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In Situ Measurements of pH Changes in β-Lactoglobulin Solutions under High Hydrostatic Pressure

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A novel in situ method, in which the spectral changes of aqueous solutions under pressure are measured using optical pH indicators in a high-pressure spectrophotometer, has been developed in order to provide a quantitative description of the pressure dependence of acid/base equilibria of proteins. The self-consistent method, insensitive to compressibility, was developed for measurement of changes in pH with pressure based on α-naphthyl red and neutral red as these indicators were found to have pressure insensitive pK'_a values. The method was validated for up to 500 MPa by measurement of the pressure-dependence of the weak acid buffers acetic acid/acetate and imidazolium/imidazole from which volumes of dissociation of $\Delta V^\circ = -11.2$ and 3.7 mL/mol, respectively, were established. Succinic acid/hydrogensuccinate was surprisingly insensitive to pressure with $\Delta V^\circ = -0.9$ mL/mol. For β-lactoglobulin B in an unbuffered aqueous solution with ionic strength of 0.05 M and pH 4, pressure up to 300 MPa increased pH up to 1.5 units depending on concentration (up to 5 mg/mL investigated), followed by a decrease to the initial pH 4 for pressure up to 500 MPa. The surprising increase in pH at pressure up to 300 MPa is suggested to be caused by an increase in the effective pK_a values of aspartic acid and glutamic acid side chain in hydrophobic compartments of the protein created by pressure denaturation, leading to a binding of water protons and an increase in free hydroxide ions. For higher pressure the carboxylic side chains in the fully denatured protein again becomes exposed to the solvent, and pH decreases to the initial pH of the aqueous system.

KEYWORDS: High pressure; pH; acid dissociation; beta-lactoglobulin

INTRODUCTION

Pressure is a fundamental physical parameter that affects chemical reactions and physiological functions of biomolecules differently than temperature, as has been demonstrated in the evolutionary adoption of high-pressure treatment of biochemical systems. Investigation of high-pressure effects on biochemical systems are usually conducted using buffered solutions, since variations in pH can have great impact on physical properties of biomolecules and consequently on reaction rates and chemical equilibria. The pH of a buffered aqueous solution is determined by the magnitude of the acid dissociation constant, K_a, which further depends on temperature, pressure, and ionic strength to a varying degree for different weak acids:

$$HA + H_2O \overset{K_a}{\rightleftharpoons} A^- + H_3O^+ \qquad (1)$$

It is known that the self-ionization of water is enhanced upon pressure, resulting in a significant increase in the ion product of water, K_w, with increasing pressure (*1*, *2*). This effect has, on the basis of Le Chatelier's principle, been interpreted as a net contraction of the system upon ionization due to the electrostriction of water ions, and thus favored by pressure. The thermodynamic description relates the change in reaction volume ΔV° with the pressure dependence of the dissociation constant:

$$\frac{\partial \ln K_a}{\partial P}\Big|_T = -\frac{\Delta V^\circ}{RT} \qquad (2)$$

This relationship applies to water ionization and to weak acids employed as buffer solutions. Hence, the dissociation of acids increases upon pressurizing if the ion-pair formation is accompanied by a substantial volume reduction. Consequently, the buffering capacity of certain buffers decreases with pressure with significant changes in pH upon pressurizing. This emphasizes the importance of a quantitative description of acid−base equilibria in order to control pH in high-pressure investigations of biochemical systems.

pH in aqueous solutions may be measured spectrophotometrically using acid−base indicators and applied in pressure studies of commonly used buffer solutions (*3*, *4*). Notably, the pressure dependence of the dissociation of the optical pH-indicators becomes important and in previous studies the pressure dependency of the indicator was measured relative to

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the pressure effect on the dissociation of acetic acid. Knowing the pressure dependence of one indicator, the pressure dependence of the dissociation of other buffers and indicators was established (*3*). We suggest a new self-consistent method, in which changes in indicator spectra due to pressure are taken into account when the pressure dependence of the indicator dissociation is established. On the basis of the pressure dependence of the indicator, the pressure dependence of the dissociation constants and the reaction volume of selected buffers were determined in order to validate the method. The self-consistent method is further used to monitor in situ pH changes in aqueous solution of the major whey protein of cow's milk β-lactoglobulin during pressure treatment as a function of pressure and protein concentration as part of our continuing studies of pressure effects on protein solutions.

MATERIALS AND METHODS

Chemicals. β-Lactoglobulin from bovine milk, genetic variant B, was isolated from acidic whey of fresh skim milk of homozygotic cows and purified according to the method described in ref *5*. 4-(Phenylazo)-1-naphthalenamine hydrochloride (α-naphthyl red) was obtained from Sigma-Aldrich (Steinem, Germany) and 3-Amino-7-dimethylamino-2-methylphenazine hydrochlorid (neutral red) was from Fluka (Germany). All other chemicals were of analytical grade, and solutions were based on highly purified water (Milli-Q Plus, Millipore Corp, Bedford, MA).

Spectrophotometry at Ambient Pressure. In order to determine the dissociation constants of the indicators, pK_i, a series of α-naphthyl red and neutral red were made in appropriate buffers with pH ranging between 2.0 and 7.4 and between 4.2 and 9.5, respectively, as measured with a glass electrode and their absorption spectra were recorded at ambient pressure and temperature. The absorption spectra of the indicator solutions at ambient pressure were recorded on an UV−visible spectrophotometer (Cintra 40, GBC Scientific Equipment Pty Ltd., Australia).

pH Measurements at Ambient Pressure. Prior to the optical measurements under pressure, the pH of the solutions was measured as a reference with glass electrode (713 pH Meter, Metrohm, Switzerland) against international activity pH standards.

High-Pressure Spectrophotometer. The intensity of light transmitted through the solutions under investigation of varying pressure was measured in situ in a thermostated high-pressure optical cell (Type 740.2006 from SITEC Sieber Engineering AG, Switzerland) equipped with a hand-operated pressure generating system (Type 750.1700 from SITEC Sieber Engineering AG, Switzerland). The optical cell has a volume of 2.2 mL and is equipped with two sapphire windows and has an optical pathway of 14 mm. A combined deuterium-halogen light source (Hamamatsu Photonics K.K., Japan) is connected to the cell by a fiber-optic cable, and the light is parallel aligned by a lens. On the other side of the optical cell, another lens focuses the light to a fiber-optic cable which transmits it to an AVS-S2000 spectrometer (Avantes, The Netherlands) covering the range from 250 to 800 nm and connected to a DAQ-700 A/D converter (Avantes, The Netherlands). The temperature of the cell is held constant by a thermostat bath, and the pressure inside the cell is measured using a pressure transducer (Type 770.6191 from SITEC, Switzerland). The pressure generating system and the optical cell were filled with the relevant solution and the intensity spectrum from 350 to 700 nm was recorded at each step of pressures between 0.1 and 500 MPa. The intensity spectra were converted to absorption spectra using $A_\lambda = -\log I_\lambda/I_\lambda^{ref}$, where I_λ and I_λ^{ref} are intensities at wavelength λ for the solution and for water as reference, respectively. The intensity spectrum of water was recorded prior to measurement of the indicator solution.

In Situ Spectrophotometry. A stock solution of α-naphthyl red ($3.6\cdot10^{-4}$ M) in water was prepared. Solutions with α-naphthyl red in its acidic, basic, or partially transformed form were prepared by adjusting aliquots to pH 2.0, 7.0, and 4.0, respectively, with appropriate amounts of 1.0 M HCl or 1.0 M NaOH, and to an ionic strength at 0.05 M with NaCl and a final α-naphthyl red concentration of $3.6\cdot10^{-5}$ M. A stock solution of neutral red ($1.0\cdot10^{-3}$ M) in water was prepared. Solutions with neutral red in its acidic, basic, or partially transformed form were prepared by adjusting aliquots to pH 3.5, 8.2, and 6.7, respectively, with appropriate amounts of 0.1 M HCl or 0.1 M NaOH, and to an ionic strength at 0.008 M with NaCl and a final neutral red concentration of $7.5\cdot10^{-5}$ M. Prior to optical measurements under pressure, as described above, the pH of the solutions at ambient pressure was measured with a glass electrode.

Pressure Dependency of Dissociation of Weak Acids. In order to study high-pressure effect on buffers, buffer solutions were made similar to the aqueous indicator solutions. Stock solutions of acetic acid, succinic acid, and imidazole were prepared according to standard prescriptions. Acetic acid solutions (0.2 M) with α-naphthyl red ($3.6\cdot10^{-5}$ M) in its acidic, basic, or partially transformed form were prepared by adjusting aliquots with appropriate amounts of 1.0 M HCl or 1.0 M NaOH to pH 2.0, 8.0, and 4.7, respectively, as measured with a glass electrode. Succinic acid solutions (0.1 M) with α-naphthyl red ($3.6\cdot10^{-5}$ M) in its acidic, basic, or partially transformed form were prepared by adjusting aliquots with appropriate amounts of 1.0 M HCl or 1.0 M NaOH to pH 2.0, 8.0, and 4.0, respectively, as measured with a glass electrode. Imidazole solutions (0.05 M) with neutral red ($7.5\cdot10^{-5}$ M) in its acidic, basic, or partially transformed form were prepared by adjusting aliquots with appropriate amounts of 1.0 M HCl or 1.0 M NaOH to pH 3.5, 8.5, and 6.7, respectively, as measured with a glass electrode. The buffer samples were filled in the optical cell of the high-pressure spectrophotometer, and a series of absorption spectra from 350 to 700 nm were recorded at increasing pressure from 0.1 to 500 MPa in steps of 50 MPa at ambient temperature.

pH of β-Lg Solution under Pressure. Purified bovine β-lactoglobulin B (β-Lg) was dissolved in water as a stock solution and stored at 5 °C overnight for equilibration. β-Lg solutions were made by adding an appropriate aliquot of α-naphthyl red (final concentrations of $3.6\cdot10^{-5}$ M) to aliquots of the stock solution giving final β-Lg concentrations of 0.00, 0.25, 0.50, 1.00, 2.50, and 5.00 mg/mL. The acidic and basic samples and solutions for the pressure studies were prepared by adjusting aliquots with appropriate amounts of HCl or NaOH to pH 2, 7, and 4, respectively, as measured with a glass electrode. The β-Lg solution samples were filled in the optical cell of the high-pressure spectrophotometer and a series of absorption spectra from 350 to 700 nm were recorded at increasing pressure from 0.1 to 500 MPa in steps of 50 MPa at ambient temperature.

IN SITU METHODOLOGY

In Situ Spectrophotometry. Using optical pH indicators, pH of a solution can be measured photometrically by means of the dissociation constant (pK_i) and the ratio of the base and acid form of the indicator:

$$HIn + H_2O \overset{K_i}{\rightleftharpoons} In^- + H_3O^+ \tag{3a}$$

$$pH = pK_i + \log\frac{\alpha}{1-\alpha} \qquad \alpha = \frac{[In^-]}{[HIn]+[In^-]} \tag{3b}$$

where HIn and In^- are the acid and base form of the indicator, respectively, and α is the degree of dissociation of the indicator.

The change in pH of a solution under increasing pressure can accordingly be determined by the knowledge of the pressure dependency of the dissociation constant and the optical measurement of the degree of dissociation of the indicator:

$$\frac{\partial pH}{\partial P} = \frac{\partial pK_i}{\partial P} + \frac{\partial \log\left(\frac{\alpha}{1-\alpha}\right)}{\partial P} \tag{4}$$

For chemical equilibria like weak acid buffer dissociation (1) and indicator dissociation (3a), the equilibrium constant (K_i) is thermodynamically defined by the activity of each components. However, it is not always experimentally possible to measure the activity of the relevant species, and approximations are often made in which activities

are replaced by concentration terms as in the so-called incomplete acid dissociation constant which depends on ionic strength:

$$K_i' = \frac{a(H_3O^+)[In^-]}{[HIn]} \quad (5)$$

The incomplete dissociation constant, K_i', is based on concentrations of the acidic and basic form of the indicator, which can be measured photometrically and hydrogen ion activity, which can be measured electrochemically relative to pH standards.

The degree of dissociation of the indicator can be measured photometrically, and is related to the measured absorbance by

$$\alpha(P) = \frac{A_x(P) - A_a(P)}{A_b(P) - A_a(P)} \quad (6)$$

where $A(P)$ is the absorbance of the partially transformed indicator (index x), and absorbance of the indicator in its acidic form (index a), and in its basic form (index b), respectively, at the respective pressure.

Thus, the pressure dependency of α was determined by measuring the absorption spectrum of the three solutions of the acidic, basic, and partially transformed indicator in the high-pressure spectrophotometer at increasing pressure. Since α only depends on the difference in absorption at each pressure and not on indicator concentration no correction of change in concentration due to compressibility is required. Thus, a constant α-value as a function of pressure indicates that the pK_i' of the indicator does not depend on pressure and that the indicator is pressure insensitive and can be used to determine the pressure effect on the acid dissociation of weak acids without complicated corrections.

Pressure Dependency of Dissociation of Weak Acid. Similar to the indicator dissociation, it is possible to determine the incomplete dissociation constant of acids used for aqueous buffers:

$$K_a' = \frac{a(H_3O^+)[A^-]}{[HA]} \quad (7)$$

and subsequently the pressure dependency:

$$\frac{\partial \ln K_a'}{\partial P}\Big|_T = -\frac{\Delta V^\circ}{RT} \quad (8)$$

ΔV° is the difference between the partial molar volumes of the products and the partial molar volumes of the reactants, all at the actual concentrations of the solution, and the pressure dependence of K_a' is accordingly governed by the partial molar volumes under the actual conditions in the equilibrium solutions.

Pressure-induced changes in pH of a buffer depend directly on the pressure-induced changes of the dissociation constant of the buffer:

$$\frac{\partial pH}{\partial P} = \frac{\partial pK_a'}{\partial P} \quad (9)$$

Combined with eq 4, the pressure dependency of the buffer pK_a' can be determined by

$$\frac{\partial pK_a'}{\partial P} = \frac{\partial pK_i}{\partial P} + \frac{\partial \log\left(\frac{\alpha}{1-\alpha}\right)}{\partial P} \Rightarrow$$

$$\Delta pK_a' = \Delta pK_i + \Delta\log\left(\frac{\alpha}{1-\alpha}\right) \Rightarrow \Delta pK_a' = \Delta\log\left(\frac{\alpha}{1-\alpha}\right) \quad (10)$$

when the dissociation of the indicator is pressure insensitive. Hence, the change in the dissociation constant of the weak acid as a function of pressure is expressed by the observable quantity $\Delta\log(\alpha/1-\alpha)$ and may be determined by measuring absorption spectra of solutions of the acidic, basic, and partially transformed indicator in a high-pressure spectrophotometer at varying pressure and constant temperature.

pH of β-Lg Solution under Pressure. The method developed was used to monitor in situ pH changes in solutions of β-lactoglobulin B under pressure by measuring the absorption spectra of β-Lg under acid and basic conditions and the β-Lg solution under investigation in a high-pressure spectrophotometer. From the relationship eq 4 the change in pH is calculated in accordance with

$$\Delta pH(P) = \Delta\log\left(\frac{\alpha(P)}{1-\alpha(P)}\right) \quad (11)$$

where $\Delta pH(P) = pH(0.1\ MPa) - pH(P)$ and $\alpha(P)$ is determined from eq 6.

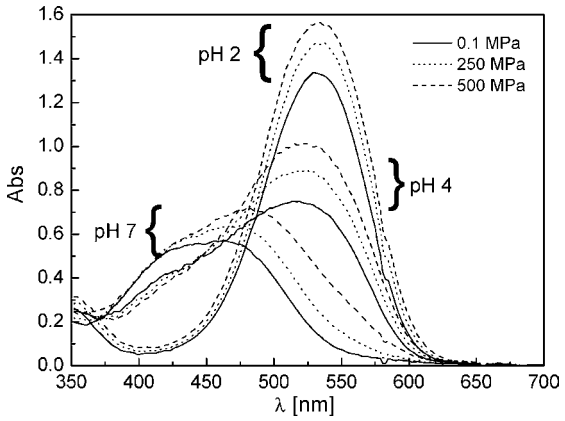

Figure 1. Absorption spectra of α-naphthyl red at acidic (pH 2.0) and basic (pH 7.0) conditions compared to the partially transformed form at pH 4.0 at varying pressure.

RESULTS AND DISCUSSION

In Situ Spectrophotometry. Spectrophotometric measurement of pH with acid−base indicators is based on differences in absorption spectra between the acidic form and the basic form of the indicator molecule and the useful range depends on pK_i, see eq 3a. However, pK_i may for constant temperature vary with pressure, and the pressure dependency of the indicator pK_i needs to be established in order to convert α in eq 3b to pH at varying pressure. Among a number of possible indicators, α-naphthyl red and neutral red were investigated in details over the spectral range 350−700 nm in solutions of their acidic and basic forms, and of a mixture around pK_i value of each indicator, since these indicators were considered to be good candidates for pressure insensitive indicators. **Figure 1** shows the absorption spectra for α-naphthyl red in water at different pH as a function of pressure. For clarity only spectra at ambient pressure and the highest pressure used and one intermediate pressure are shown. The maximum absorption at 529 nm of the acidic form of α-naphthyl red shifts to 456 nm for the basic form at ambient pressure. The small variation in absorption spectra of the base form with pressure is most likely a combined effect of compressibility of the solvent, deformation of windows of the high-pressure cell, and to a lesser degree of conformational changes of the indicator. The increase in molar absorptivity upon pressurizing reflects the concentration increase with pressure as the system is compressed and a correction could have been applied in order to convert high-pressure molarity to concentration units at ambient pressure using the solvent compressibility coefficient, κ. However, van Eldik (*6*) questions the use of correction term based on fundamental thermodynamic considerations. Our use of the absorbance differences in the pressure series of acid, basic, and partially transformed forms of the indicator solely as ratios as seen from eq 6 indirectly corrects the absorbance of the indicator forms at the respective pressure. Using indicators with sufficiently different absorption spectra for the acid and base forms as seen for α-naphthyl red in **Figure 1** ensures an accurate calculation of the degree of dissociation

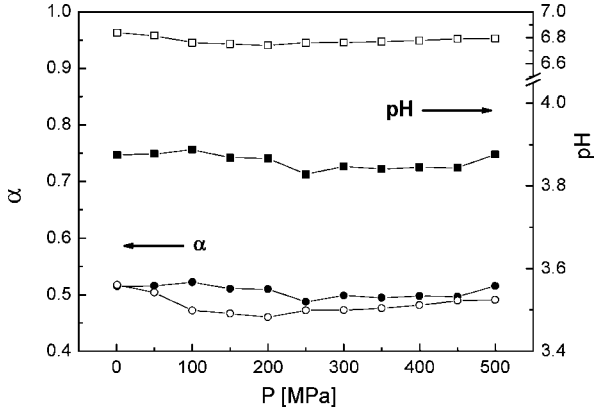

Figure 2. Pressure dependence of indicator dissociation α (●, ○) and pH (■, □) calculated from spectral data according to eqs 6 and 3b, respectively, for α-naphthyl red (solid symbols) and neutral red (open symbols).

at each pressure. From the three pH series the base fractions of α-naphthyl red and of neutral red were calculated for each pressure and from this the change in pH according to eq 3b using the pK_i' value at ambient pressure (determined to be 3.85 for α-naphthyl red and 6.81 for neutral red) could be calculated. The result presented in **Figure 2** shows clearly that the dissociation constants of α-naphthyl red and neutral red do not change with increasing pressure for up to 500 MPa and confirms that the indicators are pressure insensitive. In general, the self-ionization of water is increased upon pressurizing and could affect the equilibrium of the indicator and should accordingly be taken into account when calculating the pH in pressurized media. However, the definition of α (eq 6) is based on a hydrostatic scale and such corrections become unnecessary. Indicators, for which the acidic form is positively charged will create no net change of charges due to acid dissociation, and are accordingly the best candidates for pressure insensitive indicators (*7*). Hence, the method, as has been demonstrated in the present study for α-naphthyl red and neutral red, can be applied to measure in situ pH changes of buffer solutions and of solutions of biomolecules under pressure.

Pressure Dependency of Dissociation of Weak Acid. Weak acid dissociation is affected under pressure by changes in electrostriction. The extent of changes in dissociation of the buffer substances depends on the specific ions disappearing and produced in the actual reaction. In order to validate the developed method based on pressure insensitive indicators, the pressure dependency of acetic acid, succinic acid, and imidazole was determined with indicators present in concentrations where they did not influence the dissociation of the buffers and for pH values around the pK_i. α-Naphthyl red (indicator range pH 3.7−5.0 (*7*)) was used for the study of the pressure dependence of pK_a' for acetic acid (pK_a = 4.76) and for succinic acid (p$K_{a,1}$ = 4.21 and p$K_{a,2}$ = 5.64), while the indicator neutral red (indicator range pH 6.8−8.0 (*7*)) was used for imidazole (pK_a = 6.95).

Figure 3 shows the pressure effect on the dissociation of the three buffers investigated expressed as $\Delta pK_a'$ (eq 10). Distinctly different pressure dependencies of the pK_a' values for these acid/base pairs are observed. The decrease in the pK_a' value for acetic acid corresponds to an increase by a factor of 10.5 for the dissociation constant K_a' at a pressure of 500 MPa compared to ambient pressure. Consequently, the pH of an acetic acid/acetate buffer will decrease up to one pH unit over a pressure range of 500 MPa. Formation of an acetate ion and a hydronium ion from the electrically neutral acetic molecule

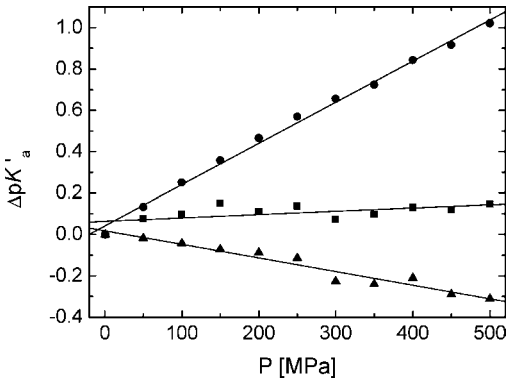

Figure 3. Pressure dependence of pK_a' for 0.2 M acetic acid (●), 0.1 M succinic acid (■), and 0.05 M imidazole (▲) expressed as $\Delta pK_a' = \Delta pK_a'$ (0.1 MPa) − $\Delta pK_a'(P)$. Lines are obtained by linear regression of eq 8 to the data.

exerts strong electrostatic contraction of the surrounding solvent water molecules, which will reduce the molar volume and, therefore, this reaction is enhanced by pressure. In contrast, dissociation of the positively charged imidazolium ion occurs without changing the number of charges and no electrostatic compression and volume decrease will result from increasing the pressure. This is confirmed by a small increase in pK_a' for increasing pressure (**Figure 3**). Hence, the dissociation of the imidazolium ion is suppressed only to a small degree by pressure and the imidazole buffer is almost pressure insensitive. The dicarboxylic succinic acid shows a remarkable lack of pressure dependency with an almost constant pK_a' value over the investigated pressure range. The initial small decrease in the pK_a' value is related to ionization of the first proton of succinic acid. The dissociation of the first proton produces a negatively charged carboxylic group, as for acetic acid, and is favored upon pressurizing. However, the second proton is shared between the two carboxylic groups and the negative charge is delocalized resulting in less electrostriction and volume changes explaining the constant pK_a' value at increasing pressure in accordance with the observed pressure independence of malic acid (*8*).

From the experimental results presented in **Figure 3**, it seems that pK_a' depends linearly on pressure up to 500 MPa and using eq 8 the following values of $\Delta V°$ were obtained by linear regression for acid dissociation: $\Delta V°$(acetic acid) = −11.2 cm^3/mol, $\Delta V°$(succinic acid) = −0.9 cm^3/mol, and $\Delta V°$(imidazole) = 3.7 cm^3/mol. Since no curvature was observed in **Figure 3** it may be concluded that the compressibility of the acid form and the base form of the buffer solutions is the same (*6*, *9*). These values are in good agreement with other published values of the volume change ranging from −9.2 to −12.5 cm^3/mol (*1*) for acetic acid and of 1.8/−2.4 cm^3/mol (*8*) for imidazole, confirming the validity of the developed method to measure changes in the dissociation constant at elevated pressures up to 500 MPa. The remarkably different values of $\Delta V°$ of the weak acid buffers imply that the extent of changes in $\Delta V°$ and the pressure-induced dissociation strongly depend on the intimate nature of the protolytic group.

pH of β-Lg Solution under Pressure. Pressure-induced changes in protein structure and function may be expected to vary considerably depending on the type and concentration of protein and on the intensity and duration of the pressure treatment. In general, most globular proteins denature under pressure due to disruption of the molecular forces responsible for maintaining the native conformation of the protein molecule. It is known, that pressure destabilization of hydrophobic, electrostatic, and van der Waals interaction unfold proteins, yet

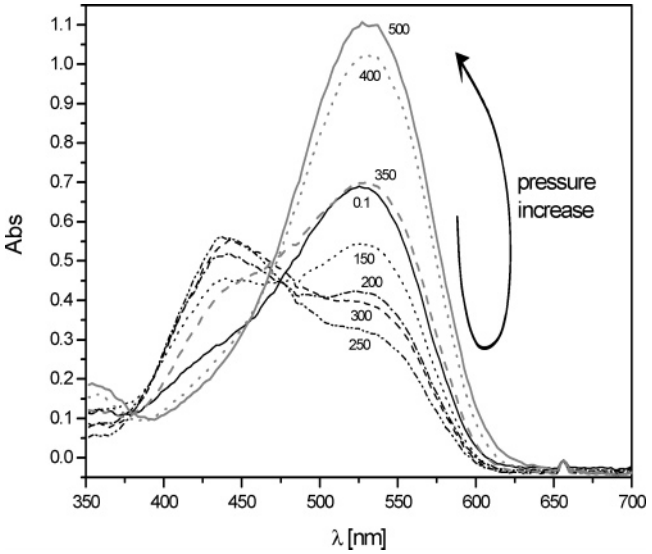

Figure 4. Pressure-induced changes in the visible absorption spectra of α-naphthyl red in an aqueous solution of β-Lg B (1.00 mg/mL) at initial pH 4.0. The number on each absorption curve denotes the pressure in MPa.

the details of pressure effects on individual interactions within the protein are not clearly understood. The developed method for in situ measurement of pH changes in aqueous protein solutions under pressure provides information which may help to understand pressure effect on electrostatic interactions important to protein conformation. Bovine β-lactoglobulin is very pressure sensitive and its denaturation process has been studied in details (*10*−*12*) and was accordingly chosen for a study of effects on proteolytic active side groups in a protein. β-Lg is a compactly folded globular protein and consists of 162 amino acid, where 53 residues have titratable side groups (*13*). In the pH region around 4 β-Lg has 10 ionisable aspartic acid (pK_a of approximately 3.9) groups and 16 ionisable glutamic acid (pK_a of approximately 4.3) groups, most of which are located on the surface of the β-Lg molecule, while only one, Glu 89, is buried in the interior of the native protein (*14*). However, the effective pK_a of the ionisable side group in the protein depends on different molecular microenvironments and may have either higher or lower value than the respective free amino acid.

Figure 4 shows the change in absorbance of α-naphthyl red in an aqueous solution of β-Lg as a function of pressure. At initial pH of 4 and pressure (0.1 MPa), the maximum absorption at 525 nm confirms that α-naphthyl red is on its acidic form. Upon pressurizing, the absorption spectra of α-naphthyl red change considerably corresponding to changes in the acid/base equilibrium of the indicator. The intensity of the absorption band of the acidic form of the indicator decreases when the pressure increase to 250 MPa followed by an increase in absorption on further pressure increase, indicating an initial pH increase for moderate pressure, followed by a pH decrease for further pressure increase. The model used to determine the change in pH is based on the assumption that the acidic form and basic form of the indicator do not interact spectrally. The characteristic absorption spectra of α-naphthyl red in the aqueous solutions of β-Lg under acidic (pH 2.0) and basic (pH 7.0) conditions both increase their molar absorptivity upon pressurizing (results not shown), but without changing spectral properties similar to the spectra of α-naphthyl red in water under acidic and basic conditions (see absorption spectra at pH 2.0 and 7.0, **Figure 1**), thus confirming the validity of using spectra of the acidic and basic β-Lg solutions as the acidic and basic form of α-naphthyl red, respectively, in the calculation of α(*P*) (eq 6). It may accordingly be assumed that the absorption spectra of α-naphthyl red probe the charged molecular microenvironment of the protolytic side groups in β-Lg and thus function as a sensor of the distribution of the effective pK_a values of protolytic active groups in the protein. This sensor effect is the result of a diffusion controlled transfer of protons from the solvent to the acidic and basic side groups of the protein in contact with the indicator. The distinct absorption spectra of the acidic and basic α-naphthyl/protein solutions increase in molar absorptivity upon pressurizing due to compressibility of the solvent confirming that the α-naphthyl molecules are not bound in the interior of the unfolded protein structure during pressurization. Thus, the spectral changes shown in **Figure 4** represent the interaction between β-Lg and α-naphthyl based on the equilibrium of eq 3a, and not a binding of α-naphthyl to the protein. This distinction is emphasized since the changes of the absorption spectra during pressurization reflect the shifted equilibrium according to the spectral changes of α-naphthyl in water at the relevant pH's (compare with the absorption spectra of α-naphthyl red at acidic (pH 2.0), basic (pH 7.0), and pH 4.0). From these spectral data pH was calculated according to eq 11 and the results are presented in **Figure 5** for five different β-Lg concentrations. Pressure treatment was found to induce marked changes in the pH of the β-Lg aqueous solutions dependent on the working pressure and a maximum change in the pH was found around 300 MPa, as may be seen in **Figure 5**.

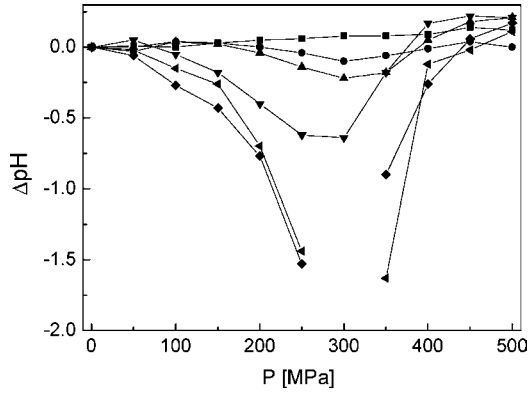

Figure 5. Pressure dependence of pH of aqueous solutions of β-Lg B at different concentrations, 0.00 mg/mL (■), 0.25 mg/mL (●), 0.50 mg/mL (▲), 1.00 mg/mL (▼), 2.50 mg/mL (◆), and 5.00 mg/mL (triangle pointing left), all with an initial pH of 4.0. The change in pH, $\Delta pH(P) = pH(0.1MPa) - pH(P)$, is calculated from spectral data according to eq 11, accordingly the negative change represent an increase in pH under pressure.

The application of high pressure to proteins in solution promotes any structural rearrangement of the macromolecules/solvent system that results in a net reduction in its volume. The magnitude of the change in volume is determined mostly by two opposing contributions. It is accepted that exposure of polar and charged groups will lead to a decrease in volume due to electrostrictive effects. Another substantial negative contribution to ΔV is the elimination of internal cavities and voids upon disruption of the native folded structure. Moderately high pressure (100−200 MPa) has been found to promote dissociation of oligomeric proteins, and is typically accompanied by negative and relatively large ΔV values between −50 to −200 cm^3/mol (*15*). In contrast, hydrophobic interactions are expected to be stabilized by elevated pressure, an effect which can be attributed to the unfavorable volume increase that results from the solvation of apolar surfaces during protein unfolding. This

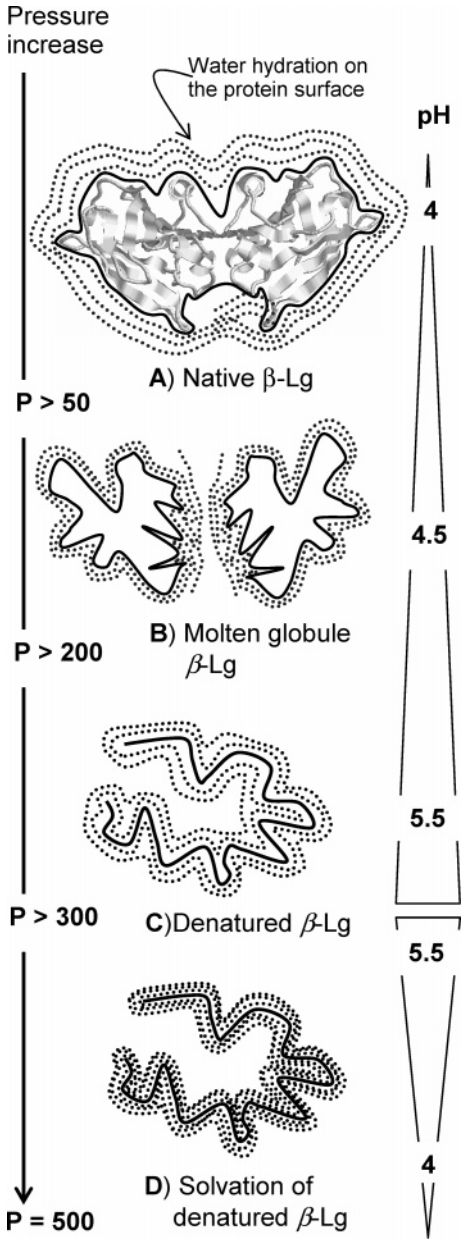

Figure 6. Changes in β-Lg B structure and pH of aqueous solution under increasing pressure. (A) Representation of the native protein as a dimer at pH 4.0, (B) the molten globule state, (C) denatured β-Lg, and (D) solvation of the denatured β-Lg molecule.

volume increase results from clathrate formation around the apolar groups when they are exposed to water (*16*). Hence, the major factors that contribute to ΔV and thus together control effects of pressure include the electrostriction of charged and polar groups, elimination of packing defects, and the solvation of hydrophobic groups.

The effects of high hydrostatic pressure on an unbuffered aqueous solution of β-Lg with an initial pH of 4 based on the observed pH-profiles shown in **Figure 5** are visualized in **Figure 6**. The dissociation of the oligomeric proteins (Figure 6) increases the accessible surface area of β-Lg molecules resulting in an increase in hydration of the protein molecules (*17*). The electrostriction will result in the contraction of solvent water due to alignment of dipolar water molecules in the electric field of an exposed charge leading to volume decrease and disruption of ion pairs in the protein molecule (*18*). The dissociation of ion pairs will facilitate motion of the side chains and polypeptide backbone which increase the conformational fluctuations of the protein and provide pathways for water to penetrate into the interior of the native protein upon applications of relatively low pressures (*19*). These fluctuations are enhanced by pressure due to increased water exchange between the protein interior and bulk solvent. As a result of water penetration and the accompanying electrostriction of water in the protein interior, the carboxylic side chains might reassociate the proton from water since the initial pH of the solution is very close to the pK_a values of these carboxylic groups, and the pK_a value may increase upon inclusion in hydrophobic pockets created during partial unfolding. This will lead to an excess amount of OH^- on the surface of the protein and also in the solution corresponding to an increase in pH as seen in **Figure 5**. The pressure-induced penetration of water into the protein interior likely leads to conformational transitions of the protein structure to adopt the conformation of a molten globule, a compact, partially folded conformation without specific tertiary structure (*15*) (**Figure 6B**). The increased hydration of the protein interior will result in two opposing effects: (i) the pressure-induced hydration of the polypeptide leads to decreases in the protein compressibility and flexibility due to electrostriction and loss of void volume resulting in decreased mobility of the polypeptide, and (ii) hydration reduces the number of intramolecular hydrogen bonds and promotes the formation of intermolecular hydrogen bonds with water thus causing increased conformational fluctuation of peripheral protein segments (*20*). These two opposing effects are optimally balanced at pressures around 300 MPa, where the maximum increase in pH is occurring (**Figures 5** and **6C**). For further increase in pressure, the tertiary structure of β-Lg is disrupted leading to a denatured protein (**Figure 6D**) with greater compressibility than the structure around 300 MPa due to increased solvation of the unfolded structure promoted by intermolecular hydrogen bonds between water and the newly exposed amino acid residues (*21*). The loss of tertiary structure results in a pH-decrease due to further contraction of solvent in the denatured state of β-Lg as depicted in **Figure 6**, which reverts the observed pH at 500 MPa to its initial value (**Figure 5**) corresponding to the dissociation of the carboxylic groups protonated under moderate high pressure.

In conclusion, the proposed method to measure pH changes under pressure has proven to be self-consistent, insensitive to compressibility and in accordance with the previous methods confirming the validity of the developed method to measure changes in the dissociation constant of weak acids at elevated pressures up to 500 MPa. The remarkable pressure resistant behavior of the succinic acid/hydrogensuccinate buffer shows that direct pressure experiments are necessary to obtain accurate measurements of pH changes of buffers because they are normally used in biochemical systems. Indeed, the results obtained in this study of the pressure unfolding and denaturation of β-Lg has been shown to result in unexpected pH variations under pressure of aqueous protein solutions. For milk and milk products such changes caused by β-Lg and possibly also of other milk proteins may be of importance for pressure effect on functional properties of milk proteins which should be included in further studies.

LITERATURE CITED

(1) Lown, D. A.; Thirsk, H. R.; Lord Wynne-Jones. Effect of Pressure on Ionization Equilibria in Water at 25 °C. *Trans. Faraday Soc.* **1968**, *64* (3), 2073−2080.

(2) Marshall, W. L.; Franck, E. U. Ion Product of Water Substance, 0−1000 °C, 1−10,000 Bars New International Formulation and Its Background. *J. Phys. Chem. Ref. Data* **1981**, *10* (2), 295−304.
(3) Neuman, R. C., Jr.; Kauzman, W.; Zipp, A. Pressure Dependence of Weak Acid Ionization in Aqueous Buffers. *J. Phys. Chem.* **1973**, *77* (22), 2687−2691.
(4) Stippl, V. M.; Delgado, A.; Becker, T. M. Development of a method for the optical in-situ determination of pH value during high-pressure treatment of fluid food. *Innovative Food Sci. Emerg. Techn.* **2004**, *5*, 285−292.
(5) Kristiansen, K. R.; Otte, J.; Ipsen, R.; Qvist, K. B. Large scale preparation of β-Lactoglobulin A and B by ultrafiltration and ion-exchange chromatography. *Int. Dairy J.* **1998**, *8*, 113−118.
(6) van Eldik, R. In *Inorganic High Pressure Chemistry. Kinetics and Mechanisms*; van Eldik, R., Ed.; Elsevier: Amsterdam, 1986; pp 2−68.
(7) Bates, R. G. Measurements of acidity with indicators. In *Determination of pH. Theory and Practise*; John Wiley and Sons, Inc.: New York, 1964; pp 131−171.
(8) Kitamura, Y.; Itoh, T. Reaction volume of protonic ionization for buffering agents. Prediction of pressure dependence of pH and pOH. *J. Solution Chem.* **1987**, *16*, 715−725.
(9) Stippl, V. M.; Delgado, A.; Becker, T. M. Ionization equilibria at high pressure. *Eur. Food Res. Technol.* **2005**, *211*, 151−156.
(10) Knudsen, J. C.; Otte, J.; Olsen, K.; Skibsted, L. H. Effect of high hydrostatic pressure on the conformation of β-lactoglobulin A as assessed by proteolytic peptide profiling. *Int. Dairy J.* **2002**, *12*, 791−803.
(11) Stapelfeldt, H.; Skibsted, L. H. Pressure denaturation and aggregation of β-Lactoglobulin studied by intrinsic fluorescence depolarization, Rayleigh scattering, radiationless energy transfer and hydrophobic fluroprobing. *J. Dairy Res.* **1999**, *66*, 545−558.
(12) Kolakowski, P.; Dumay, E.; Cheftel, J.-C. Effect of high pressure and low temperature on β-lactoglobulin unfolding and aggregation. *Food Hydrocolloids* **2001**, *15*, 215−232.
(13) Fogolari, F.; Ragona, L.; Licciardi, S.; Romagnoli, S.; Michelutti, R.; Ugolini, R.; Molinari, H. Electrostatic Properties of Bovine β-Lactoglobulin. *Proteins: Struct., Funct., Genet.* **2000**, *39*, 317−330.
(14) Qin, B. Y.; Bewley, M. C.; Creamer, L. K.; Baker, H. M.; Baker, E. N.; Jameson, G. B. Structural Basis of the Tanford Transition of Bovine β-Lactoglobulin. *Biochemistry* **1998**, 3*7*, 14014−14023.
(15) Silva, J. L.; Weber, G. Pressure stability of proteins. *Annu. Rev. Phys Chem.* **1993**, *44*, 89−113.
(16) Hvidt, A. A Discussion of Pressure −Volume Effects in Aqueous Protein Solutions. *J. Theor. Biol.* **1975**, *50*, 245−252.
(17) Paladini, A. A.; Weber, G. Pressure-Induced Reversible Dissociation of Enolase. *Biochemistry* **1981**, *20*, 2587−2593.
(18) Hamann, S. D. The role of electrostriction in high pressure chemistry. *Rev. Phys. Chem. Jpn.* **1980**, *50*, 147−168.
(19) Tanaka, N.; Ikeda, C.; Kanaori, K.; Hiraga, K.; Konno, T.; Kunugi, S. Pressure Effect on the Conformational Fluctuation of Apomyoglobin in the Native State. *Biochemistry* **2000**, *39*, 12063−12068.
(20) Cioni, P.; Strambini, G. B. Pressure effects on protein flexibility monomeric proteins. *J. Mol. Biol.* **1994**, *242*, 291−301.
(21) Prehoda, K. E.; Moobery, E. S.; Markley, J. L. Pressure Denaturation of Proteins: Evaluation of Compressibility Effects. *Biochemistry* **1998**, *37*, 5785−5790.

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