Modifications of High-Order Structures upon Heating of β-Lactoglobulin: Dependence on the Protein Concentration

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A study on the concentration dependence of the modifications ensuing from thermal treatment of bovine β-lactoglobulin was carried out by using a combination of techniques. Heat-induced changes in tertiary structure were monitored by intrinsic tryptophan fluorescence, while modifications in protein surface hydrophobicity were studied both during their occurrence and at equilibrium by using the fluorescent hydrophobic probe 1,8-anilinonaphthalenesulfonate. The association equilibria in the heated and cooled protein and the stabilization of aggregates by intermolecular disulfides were studied by gel permeation chromatography and nonreducing, denaturing electrophoresis. Results indicate that irreversible modification of the tertiary structure is not concentration dependent, while the temperature required for the occurrence of protein swelling, the initial step in the formation of associated forms of the protein, increases with the protein concentration. Stabilization of aggregates by intermolecular disulfides was dependent on concentration only at temperatures below 75 °C.

Keywords: *β-Lactoglobulin; thermal treatment; protein structure; concentration-dependent association; intermolecular disulfide formation*

INTRODUCTION

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 of this protein, its function is still not clearly understood (Hambling *et al.*, 1992; Papiz *et al.*, 1986). The crystalline structure of the homodimer of bovine β-lactoglobulin has been determined in detail, showing a similarity with the plasma retinol binding protein and the odorant binding protein (Monaco *et al.*, 1987; Pervaiz and Brew, 1985). This suggests that the role of β-lactoglobulin may be connected with transport or accumulation of lipid-soluble biological components (Robillard and Wishnia, 1972a,b).

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 (Kuwajima *et al.*, 1987; Ptitsyn and Semisotnow, 1991) and framed in the now current "molten globule" hypothesis of protein folding/unfolding (Ptitsyn, 1992). The remarkable 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 (Papiz *et al.*, 1986; de Wit and Klarenbeek, 1984). The free, highly reactive −SH group of Cys 121 has been supposed to be involved in intramolecular and intermolecular disulfide interchange with other −SH groups in treated milk (Hambling *et al.*, 1992; Papiz *et al.*, 1986).

Despite the wealth of structural information available, little is known about the monomer/monomer interface in the freely associating β-lactoglobulin dimer or upon its modification in processing (Cairoli *et al.*, 1994), and there is no information on how protein concentration may influence the structural changes consequent to possibly denaturing treatments, such as those typical of industrial processing of dairy products and byproducts.

We previously have demonstrated the occurrence and discussed the significance of changes in protein surface hydrophobicity upon heat treatment of milk and milk proteins (Eynard *et al.*, 1992; Pagliarini *et al.*, 1990). Our studies on the thermal sensitivity of β-lactoglobulin have provided some detail on the mechanism and the nature of structural protein modifications representing the initial steps of subsequent macroscopic changes such as loss of solubility and exposure of regions suitable for different kinds of interaction with other milk components. The modifications in the exposure to the solvent of hydrophobic residues and in their organization into surface hydrophobic patches upon thermal treatment at neutral pH and at low concentration were studied along with alterations in the association equilibrium of β-lactoglobulin (Cairoli *et al.*, 1994).

In this work we focus on the influence of the protein concentration on these modifications, on the premises that the protein unfolding steps are expected to be independent of concentration, while the association phenomena should be highly dependent on the protein concentration. Understanding and modeling structural changes as a function of the protein concentration may be of practical interest, since most of the thermal treatments of whey and isolated whey proteins in the industry are carried out after a preliminary concentration step to minimize energy requirement.

MATERIALS AND METHODS

Reagents and Chemicals. Chemicals were of reagent or HPLC grade. β-Lactoglobulin (BLG) was from Sigma Chemical Co. (Sigma L-0230, lot 51H7210) and was dissolved in 50 mM sodium phosphate, pH 6.8, at 3.8, 8.0, and 16.0 mg/mL.

Methods. Intrinsic fluorescence measurements were taken at fixed temperature in a Perkin-Elmer LS 50 fluorometer, using the standard thermostated cell holder. Emission spectra were recorded using λ_{ex} = 298 nm.

Spectrofluorometric titration of protein samples with the hydrophobic fluorescent marker 1,8-anilinonaphthalensul-

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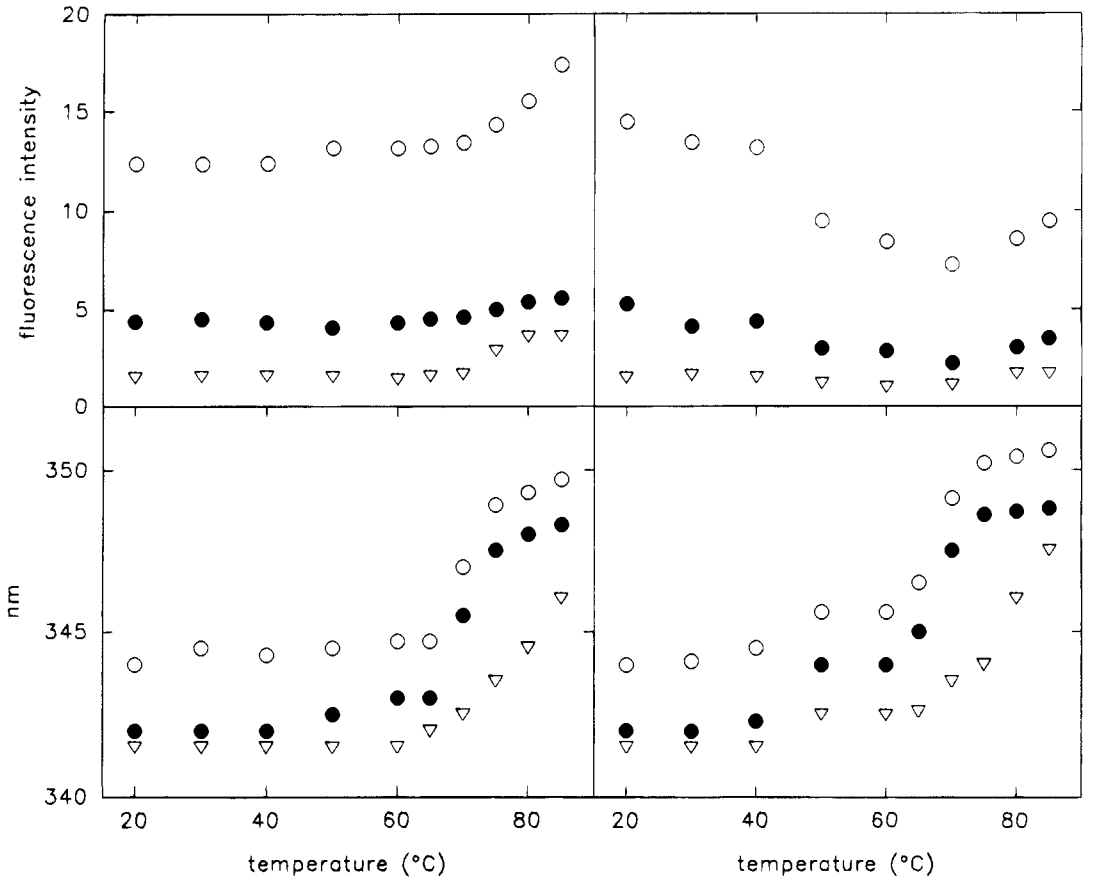

Figure 1. Intrinsic fluorescence parameters for BLG solutions of different concentrations. The protein was dissolved in 50 mM phosphate buffer, pH 6.8, at 3.8 (circles), 8.0 (dots), and 16.0 mg/mL (triangles). Individual samples were kept at the temperature given on the abscissa for 15 min before the emission spectra (λ_{ex} = 298 nm) on the hot solution (left panels) were recorded. Each sample was then allowed to cool at room temperature, and the emission spectrum was recorded on the cooled solutions (right panels). Fluorescence intensity was corrected for protein concentration.

fonate (ANS) at fixed temperature was performed as described previously (Pagliarini *et al.*, 1990) on the protein solution previously equilibrated for 15 min at the required temperature. In reversibility studies, the protein solution was titrated after equilibration for 15 min at the required temperature, followed by cooling at room temperature. Binding of ANS was monitored at λ_{ex} = 390 nm and λ_{em} = 480 nm. In titration experiments, the instrumental response was standardized with 10 nmol of ANS in 3 mL of 5% Triton X-100 (v/v) giving a response of 50 arbitrary units at appropriate instrumental settings. Binding equilibria were analyzed with the Ruzic algorithm (Ruzic, 1982).

Measurement of fluorescence changes upon isothermal heating of mixtures of BLG and ANS was performed by using a capillary cell and the electronically thermostated microcell holder already described elsewhere (Bonomi and Iametti, 1991; Eynard *et al.*, 1992; Iametti and Bonomi, 1993), fitted to a Perkin-Elmer MPF-2A fluorometer.

Gel permeation was performed on a Superdex G-75 column fitted to a Waters 625 HPLC and run in 50 mM phosphate and 0.1 M NaCl, pH 7.5. The eluate was monitored at 280 nm, and the chromatograms were analyzed quantitatively through appropriate software.

Nonreducing SDS–PAGE was performed in a 12% monomer gel by using a Bio-Rad MiniGel Protein II apparatus and by omitting 2-mercaptoethanol from the denaturing buffer. Gels were silver stained, and the intensity of individual protein bands was quantitated through an image analysis system based on CREAM 4.0 software (Kem-En-Tec, Copenhagen, Denmark).

The amount of covalently stabilized associated forms of BLG was calculated by SDS–PAGE analysis of individual peaks from the gel permeation step. To determine the total amount of individual covalently stabilized aggregates, the intensity of bands corresponding to dimers, trimers, tetramers, and high-order aggregates was compared to the total band intensity in the electropherograms and further normalized for the chromatographic distribution of each aggregate species in gel permeation experiments.

An overall "disulfide index" was calculated from the data obtained as above by using the equation

$$\text{disulfide index} = \sum(0.50M_2 + 0.66M_3 + 0.75M_4 + M_h)$$

where $M_{(2;3;4;h)}$ represents the molar fraction of covalently stabilized dimers, trimers, tetramers, and high-order aggregates with respect to total protein and the numeric coefficients represent the ratio between the minimum number of disulfide bridges (d) required to hold together a given number of monomers (m). The d/m ratio may assume the values 1/2 (dimer), 2/3 (trimer), 3/4 (tetramer), and $(n - 1)/n$ for high molecular weight aggregates involving n monomers. For the sake of simplification, we assumed $\lim_{n\to\infty}(n - 1)/n = 1$.

RESULTS

Intrinsic Fluorescence. Changes in the fluorescence of BLG heated at neutral pH are shown in Figure 1. Emission spectra were recorded both on the hot protein solution, at the end of a 15 min incubation at the required temperature, and on the same protein solution after cooling at room temperature. This approach allowed the estimation of the reversibility of the observed spectral changes. By using λ_{ex} = 289 nm, the emission spectra were dominated by the contribution of Trp residues. All of the observed spectral modification occurred within 2 s after exposure to heat, as determined by monitoring spectral changes following

the injection of small aliquots of concentrated protein into an appropriately large volume of hot buffer.

Structural modifications in the hot protein solution before cooling produced a temperature-dependent increase in λ_{em}, which occurred in a concentration-independent fashion at temperatures above 65−70 °C. That λ_{em} was increased even after the samples were cooled demonstrates that the exposure of tryptophans to the solvent was an irreversible process.

Fluorescence intensity per unit of protein concentration showed progressive quenching of the tryptophan fluorescence with increasing protein concentration at all temperatures. Fluorescence intensity showed a modest increase in hot protein solutions above 65−70 °C. Upon cooling to room temperature, a decrease in fluorescence intensity was observed in the previously heated protein solutions, and this decrease was more pronounced as the temperature of sample treatment was increased. Thus, the heating and cooling cycle had some effect on the Trp environment not only in terms of exposure to solvent but also in terms of the spatial relationships between residues. These latter modifications occur mainly upon cooling, and their amplitude is reduced at increased protein concentration. In this context, it has to be recalled that close interaction among Trp residues has been proven to result in a decrease of the fluorescence quantum yield as evinced by pronounced fluorescence quenching (Mills, 1976). Therefore, the irreversible exposure of Trp residues upon thermal treatment (indicated by the increase in λ_{em}) may result in their closer interaction upon cooling of the heat-treated protein and thus in mutual quenching of intrinsic fluorescence.

Titration with ANS. Protein solutions of different concentration were titrated with ANS both at high temperature and after a heating/cooling cycle similar to that used in intrinsic fluorescence studies. Titration curves were analyzed by the Ruzic algorithm to determine the average number and affinity of sites available on the protein surface for ANS binding. In these titrations, no significant changes in the average affinity of the ANS binding site(s) for the probe was observed either at different temperatures or after the heating/cooling cycles.

As shown in Figure 2, the number of ANS binding sites at room temperature attained a maximum value of one per dimer in very diluted protein solutions and decreased as the protein concentration was increased, likely as a consequence of concentration-dependent association of proteins, resulting in a lower accessibility of the protein surface. Heating of dilute protein solutions (<3.8 mg/mL) induced an increase in the number of ANS binding sites. This heat-induced increase is less evident in concentrated solutions and is completely reversible upon cooling.

Kinetic Studies. Monitoring the fluorescence changes during isothermal heating of mixtures of different concentrations of the protein and ANS allowed a "protein swelling" and a "protein collapse" phase to be distinguished. The protein swelling phase, leading to formation of a molten globule-like structure, was made evident by an increase in the fluorescence of the mixture, which was interpreted as being due to the exposure of hydrophobic regions previously buried in the native protein structure (Ptisyn *et al.*, 1991, 1992). The protein collapse phase only occurred as protein swelling was advanced; it was made evident by a decrease in the fluorescence of the mixture, which was interpreted as due to adhesion of the hydrophobic patches exposed in the protein swelling phase with formation of novel

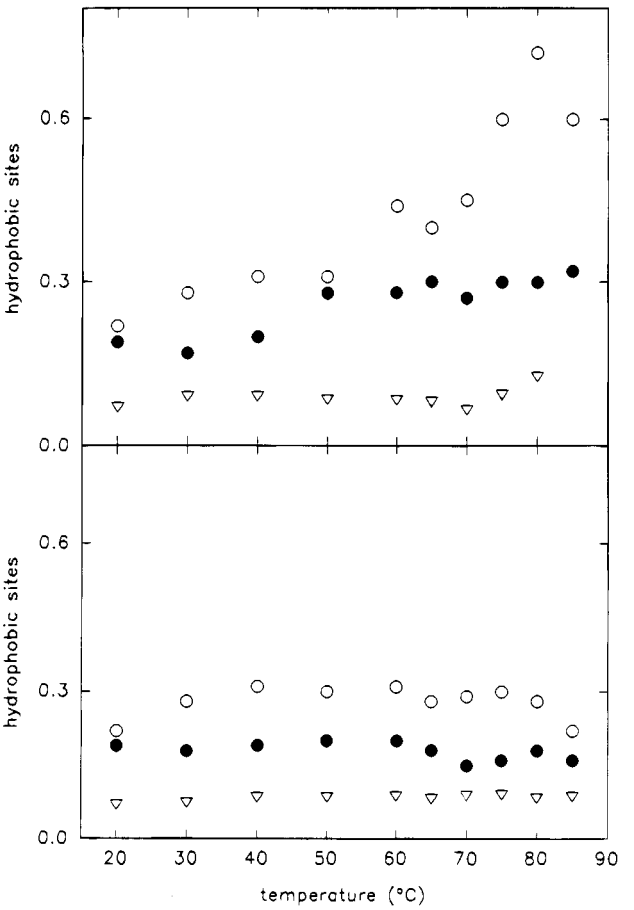

Figure 2. Temperature dependence of the number of ANS binding sites on BLG at different protein concentrations. The protein was dissolved in 50 mM phosphate buffer, pH 6.8, at 3.8 (circles), 8.0 (dots), and 16.0 mg/mL (triangles). Individual samples were kept at the temperature given on the abscissa for 15 min and titrated spectrofluorometrically with ANS while hot (upper panel). Alternatively, samples were kept at the temperature given on the abscissa for 15 min, allowed to cool, and then titrated with ANS at room temperature (lower panel). The number of hydrophobic surface sites available for ANS binding on each monomer was determined by analyzing the raw titration data according to the method of Ruzic (1982).

intermolecular bonds (possibly including covalent ones, as discussed in a later section of this paper) and to masking of ANS binding sites on the surface of the novel protein species formed upon heating (Iametti and Bonomi, 1993) by acquisition of an extremely compact conformation.

These modifications, despite the fact that they likely involve different regions of the protein and that they likely require some cooperative step(s), do occur distinctly and apparently do obey pseudo-first-order kinetics in our system. These peculiar features of our approach allowed the separate determination of the apparent rate constant (k'_{app}) for both of these subsequent events (Eynard *et al.*, 1992; Cairoli *et al.*, 1994).

The temperature dependence of k'_{app} for both the protein swelling and the protein collapse phases was analyzed through the Arrhenius plots shown in Figure 3, from which the thermodynamic parameters listed in Table 1 were calculated.

A transition temperature was observed for the protein swelling phase of the thermally induced changes in ANS binding. The temperature required for changes in E_a increased with the protein concentration, as if an increased protein concentration prevented the switching to a different swelling mechanism. Above this transition temperature, the rate constant of the protein

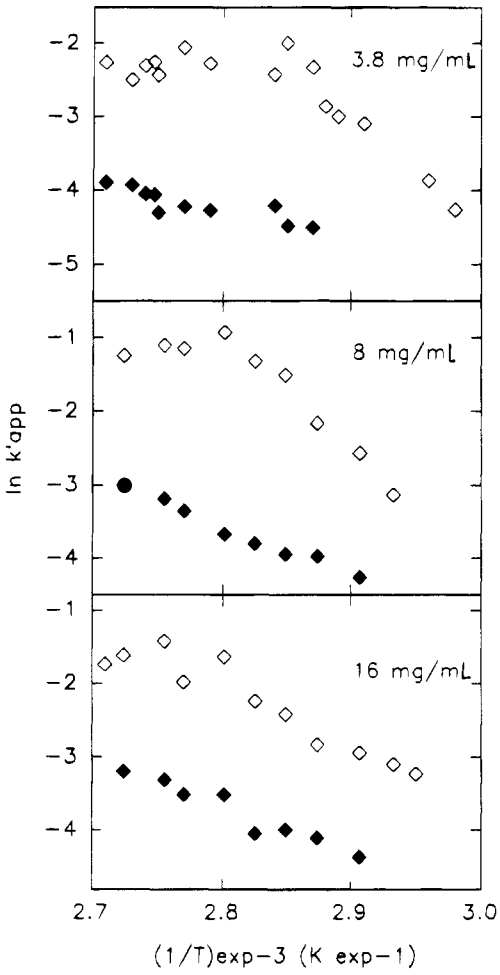

Figure 3. Arrhenius plots for the rate constants of isothermal fluorescence changes in mixtures of BLG and ANS. Aliquots of BLG at the concentration given for each panel in the presence of 0.120 mM ANS were placed in a 3 mm i.d. cylindrical microcell, which was rapidly inserted in a thermostated cell holder. Isothermal fluorescence changes were monitored over a 2-min time span from insertion of the microcell. Apparent rate constants for the initial fluorescence increase or "swelling" phase (open symbols) and for the subsequent fluorescence decrease or "collapse" phase (solid symbols) were calculated from standard semilog plots of the fractional fluorescence change versus time.

Table 1. Thermodynamic Parameters for the Modifications Observed upon Heating of Mixtures of ANS and BLG at Different Concentrations

protein concn (mg/mL)	$T_c^{swelling}$ (°C)	$E_a^{swelling}$ (kJ/mol)	$E_a^{collapse}$ (kJ/mol)
3.8	78	45.5	13.3
8.0	81	61.6	20.9
16.0	87	31.0	24.5

swelling reaction became independent of temperature, no matter what the protein concentration. Values for E_a in the temperature-dependent region of Arrhenius plots for the swelling phase did not show a clear-cut dependence on concentration. In other words, the mechanism of the swelling phenomenon was not affected by possible interprotein interactions, while the switching between high-temperature and low-temperature swelling mechanisms was concentration-sensitive.

A different picture was observed for the events resulting in collapse of the protein structure and ejection of formerly bound ANS. As already reported, no transition temperatures were detected for the protein collapse phase of heat-induced modifications, which did not occur at temperatures below 70 °C. As listed in Table 1, E_a for the collapse reaction increases with the protein concentration, suggesting that the collapse phenomenon is affected by interprotein interaction.

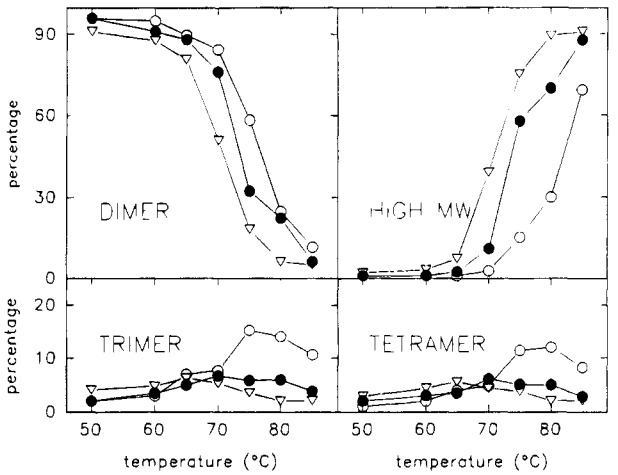

Figure 4. Irreversible thermal association of BLG monomers as a function of temperature and protein concentration. The protein was dissolved in 50 mM phosphate buffer, pH 6.8, at 3.8 (circles), 8.0 (dots), and 16.0 mg/mL (triangles). Individual samples were kept at the temperature given on the abscissa for 15 min and cooled at room temperature. A 0.2 mL aliquot was then applied on a Superdex G-75 column in 50 mM phosphate, 0.1 M NaCl, pH 7.5, fitted to a Waters 625 HPLC. The amount of protein in peaks corresponding to dimers, trimers, tetramers, and high molecular weight species is given as percent of total protein.

On the other hand, no linear relationship was observed between the rate constants for either phase of denaturation and the protein concentration (Figure 3). Thus, aggregation phenomena are not directly affecting the rate of thermally induced exposure of hydrophobic sites or the rate of their disappearance upon structural collapse.

Association/Dissociation Equilibria. Gel permeation chromatography of heat-treated and cooled BLG solutions allowed separated different associated forms of the protein (Cairoli *et al.*, 1994). Under the nondenaturing conditions used, this procedure did not discriminate between covalent aggregates and noncovalent multimeric associated forms. Figure 4 depicts the formation of trimers, tetramers, and high molecular weight soluble aggregates as a function of the temperature, along with the persistence of the dimer. As in similar previous studies carried out at neutral pH on pure BLG solutions, formation of protein precipitates was not observed.

No irreversible changes in the association/dissociation equilibrium of BLG were observed below 60 °C at any protein concentration. Above this temperature, disappearance of the native dimeric form with increasing temperature is more pronounced and more temperature-sensitive as the protein concentration is increased. No residual dimer was present in BLG solutions at concentrations higher than 3.8 mg/mL when heated at 85 °C for 15 min. Formation of high-order aggregates proceeds through transitory dimer dissociation and monomer release, which are favored by low protein concentrations. Indeed, at protein concentrations of 2.5 mg/mL trimeric forms of BLG represented the most abundant product at equilibrium (not shown). High protein concentrations favored the formation of high-order aggregates, which represented by far the most abundant form of BLG in samples at 8 and 16 mg/mL heated above 85 °C for 15 min. Accumulation of intermediate-sized associated forms was therefore only possible at intermediate protein concentrations.

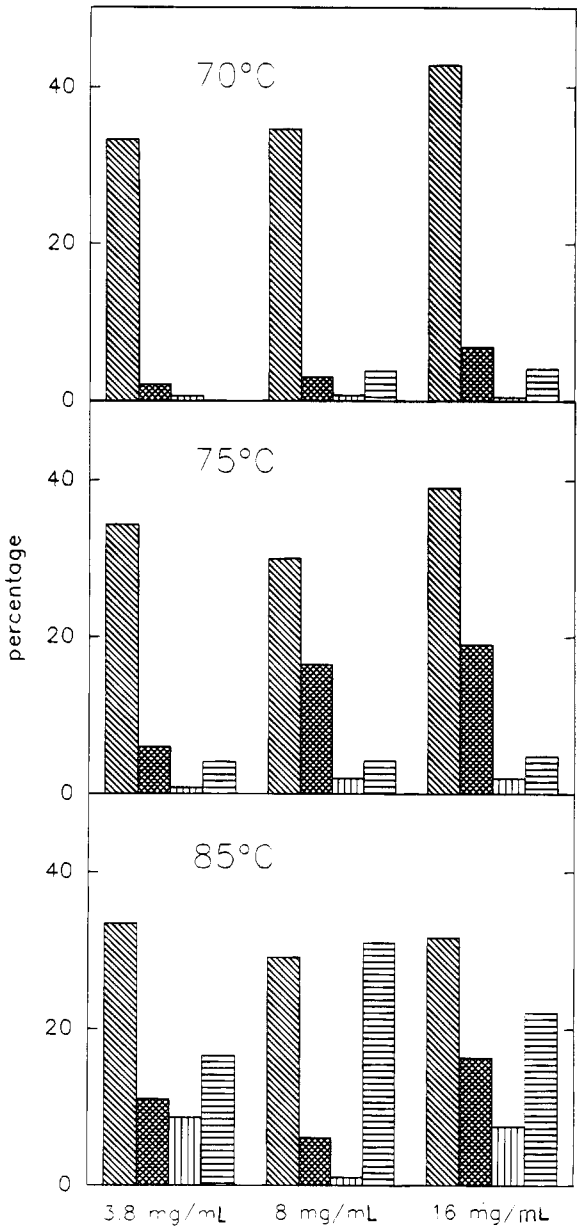

Figure 5. Stabilization by disulfide bonds of different association forms of BLG as a function of temperature and protein concentration. Protein was treated and chromatographed as detailed in the legend to Figure 4. Chromatographic fractions corresponding to individual associated form were collected and analyzed by nonreducing SDS–PAGE. Individual bands were quantitated by image analysis of silver-stained gels. For each protein concentration, columns refer to (from left to right) dimers, trimers, tetramers, and high molecular weight aggregates. The percentage of each species was calculated by comparing its distribution in SDS–PAGE with the gel permeation tracings and is reported with respect to total protein.

Stabilization of Aggregates by Disulfide Bonds. The nature of the chemical bonds involved in the stabilization of the associated forms of BLG was investigated by resolving in nonreducing SDS–PAGE the components of individual peaks obtained from the gel permeation experiments presented in the former section. By quantitating individual bands in the electropherograms, it was possible to calculate the percentage of individual aggregate forms (dimers, trimers, tetramers, and high-order aggregates) being stabilized by disulfide bonds. Results of these analyses are reported in Figure 5, where the percentage of individual aggregate forms is given with respect to total protein.

Table 2. Dependence of the Disulfide Index in Heat-Treated BLG on the Temperature and Protein Concentration

protein concn (mg/mL)	temperature		
	70 °C	75 °C	85 °C
3.8	0.67	0.96	0.96
8.0	0.75	0.94	0.94
16.0	0.93	0.94	1.01

At 70 °C, BLG dimers represented the most abundant species, and their relative abundance decreased as the concentration was increased (Figure 4). Covalently stabilized dimers increased with the protein concentration (Figure 5). At a protein concentration of 3.8 mg/mL, dimers (however held together) represented 85% of total protein, with covalently bound dimers being 33% of the total protein. Thus, 38% of the dimers were held together by intermolecular disulfide bridges. At a protein concentration of 16 mg/mL, total dimers were 51.2% of total protein and covalently stabilized dimers were 43% of total protein. This means that at this protein concentration 83% of the dimers were stabilized by intermolecular disulfide bridges. Thus, the formation of intermolecular disulfide was favored by increasing the protein concentration.

At 85 °C, high molecular weight aggregates were the dominant species, and their relative abundance increased with protein concentration (Figure 4). The amount of covalently stabilized high molecular weight aggregates did not show any meaningful dependence on concentration. At 3.8 mg/mL, 69% of the protein was present as high-order aggregates, of which 25% was stabilized by intermolecular disulfides (that is, 17% of the total protein). By comparison, at 16 mg/mL 92% of the protein was present as high-order aggregates, of which 24% was stabilized by intermolecular disulfides (that is, 22% of the total protein). Intermolecular disulfide formation therefore appears to become less concentration-dependent as the temperature of treatment is increased.

The necessity of summarizing our analysis of the nature of the stabilizing forces in associated forms of BLG suggested the introduction of a disulfide index. This parameter was calculated from separation data and represents a quantitative estimate of the molar fraction of monomers held together by a disulfide bond. Ideally, values for the disulfide index should range from 0 (as in the freely associating dimeric form of the native protein) to 0.5 for a disulfide-linked dimer to the highest possible value of 1 when all of the protein is present as an extremely large disulfide-linked aggregate.

As seen in Table 2, the highest value of the disulfide index was only reached for the most severe thermal treatment at the highest protein concentration. At 70 °C, the disulfide index showed a marked positive dependence on concentration, which was not observed at higher temperatures. Thus, formation of intramolecular disulfide bonds was particularly relevant to the stabilization of high-order aggregates, which were most abundant when both the concentration of the protein and the temperature of treatment were the highest.

CONCLUSIONS

Association of BLG in solution at neutral pH to yield freely dissociating soluble species larger than dimers is a concentration-dependent process already occurring at room temperature, as demonstrated by the concentration dependence both of the quenching of tryptophan fluorescence and of the decrease of ANS-accessible

surface hydrophobic sites. Both observations are consistent with hydrophobic interaction being the dominating force in this reversible association phenomenon.

Heating of BLG solutions at neutral pH at temperatures above 60 °C resulted in irreversible, concentration-independent modifications of the tertiary structure of the protein, as indicated by the increased exposure of tryptophan residues to the solvent. Further modifications of the protein tertiary structure occurred upon cooling and apparently led to association of exposed tryptophans into tighter clusters. In dilute protein solutions (≤3.8 mg/mL), these modifications of the tertiary structure resulted in an increase in the number of sites available for ANS binding, which normally average 0.5 per monomer (or one per dimer) at room temperature at infinite protein dilution. This increase could be interpreted as due to the release of free monomers. When studied through titration with ANS, modifications in this particular association equilibrium were apparently reversible upon cooling.

By monitoring the kinetics of changes in protein surface hydrophobicity, we observed that rate constants for the swelling and collapse phases of protein denaturation were independent of concentration, suggesting that changes in quaternary structure did not represent a rate-limiting step in the unfolding of the protein structure or in its subsequent collapse in hot protein solutions. Of course, this observation does not rule out the possibility that dissociation of dimers and formation and stabilization of aggregates, although not rate limiting, represent relevant steps in the protein swelling and protein collapse phases, respectively, of the overall denaturation process. As for the formation of associated species upon cooling of heated BLG solutions, formation of ANS-entrapping associated forms of BLG was shown to occur upon cooling of previously heated protein solutions (Cairoli *et al.*, 1994), while evidence for clustering of tryptophan residues upon the heating/cooling treatment has been discussed above. This latter event may have ensued from interactions between individual heat-denatured polypeptides, which did not occur in hot solutions but became relevant upon cooling.

For obvious methodological reasons, changes in the quaternary structure of BLG were only approachable at equilibrium on the heated/cooled protein and indicated that formation of multimeric species was promoted by increasing both temperature and protein concentration. Besides confirming the sequential polymerization mechanism already hypothesized (Cairoli *et al.*, 1994), this study also presents evidence on almost complete stabilization by intermolecular disulfide bridges of the aggregates obtained at high temperature (>75 °C). At temperatures closer to those typical of industrial milk pasteurization processes (≈70 °C) the formation of intermolecular disulfides was concentration-dependent. A possibility for explaining the concentration dependence of intermolecular disulfide formation at 70 °C is that some more or less completely denatured form of BLG may be involved in the catalysis of disulfide exchange (Dr. Ingolf Krause, Technical University of Munich, personal communication, 1994). At higher temperatures, intermolecular disulfide formation was likely fast enough not to show a dependence on concentration.

Besides their possible relevance to the understanding of the thermal unfolding of multimeric proteins, these observations may be relevant to modeling and improving the technological treatment of milk and whey. In this context, investigation of interaction among different whey proteins is currently under way in our laboratory.

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