# A $^1$H-NMR Study on the Effect of High Pressures on β-Lactoglobulin

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$^1$H NMR was used to study the effect of high pressure on changes in the structure of β-lactoglobulin (β-Lg), particularly the strongly bonded regions, the "core". β-Lg was exposed to pressures ranging from 100 to 400 MPa at neutral pH. After depressurization and acidification to pH 2.0, $^1$H NMR spectra were taken. Pressure-induced unfolding was studied by deuterium exchange. Refolding was also evaluated. Our results showed that the core was unaltered at 100 MPa but increased its conformational flexibility at ≥200 MPa. Even though the core was highly flexible at 400 MPa, its structure was found to be identical to the native structure after equilibration back to atmospheric pressure. It is suggested that pressure-induced aggregates are formed by β-Lg molecules maintaining most of their structure, and the intermolecular −SS− bonds, formed by −SH/−SS− exchange reaction, are likely to involve $C^{66}$−$C^{160}$ rather than $C^{106}$−$C^{119}$. In addition, the β-Lg variants A and B could be distinguished in a $^1$H NMR spectrum from a solution made with the AB mixed variant, by the differences in chemical shifts of $M^{107}$ and $C^{106}$; structural implications are discussed. Under pressure, the core of β-Lg A seemed to unfold faster than that of β-LgB. The structural recovery of the core was full for both variants.

**Keywords:** *Milk proteins; protein structure; nuclear magnetic resonance; high pressure*

## INTRODUCTION

One hundred years ago, Hite (1899) showed that high hydrostatic pressures reduced microorganism survival in foods. However, it has not been until recently that high-pressure treatments have been studied vigorously as an alternative method to heat processes for food preservation, as well as to improve the functionality of foods. High-pressure treatment of milk induces microbial and enzyme inactivation as well as it modifies the physicochemical characteristics of milk components (Hoover et al., 1989; Messens et al., 1997; Datta and Deeth, 1999). These modifications can change the suitability of milk for manufacturing of certain dairy products, such as improving yogurt texture (Johnston et al., 1993) or increasing cheese yield (López-Fandiño et al., 1996). High pressures induce structural changes in milk proteins, such as the dissociation of casein micelles (Desobry-Banon et al., 1994) and the formation of β-lactoglobulin (β-Lg) aggregates (Funtenberger et al., 1997).

β-Lg is a milk protein with functional applications and immunogenic characteristics. There are two major genetic variants of β-Lg, A and B, which present differences in the amino acid positions 64 and 118, being $D^{64}$ and $V^{118}$ for β-Lg A and $G^{64}$ and $A^{118}$ for β-Lg B. These amino acid substitutions cause differences in the physicochemical behavior, such as the aggregation state at acidic pH (Timasheff and Townend, 1961). In addition, β-Lg B seems to be thermally more stable than β-Lg A, as it has been shown by DSC (Imafidon et al., 1991; Boye et al.,1997). The physicochemical behavior is closely related to the functionality; thus thermally induced gels containing different β-Lg variants display dissimilar rheological properties. It has been reported that gels made with β-Lg B have a higher gelation point (Huang et al. 1994), display a higher elasticity (Allmere et al., 1998), and the gel matrix is formed by larger aggregate structures (Boye et al., 1997) than those made with β-Lg A. β-Lg gels made by pressurization exhibit different rheological properties than those made by heating (Dumay et al., 1998), and even though the effect of the genetic variants have been less studied, it has been suggested that the different aggregation behaviors of β-Lg A, B, and AB could affect the functionality of the protein (Hoseini-nia et al., 1999).

The structure of β-Lg is known from X-ray studies and consists basically of an 8-stranded, antiparallel β-barrel with an α-helix at the external face, located at the closure of the barrel (Papiz et al., 1986; Brownlow et al., 1997). This structure allows β-Lg to be very stable at acid pHs (Kella and Kinsella, 1988).

The changes that occur in the structure of β-Lg under pressurization include the monomerization of the dimeric state (Iametti et al., 1997), the decrease of α-helix and β-sheet (Hayakawa et al., 1996, Panick et al. 1999), and the formation of irreversible changes in which the formation of intermolecular disulfide bonds is involved (Funtenberger et al., 1997; Iametti et al., 1997; López-Fandiño et al., 1997; Moller et al., 1998). The irreversible denaturation, of which the reaction order has been calculated as 2.5 (Hinrichs et al., 1996), leads to the formation of aggregates linked by disulfide bonds rather than through hydrophobic interactions (Panick et al., 1999). In addition, it does not seem to be accompanied by gross structural changes, as circular dichroism studies have shown small structural losses after treatments at as high as 900 MPa (Iametti et al., 1997). Nevertheless, the nature of the intermolecular associa-

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tions seems to depend on the protein concentration and on the pressurization time (Panick et al., 1999).

$^1$H NMR is a technique that is very sensitive to structural changes and can give structural and dynamic information at an atomic level. A full $^{13}$C, $^{15}$N, and $^1$H assignment is now available for β-Lg, which has been obtained from isotopically enriched recombinant β-Lg (Uhrinova et al., 1998). The application of $^1$H NMR to $D_2O$ solutions of β-Lg has shown that the strongly associated portions of the molecule, the protein "core", are maintained at low pH and a partial resonance assignment has been obtained (Molinari et al., 1996; Belloque and Smith, 1998). $^1$H NMR combined with deuteration has been an useful way to elucidate folding and denaturation processes in proteins (Robertson and Baldwin, 1991; Roder and Wüthrich, 1986). This technique, which is based on the ability of the NH protons to exchange for D atoms upon contact the solvent ($D_2O$), demonstrates transiently unfolded states of proteins and has allowed the study of the unfolding behavior of β-Lg (Tanaka and Kunugi, 1996; Belloque and Smith, 1998). Not only can the unfolding behavior be studied by $^1$H NMR, but also the aggregation process. Aggregation decreases molecular mobility and this causes a faster transverse relaxation of the spins or a decreased $T_2$ relaxation time. A decrease in the transverse relaxation time of the solvent line and corresponding increase in line width are indicators of aggregate formation. $T_2$ relaxation time measurements have shown the presence of an aggregated state of β-Lg after heating (Lambelet et al., 1992).

We report here more detailed information about the structural effects of high-pressure treatments on β-Lg, by using one and two-dimensional $^1$H NMR, solvent $T_2$ relaxation time measurements and deuteration techniques. This work includes the unfolding and refolding of β-Lg AB, as well as the conformational differences between native β-Lg variants A and B, and their individual behavior within the AB mixed variant when subjected to high pressures.

## MATERIALS AND METHODS

**Sample Preparation.** β-Lg A, β-Lg B, and β-Lg AB were obtained from Sigma (St. Louis, MO).

Except for the samples used for $T_2$ measurements, all β-Lg solutions were analyzed at pD 2.0. This prevented additional exchange with deuterium (Wüthrich, 1986), while maintaining the structure of β-Lg, which has been shown to be the same as that at neutral pH (Uhrinova et al., 1998). Samples used for unfolding and refolding processes were pressurized at neutral pH, and after decompression, they were acidified. The samples used for denaturation were made in $D_2O$, whereas those used for refolding and $T_2$ measurement were made in $H_2O$, and then pressurized. We have assumed that the behavior of the protein in $D_2O$ is similar to that in $H_2O$.

For spectral comparison between variants, β-Lg A, β-Lg B, and β-Lg AB were dissolved to a 4% concentration in $D_2O$ and acidified with DCl to pD 2.0.

For the deuteration studies under high pressure, a 4% (w/v) solution of β-Lg AB was prepared in $D_2O$ (pD = 7.25) and aliquoted to Eppendorf tubes. One of the aliquots was kept as the control sample. The remaining tubes were sealed and pressurized at 100, 200, 300, or 400 MPa, in a 900 HP instrument (Eurotherm Automation, Lyon, France). Compression was done at a rate of 2.5 MPa/s, the final pressure was maintained for 5 min, and decompression was done at the same rate as compression. The temperature was set to 22 °C, although compression led to an increase of 2 °C per 100 MPa. Afterwards, the control and pressurized samples were acidified with DCl to pD 2.0 and kept at room temperature until analyzed.

For refolding studies, samples were prepared in a manner similar to that above. A 4% (w/v) solution of β-Lg AB was prepared in $H_2O$ (pH = 7.0). One aliquot of the solution was kept as a control, and the remaining samples were pressurized as described above together with their equivalent $D_2O$ samples (described above). After treatment, both control and pressurized samples were diluted 1:20 in $D_2O$ and the pD was adjusted to 2.0 with DCl.

For $T_2$ relaxation time measurements, a 4% solution of β-Lg AB in $D_2O$ (pD = 7), was employed. Two aliquots of this solution were taken: one was used as a control sample, and the other was pressurized at 400 MPa for 15 min. After decompression, samples were not acidified in order not to alter the aggregation state of the protein; thus $T_2$'s were measured at neutral pD.

**NMR Spectra.** $^1$H−one-dimensional (1D) NMR spectra, phase-sensitive DQF−COSY, and HOHAHA spectra were taken on a GE Ω-500 spectrometer, with a 5 mm probe (GE-NMR), controlled by Omega Spectrometer Operating Software, ver. 6.1. Unless otherwise stated, spectra were taken at 35 °C, using a spectral width of 7220 Hz, 1024−2048 points, a predelay time of 0.7 s, and a solvent presaturation time of 0.5−0.7 s.

1D spectra of the samples used for deuteration studies were done using 256 transients, whereas 1D spectra for structure recovery studies needed 4096 transients due to the dilution of the sample. DQF−COSY spectra were taken using 1024 and 512 points for $t_2$ and $t_1$ dimensions, respectively, and 96 transients. HOHAHA in the TPPI mode spectra was obtained at 40 ms mixing time, 64 transients, and 1024 and 256 points for $t_2$ and $t_1$, respectively.

HOD $T_2$ relaxation times were measured in a Varian instrument, operating at 400 MHz. The experiment was performed with a CPMG pulse sequence. For the exponential decay of the HOD line, 20 points were obtained by allowing for relaxation between 0 and 3 s, with increments of 150 ms. The $T_2$ relaxation time was calculated from the exponential decay by using Varian NMR software.

**Resonance Assignments.** Identification of resonances was done using COSY and HOHAHA spectra of unpressurized β-Lg AB and comparing them to those resonances assigned previously at pH 2.0 (Molinari et al., 1996; Belloque and Smith, 1998). These assignments were in agreement to those reported by Uhrinova et al. (1998). Identification of the resonances belonging specifically to the A and B variants was done by comparison among COSY spectra from β-Lg A, β-Lg B, and β-Lg AB.

***β*-Lg Structure Examination.** The structure of β-Lg was modeled by macromolecular visualization software, using the β-Lg coordinates obtained by X-ray crystallography, and generously supplied by L. Sawyer (Structural Biochemistry Group, Institute of Cell and Molecular Biology, University of Edinburgh).

## RESULTS

**$^1$H NMR Spectra of *β*-Lg Variants.** The spectra of independent solutions of β-Lg A and β-Lg B, at pH 2.0 in $D_2O$, revealed chemical shift (δ) differences in some resonances (spectra not shown), and the same differences could be observed simultaneously in spectra obtained from β-Lg AB solutions, containing both variants (Figure 1a and 2a). The NH/$H_\alpha$ spectral region of the DQF−COSY spectrum of β-Lg AB (Figure 2a) showed differences on the NH/$H_\alpha$ cross-peaks from residues $M^{107}$ and $C^{106}$. These resonances were recognized as they had been previously assigned for β-Lg A (Belloque and Smith, 1998). The spectrum showed that both δ(NH) and δ($H_\alpha$) of $M^{107}$ were different in the variants, whereas for $C^{106}$ only δ($H_\alpha$) was different, and

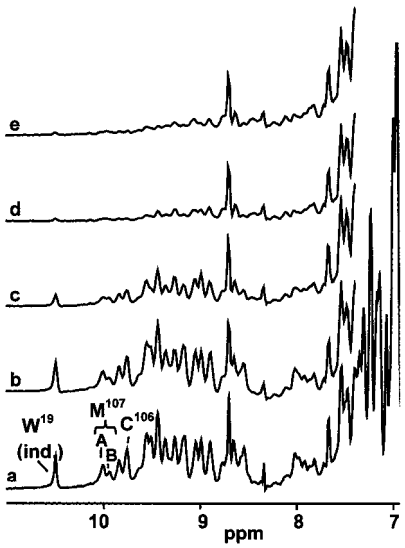


**Figure 1.** NH spectral region of 1D $^1$H NMR spectra of $\beta$-Lg AB subjected to different pressure treatments using $D_2O$ as solvent: (a) 0.1 MPa (control), (b) 100, (c) 200, (d) 300, and (e) 400 MPa. The NH indole from $W^{19}$ and the backbone NHs from $C^{106}$ and $M^{107}$ from $\beta$-Lg genetic variants A and B are labeled.

the NH resonances overlapped. Furthermore, the variants could be distinguished in a 1D spectrum by means of the $\delta$(NH) of $M^{107}$ (Figure 1a).

**Denaturation of $\beta$-Lg under High Pressure.** A $D_2O$ solution of $\beta$-Lg AB was pressurized at neutral pD, and exchange with deuterium was allowed to occur. After decompression and acidification, 1D $^1$H NMR spectra were taken. Unpressurized $\beta$-Lg showed many resonances from backbone NHs, that disappeared from the spectrum as the pressure increased up to 400 MPa (Figure 1) as a consequence of deuteration. It has been pointed out that the deuterium exchange process of $\beta$-Lg and α-La can be more complex than considering only denaturation or increased flexibility, as the contribution of pressure-induced accelerated intrinsic exchange rate has to be taken into account (Tanaka and Kunugi, 1996; Dzwolak et al., 1999). Even though the latter may contribute in some degree to deuteration, studies carried out by other techniques, such as protease accesibility or fluorescence (Dufour et al., 1995; Stapelfeldt et al., 1996) have shown that conformational changes occur during pressurization of $\beta$-Lg. Therefore, a combination of all of the above factors may contribute to the deuteration of $\beta$-Lg. Nevertheless, we have focused on the denaturation or structural flexibility since it has been shown to provide qualitative information regarding structural changes upon denaturation (Belloque and Smith, 1998).

Therefore, the loss of NH resonances was considered to be mainly the consequence of the increased flexibility acquired by the protein at higher pressures, which allowed the exchangeable protons (NHs) to become deuterated. A pressure of 100 MPa was not high enough to promote significant flexibility, as negligible deuteration was observed relative to the control sample. At 200 MPa, the structure was more mobile as deduced from the extent of deuteration. At 300 and 400 MPa, deuteration was almost complete, indicating a very flexible conformation or denatured state. It was clear that in the range of 100−300 MPa the whole protein structure became very flexible, if not denatured.

The effect of increasing pressure on particular resonances showed that the NH−indole group of $W^{19}$, which

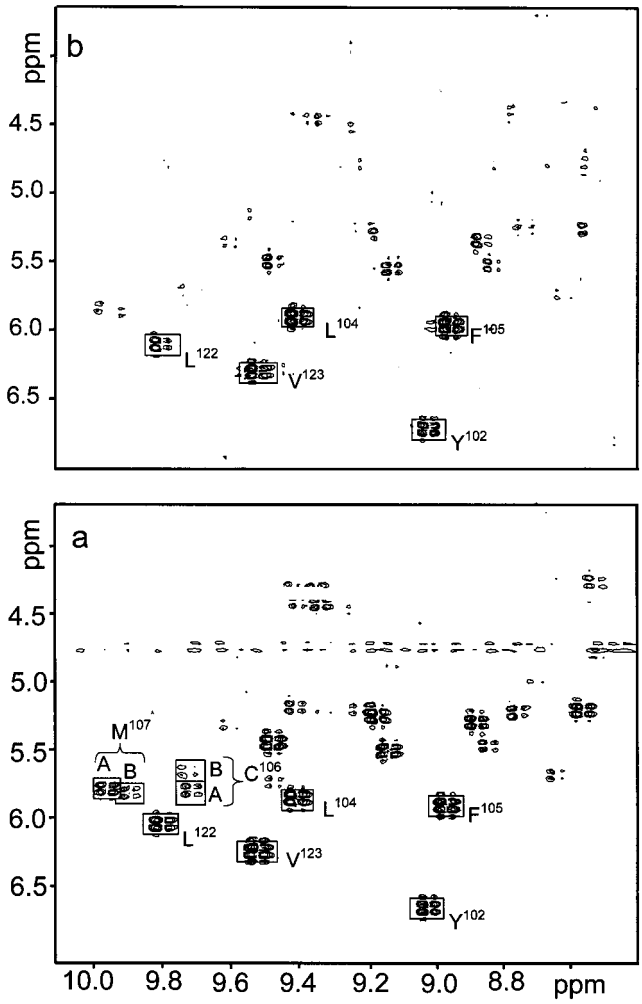


**Figure 2.** NH/Hα cross-peaks in DQF−COSY spectra of (a) a $\beta$-Lg AB control solution and (b) after pressurization at 200 MPa for 5 min, using $D_2O$ as solvent. The residues showing cross-peaks with the highest intensity are labeled.

faces the inside of the calyx in the structure of $\beta$-Lg (Papiz et al., 1986; Brownlow et al., 1997), became significantly deuterated at 200 MPa and above (Figure 1). This observation indicated exposure to the solvent of the internal face of the protein.

To reveal other structural changes that were not detectable in a 1D spectrum, a 2D DQF−COSY spectrum was obtained at pD 2.0 for both control $\beta$-Lg and $\beta$-Lg pressurized at 200 MPa and pD 7.25 (Figure 2). Among all NH/Hα resonances present in the spectrum, those belonging to the backbone of $Y^{102}$, $L^{104}$, $F^{105}$, $L^{122}$ and $V^{123}$ were the most prominent after pressurization of $\beta$-Lg at 200 MPa for 5 min (Figure 2b). These resonances also showed the highest signals in the spectrum of control $\beta$-Lg (Figure 2a). These residues are located in the $\beta$-sheet formed by FGH strands in the 3D structure of $\beta$-Lg (Papiz et al., 1986; Brownlow et al. 1997). Therefore, the FGH strands, the stronger portion of $\beta$-Lg, remained after pressurization, indicating that they are the last part to unfold during pressure processing.

The unfolding under pressure of both $\beta$-Lg A and B was simultaneously followed within the mixed AB variant by monitoring the decrease in intensity of the NH resonance of $M^{107}$ (Figure 1). In the control sample, this resonance was higher for variant A, which could be a consequence of either a stronger H-bonding or that

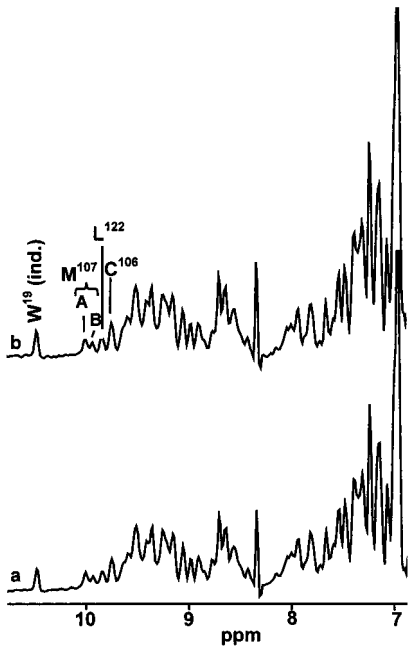


**Figure 3.** NH region of the $^1$H NMR spectra of (a) a control solution of β-Lg and (b) after pressurization at 400 MPa for 5 min followed by equilibration at atmospheric pressure, using $H_2O$ as solvent for pressure treatment followed by dilution in $D_2O$ for obtaining the spectra. Labels are as in Figure 1.

simply there was a higher concentration of A than of B. At 200 MPa, the intensity of the NH $M^{107}$ resonance decreased in both variants, but a slightly larger decrease was observed for variant A (Figure 1c). This observation indicates that the structure of the core β-Lg A became flexible more rapidly than that of β-Lg B.

**Refolding of β-Lg after Pressurization.** The spectra, obtained at pD 2.0, of both control and 400 MPa-pressurized β-Lg were practically indistinguishable when the pressurization step was done in $H_2O$ at neutral pH (Figure 3). Therefore, even though when pressurized at 400 MPa in $D_2O$, β-Lg had exchanged most H for D, suggesting denaturation, the structure of the core refolded back to its native conformation, after pressure was released. As far as the refolding of the variants is concerned, the NH resonances of $M^{107}$ from each variant of β-Lg showed, after pressurization at 400 MPa in $H_2O$, the same relative intensity as that in the native protein. Therefore, the cores of both β-Lg A and B refolded back to their native states.

**Solvent HOD $T_2$ Relaxation Time.** The $T_2$ relaxation time of the HOD line in the 4% solution of β-Lg (control) was 1.60 ± 0.05 s, whereas that measured from a sample that had been pressurized sample at 400 MPa for 15 min. was 1.96 ± 0.06 s. This increase upon pressurization did represent a molecular structural state with higher mobility than that of the control sample.

## DISCUSSION

In this study, we used $^1$H NMR to obtain more information about the effect of high pressures on the structure of β-Lg. The preservation of the structure of β-Lg at acid pH compared to that at neutral pH (Uhrinova et al., 1998) was of great advantage, since it allowed us to study the effect of pressure at neutral pH and then to analyze it under acidic conditions. The acidic conditions were not only used to prevent additional deuterium exchange but also because 2D spectra were easier to obtain since, under these conditions, β-Lg displays sharper resonances. Regarding the behavior of β-Lg in $D_2O$, it shows characteristics similar to that in $H_2O$, such as the narrowing of resonances that occurs when pH is lowered, which is consistent with the monomerization that has been observed when working with $H_2O$ solutions (Timasheff and Townend, 1961). In addition, the chemical shifts of resonances found for β-Lg in acidic solutions of $D_2O$ (Molinari et al., 1996; Belloque and Smith, 1998) are very similar to those obtained in $H_2O$ solutions (Uhrinova et al., 1998).

This study focuses on the region of ca. 7.5−11 ppm of the $^1$H NMR spectrum of β-Lg, taken at acid pH in $D_2O$ where the resonances of the exchangeable backbone NHs lie. As the spectra were taken in $D_2O$, the resonances that appeared in the spectrum belonged almost exclusively to those NHs that form secondary structures involving H-bonding to CO groups of other residues within the polypeptide chain. By comparison to spectra obtained in previous work (Belloque and Smith 1998), the observed resonances belong to the strongly bonded structure of β-Lg referred to as the core of β-Lg. The observed resonances of the core, 7.5−11 ppm, comprise sequential residues within the β-strands of the protein. This sequentiality implies that the β-strand to which they belong and the neighbor antiparallel strands are structured. It has been shown that the sequential residues which resonances are observed in the spectrum of a $D_2O$ solution of β-Lg belong to A, C, E, F, G, and H β-strands (Molinari et al., 1996; Belloque and Smith, 1998), which implies that most of the β-barrel structure of β-Lg is preserved.

**Structural Differences between β-Lg A and β-Lg B.** The major variants of β-Lg could be distinguished in 1- and 2D spectra from differences in chemical shifts that are related to their structure. The variants differ in the amino acid positions 64 and 118, being aspartate and valine for β-Lg A, and glycine and alanine for β-Lg B. As all spectra were done at pH 2.0, both A and B variants should be dissociated, and therefore, the differences found in the spectra must be conformational. A portion of the structure of β-Lg near the amino acid position 118 and the neighboring region is shown in Figure 4. This region belongs to the β-sheet formed by FGH strands, where $V^{118}$ is H-bonded to $M^{107}$ in β-Lg A. In variant B, $V^{118}$ is substituted for an alanine, whose side chain is less hydrophobic. The H-bonding in a β-sheet is the consequence of the interaction between the side chains of residues in neighbor strands. Because of the different hydrophobicity between alanine and valine, the interaction between the side chains $M^{107}$−$A^{118}$ in β-Lg B is probably weaker than that between $M^{107}$−$V^{118}$ in β-Lg A. This difference can alter the length of the H-bond that these residues share (Figure 4b). A consequence of this difference can be the alterations observed in both the $\delta(H_\alpha)$ and the $\delta$(NH) of $M^{107}$ between the variants (Figure 2a). In addition, a change in the H-bonds from $M^{107}$−$V^{118}$ would affect neighboring residues. The change in $M^{107}$−$A^{118}$ H-bond can alter the near H-bond formed by the NH group of $L^{93}$ and the CO group of $C^{106}$ (Figure 4b), causing the change in $\delta(H_\alpha)$ of $C^{106}$. The $\delta$(NH) of $C^{106}$ remained unaltered, probably due to structural rearrangements involving the dihedral angles.

In summary, a slightly different conformation of β-Lg A and β-Lg B that occurs around amino acid 118 can

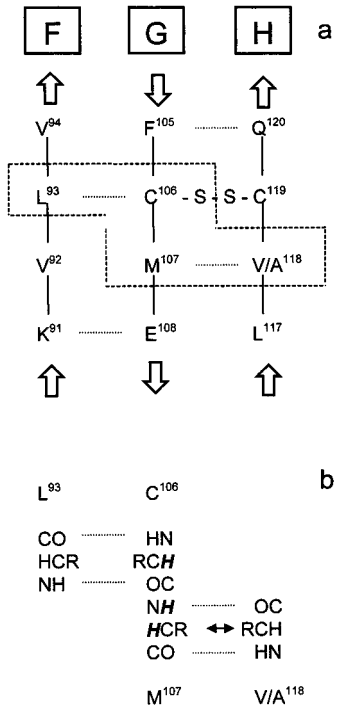


**Figure 4.** (a) Scheme of the $\beta$-sheet region of $\beta$-Lg formed by FGH strands showing the location of the residues, the H-bonds (·····) between neighboring amino acids, the disulfide bonds (−SS−), and the amino acid difference between both variants ($V/A^{118}$, V for $\beta$-Lg A, A for $\beta$-Lg B). (b) Detailed rendition of the region in the box, showing more specific H-bonds, and the point where strand interaction can be altered (arrow) depending on the variant, causing changes on some proton chemical shifts (in bold italics).

cause the changes on $\delta$(NH), $\delta(H_\alpha)$ of $M^{107}$ and the $\delta$-$(H_\alpha)$ of $C^{106}$ observed in a COSY spectrum (Figure 2a). In addition, the different $\delta$(NH) of $M^{107}$ can be used to distinguish between the variants in a 1D spectrum (Figure 1a).

**Unfolding and Refolding of $\beta$-Lg.** The denaturation process was shown to be dependent on the pressure treatment at neutral pD. At relatively low pressures, 50 MPa, some authors have claimed the existence of a "pre-denatured" state of $\beta$-Lg (Moller et al., 1998). An NMR study has also shown some degree of deuteration at this pressure (Tanaka and Kunugi, 1996). However, the degree of deuterium exchange observed in the present study, at 100 MPa, was very small (Figure 1b). As the resonances observed in $D_2O$ were only those belonging to the strongly bonded core of $\beta$-Lg, and this structure was still tight at 100 MPa, it is unlikely to represent the pre-denatured state claimed by Moller. On the other hand, trypsin and chymotrypsin have shown to increase significantly their proteolytic activity of when $\beta$-Lg is pressurized at 100 MPa, showing little further increase above this pressure (Dufour et al., 1995; Stapelfeldt et al., 1996). As proteases can act effectively only on unfolded regions of a protein, there must be some unfolding of $\beta$-Lg at 100 MPa. Therefore, as the core is still structured, the regions of $\beta$-Lg affected by the pre-denatured state are likely to comprise other regions of the protein different than the core, and these regions must be the main targets for the proteolytic activity of chymotrypsin and trypsin.

At 200 MPa, deuteration was significant, indicating that between 100 and 200 MPa, important structural changes did occur. This observation agrees with that of Moller et al. (1998), who found the half-denaturation pressure to be about 150 MPa by measurement of the exposure of the −SH group by a static method. The proteolytic activity of pepsin on $\beta$-Lg requires pressures of about 200 MPa and has been traced to the denaturation of $\beta$-Lg by fluorescence spectroscopy (Stapelfeldt et al., 1996). The increased flexibility of $\beta$-Lg found after treatments at 200 MPa and above is consistent with the increased activity of pepsin reported by Stapelfeldt (1996). This enzyme seems to act mainly on the strong core of $\beta$-Lg.

After treatment at 300 and 400 MPa, deuteration was almost complete, demonstrating a high flexibility of the entire structure of $\beta$-Lg. The FGH $\beta$-strands, which are part of the $\beta$-barrel, have been shown to be very strong and resistant to heat denaturation (Belloque and Smith, 1998). The exposure of the NH−indole group of $W^{19}$ and the backbone NHs from several residues belonging to FGH strandss upon pressurization, demonstrates that the increased flexibility involves the entire structure of $\beta$-Lg, including the strongest regions, suggesting that $\beta$-Lg exists in a completely unfolded state at these pressures. However, it was found that the conformation of the $\beta$-Lg core was identical to the native structure, once the pressure had been released. As the preservation of the core implies that most of the $\beta$-barrel is structured, a large proportion of the whole protein would have a structure similar to that of the native. If refolding were achieved to a small degree, or if there were an erroneous refolding, chemical shift changes, or decreased intensity of resonances would have been observed.

Two explanations for the complete refolding of the protein core after pressurization at 400 MPa are possible: (1) the protein unfolded during pressurization and then refolded back when it was set to ambient pressure, and (2) the protein did not unfold during pressurization, but only showed high flexibility or transient unfolding which allowed for the NHs to exchange. The presence of a refolded state of the $\beta$-Lg core after pressurization has been described previously; Iametti et al. (1997) used circular dichroism and fluorimetry and found that only 10% of the structure was lost at both 600 and 900 MPa, and that any modification of the tryptophan exposure was reversible. Moller et al. (1998) also showed that the exposure of the thiol group occurring at 150 MPa decreased after 2 days, a result that was attributed to the refolding of the molecule. If the protein was completely unfolded, it is very unlikely that the structure would be rebuilt in the same manner as the native. FT-IR studies have shown that pressures up to 10 kbar (∼1000 MPa) do not result in complete unfolding of $\beta$-Lg (Panick et al., 1999). Therefore, the existence of a flexible but still partially structured core of $\beta$-Lg, that allows the protein to refold back, is the more reasonable explanation.

**Polymerization of $\beta$-Lg.** The intermolecular associations, involving the formation of −SS− bonds, that occur in $\beta$-Lg under pressure seem to be a consequence of −SH/−SS− exchange reactions rather than of oxidation of −SH groups (Funtenberger et al., 1997). Native $\beta$-Lg contains five cysteine residues, which form two intramolecular −SS− bonds ($C^{66}$−$C^{160}$ and $C^{106}$−$C^{119}$), leaving $C^{121}$ as a free thiol group. Inspection of the structure of $\beta$-Lg shows that $C^{121}$ lies in the H strand and is protected from the solvent by the α-helix, $C^{106}$−

$C^{119}$ are part of the strong structure formed by FGH β-strands, and $C^{66}-C^{160}$ are at the external part of β-Lg, in an accessible location. We have shown that the FGH strands are very strong and refold back to the original structure, particularly the $C^{106}$ residue. Therefore the −SS− bond formed by $C^{106}-C^{119}$ is not likely to participate in −SH/−SS− exchange reactions. Otherwise, changes in chemical shifts would have been observed. On the other hand, it is rather possible that $C^{66}-C^{160}$, being very accessible, would participate in these interactions.

Another important observation was that the spectrum of the refolded β-Lg did not show any decrease in intensity or broadening of the resonances, which would occur if there was aggregation. In addition, the HOD $T_2$ increased, suggesting that the protein was more mobile, contrary to what it would be expected for an aggregated protein. Indeed, there are several mechanisms that could either increase or decrease this parameter. Previous reports have shown that, under similar conditions of pressurization, β-Lg is aggregated in amounts of ∼20% of the initial β-Lg content (Funtenberger et al., 1995; López-Fandiño et al., 1997). $T_2$ is very sensitive to protein aggregation, and this degree of aggregation would be detected. However, the native state of β-Lg is a dimer, and dissociation of the dimers into monomers can also take place during pressurization (Iametti et al., 1997), a process that would have the opposite effect on the $T_2$ value. Therefore, the dimer dissociation effect could be larger than the aggregation process and thus lead to an average increase in $T_2$. On the other hand, and as suggested above, intermolecular associations −SS− bonds could be formed at an edge of the protein, forming a polymer rather than a compact aggregate. Such a structure would not greatly affect the core structure, and could refold easily to the native conformation, maintaining the mobility of the core protons and the line width of the resonances belonging to it. In addition, the presence of a small portion of unfolded regions in the protein could also contribute to the increased solvent $T_2$ values. A good candidate for unfolding would be the α-helix, since it has a protective effect on the −SH group of $C^{121}$ in the native structure, and therefore, it must unfold to allow the thiol group to participate in the −SH/−SS− exchange reactions.

In conclusion, the β-Lg aggregates are likely to be polymers of rather structured proteins, formed by −SS− bonds produced by −SH/−SS− exchange reactions, involving the −SS− of $C^{66}-C^{160}$ and the −SH of $C^{121}$. These results agree with previous studies by FT-IR which have shown that intermolecular β-sheet formation is of less importance in pressure-induced than in thermally induced aggregation of β-Lg (Panick et al., 1999). These authors have also pointed out that these differences may be the cause for the structural differences found in gels made by pressure and those made by heating. It has been shown that pressurization produces porous gels that are less rigid than the fine-stranded gels formed by heating (Dumay et al., 1998). Indeed, if intermolecular association in pressure-induced gels was driven mainly by the formation of −SS− bonds at the edge of the protein, presenting little hydrophobic interaction, a network could be formed by very structured though covalently bonded molecules. The β-Lg units within the polymer could, therefore, have more freedom of movement than if they were hydrophobic bound aggregates, and this might reduce the rigidity of the gel.

**Comparative Behavior of β-Lg Variants under Pressure.** We have shown that $^1$H NMR is a good tool to distinguish β-Lg A from β-Lg B; thus some denaturation features of the variants could be followed simultaneously in a solution made of the mixed AB variant. The deuteration of the backbone NH of $M^{107}$ in variants A and B showed that the core of β-Lg became more flexible in β-Lg A than in β-Lg B, after pressurization at 200 MPa for 5 min. These results are consistent with previous FT-IR studies, which showed different conformational behavior upon compression and decompression of β-Lg A, B, and AB and found that the structure of β-Lg A was more sensitive than that of β-Lg B (Hosseinia et al., 1999). Our results indicate that the behavior of β-Lg A and B within the mixed AB variant is similar to that of the pure variants. The faster denaturation of the A variant has also been found during denaturation by heat (Boye et al., 1997, Imafidon et al., 1991). The relative structural lability of β-Lg A, irrespective of the denaturation means, suggests that lability arises from the structural differences existing between the variants, caused by the change of the residue $V^{118}$ in β-Lg A to $A^{118}$ in β-Lg B.

We have shown that the refolding of the core seems to be equally complete for β-Lg A or β-Lg B. as evidenced by the recovery of intensity and the chemical shift of all residues, including the two resonances of $M^{107}$ belonging to the two variants and that of $C^{106}$. This observation also agrees with the FT-IR studies carried out by Hosseini-nia and colleagues, who pointed out that most changes induced by high pressure on the secondary structure of β-Lg AB were reversible (Hosseini-nia et al., 1999). It is particularly noteworthy that the resonances of residues such as $C^{106}$ and $M^{107}$, located in and around the $C^{106}-C^{119}$ −SS− bond, did not change after pressurization in any of the variants. We therefore argued that the Cys involved in SS/SH reaction caused by pressurization were likely $C^{66}-C^{160}$. As β-Lg A seems to unfold faster than B, there may be different aggregation pathways for β-Lg A and β-Lg B. However, based on the preservation of the structure of the region around the $C^{106}-C^{119}$ −SS− bond, the proposal that $C^{66}-C^{160}$ are responsible for the −SH/−SS− exchange reaction is valid for both variants.

## ABBREVIATIONS USED

β-Lg, β-lactoglobulin; NMR, nuclear magnetic resonance, $T_2$, transverse relaxation time; 1D, monodimensional; 2D, two-dimensional; DQF−COSY, double quantum filtered correlation spectroscopy; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; TPPI, time proportional phase increment; CPMG, Carr−Purcell−Meiboom−Gill sequence; δ, NMR chemical shift; −SS−, disulfide bond; −SH, thiol group; FT-IR, Fourier transform infrared spectroscopy; DSC, differencial scanning calorimetry.

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