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Hydrophobic Probe Binding of β-Lactoglobulin in the Native and Molten Globule State Induced by High Pressure as Affected by pH, KIO_3 and *N*-Ethylmaleimide

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High hydrostatic pressure (HHP) at 500 MPa and 50 °C induces β-LG into the molten globule state. Retinol, cis-parinaric acid (CPA), and 1-anilino-naphthalene-8-sulfonate (ANS) fluorescence from pH 2.5 to 10.5 in the presence of the native and molten globule states of β-LG indicate that retinol binds to β-LG in the calyx, CPA at the surface hydrophobic site, and ANS in multiple hydrophobic sites. HHP treatment results in a decrease of β-LG affinity for retinol and CPA, suggesting conformational changes in the calyx and surface hydrophobic site of β-LG during HHP treatment. β-LG treated by HHP in the presence of *N*-ethylmaleimide (NEM) retains retinol affinity, suggesting that NEM protects the calyx conformation of β-LG during HHP treatment. HHP treatment of β-LG in the presence of KIO_3 exhibits a great decrease of CPA affinity compared to HHP-treated β-LG in the absence of KIO_3, suggesting the formation of non-native disulfide bonding at the CPA binding site.

KEYWORDS: β-Lactoglobulin; high pressure; molten globule; retinol; cis-parinaric acid; 1-anilino-naphthalene-8-sulfonate; *N*-ethylmaleimide; KIO_3

INTRODUCTION

β-Lactoglobulin (β-LG) is a 162 residue globular protein present in bovine milk. β-LG exists as a dimer at pH 3.5−8 and as a monomer below pH 3.5 or above pH 8 (*1*). Similar to retinol-binding protein, β-LG is characterized as a member of the lipocalin superfamily with a β-barrel calyx formed by eight strands of antiparallel β-sheet (*2*, *3*, *4*). Although the physiological function of β-LG is not clear, β-LG exhibits affinity for a variety of hydrophobic ligands such as retinol, retinoids, fatty acids, alkanes, aldehydes, ketones, alkanones, benzaldehyde, β-ionone, and ergocalciferol (vitamin D_2) (*5*−*11*). β-LG possesses two distinct binding sites: an internal and an external binding site (*12*). The internal hydrophobic site is located within the β-barrel as a calyx site, and the external hydrophobic site is located in a groove between the β-barrel and the α-helix (*4*, *12*, *13*).

Due to the structural similarity of bovine β-LG to human retinol-binding protein, retinol is believed to bind to β-LG in the calyx site within the β-barrel (*2*). Selective modification of amino acid residues residing in the two binding sites provides evidence that the location of the retinol binding site of β-LG is in the internal calyx site rather than the external hydrophobic site (*14*). However, fluorescence resonance energy transfer experiments suggests that the β-LG binding site for retinol and retinoic acid lies in the external hydrophobic site rather than the internal calyx site (*15*).

Retinoids and fatty acids bind independently and simultaneously to β-LG, suggesting that retinol and palmitic acid bind to β-LG at two different sites (*16*, *17*). Modification of cysteine^{121} at the external hydrophobic binding site of β-LG decreases the affinity of β-LG for fatty acids but not for retinol (*17*). These results indicate that fatty acids bind at the external hydrophobic site of β-LG and retinol binds at the internal calyx site within the β-barrel (*17*). However, X-ray structure investigation of β-LG with the fatty acid analogue 12-bromododecanoic acid demonstrates that fatty-acids bind to β-LG inside the calyx at pH 7.3 (*18*). Wu et al. (*4*) studied cocrystallization of β-LG with palmitic acid at pH 7.5 and observed that the carboxyl group of palmitic acid binds to lysine^{60} and lysine^{69} at the entrance to the cavity and the hydrophobic tail in the fully extended conformation enters the center of the calyx site. The observations suggest that palmitic acid binds within the internal calyx site of β-LG. Ragona et al. (*19*) also observed by using one-, two-, and three-dimensional NMR spectroscopy at neutral pH that palmitic acid binds within the calyx site with the methyl end of palmitic acid deeply buried in the calyx site.

cis-Parinaric acid (CPA), a fatty acid analogue, is a hydrophobic probe used to evaluate surface hydrophobicity of proteins. The exact binding site of β-LG for CPA is not clear. Alizadeh-Pasdar and Li-Chan (*20*) reported that the fluorescence of CPA bound to β-LG is three-times lower at pH 3.0 than at

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pH 7.0, indicating that CPA may lose binding to β-LG at the acidic pH (*16*).

1-Anilinonaphthalene-8-sulfonate (ANS) is a hydrophobic probe that binds to proteins. ANS exhibits two fluorescence lifetime responses when bound to β-LG, suggesting two different binding sites or binding modes to β-LG (*12*, *21*). Collini et al. (12) elucidated that a long lifetime decay (11 to 14.5 ns) of ANS fluorescence is attributed to binding at the calyx site inside the hydrophobic β-barrel. A short lifetime decay (2.5 to 3.5 ns) of ANS fluorescence is attributed to ANS binding at the external surface hydrophobic binding site.

β-LG undergoes two discrete reversible conformational transitions from pH 4 to 6 and from pH 6.5 to 9.5 that accompany changes in physical and chemical properties (*22*−*24*). The Tanford transition is described as the conformational transition occurring from pH 6.5 to 9.5 and can be related to the descriptive open calyx or closed calyx conformational structures of β-LG. Qin et al. (*25*) observed that a loop segment (amino acid residues 85−90) between β-sheet E and F of β-LG acts structurally like a lid to close the calyx site of β-LG at pH 6.2 and open the calyx site at pH 7.1 to 8.2. Uhrínová et al. (*26*) also reported that the calyx site is closed by the EF loop at pH 2.6. The carboxyl group of glutamic acid[89] in the EF loop of β-LG provides evidence that the EF loop acts like a lid to open and close the calyx because the carboxyl group of glutamic acid[89] is buried at pH 6.2 and becomes exposed at pH 7.1 and 8.2 (*18*, *22*, *24*, *25*). The conformational changes of β-LG with pH provide functional implications for reversible binding and release of ligands (*18*, *26*). The influence of pH on protein structure is associated with changes of the protonated state of titratable groups, which influences processes such as ligand affinity or release, partial or global unfolding, and protein−protein associations (*27*).

Native β-LG can convert to the molten globule state when heated or when exposed to chemical denaturants such urea and guanidine-hydrochloride, or ethanol (20−40%) (*28*−*31*). Yang et al. (*32*) reported that high hydrostatic pressure (HHP) at 600 MPa and 50 °C for 16 min induced β-LG into a stable molten globule state stabilized by the formation of disulfide bonds. The HHP induced molten globule state β-LG exhibits an increase of ANS fluorescence and a decrease of CPA fluorescence compared with native β-LG (*32*).

Retinol is a molecule with an aromatic and an aliphatic moiety. CPA is a molecule with an aliphatic structure, and ANS is a molecule with an aromatic structure. The study of the retinol, ANS, and CPA fluorescence in the presence of native and HHP-treated (molten globule) β-LG as affected by the pH may provide useful information about the location of retinol, CPA, and ANS binding sites on β-LG. Potassium iodate (KIO_3) is a reagent that promotes the formation of disulfide bonds through oxidation reactions, and *N*-ethylmaleimide (NEM) is a thiol blocking reagent that prevents the formation of disulfide bonds. The study of retinol, CPA, and ANS fluorescence of β-LG in the native and molten globule states as affected by KIO_3 and NEM may provide information about the effect of the formation of non-native disulfide bonds on β-LG affinity for retinol, CPA, and ANS.

The objectives of this research are to investigate (1) retinol, CPA, and ANS fluorescence in the presence of β-LG in the native or in the molten globule state as affected by pH and (2) retinol, CPA, and ANS fluorescence in the presence of β-LG as affected by the presence of KIO_3 and NEM before and after HHP treatment.

MATERIALS AND METHODS

Protein and Chemicals. β-LG (a mix of two genetic variants A and B, no. L-6879), 1-anilino-naphthalene-8-sulfonate (ANS) fluorescent probe (no. A-1028), retinol (no. R-7632), KIO_3 (no. P-8269), and *N*-ethylmaleimide (NEM) (no. E−3876) were purchased from Sigma Chemical Co. (St. Louis, MO). *cis*-Parinaric acid (CPA) (no. 46164) was purchased from Pierce (Rockford, IL). Sodium phosphate (monobasic, dibasic) was purchased from J. T. Baker (Philipsburg, NJ).

High-Pressure Treatment. Sodium phosphate buffer (0.01M, pH 7.0) was used to prepare β-LG solutions. The final concentration of β-LG was determined by spectrophotometry using a molar extinction coefficient of $\epsilon_{280} = 17\,600\ M^{-1}cm^{-1}$ at 280 nm (1). β-LG solutions (27 μM) were prepared in 0.01 M phosphate buffer (pH 7.0) at pH 2.0, 3.0, 9.0, and 11.0, adjusted with 0.1 M HCl or 0.1 M NaOH. HHP was applied to β-LG solutions (27 μM) with a warm isostatic pressing system (Engineered Pressure Systems, Inc., Andover, MA) with a cylindrical pressure chamber (height + 0.25 m, diameter = 0.10 m) at 600 MPa and 50 °C for a holding time of 32 min in the absence or presence of 200 μM KIO_3 or 200 μM NEM. After exposure to high pressure, the β-LG was studied immediately or stored at 5 °C for less than 5 days.

Extrinsic Fluorescence. Retinol, CPA, and ANS were used as extrinsic probes to study the binding of β-LG with hydrophobic molecules. The extrinsic fluorescence for the ligands was assayed using excitation at a wavelength of 340, 325, and 390 nm and observing emission at a wavelength of 460, 490, and 470 nm for retinal, CPA, and ANS, respectively. The fluorescence was recorded with an Aminco-Bowman J4-8961 spectrophotofluorometer (Aminco Division of Travenol Laboratories, Inc. Silver Spring, MD).

To study the pH effect during HHP treatment on β-LG, we adjusted β-LG solutions (pH 2.0, 3.0, 9.0, or 11.0) following HHP treatment by HCl (0.1 M) or NaOH (0.1 M) to pH 7.0. One-half of a milliliter of native or HHP-treated β-LG was diluted with 1.5 mL phosphate buffer (0.01M, pH 7.0) to obtain 2 mL of the β-LG solution (6.8 μM). Twenty microliters of 8.0 mM ANS in 0.01 M phosphate buffer at pH 7.0 was added to 2 mL of the β-LG solution. The extrinsic ANS fluorescence of β-LG was assayed using the spectrophotofluorometer.

To study the effect of pH on the binding of β-LG, we diluted 0.5 mL of native or HHP-treated β-LG with 1.5 mL phosphate buffer (0.01M, pH 7.0) to obtain 2 mL of the β-LG solution (6.8 μM). Twenty microliters of 0.5 mM retinol (in 95% ethanol), 10 μL of 2.0 mM CPA (in 95% ethanol containing equimolar butylated hydroxytoluene), or 20 μL of 8.0 mM ANS (in 0.01 M phosphate buffer at pH 7.0) wwas added to 2 mL of native or HHP-treated β-LG solution (6.8 μM). In assays at the pH below 7.0, 2 mL of β-LG solution was titrated with 1 M HCl or 0.5 M HCl to adjust the pH of the β-LG solution from pH 7.0 to 2.5. In assays at pH above 7.0, 2 mL of β-LG solution was titrated with 1 M NaOH or 0.5 M NaOH to adjust the pH of the β-LG solution from pH 7.0 to 10.5. After mixing, the retinol, CPA, and ANS fluorescence were determined. The titration experiment for retinol, CPA, and ANS was repeated 3 times. The pH of the β-LG solution after addition of an equivalent volume of HCl (0.5 or 1 M) or NaOH (0.5 or 1 M) was determined by separate experimentation.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis (12%) in the presence of sodium dodecyl sulfate (SDS−PAGE) was performed according to the instruction manual of Mini-Protean II Ready Gels (catalog number 161-0900). Native and HHP-treated β-lactoglobulin solutions (0.5 mg/mL) were diluted with the sodium phosphate buffer (1:1 ratio) and heated at 100 °C for 4 min. Aliquots of 50 μL of the diluted mixes were loaded on the gels. Electrophoresis was run at ambient temperature for 36 min at 200 V. Prestained SDS−PAGE standards (catolog number 161-0318, Bio-Rad, Hercules, CA) were used to calibrate the gels. The protein standards included aprotinin (6.9 kDa), lysozyme (20.7 kDa), soybean trpsin inhibitor (29.1 kDa), carbonic anhydrase (34.9 kDa), ovalbumin (52.4 kDa), bovine serum albumin (70.0 kDa), β-galactosidase (121.1 kDa), and myosin (205.0 kDa).

RESULTS AND DISCUSSION

pH Effect during HHP Treatment. HHP treatment at 600 MPa and 50 °C at pH 7.0 induced β-LG into the molten globule

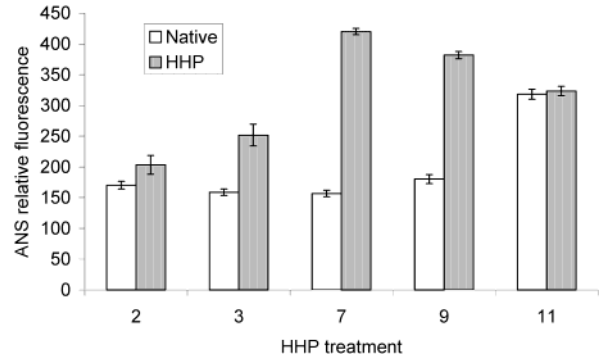

Figure 1. ANS fluorescence of β-LG assayed at pH 7.0 after HHP treatment at selected pH, 600 MPa, and 50 °C for 16 min.

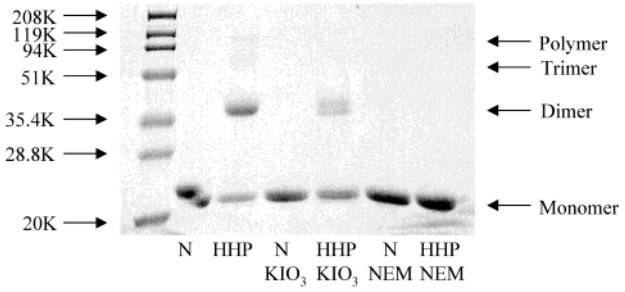

Figure 2. Gel electrophoresis of native (N) and HHP-treated β-LG (600 Mpa and 50 °C for 32 min) in the presence of KIO_3 or NEM.

state (*32*). Compared to HHP treatment at pH 2.0, 3.0, 9.0, and 11.0, HHP treatment at pH 7.0 resulted in the greatest ANS fluorescence of β-LG (**Figure 1**). Therefore, HHP treatment at neutral pH is the optimum pH to induce β-LG into the molten globule state compared to other selected pHs. HHP treatment of β-LG at alkaline pH results in greater ANS fluorescence at pH 7.0 in the presence of β-LG than HHP treatment at acidic pH, indicating that more conformational changes occur during HHP treatment for β-LG at alkaline pH than at acidic pH. Molinari et al. (*33*) observed that β-LG at pH 2.5 exhibits a highly structured β-sheet core. β-LG at pH 2.5 is more resistant to thermal denaturation at 90 °C for 30 min than at pH 4.5 or 6.5 (*34*). The highly ordered structure of β-LG at acidic pH may contribute to the resistance of β-LG to conformational changes during HHP treatment. At pH 11 ANS fluorescence is equivalent for native and HHP-treated β-LG, exhibiting irreversible alkaline denaturation of β-LG.

Polymerization during HHP Treatment. HHP treatment of β-LG resulted in formation of dimers, trimers, and polymers (**Figure 2**). The free thiol group of cysteine^{121} is buried in the interior of native β-LG and is not accessible to Ellman's reagent at pH 7.0 (*2*, *32*). During HHP treatment of β-LG, conformational changes expose the free thiol group of β-LG, increase accessibility, and promote reactivity of native disulfide bonds to form non-native inter- and intradisulfide bonds (*32*, *35*). Therefore, dimers and trimers are formed through non-native disulfide bonds in β-LG between the free thiol group of cysteine^{121} and two disulfide bonds during HHP treatment (*32*, *36*). Since oxygen may be present in the β-LG solution, both the interchange reaction and oxidizing reaction may be involved in the formation of disulfide bonds during HHP treatment of β-LG. In the presence of KIO_3, HHP treatment did not result in formation of trimers and polymers. β-LG dimers were formed after the HHP treatment of β-LG in the presence of KIO_3, as observed by gel electrophoresis (SDS−PAGE) (**Figure 2**). KIO_3 is an oxidizing agent that promotes the formation of disulfide bonds between free thiol groups of proteins such as gluten proteins in dough. The oxidation reaction may be the predominant reaction responsible for forming β-LG dimers during HHP treatment in the presence of KIO_3. In the absence of KIO_3, the interchange reaction may be the predominant reaction responsible for forming β-LG dimers during HHP treatment. Therefore, the formed non-native disulfide bonds of β-LG during HHP treatment in the absence of KIO_3 may differ from the formed non-native disulfide bonds of β-LG during HHP treatment in the presence of KIO_3. HHP treatment of β-LG in the presence of NEM did not result in the formation of β-LG dimers (**Figure 2**) because NEM blocks the free thiol group of cysteine^{121} of β-LG and the formation of non-native disulfide bonds is inhibited during HHP treatment.

Retinol Binding. Retinol fluorescence in the presence of native β-LG from pH 7.5 to 10.5 is 5 times greater than that in the presence of β-LG from pH 2.5 to 5.8 (**Figure 3**). The dramatic transition of retinol fluorescence in the presence of native β-LG occurs between pH 6.0 and 7.0. Media pH affects the conformational structure of native β-LG (*22*, *24*, *25*). The EF loop of β-LG acts like a lid to close the calyx at pH below 6.2 and to open the calyx at pH above 7.1 (*25*, *26*). The pH range for the transition of retinol fluorescence in the presence of native β-LG is coincident with the pH range for the transition of the open to the closed calyx conformation of native β-LG (**Figure 3**). The transitions of retinol fluorescence and calyx conformational structure of β-LG suggest that retinol binds to native β-LG in the accessible open calyx binding site. At pH greater than 7.0, the calyx of native β-LG is open, facilitating retinol binding and increasing the intensity of retinol fluorescence due to the hydrophobic environment in the calyx of the β-barrel. At pH less than 6.0, the calyx of native β-LG is closed, and retinol cannot access the calyx. Therefore, retinol in the presence of β-LG at pH less than 6.0 emits only about one-fifth of the fluorescence intensity of retinol in the presence of native β-LG at pH greater than 7.0.

Retinol binding to native β-LG is pH dependent. In the pH range from 2.5 to 5.8, retinol may bind to native β-LG at surface hydrophobic binding sites. Sawyer et al. (*37*) stated that both the calyx site and the surface hydrophobic site between the sole α-helix and the β-barrel are able to accommodate retinol. The accessibility of retinol to binding sites of native β-LG is hypothesized to be the calyx site at pH greater than 7.0 and the surface hydrophobic site at pH less than 6.0. Previous controversial reports that retinol binding to β-LG either within the calyx or at the surface hydrophobic site may depend on the open or the closed calyx conformational structure of β-LG under specific experimental conditions. In the open calyx conformation, β-LG may favor retinol binding within the calyx and preferentially exclude the surface hydrophobic site on β-LG. In the closed calyx conformational structure, retinol binding to β-LG may be restricted to the surface hydrophobic site of β-LG.

Retinol binding to HHP-treated β-LG is not pH dependent. Retinol fluorescence intensity in the presence of HHP-treated β-LG is constant from pH 2.5 to 10.5 and is approximately one-third the fluorescence intensity of retinol in the presence of native β-LG from pH 8.0 to 10.5 (**Figure 3**). The reduction of retinol fluorescence intensity in the presence of HHP-treated β-LG may result from conformational changes of the calyx during HHP treatment. The calyx in the native β-LG is formed by eight strands of antiparallel β-sheets (*2*). Yang et al. (*32*) reported that HHP treatment of β-LG induced secondary structure rearrangement from β-sheet to non-native α-helix (*32*). Therefore, the calyx site or β-barrel is changed significantly during HHP treatment. In addition, β-LG possesses a disulfide

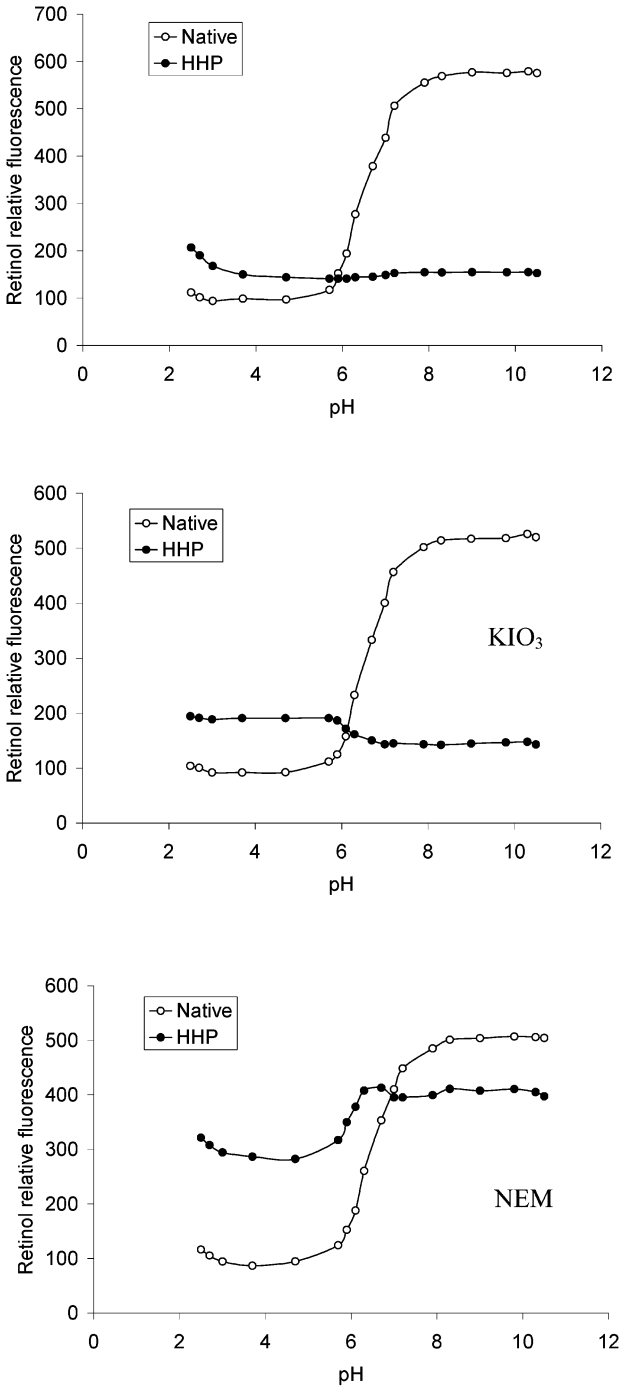

Figure 3. Retinol fluorescence of native and HHP-treated β-LG (600 Mpa and 50 °C for 32 min) in the presence of KIO_3 or NEM as affected by pH.

bond between cysteine[106] and cysteine[119], linking the β-sheet designated as H and the β-sheet designated as G of the calyx (*2*). The distance between the sulfur atoms of the cysteine[121]−free thiol and the cysteine[119] disulfide bond is 10.5 Å (*2*). An interchange reaction between the cysteine[121]-free thiol and cysteine[119] disulfide bond may be possible due to significant conformational change. HHP treatment of β-LG results in the formation of β-LG trimers and polymers (**Figure 2**), indicating that such interchanges between cysteine[119] and cysteine[121] occur during HHP treatment. Therefore, there are significant conformational changes in the calyx during HHP of β-LG. Furthermore, since non-native disulfide bonds are formed during HHP treatment (*32*), the β-LG structure altered by HHP does not reversibly fold to reform the native β-barrel or the calyx after the HHP is released. Therefore, permanent conformational changes of the calyx during HHP treatment of β-LG result in a decrease of β-LG affinity for retinol and a decrease in retinol fluorescence in the presence of HHP-treated β-LG.

HHP treatment of β-LG in the presence of KIO_3 resulted in an increase of retinol fluorescence in the pH range from 3.0 to 6.0 compared with HHP treatment of β-LG in the absence of KIO_3 (**Figure 3**). The differences in retinol binding to HHP-treated β-LG between the presence of KIO_3 and the absence of KIO_3 may result from the formation of non-native disulfide bonds through interchange or oxidization reactions during HHP treatment. In the presence of KIO_3, the oxidizing reaction between free thiol groups of β-LG and the interchange reaction between a free thiol and a disulfide bond occurred, forming inter- and intramolecular disulfide bonds, whereas in the absence of KIO_3, the interchange reaction between the free thiol groups and disulfide bonds resulted in the formation of intramolecular disulfide bonds.

In the pH range from 2.5 to 6.0, retinol fluorescence in the presence of β-LG treated with HHP in the presence of NEM exhibited 3 times greater intensity than retinol fluorescence in the presence of native β-LG (**Figure 3**). In the pH range from 7.0 to 10.5, β-LG treated with HHP in the presence of NEM retained 80% of the fluorescence intensity of retinol in the presence of native β-LG (**Figure 3**). The retention of retinol fluorescence in the presence of β-LG and NEM after HHP treatment results from the blocking of the cysteine[121]-free thiol group and inhibition of the formation of non-native disulfide bonds during HHP treatment of β-LG. After the HHP is released, the denatured β-LG refolds to a nativelike conformational structure with the calyx in an open state. The refolded calyx site of β-LG exhibits affinity for retinol, resulting in retention of 80% retinol fluorescence after HHP treatment. Since the cysteine[121]-free thiol group is located at the surface hydrophobic site, the blocked thiol group of cysteine[121] at the surface hydrophobic site does not influence retinol binding in the calyx site of β-LG. Therefore, the retention of retinol fluorescence in the presence of HHP-treated β-LG and NEM supports the assertion that retinol binds to β-LG within the calyx. Sawyer et al. (*37*) also stated that modification of the thiol group of cysteine[121] of β-LG affects fatty acid binding, but not retinol binding.

CPA Binding. The fluorescence intensity of CPA in the presence of native β-LG increases in the range of pH from 2.5 to a maximum around pH 6.0 and then decreases as the pH is increased to 10.5 (**Figure 4**). Since CPA fluorescence in the presence of β-LG decreases at both the open calyx conformation at pH above 7.0 and the closed calyx conformation at pH below 6.0, CPA binding to β-LG is not dependent on the open or closed calyx conformation, suggesting that the CPA binding site is not located in the calyx of β-LG. The CPA fluorescence intensity in the presence of native β-LG and NEM significantly decreased at pH 6.0 compared to the fluorescence intensity of CPA in the presence of native β-LG without NEM (**Figure 4**). NEM blocks the free thiol group located at the surface hydrophobic binding site of β-LG and decreases fatty acid binding at the surface hydrophobic site of β-LG (*37*). Binding of NEM to the free thiol also leads to monomer formation. The decrease of CPA fluorescence in the presence of β-LG and NEM indicates that NEM molecules sterically hinder CPA binding at the surface hydrophobic binding site. Therefore, our observations suggest that CPA binds at the surface hydrophobic site of β-LG. Although Wu et al. (*4*) and Ragona et al. (*19*) reported that the

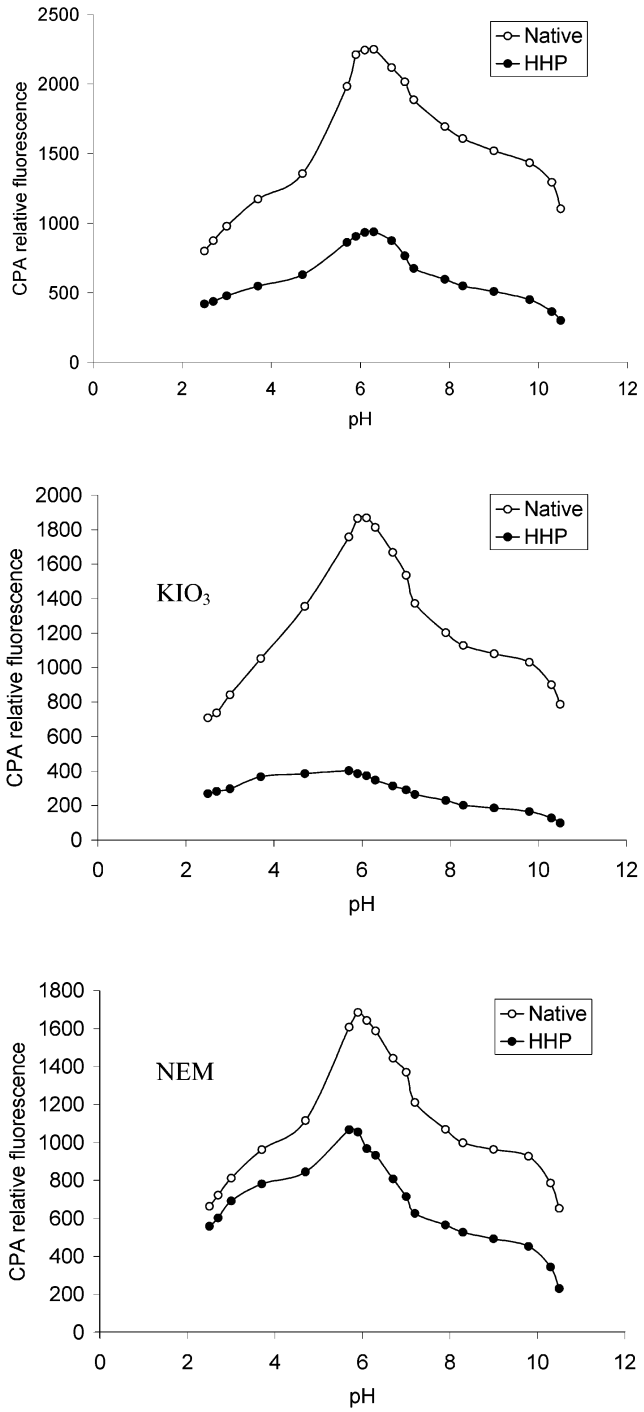

Figure 4. CPA fluorescence of native and HHP-treated β-LG (600 Mpa and 50 °C for 32 min) in the presence of KIO_3 or NEM as affected by pH.

binding site for palmitic acid on β-LG is in the calyx site, CPA may bind to β-LG at binding sites different from binding sites for palmitic acid because palmitic acid is a fully saturated ligand and CPA is conjugated.

The CPA fluorescence intensity of CPA in the presence of HHP-treated β-LG as a function of pH is similar to CPA fluorescence in the presence of native β-LG (**Figure 4**). The intensity of the CPA fluorescence in the presence of HHP-treated β-LG is about 2- or 3-fold smaller than the fluorescence intensity of CPA in the presence of native β-LG. HHP treatment of β-LG at 600 MPa and 50 °C results in the exposure of the inaccessible free thiol group of β-LG and the formation of β-LG dimers through covalent disulfide bonds (*32*). Since the free thiol group of β-LG is associated with the surface hydrophobic binding site (*2*), consequently the exposure of free thiol groups and the formation of intermolecular covalent disulfide bonds during HHP treatment reduce the binding and accessibility of CPA to the surface binding sites of β-LG. Therefore, CPA fluorescence in the presence of HHP-treated β-LG decreases compared to CPA fluorescence in the presence of native β-LG.

HHP treatment of β-LG in the presence of KIO_3 resulted in a disappearance of CPA fluorescence peak at pH 6.0 compared to HHP treatment of β-LG in the absence of KIO_3 (**Figure 4**). The difference in CPA fluorescence in the presence of HHP-treated β-LG in the absence of KIO_3 and in the presence of KIO_3 may result from chemical reactions of cysteine^{121}-free thiol groups of β-LG during HHP treatment. In the absence of KIO_3, intermolecular disulfide bonds are formed at the surface hydrophobic binding site through the interchange reaction between the free thiol groups and disulfide bonds. However, in the presence of KIO_3, intermolecular disulfide bonds are formed at the surface hydrophobic binding site through the oxidizing reaction between free thiol groups of β-LG. Intermolecular disulfide bonds among β-LG dimers formed through the oxidizing reaction during HHP treatment in the presence of KIO_3 may greatly reduce the accessibility of CPA to the surface hydrophobic binding sites compared to intermolecular disulfide bonds formed through interchange reaction during HHP treatment in the absence of KIO_3. Therefore, the peak fluorescence of CPA at pH 6.0 disappears in the presence of HHP-treated β-LG and KIO_3. The decrease of CPA fluorescence in the presence of β-LG and KIO_3 may also be due to the interaction of IO_3^- with lysine^{153} located at the surface binding site of β-LG. The interaction between IO_3^- and lysine^{153} of β-LG may sterically hinder CPA binding to β-LG.

CPA fluorescence intensity at pH 6.0 in the presence of HHP-treated β-LG and NEM was greater than the fluorescence intensity of CPA in the presence of HHP-treated β-LG in the absence of NEM (**Figure 4**). NEM blocks the free thiol group of cysteine^{121} at the surface hydrophobic site of β-LG and inhibits the formation of non-native disulfide bonds during HHP treatment. The denatured β-LG refolds to a native like conformational β-LG structure after release of pressure. Therefore, the CPA molecule can still access the surface hydrophobic site of β-LG after HHP treatment, exhibiting CPA fluorescence intensity greater than the fluorescence intensity of CPA in the presence of HHP-treated β-LG in the absence of NEM.

ANS Binding. ANS fluorescence intensity in the presence of native β-LG decreases gradually from pH 2.5 to 7.0, increases slightly from pH 7.0 to 8.0, and then decreases from pH 8.0 to 10.5 (**Figure 5**). In general, the changes in ANS fluorescence in the presence of native β-LG with changing pH are not dependent on the open or closed calyx conformation of β-LG. However, from pH 7.0 to 8.0, a small increase of ANS fluorescence in the presence of native β-LG may be due to the transition from the closed calyx conformation to the open calyx conformation. In the open calyx conformation, the hydrophobic calyx is accessible to ANS molecules, resulting in a small increase of ANS fluorescence from pH 7.0 to 8.0. D'Alfonso et al. (*21*) reported that ANS binds within the calyx site of native β-LG. ANS fluorescence intensity in the presence of native β-LG increased as pH changes from 6.0 to 2.5 (**Figure 5**). The greater ANS fluorescence in the presence of native β-LG at pH 2.5 than at neutral pH indicates that β-LG binds more ANS molecules at acidic pH. Hamdan et al. (*38*) observed that two molecules of ANS bound to β-LG at pH 7.0 and three molecules

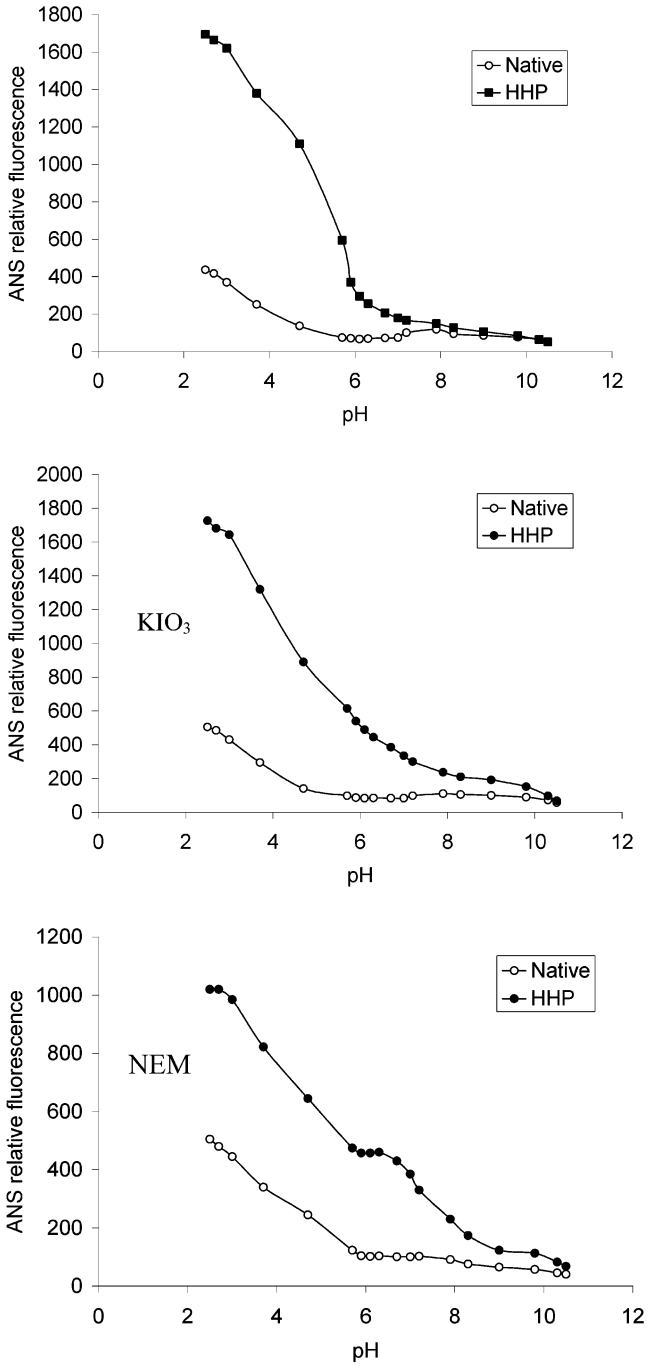

Figure 5. ANS fluorescence of native and HHP-treated β-LG (600 Mpa and 50 °C for 32 min) in the presence of KIO_3 or NEM as affected by pH.

of ANS bound to β-LG at pH 2.0. β-LG at the acidic pH (pH 2.0) and low ionic strength (0.012 M sodium phosphate) exhibits a partially folded structure with a highly structured β-sheet core and a less ordered unstructured region (*33*). The less ordered unstructured region of β-LG at pH 2.0 may provide a hydrophobic environment with greater affinity to ANS molecules than that at pH 7.0 (*38*). In addition, in acid environments, ANS is negatively charged, and β-LG is positively charged (*27*, *39*, *40*). There are about 15−20 positive charges among amino acid residues in β-LG at pH 2.0−3.0, and about 12 negative charges among amino acid residues in β-LG at pH 7.0 (*27*). The electrostatic ionic interactions among ANS and β-LG increase with a decrease in pH, resulting in an increase in the number of ANS molecules bound to β-LG at low pH. Matulis and Lovrien (*39*) reported that electrostatic interactions are the predominant interactions among ANS and bovine serum albumin, and hydrophobic affinity is a minor interaction among ANS and bovine serum albumin. The increase of electrostatic interaction with a decrease of pH may also result in an increase of ANS fluorescence intensity in the presence of β-LG at acidic pH.

ANS fluorescence in the presence of HHP-treated β-LG increases dramatically as pH is decreased from pH 6.0 to 2.5 (**Figure 5**). The ANS fluorescence intensity in the presence of HHP-treated β-LG at pH 2.5 is about 4 times greater than ANS fluorescence intensity in the presence of native β-LG. The increase in ANS fluorescence intensity between the presence of native β-LG and in the presence of HHP-treated β-LG at acidic pH may result from induction of the molten globule state of β-LG. HHP induces β-LG into the molten globule state at pH 7.0 (*32*). At acidic pH, intramolecular repulsion among positive charges induces a more disordered structure of β-LG than the molten globule state of β-LG induced by HHP at pH 7.0. Therefore, acidic pH promotes the accessibility and affinity of ANS molecules to the HHP induced molten globule states of β-LG (*32*, *38*), resulting in much greater ANS fluorescence intensity in the presence of HHP-treated β-LG than native β-LG.

When KIO_3 or NEM was added to β-LG solution before HHP treatment, ANS fluorescence intensity in the presence of HHP-treated β-LG was greater at pH 6.0 than the fluorescence intensity of ANS in the presence of HHP-treated β-LG without addition of KIO_3 or NEM (**Figure 5**). However, the addition of NEM before HHP treatment resulted in a decrease of ANS fluorescence intensity in the presence of HHP-treated β-LG at pH 2.5 compared with the fluorescence intensity of ANS in the presence of HHP-treated β-LG without the addition of NEM or with the addition of KIO_3 (**Figure 5**). KIO_3 promotes non-native disulfide bond formation through oxidization reactions, while NEM inhibits disulfide bond formation by blocking the free thiol groups of cysteine[121] in β-LG. In the absence of KIO_3 or NEM, non-native disulfide bonds are formed through interchange reactions. The observed difference in ANS fluorescence in the presence of HHP-treated β-LG in the presence or absence of KIO_3 or NEM may be attributed to the conformational structure of β-LG after HHP treatment in the presence of KIO_3 or NEM. In the presence of NEM, denatured β-LG can refold to the native conformational structure after HHP treatment and have a calyx at acidic pH similar to native β-LG. Therefore, ANS fluorescence intensity in the presence of the HHP-treated β-LG and NEM is smaller than ANS fluorescence intensity in the presence of the HHP induced molten globule state of β-LG in the absence of NEM.

CONCLUSIONS

Retinol, CPA, and ANS fluorescent probes exhibit variable binding characteristics with native β-LG. Retinol binds predominantly to native β-LG in the calyx site. CPA binds predominantly to native β-LG in the external hydrophobic site. ANS may bind to native β-LG in the internal calyx site, external surface hydrophobic sites, or other sites by either hydrophobic affinity or electrostatic interaction. β-LG in the molten globule state induced by HHP treatment at 500 MPa and 50 °C for 32 min partially decreases binding affinity of β-LG for retinol and CPA, but it increases binding affinity for ANS.

The binding of retinol, CPA, and ANS to β-LG depends on the conformational structure of the binding sites and the accessibility of these probes. The open or closed calyx conformation affects the accessibility of retinol to the calyx sites

of β-LG. The free thiol of cysteine[121] at the surface binding site is reactive to formation of non-native disulfide bonds during HHP treatment. The non-native disulfide bond formation results in conformational changes of the calyx site of β-LG, and a decrease of retinol binding to β-LG after HHP treatment. The non-native disulfide bond formation also results in reduction of accessibility to the surface hydrophobic binding site, and a decrease of CPA binding to β-LG after HHP treatment. Since the calyx conformation is protected by NEM during HHP treatment by inhibition of non-native disulfide bond formation due to blocking of the free thiol group, the presence of NEM during HHP treatment retains or increases retinol binding to β-LG at pH above 7.0 or at pH from 2.5 to 6.0 after HHP treatment, respectively. β-LG modified by a thiol-blocking agent and HHP treatment may have applications as a retinol carrier for acid foods.

Abbreviations Used. β-LG, β-lactoglobulin; ANS, 1-anilinonaphthalene-8-sulfonate; CPA, *cis*-parinaric acid; NEM, n-ethylmaleimide; HHP, high hydrostatic pressure; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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