Molecular Modifications of β-Lactoglobulin upon Exposure to High Pressure

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Irreversible modifications in tertiary structure, surface hydrophobicity, and association state of β-lactoglobulin were studied after exposure to high pressure (600 and 900 MPa) of solutions of the protein at neutral pH and at different concentrations. Only minor irreversible structural modifications were evident even for treatments as intense as 15 min at 900 MPa. The occurrence of irreversible modifications was time-progressive at 600 MPa but was complete within 2 min at 900 MPa. The irreversibly modified protein was soluble, but some covalent aggregates were formed. Formation of aggregates increased with increasing protein concentration and was prevented by blocking the free thiol moiety in each β-lactoglobulin monomer. Results are discussed in light of their practical relevance, and a unifying denaturation mechanism is envisaged for β-lactoglobulin. In the proposed mechanism, release of monomers represents one of the earliest events, while association of transiently modified monomers stabilizes the denatured forms of the protein.

Keywords: *β-Lactoglobulin; high-pressure treatments; protein structure; protein association*

INTRODUCTION

Sanitizing technologies other than heat treatment are gaining popularity in the food industry. Among them, high-pressure treatment has been recognized as a physical tool for the modification of macromolecular constituents, such as proteins (Cheftel, 1992; Hayashi, 1992; Balny and Masson, 1993), and has been proposed as an alternative to established thermal sanitation procedures.

Several studies have demonstrated changes in the structure and/or functionality of proteins under pressure (up to 1000 MPa) (Zipp and Kautzmann, 1973; Taniguchi and Suzuki, 1983; Pittia *et al*., 1996), and the effects of high-pressure treatment have been the subject of several reviews (Weber and Drickhamer, 1983; Balny *et al.*, 1989; Silva and Weber, 1993).

High pressures act by altering the balance of intramolecular and solvent−protein interactions. Pressure-induced denaturation is a complex phenomenon, mainly ensuing from the disruption of both hydrophobic bonds and salt bridges. The extent of pressure-induced changes in proteins depends on factors such as temperature, pH, solvent, and ionic strength, as well as on the native protein structure and on the applied pressure. Reversible effects such as dissociation of polymeric structures or partial unfolding are observed below 100−200 MPa (Jaenicke, 1991). Higher pressures (>200 MPa) cause nonreversible and more extensive effects on proteins. These include unfolding of monomeric proteins and aggregation (Weber and Drickamer, 1983; Zipp and Kautzmann, 1973; Silva *et al*., 1989), as well as the formation of gel structures (Van Camp and Huyghebaert, 1995).

In general, studies on the effects of high pressures have been undertaken with globular proteins, including β-lactoglobulin. Partial unfolding of β-lactoglobulin during treatment has been detected at pressures as low as 50 MPa by deuterium exchange and proteolytic sensitivity studies (Tanaka and Kunugi, 1996; Stapelfeldt *et al.*, 1996; Dufour *et al.*, 1995; Van Willige and Fitzgerald, 1995; Hayashi *et al*., 1987). Aggregation effects have been observed for β-lactoglobulin upon treatment between 200 and 600 MPa (Dumay *et al.*, 1994; Nakamura et al., 1993), and formation of intermolecular disulfides at neutral pH values and at pressures up to 450 MPa has been reported (Funterberg *et al.*, 1995; Tanaka *et al*., 1996).

Detailed information is available on the crystalline structure of β-lactoglobulin (Pervaiz and Brew, 1985; Papiz *et al.*, 1986; Monaco *et al*., 1987; Hambling *et al.*, 1992), on the occurrence of molten-globule-like intermediates in the refolding of its tertiary structure from chaotrope solutions (Kuwajima et al., 1987; Ptitsyn, 1992), and on the involvement of the free, highly reactive −SH group of Cys121 in intramolecular and intermolecular disulfide interchange with other −SH groups in treated milk (DeWit and Klarenbeek, 1984; Shimada and Cheftel, 1989; Griffin *et al*., 1993). There is little information on the mechanistic details of structural changes in β-lactoglobulin consequent to the possibly denaturing treatments typical of the industrial processing of dairy products and byproducts, such as whey and sweet whey (Watanabe and Klostermayer, 1976; Roefs and De Kruif, 1994).

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Some of us 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 (Bonomi and Iametti, 1991; Eynard *et al.*, 1992; Cairoli *et al.*, 1994; Iametti *et al.*, 1995, 1996). Studies on the thermal sensitivity of β-lactoglobulin at neutral pH focused on the modifications in the exposure to the solvent of hydrophobic residues. Heat-induced changes in the organization of hydrophobic side chains into surface hydrophobic patches were investigated at neutral pH and at low protein concentration, concomitantly with nonreversible alterations in the association equilibrium of β-lactoglobulin. Details on the mechanism of heat-induced polymerization of the protein and on the nature of the chemical bonds involved (Cairoli *et al.*, 1994; Iametti *et al.*, 1995, 1996) were in agreement with those proposed by others (Shimada and Cheftel, 1989; Griffin *et al.*, 1993; Roefs and De Kruif, 1994). These studies indicated that monomer dissociation was a necessary step for formation of stable protein polymers. Polymerization occurred initially through adhesion of exposed hydrophobic regions of the protein to give aggregates. Above a certain temperature threshold these aggregates were stabilized into polymers by intermolecular disulfide exchange.

In this study we report on the application of some of the methodologies developed for studying the heat-induced modifications of tertiary and quaternary structures of β-lactoglobulin to the characterization of the high-pressure-treated protein. Our results are discussed in terms of similarities and differences in the irreversible structural modifications induced by treatments of different nature but resulting in approximately equivalent sanitizing effects.

MATERIALS AND METHODS

Reagents and Chemicals. Chemicals were of reagent or HPLC grade. Bovine β-lactoglobulin (BLG) was from Sigma. Each protein lot was tested as received for the presence of multimeric forms or of disulfide-linked dimers, by using HPLC gel permeation and SDS−PAGE under nonreducing conditions as indicated below.

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

Methods. High-pressure treatments were performed on BLG solutions at concentrations of 2.5, 5, and 10 mg/mL in buffer. For each treatment, 150 mL of the protein solution was sealed in plastic bags before pressure treatment. An aliquot of the sample was retained as a control. Pressure treatment was carried out in water in an isostatic high-pressure generator ABB Model QFP-6 (ABB, Sweden) with pressure settings of 600 and 900 MPa (maximum pressure = 900 MPa). The isostatic pressure generator was not equipped with a temperature control system, so the temperature of the solution increased during the compression step of the treatment, until the prefixed pressure was reached. Under our conditions, the temperature increase was about 2 °C/100 MPa, so the highest temperature (about 37−38 °C) was reached for 900-MPa-treated samples. The pressurization step of the samples took between 60 and 90 s depending on the final pressure achieved. During the holding time (which is referred to as the actual treatment time), and the following instantaneous decompression, the temperature of the samples decreased to a final value of 25 ± 2 °C. Immediately after the pressure treatment, the solutions were refrigerated and kept at 0−4 °C until analysis.

Intrinsic fluorescence measurements were taken at room temperature in a Perkin-Elmer LS 50 spectrofluorometer. Emission spectra were recorded using λ_{ex} = 298 nm.

Spectrofluorometric titration of protein samples with the hydrophobic fluorescent marker 1,8-anilinonaphthalenesulfonate (ANS) was performed at room temperature as described previously (Pagliarini *et al.*, 1990). 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 the appropriate instrumental settings. Binding equilibria were analyzed with the Ruzic algorithm (Ruzic, 1982).

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

When required, the sulfhydryl reagent, dithiobis(2,4-nitrobenzoate) (DTNB; Ellman, 1959) was added prior to the pressure treatment. Reacted −SH groups were determined spectrophotometrically after the pressure treatment by using A_{412} = 13 600 for the nitrobenzoate thiolate anion. An untreated mixture of DTNB and BLG was used as reference.

Nonreducing SDS−PAGE was performed in a 12% monomer gel by using a Bio-Rad MiniGel Protein II apparatus, omitting 2-mercaptoethanol from the denaturing buffer. Gels were stained with Coomassie Brilliant Blue R-250, and the intensity of individual protein bands was evaluated quantitatively through an image analysis system based on CREAM 4.0 software (Kem-En-Tec, Copenhagen, Denmark).

Near-UV circular dichroism (CD) spectra were recorded at room temperature on a Jasco J500 A spectropolarimeter and analyzed by means of the Jasco J700 A software.

RESULTS AND DISCUSSION

Modification in the Tertiary Structure As Detected by Circular Dichroism, Intrinsic Fluorescence, and Surface Hydrophobicity Measurement. The irreversible effects of high-pressure treatment on the secondary and tertiary structures of BLG were investigated by spectroscopic and ligand-binding measurements on the protein treated for different times at different pressures. In these experiments pressure was kept above the minimum threshold required for a microbiological effect equivalent to that of pasteurization. In previous studies on thermal denaturation of BLG modifications in the near-UV CD spectra of the protein, in its intrinsic fluorescence and in the distribution of hydrophobic patches on its surface, were considered. Using the same experimental approaches in the present study allowed direct comparison with previous results.

The CD spectra of BLG before and after treatment at different pressures are shown in Figure 1. The spectra presented in Figure 1 represent the structural equilibrium attained after equilibration, since no further changes were observed with prolonged high-pressure treatment. Spectral modifications at equilibrium are evidenced by a decrease in the overall intensity of the signals similar to that observed upon heating (Iametti *et al.*, 1996). Irreversible structural modifications are induced only on a rather minor fraction of the BLG molecules. A quantitative analysis of the CD data showed that the fraction of protein having lost elements of its tertiary structure was about 10% at both 900 and 600 MPa. No distinction could be made among variations attributable to different individual contributions to the CD signal in the treated samples (Strickland, 1974). In other words, all of the different amino acid residues contributing to the near-UV CD signal of BLG seemed to be affected by pressure to the same extent.

As shown in Figure 2, the irreversible changes in structure observed after treatment at 600 MPa were time-dependent and were essentially complete after 10 min. The same type of modification appeared to be complete to the same extent after only 2 min when the

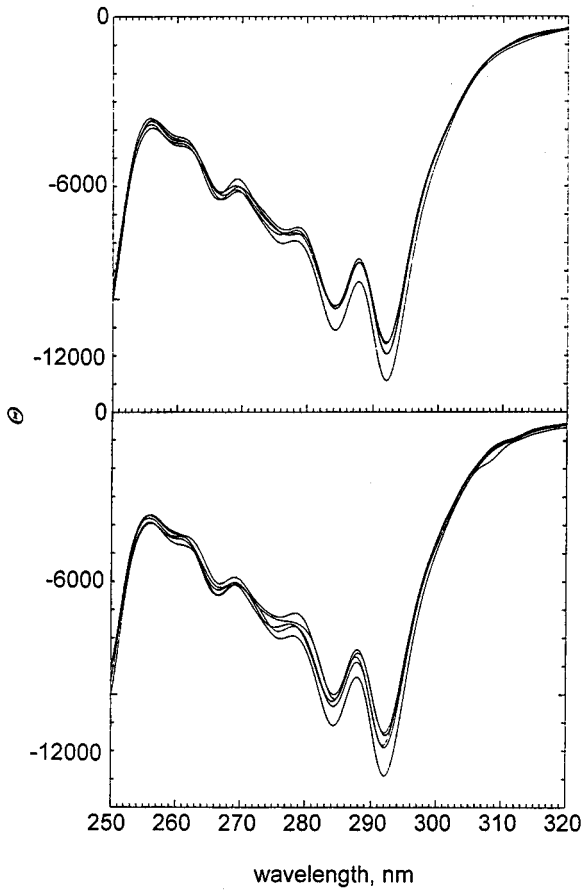

Figure 1. Near-UV CD spectra of BLG exposed to different pressures. Spectra were recorded on aliquots of BLG solutions (2.5 mg/mL in 50 mM sodium phosphate, pH 6.8) treated at 600 (top) or 900 MPa (bottom) for different times. The spectrum of untreated BLG is given in each panel as the bottom line at 292 nm. From bottom to top at 292 nm, spectra are those of samples treated for 5, 10, 15, and 20 min (600 MPa) or for 1, 2, 5, and 10 min (900 MPa).

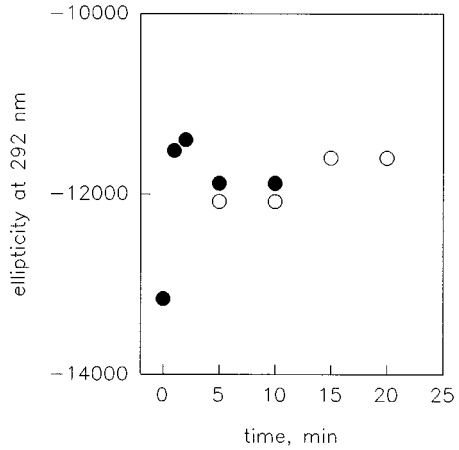

Figure 2. Time course of CD variations at different pressures. Data were taken from the spectra shown in Figure 1, for samples treated at 600 (open symbols) or at 900 MPa (solid symbols).

protein was treated at 900 MPa. Treatments of BLG at lower pressures (450 MPa), as reported by other authors (Dumay *et al.*, 1994; Funterberg *et al.*, 1995; Tanaka and Kunugi, 1996), also resulted in minor structural changes and required long treatment times. These observations indicate that a dependence of irreversible structural modifications on the duration of the treatment is observed only in a definite pressure range.

Table 1. Concentration Dependence of the Molecular Changes Ensuing from Treatment of BLG at 900 MPa for 2 min[a]

protein concn, mg/mL	no. of surface hydrophobic sites, % of control	residual total dimer, % of control	disulfide-linked dimer, % of control	molar ellipticity at 292.5 nm, % of control
2.5	78	88	13	88
5	25	82	14	67
10	25	83	16	69

[a] Protein was dissolved in buffer at the concentrations given, and measurements were performed on individual samples before (control) and after treatment at 900 MPa for 2 min. Spectrofluorometric titrations with ANS were repeated in triplicate, and the average value of the number of sites available for titration is given. Residual total dimer was determined by gel permeation chromatography and disulfide-linked dimer by SDS−PAGE analysis under nonreducing conditions. Results are given as a percentage of the values obtained for untreated solutions of identical concentrations.

The treated proteins were also compared to the native ones in terms of modification of their tryptophan fluorescence as a consequence of alterations in the chemical environment of the tryptophan side chain (Cairoli *et al.*, 1994; Iametti *et al.*, 1995). No significant modifications in the position of the emission maximum or in the fluorescence intensity were evident after high-pressure treatment. Thus, any modification in the exposure of tryptophan residues to the solvent during high-pressure treatment was reversible, in a fashion similar to that observed when BLG was heated below 60 °C while changes in intrinsic fluorescence were continuously monitored (Cairoli *et al.*, 1994; Iametti *et al.*, 1995). The isostatic apparatus used in the present study did not allow real-time observation of changes during the treatment, but occurrence of reversible structural modifications during exposure to high pressure has been described for a number of proteins (Dumay *et al.*, 1994; Nakamura *et al.*, 1993). In the case of BLG, fluorescence measurements taken during the exposure at high pressure indicated that significant transitions in the quantum yield of tryptophan fluorescence and in the emission maximum occurred with a midpoint in the 150−200-MPa range for treatments lasting between 30 and 60 min (Dufour *et al.*, 1995; Stapelfeldt *et al.*, 1996).

Residues other than tryptophan are thought to be relevant to denaturation of BLG, especially for the monomer/monomer interface in the freely associating dimer, where other hydrophobic amino acids play a prominent role. Hydrophobic amino acids often are found associated into discrete patches on the surface of proteins. Physical denaturing agents, including those commonly used in food processing, were reported to modify significantly the number and the properties of these surface hydrophobic patches (Iametti and Bonomi, 1993; Bonomi and Iametti, 1994).

The possible irreversible modification of protein hydrophobic surfaces in pressure-treated BLG was addressed by spectrofluorometric titration of the treated protein with the fluorescent hydrophobic probe ANS. At a BLG concentration of 2.5 mg/mL (used also for CD and fluorescence studies) only minor time-dependent modifications were observed in surface hydrophobicity in protein samples treated at 600 MPa (not shown). The modifications observed after 2 min at 900 MPa (Table 1) could be interpreted in terms of a decrease of the number of accessible surface hydrophobic sites, with a concomitant decrease (from 420 to 290 nM) of their average affinity for the hydrophobic probe used in these

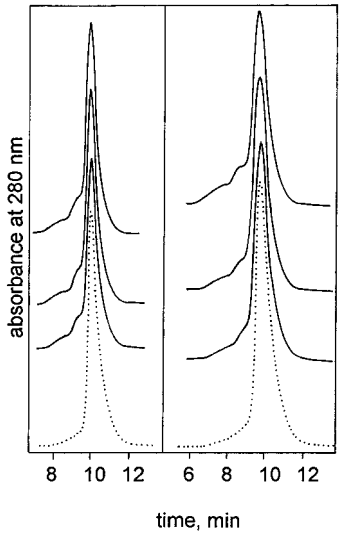

Figure 3. Aggregation of BLG upon high-pressure treatment. Aliquots of BLG solutions (2.5 mg/mL in 50 mM sodium phosphate, pH 6.8) were loaded before (dots) or after high-pressure treatment (solid line) on a Superdex G-75 column fitted to a Waters 625 HPLC or to a Pharmacia FPLC and run in 50 mM phosphate/0.1 M NaCl, pH 7.5. Treatment conditions were (from bottom to top) 1, 2, and 10 min at 900 MPa (left) or 5, 10, and 20 min at 600 MPa (right).

studies. This behavior is consistent with formation of covalent and noncovalent polymeric forms of the protein. In these polymers hydrophobic patches are located at the contact points between associating proteins rather than on the surface of individual protein molecules, so that they are less accessible to the fluorescent hydrophobic probe.

Formation of associated forms of BLG stabilized by different types of chemical bonds was observed previously for thermal treatments (Cairoli *et al.*, 1994). The nature of the chemical bonds involved in this association will be discussed in the next section of this paper.

Modifications in the Association Equilibria of Pressure-Treated BLG. Irreversible modifications in the association equilibrium of BLG monomers were studied by combining chromatography and electrophoresis. This combination of methodologies allowed some insights into the nature of the bonds that stabilize polymeric forms of the protein formed upon processing.

As in previous studies on the effects of heat treatment on pure BLG solutions at neutral pH, formation of protein precipitates was not observed upon high-pressure treatment of BLG at the concentrations used here.

Gel permeation chromatography of high-pressure-treated BLG solutions allowed separation of different associated forms of the protein, including one with an elution time indicating that it was formed by association of three monomers (Cairoli *et al.*, 1994; Iametti *et al.*, 1995). Under the nondenaturing conditions used for chromatography, this technique did not discriminate between covalent aggregates and noncovalent multimeric associated forms. Figure 3 depicts the formation of soluble aggregates as a function of the pressure and length of the treatment and shows persistence of the dimer.

Some of the species formed during the treatment were stabilized by intermolecular disulfide bonds, as indicated by the SDS−PAGE tracings in Figure 4. Dependence on the pressure and time for the formation of covalently bound dimers appeared to parallel that seen for CD modifications, as shown by the quantitative

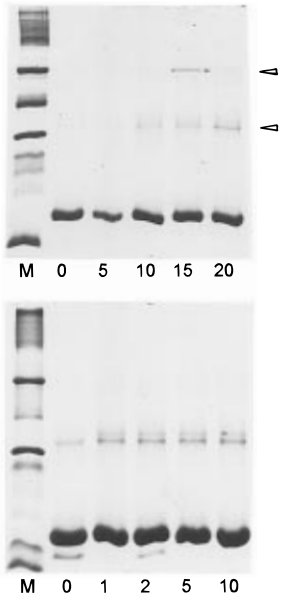

Figure 4. Formation of covalently stabilized aggregates as a function of pressure and treatment time. Aliquots of BLG solutions (2.5 mg/mL in 50 mM sodium phosphate, pH 6.8) treated at 600 (top) or 900 MPa (bottom) for the times (minutes) given below each lane were denatured and analyzed by nonreducing SDS−PAGE. An identical amount of protein was loaded in each sample lane. Covalent dimer and larger aggregates are indicated by arrows. M: molecular size markers.

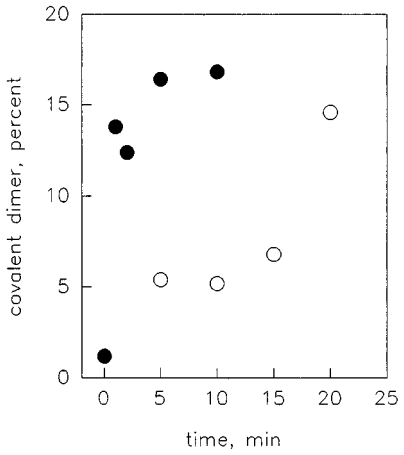

Figure 5. Time dependence of covalent dimer formation at different pressures: (open symbols) 600 MPa; (solid symbols) 900 MPa. Data were derived from computerized image analysis of the SDS−PAGE tracings shown in Figure 4, by assuming identical staining properties for the different aggregation forms of the protein.

analysis reported in Figure 5. These observations provide further circumstantial evidence that formation of covalent aggregates is relevant to the nonreversibility of structural changes in the course of BLG denaturation and confirm the possible role of shear forces in intermolecular disulfide exchange. In our conditions, covalent aggregation was time-dependent at 600 MPa and was complete in 5 min at 900 MPa. Covalent dimerization of BLG after treatment for 1 h at 450 MPa has been reported in other studies (Tanaka *et al.*, 1996).

The presence of species migrating with a size equivalent to that of an odd-numbered aggregate of BLG monomers indicated release of individual monomers upon processing and their involvement in a sequential polymerization mechanism. The monomeric species of BLG released as an active intermediate upon thermal treatment could be trapped by carrying out denatur-

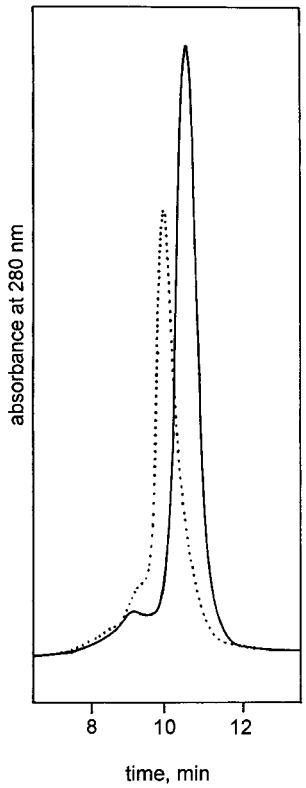

Figure 6. Formation of stable monomeric forms of BLG upon treatment at high pressure in the presence of DTNB. Solutions of BLG (2.5 mg/mL in 50 mM sodium phosphate, pH 6.8) were treated at 900 MPa for 2 min in the absence (dots) or in the presence of 2 mM DTNB (solid line). Aliquots of the treated proteins were loaded on a Superdex G-75 column fitted to a Waters 625 HPLC and run in 50 mM phosphate/0.1 M NaCl, pH 7.5.

ation in the presence of the thiol reagent, dithiobis(2,4-nitrobenzoate) (DTNB) (Iametti *et al.*, 1996). The same procedure was applied for high-pressure treatment. Gel permeation analysis of the pressure-treated protein indeed showed that free-standing monomers were the most abundant species left after treatment in the presence of DTNB (Figure 6). Similar results were achieved by other investigators when treating BLG at 450 MPa for 1 h in the presence of *N*-ethylmaleimide (Tanaka *et al.*, 1996).

Spectrophotometric measurements of the release of the DTNB anion (formed upon reaction with accessible protein −SH) indicated titration of 0.89 −SH group per monomer in the pressure-treated protein. Thus, as it was observed for thermal denaturation studies, release of the free monomer appears as a mandatory step in the sequence of molecular events leading to structural modifications of BLG also in the case of high-pressure treatment. Release of the free monomer is made evident by access of DTNB to the sulfhydryl moiety of Cys121, which is normally buried at the monomer/monomer interface in the native dimer. The impossibility of the DTNB-reacted monomer to reassociate, as the interface is sterically blocked by the bulky thiolate, shifts the equilibrium during either thermal or high-pressure treatment toward complete dimer dissociation. In the case of high-pressure treatment, complete dimer dissociation was found after 2 min at 900 MPa. For comparison, the heat-induced dissociation of BLG in the presence of DTNB had reaction half-times ranging from 38 s at 60 °C to 7 s at 70 °C (Iametti *et al.*, 1996).

Concentration Dependence of the Observed Modifications. In an attempt to understand the effects of interprotein interactions on the behavior of the protein and to model treatment of concentrated solutions that could be of practical interest, the approaches so far described have been applied to BLG solutions of different concentrations. Protein concentration has been shown to affect response of BLG to physical denaturants (Iametti *et al.*, 1995), including high pressure (Stapelfeldt *et al.*, 1996).

Concentration dependence experiments were performed at a pressure high enough to induce changes in a minimum time, to avoid prolonged exposures and time course effects, that is, at 900 MPa for 2 min. No substantial dependence on concentration was observed in intrinsic fluorescence measurements on the treated proteins. Modifications in their overall surface hydrophobicity, in the aggregation state, and in the CD spectra are summarized in Table 1.

Pressure-induced changes in the structural features responsible of the near-UV CD of BLG were sensitive to protein concentration, at least below the 5 mg/mL threshold. Apparently, increasing the protein concentration did not protect against irreversible structural damage by pressure treatment. Some modifications were also observed in the spectral shape of these samples (not shown) and will be the subject of further investigation.

The number of surface hydrophobic sites accessible on each BLG molecule to the hydrophobic probe ANS is known to decrease with increasing protein concentration, likely as a consequence of the existence of labile multimeric forms of BLG in solution. It attains a calculated maximum value of one hydrophobic site per BLG dimer in infinitely dilute protein solutions. It is interesting to note that the decrease in the number of surface hydrophobic sites upon pressure treatment is more pronounced as the protein concentration increases. No substantial modifications were observed for the apparent affinity constant of the probe for the protein. As discussed above, these results could be framed in the scheme of treatment-induced formation of stable associated species.

Association was slightly more pronounced when the most concentrated protein solutions were treated. This result is presented in Table 1 as the disappearance of the dimeric form of BLG, which was converted into higher order associated forms. The increased association at higher protein concentration could also explain the loss of tertiary structure inferred by CD studies.

Finally, concentration effects were evident also for covalent dimer formation, thus confirming that intermolecular disulfide exchange seems to depend on the structural modification of individual, dissociated monomers which behave as the active species in determining the occurrence of irreversible structural modifications (Roefs and De Kruif, 1994; Iametti *et al.*, 1996).

CONCLUSIONS

The intensities of the pressure treatments used in the present study were well above the recognized threshold for milk sanitation. Nevertheless, irreversible structural modifications of BLG under conditions (pH and concentration) similar to those occurring in milk appeared to be quite limited. The modifications were equivalent, in structural terms, to those ensuing from heating at 60 °C (Iametti *et al.*, 1995). The temperature threshold for irreversible structural modifications of BLG in the same conditions was reported at 65−70 °C, while reversible effects on intrinsic fluorescence and on

surface hydrophobicity of the protein were observed at much lower temperatures (Cairoli *et al.*, 1994). The midpoint for transient structural modification of BLG during prolonged high-pressure treatment was indicated at 150−200 MPa (Dufour *et al.*, 1994; Stapelfeldt *et al.*, 1996).

The present study does not provide a definite pressure threshold for irreversible modification of BLG upon high-pressure treatment. Nevertheless, the preliminary results presented here could be useful for defining an operating range in the development of production processes. They indicate that factors other than the length of the exposure and pressure itself are relevant to the nature of treatment-induced modifications.

Irreversible structural modifications and changes in the aggregation state of the protein became more appreciable upon treatment at high pressure of BLG solutions of increasing concentration. Some of the features that appeared most sensitive to concentration effects upon processing were surface hydrophobicity and the aggregation state of the protein. These features could be relevant to the technological behavior of the treated protein and deserve consideration in the development of high-pressure treatments of concentrated sweet whey and related materials.

The increased trend toward aggregation and the increased extent of structural modification upon treatment of protein solutions of increasing concentration is also of interest from a mechanistic standpoint (Cairoli *et al.*, 1994; Iametti *et al.*, 1995, 1996). As in thermal treatment, dissociation of the BLG dimer appears to be an early event in pressure-induced modifications, and blocking the free reactive thiol on each dissociated monomer prevents aggregation, even through noncovalent bonds. Another similarity between the two treatments is that the stabilization of aggregate forms of BLG apparently paralleled the appearance of irreversible modifications in the tertiary structure of the protein (Roefs and De Kruif, 1994; Iametti *et al.*, 1996).

Thus, a situation can be envisioned for high-pressure treatment of BLG solutions, which is similar in many aspects to what apparently occurs during thermal treatment of the protein. Modest, reversible structural modifications lead to transient dimer dissociation. Isolated monomers are further modified and, above a certain threshold of treatment intensity, they interact to give aggregates stabilized by either covalent and noncovalent interactions. Formation of stable polymers made of denatured monomers prevents reversibility of the denaturation process (Iametti *et al.*, 1996; Tanaka *et al.*, 1996). Dimeric forms of the protein apparently play no role in the sequential polymerization mechanism outlined above.

When one starts from solutions of high protein concentration, the probability of interaction between transiently modified monomers increases, favoring the formation of stable aggregates of denatured protein. At low protein concentrations, the transiently modified monomers have a lesser chance of finding an aggregation "partner" and thus a better chance of reverting to the native monomer structure (and eventually to native dimers) when the particular modifying agent is no longer acting. The occurrence of almost identical mechanisms for the denaturation of BLG in response to different physical modifying agents is not surprising, in view of the similarity in the action of high pressure and heat, which involve major modifications in the solvent structure.

Validation of the hypotheses advanced above and defining the thermodynamics of the process will require further work. In the case of high-pressure treatments, an expanded range of pressure and protein concentrations is currently being investigated, along with simultaneous modification of pressure and other factors. This approach is expected to improve the understanding of the detailed mechanism of process denaturation of BLG and to provide the molecular background for the possible development of novel processes and products.

ABBREVIATIONS USED

BLG, a mixture of the A and B forms of bovine β-lactoglobulin; DTNB, dithiobis(2,4-nitrobenzoic acid); ANS, 1,8-anilinonaphthalenesulfonic acid, sodium salt; CD, circular dichroism.

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