Pressure-Induced Denaturation of Monomer β-Lactoglobulin Is Partially Irreversible: Comparison of Monomer Form (Highly Acidic pH) with Dimer Form (Neutral pH)

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This study was conducted to assess the effect of high hydrostatic pressure on monomer β-lactoglobulin (BLg) at acid pH by fluorescence spectroscopy under pressure and by circular dichroism (CD) and ^{1}H NMR spectroscopies after release of pressure. The intrinsic (tryptophan) fluorescence measurement and the study of 8-anilinonaphthalene-1-sulfonate (ANS) binding to BLg indicated that at pH 2.0 the recovery of center of spectral mass or ANS fluorescence was almost complete upon pressure release. No difference in ^{1}H NMR spectra was observed between pressurized and unpressurized BLg. In addition, NMR detection of the H/D exchange of aromatic protein indicated that the conformation of the vicinity of tryptophan residues could be refolded almost completely after release of pressure. These results seemingly confirm that the pressure-induced denaturation of BLg at pH 2.0 is reversible. However, *cis*-parinaric acid binding ability of pressurized BLg was largely lost, although its retinol binding ability was the same as its unpressurized one. Furthermore, CD spectra of the far-UV region and 2D NMR spectra evidently revealed the difference in the conformation of the molecule between unpressurized and pressurized BLg. These results are interpreted as an existence of partially fragile structure in the BLg molecule by high pressure.

Keywords: *β-Lactoglobulin; high pressure; denaturation of protein; NMR; hydrogen/deuterium exchange*

INTRODUCTION

Food functionalities such as gel-forming ability, emulsifying activity, or foaming capacity in food processing could be considered to be basically dependent on the structure of food proteins and their amino acid constituents (*1*). For example, the gel formation of solubilized protein on heating, one expression of functionality, is directly attributable to a change in the molecular structure of protein induced by heat, namely, thermal protein denaturation. Heat has been progressively used for food processing throughout history. Recently, high-pressure technology has attracted interest because hydrostatic pressure denatures proteins in a way that is thermodynamically similar to heat. High pressure could be used to improve food texture through pressure-induced gel formation, to produce valuable materials by controlled enzymatic reaction, or to eliminate food allergens by limited digestion under high pressure.

Whey protein isolates (WPI), prepared industrially from bovine skimmed milk by ion-exchange chromatography, is widely utilized in processed foods due to its excellent functionalities as described above. Recently, it has been reported that when WPI (>10%) at neutral pH is subjected to high pressure at 300−400 MPa, it forms a gel, which is weaker, less elastic, and more exudative than corresponding thermal gel, but pressure-induced gel formation does not readily happen at acidic pH. These properties of pressure-induced WPI gel are considered to be mostly dependent on the change in the property of β-lactoglobulin (BLg), which comprises about half of WPI. Other research on the effects of high pressure on WPI and BLg has been conducted from the standpoint of food science (*2*, *3*) and was reviewed by Cheftel and Dumay (*4*).

BLg is a useful protein for the study of unfolding/refolding mechanisms for two reasons: (1) it is easy to purify in a large quantities through simple ion-exchange chromatography (*5*), and (2) its primary, secondary, and tertiary structures are well-established (*6*−*9*). In addition, recent pressure techniques have been utilized as powerful tools for this kind of study. From several studies in which in situ observations of BLg behavior under pressure were attempted, it was suggested that the unfolding of dimeric BLg at neutral pH is irreversible because of misfolding of dimers by the formation of a novel disulfide bond (*10*−*12*). Dufour et al. (*10*) also found from the measurements of fluorescence of BLg−retinol and BLg−*cis*-parinaric acid complexes that at highly acidic pH the pressure-induced denaturation of monomer BLg is partially irreversible. Their results suggested that the conformation of the *cis*-parinaric acid (PnA) binding site in the BLg molecule is more susceptible to pressure than the retinol binding site in β-sheet structures of β-barrel (*6*, *10*).

Despite some reports, the irreversibility of BLg at highly acidic pH by high pressure remains uncertain. Also, from a viewpoint of food science, the structure

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changes of BLg by pressure treatment are of interest in connection with the gel formability of pressurized BLg at neutral and acidic pH values. BLg is expected to exist as dimer or oligomers above pH 3.5 and as a monomer below pH 3.0 (*13*). The objectives of this study were to examine in further detail the structural change of monomeric BLg after release of pressure by ^{1}H NMR, circular dichroism (CD) spectra analysis, and fluorescence spectroscopic methods and to elucidate whether the unfolding of BLg induced by a high pressure of 600 MPa at pH 2.0 is reversible or irreversible.

MATERIALS AND METHODS

Materials. Crystallized bovine milk BLg was purchased from Sigma (St. Louis, MO) and further purified by DEAE-cellulose ion-exchange chromatography to obtain BLg variant B according to the procedure of Piez et al. (*5*). The purity of the protein was tested by native PAGE electrophoresis. Before use for measurement, samples were centrifuged at 90000*g* for 1 h to remove a trace of aggregation in them.

all-trans-Retinol, PnA, and 1-anilinonaphthalene-8-sulfonic acid (ANS) were also purchased from Sigma. Heavy water (99.9 atomic % D) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other reagents used were of analytical grade. Distilled water was further purified by a Millipore system.

Concentrations of BLg solutions in aqueous solution were determined spectophotometrically by using a molecular absorption coefficient of 17600 M^{-1} cm^{-1} at 280 nm. The following molecular absorptions were used to calculate ligand concentration: ϵ_{325} = 46000 M^{-1} cm^{-1} for retinol in 100% ethanol and ϵ_{304} = 71400 M^{-1} cm^{-1} for PnA in 100% ethanol (*10*). The concentration of ANS was determined using the molar extinction coefficient of 5000 M^{-1} cm^{-1} at 350 nm in 20 mM sodium phosphate buffer at pH 7.0 (*14*).

All measurements were carried out in phosphate buffer, although its pK_a was pressure dependent. Over the pressure range used in this experiment, the pH of neutral phosphate buffer was estimated to vary from 7.0 at 0.1 MPa to ~5.5 at 400 MPa (*15*). Also, the pH of acidic phosphate buffer used was presumed to be below 2.0 under high pressure. A change in pH by pressure might have serious effects on the results, so that it might be necessary to examine the effects by using a different buffer such as Tris buffer. However, significant effects were not observed on our results (in phosphate buffer; data not shown) when compared to the study of Dufour et al. (in Tris buffer) (*10*). For this reason, possible pH changes under pressure were not monitored or controlled.

High-Pressure Apparatus. Two different types of high-pressure devices were used for this study. One was a device consisting of a thermostated high-pressure vessel equipped with sapphire windows and a pump capable of elevating pressure up to 400 MPa (Teramecs Co., Kyoto, Japan). The vessel was placed in the light beam of a Hitachi F2000 spectrofluorometer. A quartz cuvette containing sample solutions was placed inside the vessel.

The other device was the same apparatus as a cold isostatic pressing (CIP) used conventionally for molding of ceramics, and pressurization was achieved by filling the pressure vessel with water (Nikkiso Co. Ltd., Tokyo, Japan). Each sample solution was vacuum-sealed in a polyethylene bag and transferred to a large polyethylene bag, and then the bag was placed in the pressure vessel. This pressure device could apply pressure from 0.1 to 650 MPa.

Fluorescence Spectroscopy. Fluorescence measurements were carried out on a Hitachi F2000 fluorospectrophotometer, in which the high-pressure vessel was placed. The fluorescence spectra were quantified by specifying center of spectral mass ($\langle\nu\rangle = \sum\nu_i F_i / \sum F_i$; ν_i is the wavenumber and F_i is the fluorescence intensity at ν_i) (*16*). The excitation wavelength for the intrinsic fluorescence spectrum was 295 nm, which excites tryptophan residues in the BLg molecule. ANS is frequently used as a hydrophobic probe, and it is very sensitive to changes in its microenviroments with a large increase in fluorescence quantum yield (*17*). Then, the fluorescence spectra of BLg solutions saturated by ANS were recorded between 420 and 560 nm with excitation of 350 nm under pressure. The process of pressure-induced denaturation of BLg was investigated by measuring in situ the intrinsic and ANS binding fluorescences.

Fluorescence intensities of retinol and PnA (fluorescent fatty acid analogue) are enhanced when they are bound to BLg due to microenvironmental solvent effect on the fluorescence quantum yields of these probes. Measurements of retinol and PnA fluorescences were performed at 487 nm (excitation = 354 nm) and 414 nm (excitation = 326 nm) upon binding to the pressurized BLg, respectively. In practice, the reaction mixtures of BLg and these probes, in which the concentration of ethanol was 2% (v/v), were permitted to stand for 2 h prior to measurements taking sufficient care to shield from light. It should be emphasized here that retinol and PnA were added to the BLg solution after pressure treatment.

CD Measurement. CD spectra of BLg after pressure treatment were recorded on a JASCO J-725 spectropolarimeter at 20 °C using a quartz cell with a 1 mm light path for far UV (200−250 nm) or a 10 mm light path for near UV (250−340 nm). The mean residue ellipticity, $[\theta]$ (degree cm^2 $dmol^{-1}$), was calculated using a molecular weight of 18362 and 162 residues.

NMR Measurement. All one-dimensional ^{1}H NMR spectra were measured on a Bruker DPX-400 spectrometer at 298 K using a standard 5 mm ^{1}H probe. The ^{1}H NMR spectra were referenced relative to sodium 4,4-dimethyl-4-silapentane-sulfonate (DSS) used as an internal standard. The BLg was contained in a 5 mm NMR microtube, which was very useful for the NMR measurement of sample in aqueous solution (Shigemi Co., Tokyo, Japan). The presaturation procedure was adopted to suppress the signals of solvent. The ^{1}H NMR spectra were recorded with data points of 64K and 512 scans.

Usually ~5 wt % BLg is required for ^{1}H NMR measurement, but such a high concentration may produce protein aggregation at high pressure. Therefore, a sample solution for ^{1}H NMR spectrum measurement was prepared at 270 μM (~0.5 wt %) to prevent aggregation, as had been reported by Tanaka and Kunigi (*18*).

Sample Preparation for H/D Exchange Reaction at pH 2.0. The H/D exchange reactions under high pressure (600 MPa) were performed in a CIP type pressure vessel as mentioned above. The BLg samples lyophilized after NMR measurement or release of pressure were dissolved again either in a mixed solvent of 90% H_2O/10% D_2O or in 100% D_2O, and then the H/D-exchanged protein sample was transferred into an NMR tube. Both solvents contained 20 mM H_3PO_4 at pH 2 or pD 2 (as direct pH meter reading), respectively. The pH value of this buffer should be further decreased at 600 MPa, but BLg could exist as a monomer in such a condition.

RESULTS AND DISCUSSION

In Situ Observation of Intrinsic Fluorescence. Figure 1 shows the effect of hydrostatic pressure from 0.1 to 400 MPa on the center mass ($\langle\nu\rangle$) of the intrinsic fluorescence spectrum of BLg at pH 2.0 and 7.0 (inset in Figure 1). As can be seen, both $\langle\nu\rangle$ values decreased as pressure increased. This indicates that the environment polarity of the tryptophan residues, Trp19 and Trp61, in BLg became stronger due to the pressure-induced unfolding of the molecule. The curves upon pressure decreased sharply from 150 to 300 MPa and reached plateaus on a further rise in pressure. The $\langle\nu\rangle$ of BLg at pH 2.0 almost returned to the initial value after release of pressure, although the $\langle\nu\rangle$ at a pressure during decompression was lower than that at the same pressure during compression. In other words, a hysteresis in the recovery of fluorescence parameters was observed upon decompression of BLg existing in the state of monomer. The hysteresis under high-pressure

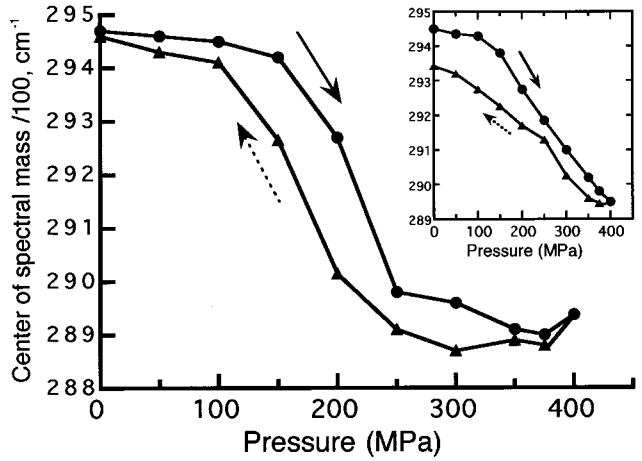

Figure 1. Pressure dependence of the center of spectral mass of BLg intrinsic fluorescence at pH 2.0 and 7.0 (inset): (●) compression; (▲) decompression. The fluorescence spectra of BLg were recorded under different pressures and then quantified by specifying the center of spectral mass. Excitation = 295 nm. Emission range = 310−380 nm. Protein concentration = 20 μM in 20 mM H_3PO_4/NaOH buffer. All measurements were carried out at 20 °C. Symbols represent averages of two measurements.

conditions has been well reported for BLg at pH 7.0 or for other dimer proteins (*10*, *12*, *19*).

The midpoint of the denaturation transition at pH 7.0 was 240 MPa, which was higher than that of the previously reported data (*10*, *12*). The difference in the midpoint may be due to differences in the buffer compositions used (Tris-HCl and phosphate buffers) because Griko and Privalov (*20*) reported that increasing phosphate ion concentration in the solution increases the heat stability of BLg. At pH 2.0 the midpoint shifted by 20 MPa toward lower pressure values compared to that at pH 7.0. As far as these results are concerned, at pH 2.0 and 20 °C the pressure-induced denaturation of BLg is reversible, whereas at pH 7.0 and 20 °C it is irreversible. This conclusion is consistent with that of Dufour et al. (*10*). The irreversibility at pH 7.0, at which BLg is mainly dimeric, was proposed to be due to the formation of a novel intermolecular disulfide bond through SH/S−S interchange reactions (*11*, *21*).

In Situ Observation of ANS Binding Fluorescence. The fluorescence of ANS has been used to detect the conformational changes of the hydrophobic region in the protein molecule. Parts A and B of Figure2 show the fluorescence spectra of BLg binding ANS at pH 7.0 and 2.0 under different pressures, respectively. The ANS emission maximum was 470−480 nm, which was different from the specific emission maximum (530 nm) of free ANS in aqueous solution, and the fluorescence of ANS was enhanced when it bound to the pressure-denatured BLg (*22*). At pH 7.0 the fluorescence intensity at 470 nm reached a peak at 200 MPa and decreased upon further pressure increase from 200 to 400 MPa. The reason for this unusual behavior of fluorescence is unclear. After release of pressure, it did not return to the original value, in agreement with the result of the inset of Figure 1. On the other hand, the fluorescence intensity (at 480 nm) of ANS−BLg at pH 2.0 increased with increasing pressure intensity and returned to its original value, tracing almost the same curve in the compression direction as when the pressure was gradually released. In ANS measurement a hysteresis was not observed, unlike the case of intrinsic fluorescence

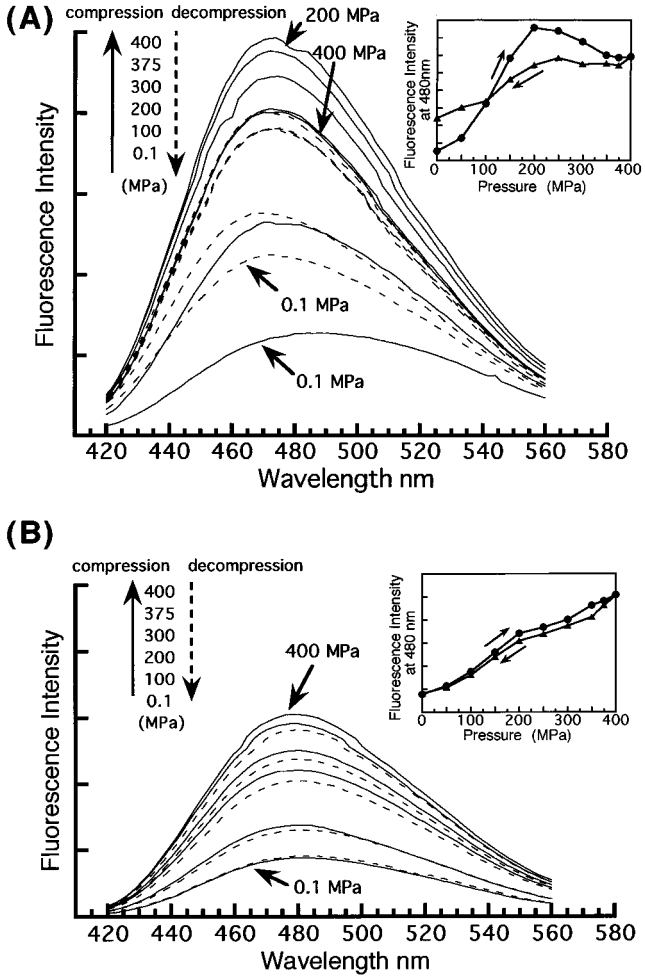

Figure 2. Fluorescence spectra of ANS−BLg at pH 7.0 (A) and 2.0 (B) under different pressures. Inset represents the ANS fluorescence intensity at 480 nm: (●) compression; (▲) decompression. Excitation = 350 nm. Emission range = 420−560 nm. Protein concentration = 20 μM in 20 mM H_3PO_4/NaOH buffer. ANS concentration = 100 μM. The experiment was duplicated, and almost the same results were obtained.

(Figure 1). The facts revealed by intrinsic tryptophans (Trp19 and Trp61) and ANS fluorescence demonstrate almost complete refolding of BLg pressurized at acidic pH.

Retinol and PnA Binding Abilities of the Pressurized BLg. Dufour et al. (*10*) have reported that the dissociation of BLg−retinol complexes at pH 3.0 was reversible between 0.1 and 400 MPa, PnA complexes dissociated irreversibly under pressure >200 MPa. This indicated that the binding of hydrophobic compounds by BLg was sensitive to pressure unfolding. We also confirmed their in situ observation using a phosphate buffer (data not shown). However, hydrophobic probes such as PnA are likely to become insoluble in aqueous solution (i.e., decrease in solubility under pressure) once they dissociate from the protein under pressure. Also, the possibility that the binding of these probes themselves could affect the pressure stability of BLg is ruled out. If so, it would be difficult to analyze the interaction between protein and ligand under pressure. Alternatively, we examined changes in retinol and PnA binding abilities of BLg after exposed to a pressure of 400 MPa at pH 7.0 or 2.0 (ex situ observation). The retinol and PnA bound to BLg were assessed by determining the fluorescence from chromphores in the molecule at 354

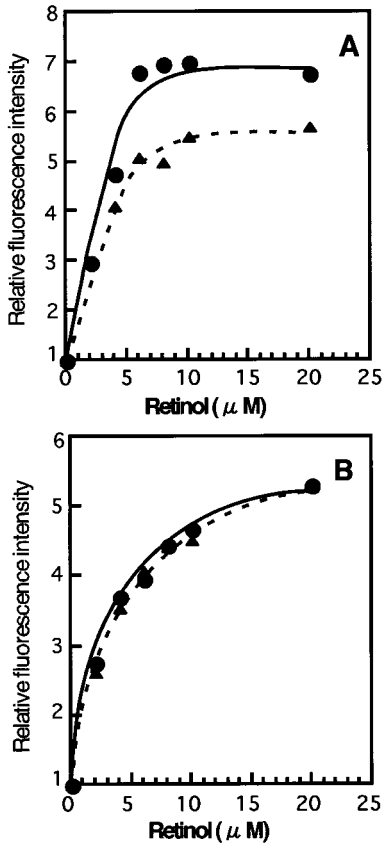

Figure 3. Measurements of retinol binding ability of BLg immediately after release of pressure at pH 7.0 (A) and 2.0 (B). The sample containing 270 μM BLg was first pressurized at 400 MPa for 15 min. After release of pressure, retinol in ethanol was added to 2.0 mL of the pressurized BLg in a cuvette. Fluorescence was measured with excitation at 330 and emission at 470 nm: (●) native BLg; (▲) pressurized BLg. Final protein concentration = 8.5 μM in 20 mM H_3PO_4/NaOH buffer. Total retinol concentrations are expressed in the abscissa. Symbols represent averages of two measurements.

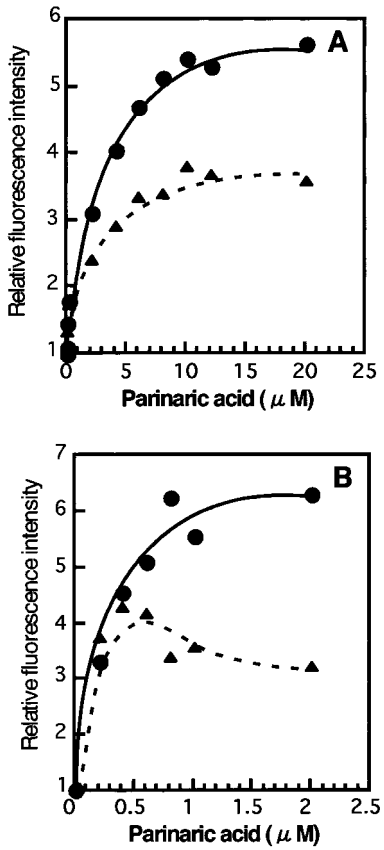

Figure 4. Measurements of PnA binding ability of BLg immediately after release of pressure at pH 7.0 (A) and 2.0 (B). The sample containing 270 μM was first pressurized at 400 MPa for 15 min. After release of pressure, PnA in ethanol was added to 2.0 mL of BLg in a cuvette. Fluorescence was measured with excitation at 326 and emission at 414 nm: (●) native BLg; (▲) pressurized BLg. Final protein concentration = 8.5 μM for pH 7.0 and 1 μM for pH 2.0. Total PnA concentrations are expressed in the abscissa. Symbols represent averages of two measurements for pH 7.0 and averages of three measurements for pH 2.0, respectively.

and 326 nm excitation, respectively. As can be seen in Figure 3, BLg pressurized at pH 7.0 lost considerable retinol binding ability, consistent with the results of Valente-Mesquita et al. (*12*), but BLg pressurized at pH 2.0 showed a binding ability similar to that of unpressurized BLg. In the case of PnA, the situation was quite different; namely, the PnA binding ability of pressurized BLg was lost at pH 7.0 and also at pH 2.0 (Figure 4) and was unstable at pH 2.0, as evidenced by the decline in fluorescence intensity when the added PnA was >0.5 μM. Thus, it seems that BLg binding is not always reversible when pressurized at acid pH, contrary to the results of Figures 1 and 2. Our present data also suggest that at acid pH the conformation of the PnA binding site is much more sensitive to pressure than is the retinol binding site, which may be located at the central hydrophobic pocket formed by the β-barrel (*7*, *8*, *10*). At neutral pH, the pressure is presumed to cause unfolding and collapse of the inner calyx of BLg, leading to impaired binding of PnA (*21*).

CD Spectra of the Pressurized BLg. The far- and near-UV CD spectra measurements were performed to compare the secondary structures of the pressurized BLg at pH 7.0 and 2.0. The far-UV (200−250 nm) and near-UV (250−340 nm) CD spectra of BLg after the elevated pressure at pH 7.0 was released are shown in part A and B of Figure 5, respectively. From the far-UV CD spectra (Figure 5A), a negative peak had a blue shift from 216 to 208 nm, and its mean residue ellipticity increased with increasing pressure intensity (0.1 MPa, $[\theta]_{216} = -6217$ deg cm^2 dmol^{-1}; 400 MPa, $[\theta]_{208} = -6794$ deg cm^2 dmol^{-1}). According to the report on thermal denaturation of BLg at neutral pH, a blue shift together with a high negative $[\theta]$ value in the far-UV CD spectra was observed only at temperatures >70 °C (*23*). This observation is in contrast with the data of Figure 5A showing the increase in negative $[\theta]$ value following a blue shift dependent on pressure intensity. The difference at 204−210 nm can be regarded as reflecting the reduction of the amount of β-structure in the protein (*24*). Practically, the deconvolution of the CD spectrum for the pressurized BLg at pH 7.0 and 400 MPa indicated an increase in the random-coil content (*25*). The negative peak at 286 and 293 nm lost a great deal of its ellipticity at 400 MPa (Figure 5B). The difference in the near-UV region is presumed to change the local environments of the tryptophan residues of the pressurized BLg at pH 7.0.

Pressurization at pH 2.0 affected the CD spectra of the far-UV region to some degree as illustrated in Figure 5C (0.1 MPa, $[\theta]_{217} = -5239$ deg cm^2 dmol^{-1}; 400 MPa, $[\theta]_{215} = -5768$ deg cm^2 dmol^{-1}), indicating the slight increase in the disordered region, whereas in the near-

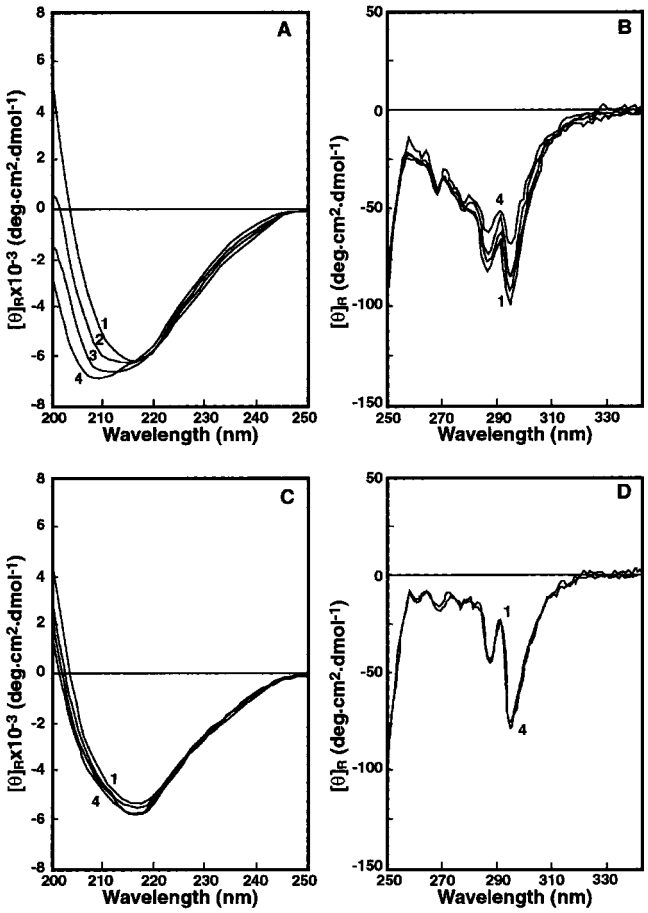

Figure 5. Changes in the CD spectrum of BLg pressurized at various pressures and 20 °C for 15 min: (A) far-UV region (200−250 nm) at pH 7.0; (B) near-UV region (250−340 nm) at pH 7.0; (C) far-UV region (200−250 nm) at pH 2.0; (D) near-UV region (250−340 nm) at pH 2.0. Curves 1−4 correspond to the samples pressurized at 0.1, 200, 300, and 400 MPa, respectively. The samples containing 270 μM BLg in 20 mM H_3PO_4/NaOH buffer were first pressurized and diluted to a final concentration of 20 μM with the same buffer after the release of pressure.

UV region little change in the CD spectrum was observed (Figure 5D). This fact suggests that the secondary structure around the tryptophan residues is well retained and the original structure recovered after release of pressure. Thus, the PnA binding site of BLg is not present in the vicinity of tryptophan residues (Trp19 and Trp61), considering in relation to the result of loss in its binding ability of BLg pressurized at pH 2.0 (see Figure 4B). Rather, it may be present at the hydrophobic surface patch located in a groove between the strands and the helix (*7, 10*).

At highly acidic pH the change observed in the far-UV CD spectra but not in the near-UV CD spectra clearly represents the indication for partial denaturation of pressurized BLg. However, its change appears to be not so large as the structure of retinol binding site is impaired.

^{1}H NMR Spectra of the Pressurized BLg. There are differences in the state of BLg in solution that are dependent on pH. That is, at neutral pH BLg is present as a dimer, whereas under a strong acidic condition it is present as a monomer due to electrostatic repulsion (*26*). The difference is reflected in the ^{1}H NMR spectrum. Figure 6, parts A and B, show ^{1}H NMR spectra of BLg pressurized at pH 7.0 and 2.0, respectively. The aliphatic proton resonances appear at ∼0−4 ppm, whereas the amide and aromatic proton resonances characteristically appear between 6 and 11 ppm. As can be seen in this figure, the ^{1}H NMR spectrum at pH 7.0 and 0.1 MPa showed, on the whole, broadening of the resonance lines and relatively low signal intensity compared to that at pH 2.0. On the contrary, at pH 2.0 sharp signals were observed in the aliphatic (high-field) and aromatic (low-field) regions, which probably originated from highly mobile regions of the molecule because BLg was suggested to be monomeric under highly acidic pH and locally unfolded (*9, 23*). In the spectrum at pH 2.0, the clearly detectable resonances were the singlet resonances at 10.09 and 10.42 ppm, which must originate in indole amide protons of two tryptophan residues. The ^{1}H NMR spectrum at pH 2.0 (control, 0.1 MPa) was, on the whole, identical to that reported by Molinari et al. (*23*) and Civera et al. (*27*). Pressurization of BLg (>300 MPa) at pH 7.0 led to a reduction of the signal intensity and a further broadening of the resonance lines (Figure 6A). On the contrary, at pH 2.0 there was no obvious difference in the ^{1}H NMR spectra at both aliphatic and aromatic fields among the pressurized samples (Figure 6B). As far as can be judged from these spectra, the pressure denaturation of BLg at acidic pH condition is seemingly reversible.

Stapelfeldt and Skibsted (*21*) demonstrated that Rayleigh scattering intensity of BLg at neutral pH increased with increasing pressure, indicating the aggregation reaction under pressure. An extensive reduction of ^{1}H NMR signal intensity of the pressurized BLg at pH 7.0 as shown in Figure 6A also suggests the possibility that pressure promoted the formation of aggregation among molecules (Figure 6A). To explore this hypothesis, the sample was subjected to pressure treatment at pH 7.0, dialyzed against phosphate buffer at pH 2.0, and then examined for ^{1}H NMR measurements (Figure 7). As a result, the NMR spectra of pressurized BLg were not identical to that of unpressurized BLg (control). For example, the signal intensities of 10.09 and 10.42 ppm were reduced and became broad. Therefore, it was found that the aggregation once formed by pressure at pH 7.0 was not easy to dissociate even if the pH condition was favorable for the formation of monomer. This appears to be attributable to the formation of the novel S−S bond among the molecules as has already been suggested by Tanaka et al. (*11*).

H/D Exchange Measurement with NMR and 2D NMR Measurement. The data at acid pH revealed by the near-UV CD, fluorescence, and NMR experiments suggested that BLg was unfolded to expose intrinsic tryptophan residues under pressure and was refolded right after release of pressure. Support for this idea comes from the NMR detection of the H/D exchange reaction. Figure 8 shows NMR spectra of BLg that has been H_2O/D_2O exchanged.

When BLg was pressurized at 600 MPa for 60 min in the presence of 100% D_2O, the signals in low-filed region (6−10 ppm), which is mainly composed of amide protons and aromatic protons, mostly disappeared (Figure 8B). According to the report of Tanaka and Kunugi (*18*), the extensive disappearance of these signals of BLg at pH 7.0 occurs at 300 MPa for 3 h. It was noteworthy that the two sharp singlet resonances at 10.42 and 10.09 ppm, which are likely to originate from the indole proton of two tryptophan residues (Trp19 and Trp61) in the

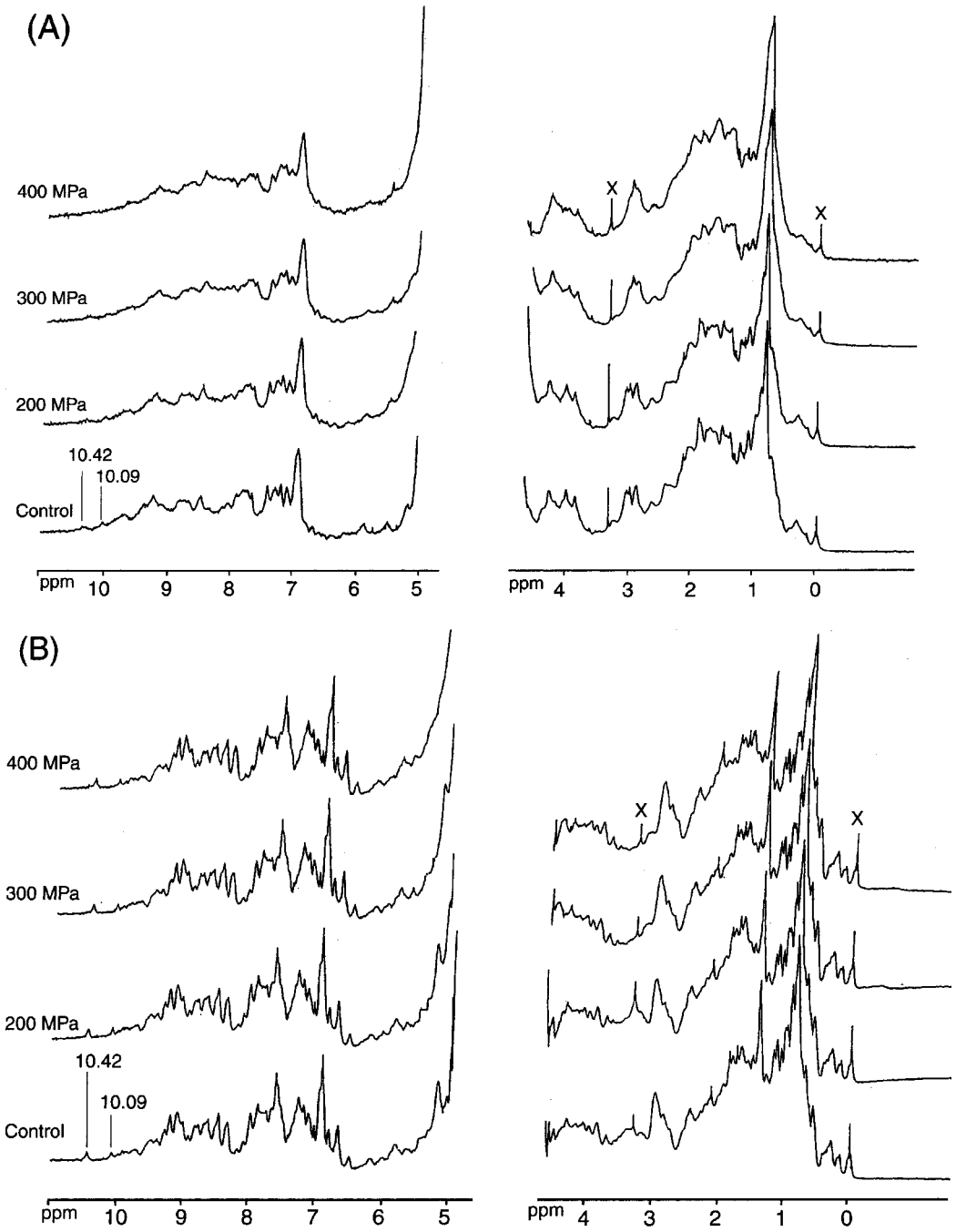

Figure 6. ^{1}H NMR spectra in a solution of BLg pressurized for 15 min at various pressures and pH 7.0 (A) or 2.0 (B). Pressurization and NMR measurements were carried out at 20 °C. The experimental conditions (including the number of scans or scaling) and data processing were identical for all samples. The sample containing 270 μM BLg was dissolved in 50 mM phosphate buffer, pH 7.0 or 2.0, and 90% H_2O/10% D_2O: (left) low-field (aromatic) region; (right) high-field (aliphatic) region. X indicates an impurity signal.

BLg molecule, disappeared completely in the pressurized sample spectrum. This occurred because pressure induced exposure of the indole protons in tryptophan residues to the solvent followed by replacement with deuterium atoms. On the other hand, labile amide protons in the region between 7.5 and 10 ppm did not completely exchange with D_2O. Probably, they were not freely accessible to the solvent (D_2O) even under a high pressure of 600 MPa as with the results of the unfolding of BLg induced by 8 M urea (*27*). Ragona et al. (*28*) demonstrated the presence of the buried cluster in BLg that was very stable at high temperature.

After lyophilization of BLg pressurized in D_2O, the sample was dissolved in H_2O and then was employed to measure the NMR spectrum (Figure 8C). Interestingly, only the signal at 10.09 ppm appeared. This indicates that the H/D exchange reaction occurs promptly when the indole proton of Trp61, which is located at the relatively molecular surface and had higher mobility, is exposed to the solvent (H_2O) (*23*). The other tryptophan residue, Trp19 (10.42 ppm), seems to be buried in the bottom of the calyx, as judged from the behavior of the indole amide proton, which is protected from exchange (*8*, *23*).

To clarify whether the tryptophan regions were reversible or not in the refolding pathway, the sample was pressurized again at 600 MPa for 60 min and then was dissolved in H_2O after lyophilization. The NMR

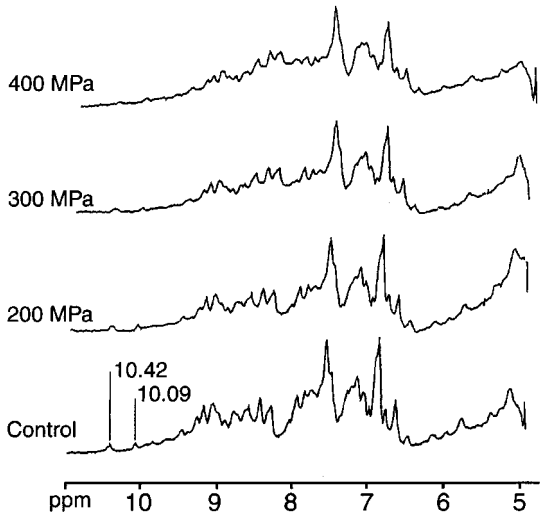

Figure 7. Aromatic region of ^{1}H NMR spectrum of BLg dialyzed against buffer at pH 2.0 after the protein was subjected to various pressures at pH 7.0. Other experimental conditions were same as those described in Figure 6.

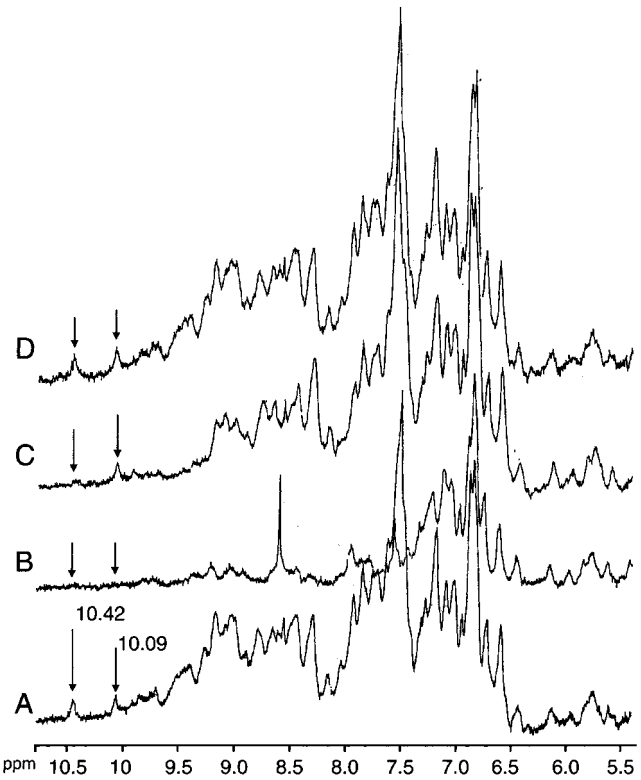

Figure 8. Aromatic region of ^{1}H NMR spectra of hydrogen/deuterium exchanged BLg at pH 2.0 and 20 °C: (A) intact; (B) H/D exchanged in D_2O under pressure of 600 MPa for 60 min; (C) dissolved in H_2O after lyophilizing sample B; (D) H/D exchanged in H_2O under pressure of 600 MPa for 60 min following step C; (A, C, and D) 90% H_2O/10% D_2O; (B) 100% D_2O.

spectrum is illustrated in Figure 8D. As can be seen in this spectrum, the signal at 10.42 ppm was observed together with that at 10.09 ppm and a spectrum feature returned to that of control sample (Figure 8A, D). From these results, it seems that at acidic pH condition the tryptophan regions are at least refolded almost perfectly after release of pressure. In other words, it may be said that the conformation in the vicinity of the retinol binding site probably involving Trp19 is resistant to pressure.

We do not have a satisfactory explanation for the irreversibility of BLg pressurized at highly acidic pH, although Ragona et al. (*29*) reported that the urea-induced denaturation of BLg was reversible up to 6 M urea. We examined the measurements of 2D NMR (NOESY, nuclear Overhauser enhancement and exchange spectroscopy) to detect the change in the fine structure of BLg induced by pressure. Although data are not shown here, a difference in the NOESY spectra in aliphatic and aromatic regions between unpressurized and pressurized BLgs at pH 2.0 was observed, obviously suggesting the possibility of a delicate change in the tertiary structure of BLg under high pressure at pH 2.0. However, this time assignment of the spectrum has not been performed because of the limited resolution in the spectrum.

As was mentioned above, it is clear that monomer BLg at acidic pH condition is partially irreversible when exposed to high pressure on the basis of the results of the changes in the far-UV CD spectrum, incomplete recovery of the PnA binding ability, and 2D NMR spectrum (data not shown). The reason for this is not completely understood, but one possibility is that the conformation of a certain hydrophobic surface in the BLg molecule, which may be the PnA binding site, collapses partially by misfolding (*7*, *10*). Our present consequence of 2D NMR spectra still presented many open questions. Therefore, further detailed 2D NMR analysis will be needed to detect changes in the structure, more precisely, in the refolding process of the protein after release of pressure.

Finally, the different states, monomer and dimer (or oligomer), of BLg differed distinctly in their pressure sensitivities. That is, BLg denatured irreversibly and aggregated at neutral pH under high-pressure treatment, as evidenced by data such as the ^{1}H NMR or CD spectra, whereas it denatured partially and did not aggregate at acidic pH. Because the BLg content of WPI is high, the difference in pressure-induced denaturation of BLg may be responsible for the pH dependency of the gel formation ability of pressurized WPI.

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