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# Modifications occur at different structural levels during the heat denaturation of β-lactoglobulin

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Heat-induced modifications in the tertiary and quaternary structure of β-lactoglobulin were followed at neutral pH for the protein at high temperature and for the protein that was heated and cooled. Fast changes in the environment of aromatic amino acids were apparent from near-ultraviolet-CD spectra of the heated protein and their intensity increased with increasing temperature. These modifications were irreversible only at temperatures higher than 65–70°C. Addition of iodoacetamide during the heating/cooling cycle greatly reduced the extent of irreversible modification of the tertiary structure of the protein. Reaction of the native β-lactoglobulin dimer with iodoacetamide or dithiobis(2-nitrobenzoic acid) was only observed upon heating at temperatures higher than 40°C and resulted in progressive reaction of the unique sulfhydryl group in each of the two protein monomers. The sulfhydryl reagents induced release of a monomeric protein species that was no longer able to aggregate to the native dimeric form or to sequentially form polymers as found in the protein after heating at high temperature. Dimer dissociation was identified as the rate-limiting step in the reaction of β-lactoglobulin with sulfhydryl reagents. It occurred at temperatures much lower than those required for appreciable modification of the tertiary structure of the protein, and had an extremely high activation energy ($E_a$ = 213 kJ/mol). These results are compared with other published data, and a general mechanism for the formation of early reactive species in heat-treated β-lactoglobulin at neutral pH is proposed which stresses the relevant role of a highly hydrophobic, molten-globule-like free monomer that has an exposed sulfhydryl group on its surface.

*Keywords:* β-lactoglobulin; heat denaturation; sulfhydryl groups.

The globular protein β-lactoglobulin is found in the whey fraction of the milk of many mammals. In spite of numerous physical and biochemical studies, its function is not clearly understood [1, 2]. The crystal structure of bovine β-lactoglobulin has been determined and shows similarities to the plasma retinol-binding protein and the odorant-binding protein [3, 4]. This finding suggests that the role of β-lactoglobulin may be connected with transport or accumulation of lipid-soluble biological components [5, 6].

Refolding of the tertiary structure of β-lactoglobulin from the chaotrope-denatured form has been investigated extensively at low pH, where the association of monomers into multimeric forms is minimal [7, 8] and refolding conforms to the molten-globule hypothesis of intermediate formation in protein folding/unfolding [9]. The high stability of β-lactoglobulin at low pH has been explained by the strong stabilizing action of the two disulfide bonds present in its tertiary structure [2, 10]. The free, highly reactive -SH group of Cys121 in each monomer has been shown to be involved in intramolecular and intermolecular disulfide interchange with other -SH groups in treated milk [11–13].

Despite the large amount of structural information available, little is known about the monomer/monomer interface in the freely associating β-lactoglobulin dimer, especially with regard to its modification during the folding/unfolding processes at neutral pH. There is also little information on the mechanism of the structural changes that occur after the possibly denaturing heat treatments that are typical of the industrial processing of dairy products and by-products, such as whey and sweet whey [11–14].

We previously have demonstrated the occurrence, and discussed the significance, of changes in parameters related to protein-surface hydrophobicity and to overall protein folding upon heat treatment of proteins [15–18]. Studies on the thermal sensitivity of β-lactoglobulin at neutral pH focused on the mechanism and the nature of structural protein modifications which represent the initial steps of subsequent macroscopic changes such as loss of solubility and exposure of regions suitable for different kinds of interaction with other components in complex systems. Modifications of the exposure of hydrophobic residues to the solvent, the organization of these residues into surface hydrophobic patches upon thermal treatment at neutral pH and at low concentration, and non-reversible alterations in the association equilibrium of β-lactoglobulin were studied. Details of the mechanism of heat-induced polymerization of the protein and insights into the nature of the chemical bonds involved were provided [16, 17] and were in good agreement with those proposed by others [11–13]. Monomer dissociation was found to be necessary for polymerization, which occurred through adhesion of exposed hydrophobic regions to form aggregates that

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*Abbreviation.* $Nbs_2$, dithiobis(2-nitrobenzoic acid).

were then stabilized, at least above a certain temperature threshold, by intermolecular disulfide exchange.

Heat-induced polymerization of β-lactoglobulin is of general relevance to the processing of proteins in industry since fouling of thermal sanitization plants by denatured β-lactoglobulin polymers is often considered as the prototype of heat-induced fouling during the processing of protein solutions. Previous application-oriented work on heat denaturation of proteins showed the necessity for better understanding, at the molecular level, of the nature of reversible and irreversible events during the denaturation process [19].

In this work we focus on the analysis of reversible and irreversible modifications that occur in heated solutions of β-lactoglobulin at neutral pH and which involve the tertiary and quaternary structure of the protein. Multiple approaches were used which combined protein chemistry with spectroscopic and separative techniques, on the basis that the different protein-unfolding steps revealed by individual approaches should be correlated. Particular attention was paid to the role of modifications in the tertiary structure in the determination of the association equilibria of the protein and to the possible role of the free -SH group in each protein monomer in the heat-induced formation of polymers.

## MATERIALS AND METHODS

Chemicals were reagent grade or HPLC grade. Bovine β-lactoglobulin was from Sigma. Each protein batch was tested upon receipt for the presence of multimeric forms or of disulfide-linked dimers by means of HPLC gel permeation and SDS/PAGE under non-reducing conditions as indicated below.

Unless otherwise specified, the buffer used was 50 mM sodium phosphate, pH 6.8.

In the experiments that involved blocking of the protein -SH groups with iodoacetamide, the protein was heated at the required temperature in the presence of a greater than 20-fold molar excess of the reagent. Excess iodoacetamide was removed after cooling to room temperature by rapid gel filtration on syringe-type Sephadex G-25 columns.

The time-course of the reaction between the protein and the sulfhydryl reagent dithiobis(2-nitrobenzoic acid) ($Nbs_2$; [20]) was followed at 412 nm in 10-mm (0.25 mg and 2.5 mg β-lactoglobulin/ml) or 1-mm (20 mg β-lactoglobulin/ml) cuvettes. The protein (0.01−0.05 ml) was added from a stock solution (125−280 mg/ml) at room temperature to a much larger volume of temperature-regulated buffer containing 2−5 mM $Nbs_2$ in the measurement cuvette in a temperature-regulated holder. Time-course measurements were started by addition of the protein. No correction was performed for the negligible decrease in temperature due to the addition of the small volume of protein solution. Time-courses were analyzed for their thermodynamic and kinetic parameters as described elsewhere [15−18].

Gel-permeation-HPLC experiments were performed in 50 mM sodium phosphate, 0.1 M NaCl, pH 7.5, on a Superdex G-75 10/30 column attached to a Waters 625 chromatograph equipped with a Waters 490 variable-wavelength detector set at 280 nm.

Non-reducing SDS/PAGE was performed as in [17] in a 12% monomer gel by means of a Bio-Rad MiniGel Protein II apparatus, and 2-mercaptoethanol was omitted from the denaturing buffer.

Near-ultraviolet CD spectra were recorded in temperature-regulated cells on a Jasco J500 A spectropolarimeter and analyzed by means of the Jasco J700 A software.

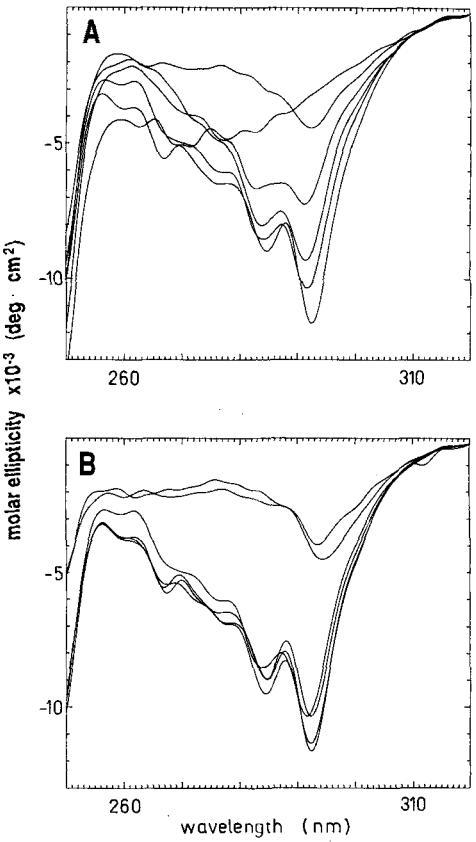



**Fig. 1. CD spectra of β-lactoglobulin at different temperatures.** (A), β-lactoglobulin (2.5 mg/ml in 50 mM sodium phosphate, pH 6.8) was placed in a temperature-regulated bath at the given temperature for 8 min. The protein was then placed in a temperature-regulated cell at the same temperature and its spectrum was recorded. Acquisition of the spectrum took 7 min. Spectra were taken at 30, 50, 60, 65, 70 and 80°C (from bottom to top at 292 nm). (B), spectra were taken on the same samples as in (A), after allowing them to cool at room temperature. The heating temperatures were 30, 50, 60, 65, 70 and 80°C (from bottom to top at 292 nm).

## RESULTS

**Modifications of the tertiary structure of heated β-lactoglobulin.** Reversible and irreversible modifications induced in the tertiary structure of β-lactoglobulin were studied through near-ultraviolet CD spectra of the protein, which was kept at the required temperature after equilibration for 8 min, and of the same protein sample after cooling to room temperature. Since acquisition of each spectrum took approximately 7 min, the total length of the heat treatment was 15 min. Modifications in the near-ultraviolet CD spectra of the protein at high temperature were only evident at temperatures above 50°C (Fig. 1 A), in agreement with previous reports [12]. The spectra of the heated and cooled protein (Fig. 1 B) revealed that only the modifications which occur above 70°C were irreversible. All these modifications were complete within seconds, and thus we could not follow their kinetics on our instrumentation.

The native protein displayed signals at 292.5, 285, 275, 266 and 259 nm that could be attributed to well-known transitions of aromatic residues [21]. The spectral feature at 266 nm, which has been interpreted also as a contribution from the sulfur-containing amino acids of the protein [22], seemed the most heat sensitive. The overall spectral shape changed dramatically in samples kept at temperatures above 70°C. The tryptophan transition shifted from 292.5 nm to 290 nm and strongly decreased in intensity, while most other transitions disappeared. As stated

above, the spectral features of the heat-modified protein were retained upon cooling only when the protein was heated above 70°C.

**Effects of sulfhydryl reagents on loss of high-order structure in heated β-lactoglobulin.** At temperatures higher than 70°C and at neutral pH, the study of structural modifications in β-lactoglobulin is complicated by the formation of irreversibly aggregated forms of the protein [11, 16, 17]. Dimer dissociation is required for formation of aggregates, which occurs by a sequential polymerization mechanism [12, 13, 16, 17]. Intermolecular disulfide exchange is important for the covalent stabilization of these aggregates, in a reaction where the free -SH group on each β-lactoglobulin monomer probably plays an important role. The detailed role of intramolecular disulfide exchange in heat-induced modifications and in polymerization has to be ascertained [11].

We attempted to discriminate between changes in tertiary structure and formation of covalent bonds during the aggregation process at neutral pH by means of similar CD studies to those described above in the presence of the -SH reagent, iodoacetamide.

Iodoacetamide had no effect on the near-ultraviolet CD spectra of β-lactoglobulin at room temperature (Fig. 2). However, CD spectra of the protein isolated at room temperature after being heated in the presence of iodoacetamide at temperatures above 65°C were different from those recorded for the protein heated at the same temperature in the absence of the -SH reagent and then cooled. The apparent protection by iodoacetamide against irreversible heat-induced modification of the CD spectra was particularly evident for transitions ascribable to tyrosine and phenylalanine residues, which indicates that a decreased number of aromatic side chains was exposed to a modified environment in the presence of iodoacetamide compared with the unblocked protein. The temperature-dependence curves (Fig. 2B) indicate that reaction with iodoacetamide did not modify the threshold temperature for irreversible denaturation of β-lactoglobulin, but reduced the number of irreversibly denatured molecules present after cooling of the heated protein.

To ascertain whether tyrosine or phenylalanine residues were involved in the formation of non-covalent aggregates, after possible exposure of hydrophobic surfaces during the heat exposure and the subsequent interaction of these surfaces, the iodoacetamide-treated protein was analyzed by gel-permeation chromatography. Addition of iodoacetamide to β-lactoglobulin during the heating/cooling cycle prevented the formation of high-order aggregates and resulted in the appearance of high amounts of free β-lactoglobulin monomer (Fig. 3).

The effects of blocking the reactive -SH group on each β-lactoglobulin monomer are also evident upon SDS/PAGE (Fig. 4). In Fig. 4, samples heated at different temperatures in the presence and absence of iodoacetamide are compared. Heating in the absence of iodoacetamide led to formation of covalently bound dimers, trimers and tetramers, which were found in increasing proportion with increasing temperature. This finding confirms the sequential polymerization mechanism previously suggested [13, 16, 17]. In contrast, heating β-lactoglobulin in the presence of iodoacetamide led to the disappearance of the modest amount covalent dimer found in some batches of commercial β-lactoglobulin used in these studies, probably through intramolecular disulfide-exchange processes.

The results of the gel-permeation and the SDS/PAGE experiments and the published data indicate that covalent and non-covalent bonds are relevant to aggregation in the absence of -SH reagents, and that blocking the -SH group on each β-lactoglobulin monomer prevents heat-induced polymerization through either a covalent or a non-covalent mechanism.

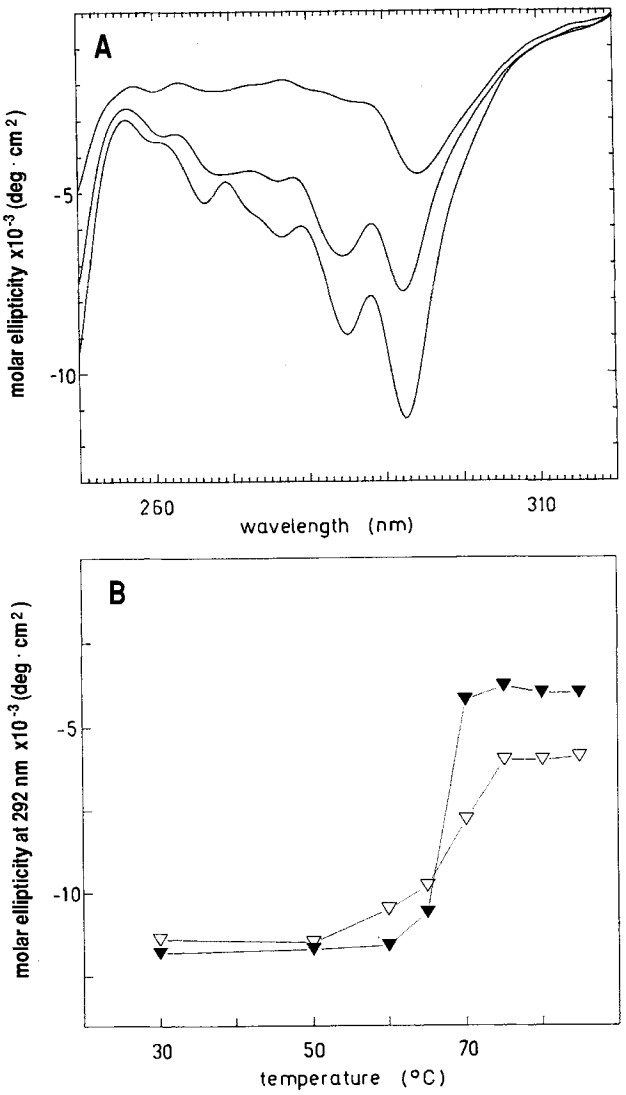



**Fig. 2. Effect of iodoacetamide on the CD spectrum of heated β-lactoglobulin.** (A), β-lactoglobulin (2.5 mg/ml in 50 mM sodium phosphate, pH 6.8) was heated in a temperature-regulated bath at 70°C for 15 min in the presence (middle line at 292 nm) and absence (top line at 292 nm) of 2.8 mM iodoacetamide. The proteins were cooled at room temperature and excess iodoacetamide was removed by rapid gel permeation on a syringe-type Sephadex G-25 column before the spectra were recorded. For comparison, the spectrum of β-lactoglobulin reacted with iodoacetamide for 15 min at room temperature is shown (bottom line at 292 nm). (B), molar ellipticity at 292 nm as a function of the treatment temperature in the absence (closed symbols) or presence of iodoacetamide (open symbols). Sample treatment was performed as for (A).

**Exposure of the reactive -SH group in β-lactoglobulin relates to dimer dissociation.** To understand in more detail the relationship between the exposure (and blockage) of the -SH group in β-lactoglobulin and other structural changes, a more detailed analysis of the reaction of β-lactoglobulin with -SH reagents was required. $Nbs_2$ was preferred to iodoacetamide for these studies since its intense absorbance at 412 nm allowed real-time monitoring of its reaction with the protein during exposure to heat.

Fig. 5 shows the time-course of the absorbance increase at 412 nm upon addition of β-lactoglobulin to a solution of $Nbs_2$ at different temperatures. The progressive formation of the yellow thiolate anion from the -SH reagent upon addition of the protein

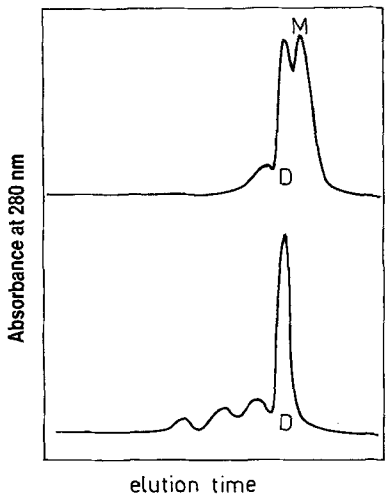



**Fig. 3. Effect of iodoacetamide on the aggregation state of heated β-lactoglobulin.** β-lactoglobulin (2.5 mg/ml in 50 mM sodium phosphate, pH 6.8) was heated in a temperature-regulated bath at 70°C for 15 min in the presence (top) and absence (bottom) of 2.8 mM iodoacetamide. The proteins were cooled at room temperature and excess iodoacetamide was removed by rapid gel permeation on a syringe-type Sephadex G-25 column before the protein was applied to a Superdex G 75 10/30 column equilibrated in 50 mM sodium phosphate, 0.1 M NaCl, pH 7.5. Chromatography was performed on a Waters 625 HPLC system equipped with a Waters 490 variable-wavelength detector set at 280 nm. M, monomer; D, dimer.

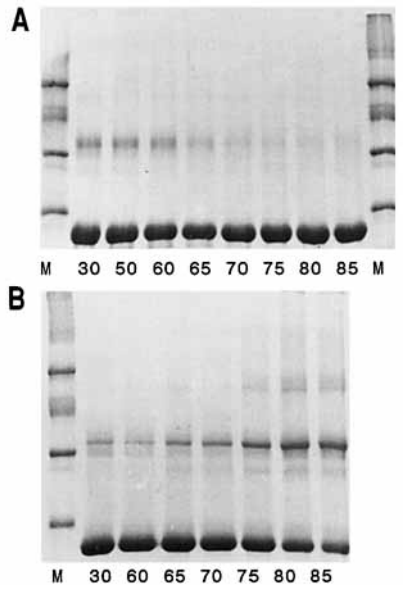



**Fig. 4. Effect of iodoacetamide on covalent stabilization of heat-induced β-lactoglobulin aggregates.** β-lactoglobulin (2.5 mg/ml in 50 mM sodium phosphate, pH 6.8) was heated in a temperature-regulated bath for 15 min at the temperature given for each lane (°C) in the presence (A) and absence (B) of 2.8 mM iodoacetamide. The samples were cooled at room temperature and excess iodoacetamide was removed by rapid gel permeation on a syringe-type Sephadex G-25 column. The protein was denatured in the absence of 2-mercaptoethanol and analyzed by SDS/PAGE. The same amount of protein was loaded in each lane. M, markers.

indicated time-dependent exposure of a rapidly reacting -SH group. Control experiments showed that reaction of $Nbs_2$ with generic thiols (such as 2-mercaptoethanol, cysteine and glutathione) at concentrations equivalent to that of β-lactoglobulin was

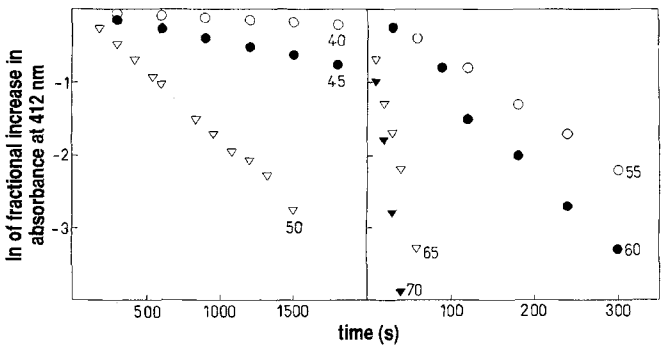



**Fig. 5. Time-course of the absorbance increase in heated mixtures of β-lactoglobulin and $Nbs_2$.** β-lactoglobulin (0.02 ml, 125 mg/ml, room temperature) was injected into 1 ml 2 mM $Nbs_2$, 50 mM sodium phosphate, pH 6.8, at the temperature given for each set of symbols. The natural logarithm of the fractional absorbance increase at 412 nm is given as a function of time.

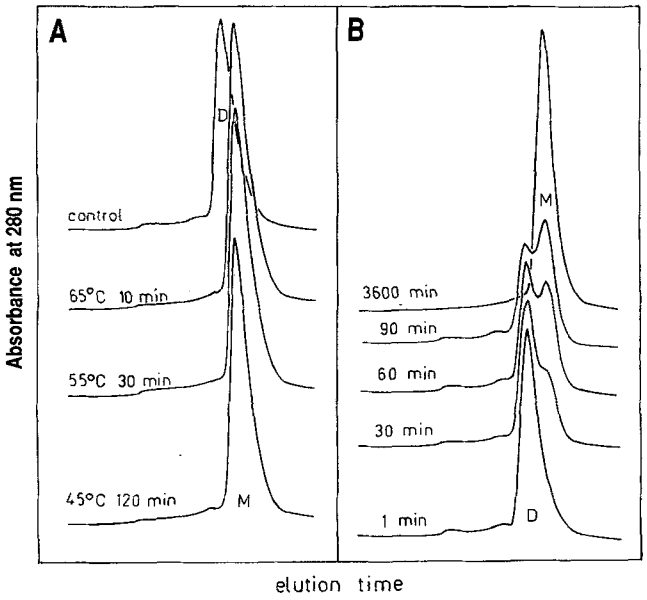
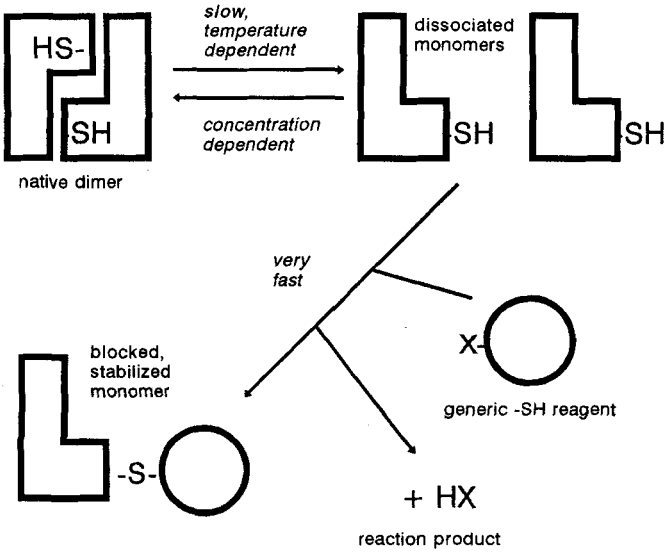



**Fig. 6. Stabilization of monomeric forms of β-lactoglobulin upon reaction with $Nbs_2$.** β-lactoglobulin (2.5 mg/ml in 50 mM sodium phosphate, pH 6.8) was heated in a temperature-regulated bath for the given time and temperature in the presence of 2 mM $Nbs_2$ and cooled at room temperature before analysis as in the legend to Fig. 3. M, monomer; D, dimer. (A), equilibrium measurements. The control sample was heated for 8 h at 40°C in the absence of $Nbs_2$. (B), time-course of monomer formation in β-lactoglobulin/$Nbs_2$ mixtures heated at 40°C for the given times.

instantaneous on our instrument time-scale at all temperatures. The reaction between β-lactoglobulin and $Nbs_2$ was negligible at room temperature (20–25°C) but became appreciable at temperatures around 40°C and was too fast to be followed at temperatures above 70°C. Despite the large differences in rate, the absorbance values at equilibrium were identical at all temperatures and indicated a titration of one -SH group/β-lactoglobulin monomer. At the highest temperature and with the highest protein concentration this figure decreased to approximately 0.9 -SH/mol monomer, probably due to competition with interprotein disulfide exchange and to the formation of aggregates no longer accessible to the -SH reagent.

β-lactoglobulin monomers were the only species present in β-lactoglobulin/$Nbs_2$ mixtures at equilibrium (Fig. 6A). The rate

**Scheme 1. Putative mechanism by which -SH reagents may block β-lactoglobulin association.**




of monomer release increased with increasing temperature and could be measured in mixtures at temperatures low enough to allow sampling at intervals (Fig. 6B). The time-course of monomer release, when measurable by HPLC, was found to parallel the time-course of exposure of -SH groups, as measured spectrophotometrically with $Nbs_2$. Residual dimers, when present, did not show stabilization by intermonomer disulfides, as indicated by non-reducing SDS/PAGE (data not shown). These observations suggest that reaction with $Nbs_2$ stabilized the monomer in that it prevented its re-association with other monomers and prevented disulfide exchange that could lead to formation of covalent aggregates. Some formation of disulfide-linked high-order aggregates only occurred at the highest temperature and with the highest protein concentration used in these studies (20 mg/ml, 70°C, data not shown). Under these conditions, perhaps because the concentration of $Nbs_2$ was limited to 5 mM to prevent artifacts, it could have been possible that interprotein disulfide exchange was faster than the reaction of the protein with $Nbs_2$.

The results obtained with either -SH reagent could be interpreted as in Scheme 1. According to the scheme, dimer dissociation promoted exposure of the free -SH groups (one/monomer) buried at the dimer interface [2, 4]. Blocking of these residues prevented re-association of the dissociated monomers and shifted the equilibrium towards complete disassembly of the native dimer. This occurred also at low temperatures (40–45°C) where only minimal and fully reversible modifications of the protein tertiary structure could be detected by the techniques used in the present study or in previous reports. These findings confirm the location of the free -SH group at the dimer interface and suggest that steric changes in this region (such as those determined by binding of iodoacetamide or Nbs to the protein) impair re-association of otherwise unmodified monomers. Non-covalent association of 8-anilino-1-naphthalenesulfonate to the heated protein had no irreversible effects on its associative behaviour [17].

The experiments illustrated in Fig. 5 were repeated at different protein concentrations since irreversible association of the protein heated in the absence of -SH reagents was shown to increase with the protein concentration [17]. The reaction with $Nbs_2$ followed pseudo-first-order kinetics. The rate constant of monomer release was determined over a range of concentrations and temperatures and had a pronounced temperature dependence, with an $E_a$ of 213 kJ/mol in the temperature range 40–70°C. No transitions were evident in the Arrhenius plot for dimer dissociation in this temperature range. The value of $E_a$ was independent of the protein concentration over a broad range of protein concentrations (0.25–20 mg/ml), as expected for measurements of the fractional rate of the dimer dissociation half-reaction, according to the assumptions that were made in Scheme 1.

## DISCUSSION

Thermal denaturation of β-lactoglobulin occurs through different steps that are characterized by well-defined temperature thresholds and by the temperature dependence of their rate constants. These steps could lead to reversible and irreversible modifications. Some of them appear to be sequential while others are non-correlated.

Kinetic information is not available for modifications observed at neutral pH in the absence of an external probe, such as those obtained in the near-ultraviolet CD studies presented here or those obtained in earlier intrinsinc-fluorescence studies [16]. Reversible and irreversible modifications in tertiary structure, when detectable by CD, occurred on a very short timescale. This finding agrees with the results observed in folding/unfolding studies carried out by means of the same spectroscopic techniques and chemical denaturants on the monomeric form of the protein that prevails at low pH. These studies required stopped-flow techniques and showed that aromatic residues were among the most immediate targets in the unfolding process, independent of how unfolding was induced. Heat-induced changes in the environment of tryptophan residues, revealed by intrinsic fluorescence at neutral pH, also were too fast to be followed with our equipment [16].

The extent of modification of the tertiary structure of the protein increased with temperature and resulted in progressive exposure of its hydrophobic interior. As the temperature of treatment was increased above 65–70°C, the molar fraction of the protein that underwent irreversible modification of the tertiary structure increased correspondingly.

70°C was shown to represent a temperature threshold for irreversible occurrence of most of the detectable conformational changes observed upon heating of β-lactoglobulin at neutral pH. For example, the structural collapse of the protein, detected by a decrease in the number of hydrophobic sites accessible to the hydrophobic fluorescent probe 8-anilino-1-naphthalenesulfonate, was only evident at temperatures above 70°C [16]. The heat-induced structural collapse observed in those experiments always followed a phase of protein swelling, in which the interior of the protein became more accessible to the hydrophobic fluorophor, such that the protein had most of the features characteristic of the molten-globule state [8, 9, 15, 16]. Formation of the swollen form occurred at temperatures much lower than those required for structural collapse of the protein or for its insolubilization [14, 16]. Non-reversible changes in the emission wavelength of tryptophans, detected by intrinsic fluorescence, also required temperatures above 70°C [16]. No irreversible aggregate forms were detected upon gel-permeation chromatography of β-lactoglobulin heated below 65–70°C at protein concentrations in the range 1–20 mg ml [16, 17].

We discuss here evidence that irreversible modifications of the tertiary structure at temperatures higher than 65–70°C depends on prior modification of the quaternary structure of the protein, that is, on temperature-dependent dissociation of the di-

**Table 1. Reaction half-times for some of the structural modifications in heated β-lactoglobulin.** Half-times in seconds, unless otherwise specified. n.d., not detectable; n.a., not available.

| Methodology used | Observed modification | Half-time at | | | | | | | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 40°C | 45°C | 50°C | 55°C | 60°C | 65°C | 70°C | 75 °C | 80°C | 85°C | 90°C | 95°C | |
| | | s | | | | | | | | | | | | |
| Near-ultraviolet CD | tertiary structure | n. d. | n. d. | n. d. | n. d. | short [1] | short [1] | short [1] | short [1] | short [1] | short [1] | short [1] | short [1] | this study |
| Intrinsic fluorescence | tryptophan exposure | n. d. | n. d. | n. a. | n. a. | short [1] | short [1] | short [1] | short [1] | short [1] | short [1] | short [1] | short [1] | [16] |
| Heating in the presence of 8-anilino-1-naphthalenesulfonate | increase in accessible hydrophobic sites (protein swelling) | n. a. | n. a. | n. a. | 123 | 51 | 27 | 7 | 5 | short | short | short | short | [16] |
| Heating in the presence of 8-anilino-1-naphthalenesulfonate | decrease in accessible hydrophobic sites (protein collapse) | n. d. | n. d. | n. d. | n. d. | n. d | n. d. | n. d. | 110 | 76 | 53 | 48 | 34 | [16] |
| Heating in the presence of $Nbs_2$ | access to reactive-SH, release of blocked monomers | 180 min | 25 min | 360 | 110 | 38 | 27 | 7 | short | short | short | short | short | this study |
| Gel permeation of heated-cooled protein | irreversible association and disappearance of native dimer | n. d. | n. d. | n. d. | n. d. | n. d. | n. d. | n. d. | 120 | 106 | 76 | 55 | n. a. | [17] |

[1] reversible below 65°C, irreversible above 70°C.

mer into monomers. Dimer dissociation did not require extensive modification of the tertiary structure of the protein and its rate constant did not show a transition temperature in the range 20–70°C. The dissociated monomer exposed large hydrophobic surfaces and a reactive -SH group, both being previously hidden at the interface between monomers in the native protein. Chemical blocking of the -SH group prevented covalent and non-covalent reassociation of monomers at all temperatures.

Blocking the free -SH exposed upon dimer dissociation reduced the molar fraction of the protein that underwent irreversible modifications in its tertiary structure at high temperature, as indicated by CD studies in the presence of iodoacetamide. This result may be due to the lack of stabilization of modified monomers through their hydrophobic association into non-covalent polymers, which was blocked by steric hindrance from the -SH reagent side chain. According to CD data, 96% of the protein heated at 70°C for 15 min in the absence of -SH blockers is irreversibly denatured (Fig. 2B). Analysis of intermolecular interactions [17] showed that the treated protein was a mixture of 85% dimer and 15% aggregate forms (larger than the dimer). Approximately 33% of the dimers was stabilized by disulfide bonds, such that covalently stabilized dimers accounted for 28% of the total protein [17]. Under identical conditions, but in the presence of iodoacetamide, only 50% of the protein was irreversibly denatured (Fig. 2B). This corresponded to the fraction of dimers, observed in gel-permeation experiments (Fig. 3), which was not stabilized by intermolecular disulfides (Fig. 4).

These observations could be combined in a reaction scheme in which the following events take place in order: heat-induced dimer dissociation; modification of the tertiary structure of the free monomer; re-association of the modified monomers through hydrophobic interactions; and stabilization of non-covalently associated polymeric forms by means of disulfide exchange. The last two steps may be experimentally indistinguishable, especially at high temperatures. Occurrence of the third step in this sequence prevents reversibility of modifications in the tertiary structure. Blocking the -SH group will prevent to some extent the occurrence of this step, thus resulting in a decreased proportion of irreversibly denatured protein.

Further support for the reaction scheme proposed above could be provided by comparison of the time-courses of different heat-induced structural changes. Table 1 compares the reaction half-times of some of the phenomena observed during the exposure of β-lactoglobulin to heat at neutral pH. This approach does not represent an accurate thermodynamic interpretation of the process but could provide evidence that heat-induced denaturation of β-lactoglobulin requires a number of steps, which may be different at different temperatures and may differ in the temperature sensitivity of their rate constants.

As discussed above, the reversible modifications induced by heating at temperatures below the 70°C threshold resulted in dimer dissociation with simultaneous exposure of the reactive -SH and of novel hydrophobic surfaces characterized by a low content of aromatic residues. When we compared measurable rates, a very good correlation was found between the rate constant of protein swelling, as detected by binding of hydrophobic probes in the absence of other reagents, and that of the reaction between the protein and $Nbs_2$ at temperatures up to 70°C. These events had similar rate constants, which showed a very similar temperature dependence, which confirms the close relationship between the two events.

Another matching pair of events, as suggested by the identity of their half-times and by the closely similar temperature dependence of their rate constants, could be represented by the structural collapse of the protein and by the irreversible formation of polymeric forms of the protein at temperatures above the 70°C threshold. We interpreted structural collapse as the decrease in fluorescence of mixtures of β-lactoglobulin and fluorescent hydrophobic probes, which only occurred upon completion of a protein-swelling phase that led to the exposure of previously buried hydrophobic regions to give a molten-globule-like structure. Structural collapse and aggregation were found to become faster at higher protein concentrations. These results suggest that the hydrophobic surfaces exposed during the swelling phase are

used in later phases of heat-induced modifications, in particular during structural collapse/polymerization, as intermolecular binding sites which participate in the stabilization of aggregates.

As discussed above and elsewhere [11–13, 17], the -SH group made available by protein swelling could be used in further stabilization of the hydrophobically aggregated forms of the protein through a disulfide-exchange mechanism. This proposal confirms the primary role played in the overall mechanism of β-lactoglobulin denaturation and aggregation by an activated monomer form [13, 17], which we believe to have identified in this study as a free modified monomer that simultaneously exposes a reactive -SH group and adhesive hydrophobic surfaces.

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## REFERENCES

1. Hambling, S. G., McAlpine, A. & Sawyer, L. (1992) β-Lactoglobulin, in *Milk proteins* (Fox, P. F., ed.) pp. 141–190, Elsevier, London.
2. Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadarao, R., Jones, T. A., Newcomer, M. E. & Kraulis, P. J. (1986) The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein, *Nature 324*, 383–385.
3. Monaco, H. L., Zanotti, G., Spadon, P., Bolognesi, M., Sawyer, L. & Eliopoulos, E. E. (1987) Crystal structure of the trigonal form of bovine β-lactoglobulin and its complex with retinol at 2.5 Å resolution, *J. Mol. Biol. 197*, 695–706.
4. Pervaiz, S. & Brew, K. (1985) Homology of β-lactoglobulin, serum retinol-binding protein and protein HC, *Science 228*, 335–337.
5. Robillard, K. A. Jr & Wishnia, A. (1972) Aromatic hydrophobes and β-lactoglobulin A. Thermodynamics of binding, *Biochemistry 11*, 3835–3840.
6. Robillard, K. A. Jr & Wishnia, A. (1972) Aromatic hydrophobes and β-lactoglobulin A. Kinetics of binding by nuclear magnetic resonance, *Biochemistry 11*, 3841–3845.
7. Kuwajima, K., Yamaya, H., Miwa, S., Sugai, S. & Nagamura, T. (1987) Rapid formation of secondary structure framework in protein folding studied by stopped-flow circular dichroism, *FEBS Lett. 221*, 115–118.
8. Ptitsyn, O. B. & Semisotnov, G. V. (1991) The mechanism of protein folding, in *Conformations and forces in protein folding* (Nall, B. T. & Dill, K. A., eds) pp. 155–168, American Association for the Advancement of Science, Washington.
9. Ptitsyn, O. B. (1992) The molten globule state, in *Protein folding* (Creighton T. E., ed.) pp. 243–300, Freeman, New York.
10. DeWit, J. N. & Klarenbeek, G. (1984) Effects of various heat treatments on structure and solubility of whey proteins, *J. Dairy Sci. 67*, 2701–2710.
11. Shimada, K. & Cheftel, J. C. (1989) Sulfhydryl group/disulfide bond interchange reactions during heat-induced gelation of whey protein isolate, *J. Agric. Food Chem. 37*, 161–168.
12. Griffin, W. C., Griffin, M. C. A., Martin, S. R. & Price, J. (1993) Molecular basis of thermal aggregation of bovine betalactoglobulin A, *J. Chem. Soc. Faraday Trans. 89*, 3395–3405.
13. Roefs, S. P. F. M. & De Kruif, K. G. (1994) A model for the denaturation and aggregation of β-lactoglobulin, *Eur. J. Biochem. 226*, 883–889.
14. Watanabe, K. & Klostermeyer, H. (1976) Heat-induced changes in sulphydryl and disulfide levels of β-lactoglobulin A and the formation of polymers, *J. Dairy Res. 43*, 411–418.
15. Eynard, L., Iametti, S., Relkin, P. & Bonomi, F. (1992) Surface hydrophobicity changes and heat-induced modifications of α-lactalbumin, *J. Agric. Food Chem. 40*, 1731–1736.
16. Cairoli, S., Iametti, S. & Bonomi, F. (1994) Reversible and irreversible modifications of β-lactoglobulin upon exposure to heat, *J. Protein Chem. 13*, 347–354.
17. Iametti, S., Cairoli, S., De Gregori, B. & Bonomi, F. (1995) Modifications of high-order structures upon heating of β-lactoglobulin: dependence on the protein concentration, *J. Agric. Food Chem. 43*, 53–58.
18. Bonomi, F. & Iametti, S. (1991) Real-time monitoring of the surface hydrophobicity changes associated with isothermal treatment of milk and milk protein fractions, *Milchwissenschaft 46*, 71–74.
19. Arnebrant, T., Barton, K. & Nylander, T. (1987) Adsorption of α-lactalbumin and β-lactoglobulin on metal surfaces versus temperature, *J. Colloid Interface Sci. 119*, 383–390.
20. Ellman, G. L. (1959) Tissue sulfhydryl groups, *Arch. Biochem. Biophys. 82*, 70–77.
21. Strickland, E. H. (1974) Aromatic contribution to circular dichroism spectra of proteins, *CRC Crit. Rev. Biochem. 2*, 113–175.
22. Holladay, L. A., Savage, C. R., Kohen, S. & Puett, E. D. (1976) Conformation and unfolding thermodynamics of epidermal growth factor and derivatives, *Biochemistry 15*, 2624–2633.