aggregation of β-lg and a more reliable determination of kinetic parameters. We have observed that temperatures in the range 62−66 °C are most suitable for unraveling the different reactions involved in the denaturation/aggregation process (Hoffmann et al., 1997a).

MATERIALS AND METHODS

Materials. In all experiments we used a purified bovine β-lg sample, containing the genetic variants A and B, which was prepared at the pilot plant of NIZO from whey, basically following the procedure of Maubois et al. (1987). The composition of this sample was 92% β-lg, 2% α-lactalbumin, 2% nonprotein nitrogen compounds, 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 4.0% moisture (Hoffmann et al., 1996).

N-Ethylmaleimide (NEM) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were of analytical grade and were obtained from Sigma Chemical Co.

Preparation of β-lg Solutions. β-Lg was dissolved in double-distilled water in a concentration of 10, 30, or 50 g of dry matter/L and stirred at room temperature for 2 h. Unless otherwise stated the pH was near neutral (pH 6.9 ± 0.1). In the experiments with NEM several amounts of a freshly prepared stock solution of NEM in double-distilled water were added to a more concentrated β-lg solution, resulting in a final β-lg concentration of 10, 30, or 50 g of dry matter/L (pH 6.9 ± 0.1), and the solutions obtained were stirred at room temperature for at least 30 min. All solutions were stored at 4 °C and used for further experiments within 24 h of preparation. In separate experiments the pH of a 10 g of dry matter/L β-lg solution was adjusted to pH values in the range 6.0−8.0 with 0.1 M HCl or 0.1 M NaOH.

Decrease in Concentration of Native β-lg. For the determination of the rate of decrease in concentration of native β-lg on heating, a series of test tubes containing ca. 5 mL of a β-lg solution was heated at 65 °C for several time periods. The tubes were cooled in ice−water, and then the pH was adjusted to pH 4.6 ± 0.1, at which denatured protein precipitates (De Wit, 1990). After centrifugation at 20 000*g* for 30 min, the native β-lg concentration present in the supernatant was determined by high-performance size-exclusion chromatography (HP-SEC) (Hoffmann et al., 1996).

Gel Electrophoresis. The heated samples were analyzed by polyacrylamide gel electrophoresis (PAGE), using the Pharmacia Phast System; 30 and 50 g/L samples were diluted to 10 g/L β-lg with double-distilled water. Then the samples for sodium dodecyl sulfate (SDS)−PAGE were diluted 1:1 with SDS−PAGE sample buffer, containing 20 mM Tris/HCl (pH 8.0), 2 mM EDTA, and 5% (w/v) SDS. The SDS−PAGE sample buffer for reducing conditions also contained 2% (w/v) dithiothreitol. The resultant mixtures were incubated overnight at room temperature before electrophoresis on Pharmacia polyacrylamide 8−25% gradient gels. For native (nondissociating) PAGE the 10 g/L β-lg samples in double-distilled water were electrophoresed on Pharmacia polyacrylamide 20% homogeneous gels. After electrophoresis the proteins on the gel were stained with 0.1% Coomassie brilliant blue R250 according to the instructions of the manufacturer.

DSC. The effect of NEM on the thermal denaturation of β-lg was monitored by differential scanning calorimetry (DSC) with a Perkin Elmer DSC7 within the temperature interval of 35−120 °C at a scan rate of 10 °C/min. Approximately 20 mg of sample solution (100 g of dry matter/L β-lg containing several amounts of NEM) was weighed into coated aluminum pans (Du Pont). As reference the same amount of already denatured β-lg solution was used. The peak temperature (i.e., the temperature corresponding to maximum excess heat capacity) was determined from three replicate runs and varied by not more than 0.5 °C.

Binding of NEM to β-lg. The decrease in concentration of unbound NEM, due to the reaction of NEM with β-lg, was studied by measuring the decrease in UV absorbance at 300 nm (Alexander, 1958; Leslie et al., 1962; Narang et al., 1967); 10 g of dry matter/L β-lg solutions with molar ratios NEM/β-lg monomer of 0.6, 1.0, and 1.8 were heated for several time periods at 65 °C. After the tubes cooled in ice−water, 2.5 mL of solution was placed in a 10 mm quartz cuvette, and the UV absorbance at 300 nm was measured at 20 °C in a Perkin Elmer Lambda 2 UV spectrophotometer. As blank a 10 g of dry matter/L β-lg solution that had been heated for the same time without NEM was used. To provide the correction needed for the spontaneous hydrolysis of NEM, control solutions containing different concentrations of NEM were heated for several time periods at 65 °C and after cooling the absorbance at 300 nm was measured at 20 °C, using double-distilled water as a blank. Except for the highest molar ratio, this correction was very small. The decrease in absorbance could be related to the decrease in the concentration of free NEM by using a cysteine calibration curve.

Reactivity of the Thiol Group. The reactivity and the accessibility of the free thiol group in β-lg was determined by reaction with DTNB, which reacts with thiol compounds to produce 1 mol of *p*-nitrothiophenol anion/mol of thiol (Ellmann, 1959; Phillips et al., 1967; Beveridge et al., 1974). DTNB was dissolved in a 0.05 M Tris/HCl buffer containing 1 mM EDTA (pH 7.0, 7.5, or 8.0), at a concentration of 107.5 mg/100 g of buffer; 2.70 mL of 0.05 M Tris/HCl buffer (pH 7.0, 7.5, or 8.0) and 0.25 mL of DTNB solution were placed in a 10 mm quartz cuvette. The cuvettes for the blank measurements contained 2.75 mL of 0.05 M Tris/HCl buffer (pH 7.0, 7.5, or 8.0) and 0.25 mL of DTNB solution. The six cuvettes were placed in a Varian Cary 1E UV−vis spectrophotometer thermostated at 20 or 65 °C, and after the experimental temperature was reached, 0.05 mL of a 50 g of dry matter/L β-lg solution in double-distilled water was added to the three sample cuvettes; the solutions were mixed rapidly, and the absorbance at 412 nm was recorded *in situ* as a function of time.

RESULTS AND DISCUSSION

Formation of Disulfide-Linked Aggregates. To verify that disulfide bond formation is involved in the aggregation of β-lg, heated solutions (10 g/L β-lg) were analyzed by SDS−PAGE under nonreducing and reducing conditions. The buffer system used with SDS−PAGE under nonreducing conditions presumably disperses noncovalently linked protein aggregates into monomers while aggregates linked through disulfide bonds remain intact. Under these conditions (Figure 1A) in the unheated sample a monomer and a faint dimer protein band could be seen. During heating the intensity of the dimer band increased and additional bands occurred: a trimer band after 4.0 h and a tetramer band after 6.75 h of heating. The "streaking" observed after longer heating times (24 and 48 h) in the high molecular mass range of the separating gel indicated that, in addition to the discrete bands of small oligomers of β-lg, larger aggregates were also formed. The high molecular mass aggregates on top of the gel failed to migrate into the stacking and separating gels and, as such, will have molecular masses larger than about 300 kDa. The increase of higher β-lg bands coincided with a decline in monomeric β-lg in time. When separated under reducing conditions (results not shown) all the bands in the heated β-lg samples migrated as the unheated sample, suggesting that disul-

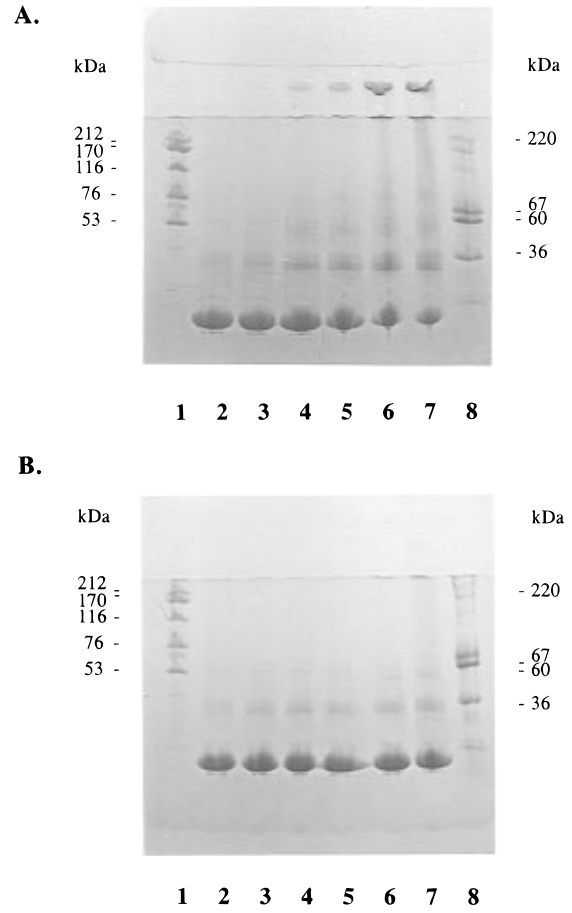

Figure 1. Nonreducing SDS−PAGE analysis (8−25% gel) of 10 g/L β-lg solutions (pH 7.0) heated for several time periods at 65 °C without (A) or with (B) NEM (molar ratio of NEM/β-lg monomer = 1). The molecular masses of the standards are indicated: lane 1, standard; lane 2, unheated; lane 3, 0.67 h; lane 4, 4.0 h; lane 5, 6.75 h; lane 6, 24 h; lane 7, 48 h; lane 8, standard.

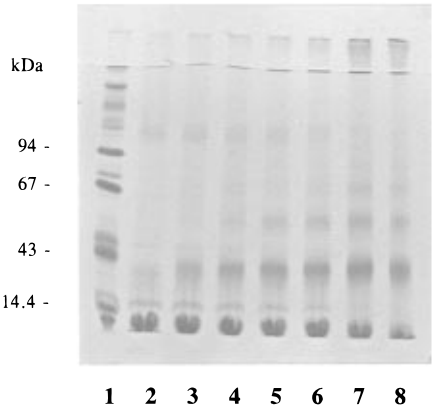

Figure 2. Native PAGE analysis (20% gel) of 10 g/L β-lg (pH 7.0) heated for several time periods at 65 °C: lane 1, standard; lane 2, unheated; lane 3, 0.67 h; lane 4, 2.25 h; lane 5, 4.0 h; lane 6, 6.75 h; lane 7, 24 h; lane 8, 48 h.

fide bonds were responsible for the intermolecular interactions of β-lg induced by heating. Native PAGE analysis of the heated solutions gave the same pattern as obtained with SDS−PAGE under nonreducing conditions (Figure 2). Although we did not quantify the aggregates on the PAGE gels, these results show that the observed aggregates were mainly held by intermolecular disulfide bonds. Further proof of this was obtained by quantitation of the amount of aggregated protein in heated solutions with SEC. Treatment of heated samples with 6 M urea or 6 M urea + 10 mM dithiothreitol prior to SEC analysis showed that the aggregates were mainly held together by intermolecular disulfide bonds. Further details of these experiments are given elsewhere (Hoffmann et al., 1997b).

On heating of 30 and 50 g/L β-lg solutions, small and large aggregates were again observed with SDS−PAGE under nonreducing conditions and with native PAGE, but compared with 10 g/L β-lg, where the small aggregates constituted a considerable part of the aggregates formed, for these higher concentrations the larger aggregates predominated. To compensate for the fact that the rate of conversion of native β-lg into aggregates increases with increasing initial β-lg concentration, we compared the intensity of the bands in heated samples in which about the same fraction of native β-lg had been converted into aggregates (see next paragraph). At each degree of conversion, the intensity of the small aggregate bands decreased with increasing concentration, whereas the intensity of the high molecular mass aggregates increased with increasing concentration. This is in line with the polymerization model proposed by Roefs and De Kruif (Roefs and De Kruif, 1994; Hoffmann et al., 1996). This model predicts that the weight-averaged mass of the aggregates should increase with increasing initial β-lg concentration.

Aggregation of β-lg in the Presence of NEM. Further insight into the role of the thiol group of β-lg in aggregate formation was obtained by heating β-lg (10 g/L) in the presence of NEM (molar ratio of NEM/β-lg monomer = 1) in order to block free thiol groups. With SDS−PAGE under nonreducing conditions, no polymers were observed during the first 24 h of heating (Figure 1B), giving further proof that polymerization must result from thiol-induced disulfide exchange reactions or from oxidation of thiol groups followed by intermolecular disulfide formation.

However, β-lg thiol blocked with NEM appeared to aggregate via a different route, and with native PAGE we observed large aggregates on top of the gel (results not shown). These high molecular mass aggregates were not observed with SDS−PAGE, demonstrating that they must be noncovalently linked. This is in accordance with the results of Sawyer (1968) and Xiong et al. (1993). The decrease in thiol content, and the subsequent decrease in disulfide bond formation in the presence of NEM, may increase molecular flexibility and enhance interactions via nonspecific bonding (Xiong et al., 1993). So, NEM favors aggregation by noncovalent interactions, and the question remains whether some noncovalently linked aggregates are also formed when β-lg is heated without addition of NEM.

The effect of NEM on the decrease in concentration of native β-lg on heating at 65 °C is shown in Figure 3. Without NEM the concentration decrease followed order 1.5 reaction kinetics (average rate constant = $5 \pm 1 \times 10^{-6}$ $(g/L)^{-0.5} \cdot s^{-1}$), as predicted by the model described (Roefs and De Kruif, 1994). The addition of NEM (molar ratio of NEM/β-lg monomer = 1) resulted in a faster initial decrease of the native β-lg concentration. For the 10 g/L β-lg solution heated in the presence of NEM, a sudden change in rate of conversion can be seen after the fast initial decrease (after ±1.5 h of heating). This fast initial conversion of native β-lg in the presence of NEM was accompanied by a considerable decrease in pH. The pH reached more or less a plateau value (pH 6.6) at the same moment as the change in the rate of conversion of native β-lg occurred. In the pH range 6−7

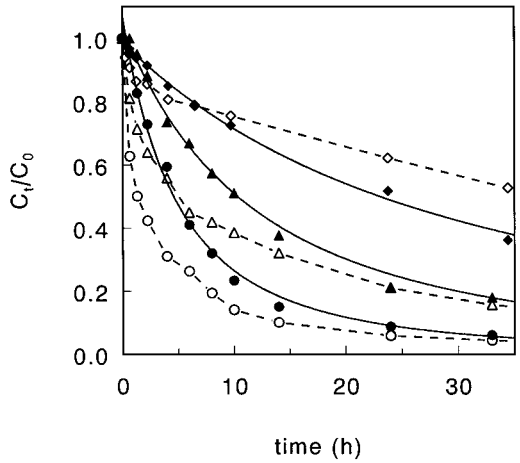

Figure 3. Decrease in relative concentration (C_t/C_0) of native β-lg as a function of time. Solutions of 10, 30, and 50 g/L β-lg in water (pH 6.9 ± 0.1) were heated at 65 °C without NEM (closed symbols) and in the presence of NEM (molar ratio of NEM/β-lg monomer = 1) (open symbols): (◆,◇) 10 g/L β-lg, (▲,△) 30 g/L β-lg, (●,○) 50 g/L β-lg. The full lines represent curves with order 1.5 reaction kinetics fitted to the experimental points of β-lg heated without NEM.

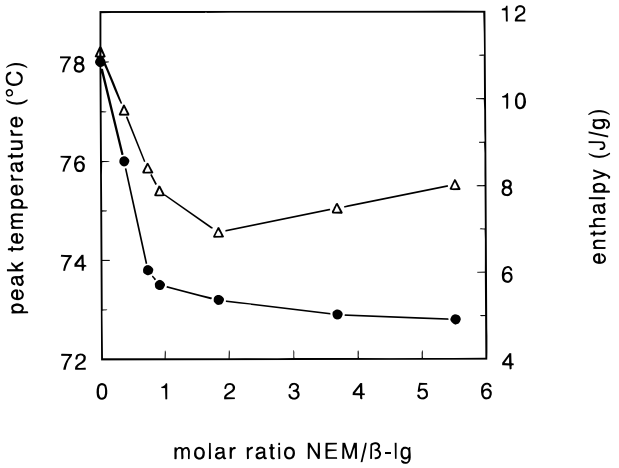

Figure 4. Peak temperature (●) and reaction enthalpy (△) determined by DSC for a 100 g/L β-lg solution as a function of the molar ratio of NEM/β-lg monomer. Results are averaged values of three different DSC runs.

the conversion rate decreases strongly with decreasing pH (see later), and the sudden change in conversion rate is presumably due to the decrease in pH of the reaction medium. For 30 and 50 g/L β-lg no sudden change in conversion rate occurred and also no change in pH was observed, owing to the larger buffering capacity of these more concentrated protein solutions.

We also studied the effect of other concentrations of NEM on the concentration decrease of native β-lg. For all β-lg concentrations studied (10, 30, and 50 g/L) the initial rate (i.e., the initial slope of C_t/C_0 versus time, see Figure 3) increased with increasing NEM concentration until a molar ratio of NEM/β-lg of 1 was reached. For higher ratios no additional effect was seen.

This corresponded with the effect of several molar ratios of NEM on the thermal stability of β-lg (100 g/L), as studied by DSC (Figure 4). Peak temperature and enthalpy decreased until a molar ratio of 1 was reached, and at higher ratios more or less constant values were obtained. This decrease in stability of β-lg on blocking the thiol group with NEM was also noticed by Ralston (1972) using optical rotation measurements. The results were interpreted in terms of steric interference and

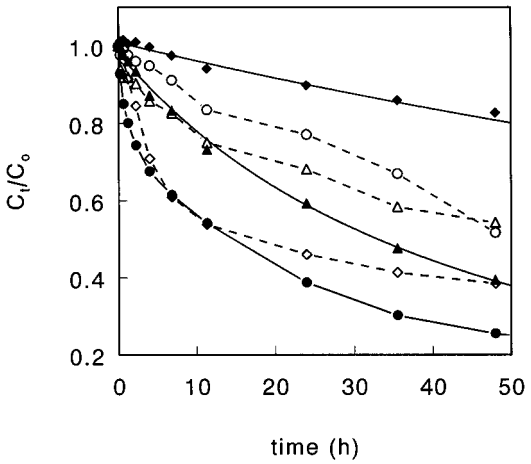

Figure 5. Decrease in relative concentration (C_t/C_0) of native β-lg at several pH values. A 10 g/L β-lg solution without (closed symbols) or with NEM (molar ratio of NEM/β-lg monomer = 2) (open symbols) was heated at 65 °C at three different pH values: (◆,◇) pH 6.0, (▲,△) pH 7.0, (●,○) pH 8.0.

disruption of noncovalent interactions, due to introduction of the modifying group. Disruption of the noncovalent interactions by means of modification would then favor the production of the activated state and hence would increase the overall rate of unfolding.

The effectiveness of the reaction between NEM and the thiol group of β-lg was studied by measuring the decrease in UV absorbance of NEM, due to the binding of NEM to β-lg. If NEM was added to a 10 g/L β-lg solution in a molar ratio of 0.6 or 1.0, a plateau value was obtained after ±1.5 h of heating. This time corresponded with the time duration of the fast initial conversion measured by HP-SEC (Figure 3) and the accompanying decrease in pH. Using a cysteine calibration curve ($\epsilon = 6250\ M^{-1}\cdot cm^{-1}$) we determined that the decrease in UV absorbance corresponded exactly to the amount of NEM present in the solution. When the same β-lg solution was heated in the presence of higher concentrations of NEM, the thiol groups were blocked in a shorter time but afterwards no plateau value was obtained. This may be due to nonspecific reactions between the excess NEM and the protein (Brewer and Riehm, 1967; Smyth et al., 1960; Habeeb, 1960) or to spectral shifts in the reaction of NEM with proteins (Leslie, 1965).

If we presume that the side reactions, which are much slower than the reaction of NEM with the thiol group (Brewer and Riehm, 1967), became important only at molar ratios larger than 1, then NEM appeared to react in a 1:1 ratio with the thiol group of β-lg until all thiol groups were blocked at the molar ratio of 1. This then is consistent with the DSC thermograms and the concentration decrease of native β-lg, where we have seen that NEM affected the thermal stability and the rate of the aggregation reaction of β-lg until a molar ratio of 1 was reached.

Effect of pH on the Aggregation of β-lg. So far we have seen that thiol/disulfide exchange reactions play an important role in the aggregation of β-lg at pH 7.0. The reactivity and accessibilty of the thiol group will depend strongly on the pH, and to investigate how the aggregation of β-lg is affected by pH, we performed experiments with 10 g/L β-lg solutions in water, of which the pH was set at values in the range 6.0−8.0 prior to heating at 65 °C.

The effect of pH on the decrease of native β-lg during heating at 65 °C is shown in Figure 5. At pH 6.0 the

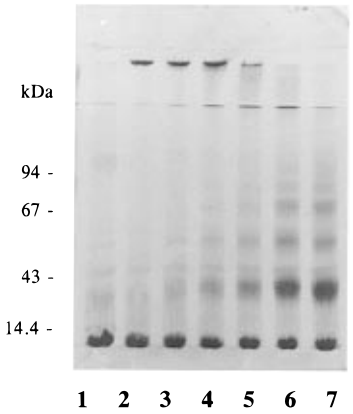

Figure 6. Native PAGE gel (20% gel) of 10 g/L β-lg solutions of several pH values heated for different time periods at 65 °C such that about 50% of β-lg had been converted into aggregates ($C_t/C_0 \approx 0.5$): lane 1, unheated, pH 7.0; lane 2, pH 6.0; lane 3, pH 6.4; lane 4, pH 6.7; lane 5, pH 7.0; lane 6, pH 7.5; lane 7, pH 8.0.

conversion of native β-lg occurred much more slowly compared with pH 7.0, whereas at pH 8.0 the reaction was considerably accelerated. Figure 6 shows a native PAGE gel of 10 g/L β-lg solutions of several pH values heated for different time periods at 65 °C such that in all solutions about 50% of the initial β-lg concentration had reacted (determined with HP-SEC). At pH 8.0 only small aggregates were observed, with no high molecular mass aggregates on top of the gel. With decreasing pH the contribution of the small aggregates to the total amount of aggregates decreased, and at pH values below 6.7 mainly high molecular mass aggregates on top of the gel could be seen. With SDS−PAGE analysis under nonreducing conditions, much the same pattern was obtained, indicating that even at pH 6.0 aggregates are partially held together by intermolecular disulfide bonds.

When 10 g/L β-lg was heated in the presence of NEM (molar ratio of NEM/β-lg monomer = 2) with native PAGE, no aggregates were observed in the solution of pH 8.0, whereas at pH 6.0 the intensity of the aggregate bands on top of the native PAGE gel increased slightly compared with β-lg heated without NEM at that pH. These effects of NEM on the aggregates formed agree with the effect of NEM on the decrease in concentration of native β-lg at the different pH values (Figure 5). When 10 g/L β-lg was heated without NEM, the rate of decrease in concentration increased with increasing pH, whereas with NEM (molar ratio = 2) it decreased with increasing pH. Without addition of NEM the increase in reaction rate can be ascribed to an increase in reactivity of the thiol group with increasing pH (see below). Furthermore, the electrostatic repulsion between the negatively charged β-lg molecules increased with increasing pH, resulting in a smaller contribution of noncovalent interactions to the overall aggregation reaction. At pH 8.0 the aggregation is completely chemical, and when β-lg is heated in the presence of NEM the formation of these disulfide-linked aggregates is prevented, whereas at this pH aggregation via noncovalent interactions is very unfavorable, resulting in a retarding effect of NEM on the decrease in concentration of native β-lg and on the formation of aggregates. The aggregates formed at pH 6.0 are substantially formed by noncovalent interactions, although, as mentioned above, intermolecular disulfide bonds may also play a role. Since NEM favors the formation of noncovalently linked aggregates, the decrease in concentration of native β-lg is accelerated by NEM. pH 7.0 is in between these two extremes, resulting in a less pronounced effect of NEM on the conversion of β-lg.

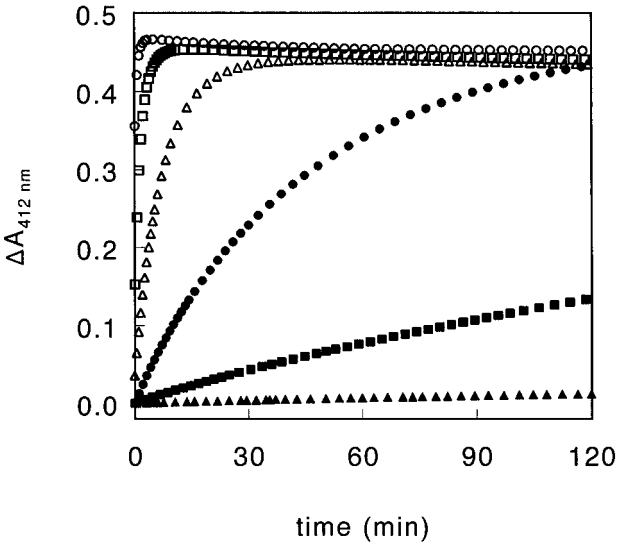

Figure 7. Effect of pH on the reaction of DTNB with the thiol group of β-lg. The increase in UV absorbance at 412 nm (ΔA_{412nm}) was measured for 0.8 g/L β-lg in the presence of DTNB (molar ratio of DTNB/β-lg monomer = 5) at 20 °C (closed symbols) and 65 °C (open symbols) and at three different pH values: (▲,△) pH 7.0, (■,□) pH 7.5, (●,○) pH 8.0.

From the above-mentioned results we can conclude that pH strongly affects the rate of the aggregation reaction and the size of the aggregates formed at 65 °C. By studying the reaction of DTNB with the thiol group of β-lg, we tried to gain more insight into the pH dependent reactivity and accessibility of the free thiol group. The UV absorbance of β-lg in the presence of DTNB (molar ratio of DTNB/β-lg = 5) at 412 nm was measured at 20 and 65 °C as a function of time (Figure 7). At 20 °C and pH 8.0 the increase in absorbance reached more or less a plateau value within ±3 h. Using ϵ = 13 600 $M^{-1}\cdot cm^{-1}$, we calculated from this plateau value that about 90% of all free thiol groups in the β-lg solution had reacted. So at pH 8.0 the thiol group in β-lg is accessible for reaction, even at room temperature. However, not all thiol groups reacted with DTNB; this may be due to the fact that some thiol groups have disappeared via oxidation and thiol/disulfide exchange reactions and are involved in intermolecular disulfide bonds. At pH 7.5 the reaction proceeded much more slowly, and at pH 7.0 almost no reaction could be observed at 20 °C, demonstrating that at room temperature and neutral pH the thiol group is buried in the interior of the molecule and is not available for reaction. Upon heating at 65 °C β-lg undergoes conformational changes, and for all three pH values within 40 min more than 80% of all thiol groups had reacted.

Although the reaction between DTNB and the thiol group of β-lg is known to follow second-order kinetics, pseudo-first-order kinetics is apparent under conditions where DTNB is in excess (Shimada and Cheftel, 1989; Phillips et al., 1967). In all cases the pseudo-first-order plots were linear up to the time where the maximum absorbance was reached (see Figure 7). The pseudo-first-order rate constants calculated from the measurements at 20 °C increased over 100-fold going from pH 7.0 to 8.0 (i.e., from 2.5×10^{-6} to 3.4×10^{-4} s^{-1}), whereas at 65 °C this increase was about 10-fold (i.e., from 2.3×10^{-3} to 2.2×10^{-2} s^{-1}).

The higher reactivity of the thiol group of β-lg in the reaction with DTNB at pH 8.0 compared with pH 7.0 may be due to a difference in the concentration of the ionized thiol or to the better accessibility of the thiol group of β-lg to DTNB, or to a combination of these two effects. According to Kella and Kinsella (1988) the free thiol group is buried in a hydrophobic environment at neutral pH. Dunnil and Green (1965) related the rate of the reaction between *p*-chloromercuribenzoate and the thiol group of β-lg at several pH values to the N ⇌ R conformational change in β-lg. Tanford et al. (1959) have shown that β-lg undergoes a reversible change of conformation between pH 6 and 8.5. At lower pH the N-state prevails, and in this conformation the thiol groups are buried in the β-lg dimers, presumably in the region of contact between the monomer subunits. At higher pH, where the R-state prevails, a refolding of the protein chains appears, resulting in a higher reactivity of the thiol group. So, at pH 8 the thiol group of β-lg is available for reaction with thiol reagents such as NEM and DTNB, whereas at pH 7 and lower values the molecule has to be heated, or unfolded in another way, in order to expose the thiol group for reaction. McSwiney et al. (1994a) also observed an increase in the rate of polymerization via thiol/disulfide exchange reactions as the pH was increased from 6.0 to 9.0 due to increased accessibility and deprotonation of the thiol group at higher pH values.

The formation of a large amount of dimers, trimers, and tetramers at pH 8.0 may be ascribed to the fact that even at early stages of the heat treatment a relatively large number of reactive intermediates, with an exposed, reactive thiol group, is formed. This increases the probability of termination reactions resulting in the formation of smaller disulfide-linked β-lg aggregates, without a reactive thiol group (Roefs and De Kruif, 1994). The large aggregates observed at pH 6.0 may be formed by secondary, noncovalent interactions of primary, disulfide-linked aggregates. These results are consistent with the effect of pH on the apparent Stokes−Einstein diameter of the aggregates formed at 68.5 °C as studied with *in situ* light scattering (Hoffmann et al., 1996). A more extensive study of the influence of pH on the kinetics of the different reaction steps in the reaction scheme of Roefs and De Kruif is currently being undertaken (Hoffmann et al., manuscript in preparation).

CONCLUSIONS

The formation of aggregates via noncovalent interactions or via covalent intermolecular disulfide bonds may occur simultaneously or sequentially. Experimentally it is very difficult to distinguish between these processes and to determine which interactions are involved in the initial stages of aggregate formation. However, based on the results obtained in this investigation, we think that in water at neutral and elevated pH values, the thiol group plays a crucial role in the heat-induced aggregation of β-lg by acting as an initiator of thiol/disulfide exchange reactions. The formation of intermolecular disulfide bonds prevents the reversibility of modifications in the tertiary structure of native β-lg and of (eventual) association of these modified monomers via noncovalent interactions. Recently Iametti et al. (1996) have also shown that an activated monomer form, with an exposed thiol group, plays a primary role in the heat-induced aggregation of β-lg at neutral pH. Furthermore, Cho et al. (1994) used site-directed mutagenesis to introduce an additional cysteine residue within 5−10 Å of the free thiol group at C121, thus accommodating the formation of a disulfide linkage with the existing free thiol. The mutant proteins did not polymerize at 80 °C (pH 8.0), giving further proof of the crucial role of the thiol group in promoting interchange reactions.

On the basis of the results obtained, we conclude that β-lg, dispersed in water at neutral pH, on heating at 65 °C mainly forms aggregates by intermolecular disulfide cross-linking. Aggregation by noncovalent interactions may be involved, but only to a lesser extent. So far, these results confirm the essence of the model as developed by Roefs and De Kruif (1994). However, the presently described model may be expanded to include (secondary) aggregation by noncovalent interactions, and further refinement is needed, in particular when reaction conditions (e.g., ionic strength and pH) are varied. Further research on these topics is in progress.

ABBREVIATIONS USED

β-lg, β-lactoglobulin; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HP-SEC, high-performance size-exclusion chromatography; DSC, differential scanning calorimetry.

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