Thiol Reactivity in Pressure-Unfolded β-Lactoglobulin. Antioxidative Properties and Thermal Refolding

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Pressure treatment of β-lactoglobulin (0.11 mM in aqueous 0.16 M NaCl, pH 7.61, at 15 °C for 30 min, up to 400 MPa investigated) induces antioxidative properties as shown for linoleic acid peroxidation in oil-in-water emulsions. The antioxidative properties obtained through pressure treatment are gradually lost at ambient pressure and paralleled by a decrease in thiol exposure and reactivity, as determined with Ellman's reagent, in an entropy-controlled ($\Delta S^{\ddagger} = -247 \pm 7$ J mol^{-1} K^{-1}) first-order renaturation process (half-life of 3.1 h at 25 °C, pH 7.61, independent of pressure used for denaturation at least up to 250 MPa) with a modest temperature dependence ($\Delta H^{\ddagger} = 23 \pm 2$ kJ mol^{-1}). The reactivity of the thiol group toward Ellman's reagent was studied kinetically by stopped-flow spectrometry. The apparent second-order rate constant for this reaction at pH 7.61 and 25 °C changes from 5.7×10^2 L mol^{-1} s^{-1} for native β-lactoglobulin to 1.6×10^5 L mol^{-1} s^{-1} for β-lactoglobulin pressure-denatured at 200 MPa. Half-denaturation occurred at ∼50 MPa. The degree of exposure of the thiol group corresponds to half-denaturation around ∼140 MPa with a reaction volume, $\Delta V°$, for denaturation of -61 ± 3 mL·mol^{-1}, a difference in half-denaturation pressure which may indicate that pressure denaturation is a stepwise process.

Keywords: *β-Lactoglobulin; thiols; Ellman's reagent; renaturation; high pressure*

INTRODUCTION

The major whey protein of cows' milk β-lactoglobulin has been found to be very pressure sensitive, having a reaction volume of −98 mL/mol at pH 7.4 and 25 °C, corresponding to a half-denaturation pressure of 110 MPa, when determined from quantum yield of intrinsic protein fluorescence (Stapelfeldt et al., 1996). β-Lactoglobulin contains one free thiol group (Cys-121; Monaco et al., 1987) of which the reactivity is markedly increased by denaturation by heat (Sawyer, 1968) or pressure (Tanaka et al., 1996). The reactivity of this thiol is involved in heat-induced gelation of whey proteins (Shimada and Cheftel, 1989) and provides oxidative stability to milk powders made from preheated milk because of the ability of the solvent-exposed thiol group to react with active oxygen species and lipid peroxyl radicals by donation of an H atom (Walstra and Jenness, 1984). High-pressure treatment has been investigated as an alternative to heat-induced whey protein gel formation. Studies show that heat-set gels, for equal protein concentrations, are firmer than pressure-induced gels (Van Camp and Huyghebaert, 1995) and that increase in pressurization temperature reduces the pressure needed to obtain the same gel strength (Van Camp et al., 1996). Funtenberger et al. (1997) further found that pressure-induced aggregation of β-lactoglobulin was caused by thiol/disulfide interchange reactions by a mechanism corresponding to the theoretical models proposed for heat-induced aggregation (Roefs and de Kruif, 1994). The functional properties of β-lactoglobulin can thus be modified using pressure treatment or combinations of pressure and heat treatment with the perspective of new applications of whey proteins in foods. However, a more quantitative description of the kinetics and thermodynamics of the different step in pressure denaturation is clearly neeeded prior to any practical use. In this context it seems important to consider pressure denaturation of β-lactoglobulin as a stepwise process and to establish which of the denaturation steps are reversible and at which step pressure irreversible denaturation occurs. Intrinsic fluorescence of β-lactoglobulin solution of low concentrations has thus been found to lack pressure hysteresis, while the increase in rate of trypsin digestion of pressure-treated β-lactoglobulin, compared to native β-lactoglobulin, was found to persist for at least 3 h after depressurization, indicating slow refolding for at least some protein domains (Stapelfeldt et al., 1996).

The thiol reactivity of β-lactoglobulin toward Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid); Ellman, 1959] increases with pH (Stapelfeldt et al., 1997a). In the present study, we have measured the reactivity of the free thiol group of β-lactoglobulin subjected to modest high pressure toward Ellman's reagent at fixed pH and using pressure-insensitive buffer for the pressurization (Neuman et al., 1973). We have combined both dynamic and static methods for evaluating thiol reactivity and have, moreover, focused on protein antioxidant properties. Protein refolding following pressure treatment obviously has technological consequences, and we have followed the thiol reactivity for up to 2 days after pressure treatment.

MATERIALS AND METHODS

Materials. β-Lactoglobulin from bovine milk (90% purity as determined by size exclusion HPLC, the remaining part identified as caseinomacropeptide), being a mixture of the A

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and B genetic variants, was obtained from MD-Foods Ingredients (Videbaek, Denmark). Ellman's reagent and L-cysteine hydrochloride monohydrate were purchased from Merck (Darmstadt, Germany), and equine metmyoglobin (type III) and linoleic acid (99% purity) were purchased from Sigma (St. Louis, MO). Other chemicals used were of analytical grade, and water used for experiments was purified through a Milli-Q unit from Millipore (Bedford, MA).

pH Measurements. Solution pH was measured using a 6.0234.110 combination glass electrode from Metrohm (Herisau, Switzerland) relative to concentration standards in the 0.16 M NaCl medium employing the definition pH = −log-[H^+] throughout, e.g. 0.0100 M HCl + 0.150 M NaCl having pH 2.000.

High-Pressure Treatment. Samples of β-lactoglobulin (molecular weight 18 300) solutions [0.11, 0.22, or 0.27 mM in aqueous 0.16 M NaCl with pH 7.61 (50 mM Tris buffer)] were pressurized in a thermostated hydraulic high-pressure reactor (PSIKA Systems Ltd., Stanford, U.K.; 0.25-L working volume, pressure up to 1000 MPa). These samples were pressurized in completely filled, closed 7-mL polyethylene centrifuge tubes using water as pressure-transmitting medium. Samples were held for 30 min at 15.0 °C at the stated pressures, and pressure was raised/decreased by ≈100 MPa/min, thereby minimizing the degree of adiabatic heating.

Heat Treatment. Solutions (7 mL) similar to those used for pressure treatment were placed in a thermostated water bath, held at the elevated temperature (in the range from 50 to 95 °C) for 5 min, and cooled immediately after. Because of the small sample volume, the times for heating to the desired temperature and subsequent cooling to 25 °C were considered negligible.

Analysis of Exposed Thiol Groups. The previously described method based on Ellman's reagent was employed (Stapelfeldt et al., 1997a), using an HP 8452A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA) for absorbance measurements at 412 nm. Solutions were made by mixing 3.5 mL of the pressure- or heat-denatured samples thermostated at 25.0 °C with 70 μL of a 9.49 mM solution of Ellman's reagent for 3 min prior to measurement. Standard curves were constructed using L-cysteine with rigorous pH control, as thiol reactivity increases with increasing pH. Analyses of pressure- or heat-denatured β-lactoglobulin were performed no later than 18 min after treatment (corresponding to <5% refolding).

Renaturation Experiments. β-Lactoglobulin solutions subjected to 150, 200, or 250 MPa for 30 min at 15.0 °C were thermostated at 5−27 °C immediately after depressurization, and the thiol exposure was determined with Ellman's reagent at regular intervals for up to 2 days [cf. Stapelfeldt et al. (1997a)]. After renaturation for 2 days, a sample originally pressure denatured (150 MPa, 15 °C for 30 min) was exposed to high pressure at the same conditions once again, to investigate the reversibility of the pressure denaturation/thermal renaturation cycle.

Stopped-Flow Kinetic Experiments. The reactivity of the thiol group was characterized by determination of the rate of reaction between Ellman's reagent and β-lactoglobulin using a DX-17MV stopped-flow spectrofluorometer (Applied Photophysics Ltd., London, U.K). The first syringe contained a 0.109 mM β-lactoglobulin solution (0.16 M NaCl, pH 7.61, 50 mM Tris buffer), and the other syringe contained a 2.0 mM solution of Ellman's reagent in the same buffer; the solutions were mixed in equal volume. The stopped-flow instrument was used in the absorbance mode using λ = 412 nm as monitoring wavelength (Stapelfeldt et al., 1997a).

Oxygen Consumption. Antioxidative activity of β-lactoglobulin was determined according to the method of Mikkelsen et al. (1992) using a mixture consisting of 500 μL of a Tween-20-stabilized linoleic acid emulsion (21 mM in 5 mM phosphate buffer, pH 6.8), 500 μL of a solution of native or pressure-treated β-lactoglobulin (0.11 mM), and 500 μL of metmyoglobin (0.2 mM). Immediately after mixing, this mixture was injected into a 70-μL temperature-controlled (25.0 °C) measuring cell (Chemiware, Viby J, Denmark) with no headspace. The relative oxygen consumption was measured with a Clark electrode (Radiometer, Copenhagen, Denmark) connected to a multichannel analyzer. The electrode was calibrated with air-saturated buffer thermostated at 25.0 °C, and the relative oxygen concentration was recorded at time intervals of 5 s.

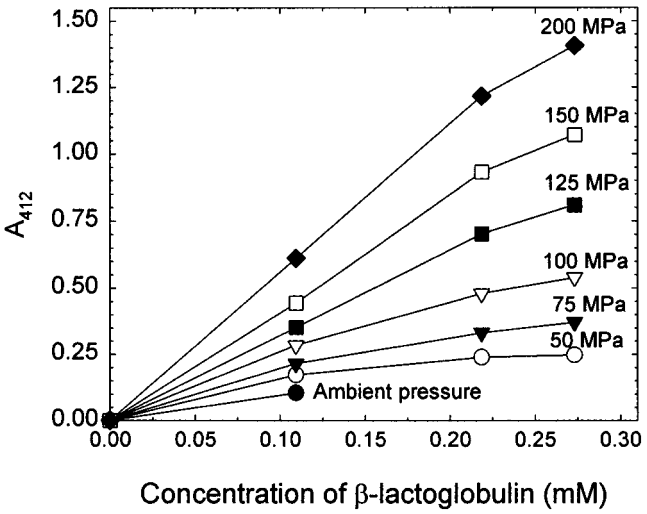

Figure 1. Absorbance at 412 nm (1 cm light path) following reaction between pressure-treated β-lactoglobulin (pressure as indicated for 30 min at 15 °C) and Ellman's reagent for various β-lactoglobulin concentrations as described under Materials and Methods. For a standard curve also valid for ambient pressure, see Stapelfeldt et al. (1997a).

Calculations. The nonlinear regression analysis was based on the Marquardt−Levenberg algorithm and performed within the framework of Sigma Plot 3.0 (Jandel, Erkrath, Germany).

RESULTS AND DISCUSSION

Of the five cysteines of bovine β-lactoglobulin, four form two intramolecular disulfide bridges and only Cys-121 is present as a free thiol group (Monaco et al., 1987). In native β-lactoglobulin, this thiol is buried in the hydrophobic interior of the protein and is only marginally reactive with reagents such as the classic Ellman's reagent (Ellman, 1959). However, pressure treatment exposes the thiol group to the solvent and, as seen from the result presented in Figure 1, even a moderately high pressure such as 50 MPa increases the reactivity toward Ellman's reagent significantly. The analytical reaction is a disulfide interchange between the reagent and the protein:

$$\text{R}-\text{S}-\text{S}-\text{R (Ellman's reagent)} + \text{peptide}-\text{S}^- \rightleftarrows \text{R}-\text{S}-\text{S}-\text{peptide} + \text{R}-\text{S}^- \quad (1)$$

and it is noteworthy that for native β-lactoglobulin and β-lactoglobulin exposed to moderately high hydrostatic pressure, the absorbance due to $R-S^-$ (the yellow tautomeric thioquinone form of the 5-thio-2-nitrobenzoate anion) shows significant deviation from Lambert−Beer's law (Figure 1), which for the native protein may indicate aggregation. From the reaction volume of −98 mL mol^{-1} (Stapelfeldt et al., 1996) for the pressure denaturation process, 225 MPa corresponds to 99% denaturation, which is considered fully denatured with the thiol group exposed. For increasing pressure the expected linearity is extended to an increasing protein concentration range, providing evidence of formation of partly pressure-denatured states of β-lactoglobulin at lower pressure, which may be considered as precursors of the fully pressure-denatured β-lactoglobulin, and in

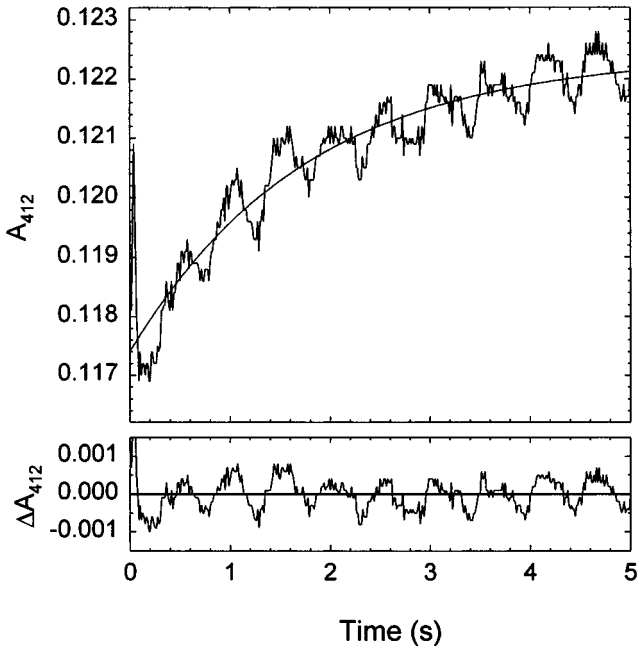

Figure 2. Absorbance at 412 nm during reaction between 0.0505 mM native β-lactoglobulin and 1.00 mM Ellman's reagent in aqueous 0.16 M NaCl with pH 7.61, 50 mM Tris buffer at 25.0 °C using the stopped-flow technique. Lower panel shows residuals ($\Delta A_{412} = \Delta A_{412,\text{actual}} - \Delta A_{412,\text{fit}}$) from nonlinear regression analysis: $A(t) = a + b \exp(-k'_{\text{obs}}t)$ corresponding to the smooth curve in the upper panel from which a pseudo-first-order rate constant of $k'_{\text{obs}} = 0.57 \pm 0.03$ s^{-1} is obtained.

which the thiol is only partly accessible to reagents in the solvent. The dynamic method previously developed for optimization of Ellman's method (Stapelfeldt et al., 1997a) based on stopped-flow absorption spectrometry was used to further characterize the different degree of exposure of the thiol group. Even for native β-lactoglobulin, where the degree of exposure is very limited, a meaningful time trace corresponding to the increasing absorption at 412 nm was obtained, which is further seen (Figure 2) to adequately described a (pseudo) first-order rate constant, k_{obs}:

$$\text{d[thioquinone]/d}t = k_{\text{obs}}[\beta\text{-lg}][\text{R}-\text{S}-\text{S}-\text{R}] = k'_{\text{obs}}[\beta\text{-lg}] \quad (2)$$

For conditions of excess of Ellman's reagent, [R−S−S−R] ≫ [β-lg], the pseudo-first-order rate constant is converted to a second-order rate constant

$$k_{\text{obs}} = k'_{\text{obs}}/[\text{R}-\text{S}-\text{S}-\text{R}] \quad (3)$$

which, notably, only applies to the actual pH (Stapelfeldt et al., 1997a). For β-lactoglobulin exposed to increasing hydrostatic pressure, the thiol group becomes increasingly reactive with respect to both rate and degree of reaction (Figure 3). As may be seen from the residuals in Figure 3 for the dynamic characterization, the kinetics was, for all pressures investigated, described by a first-order reaction. The observed second-order rate constant for the reaction between Ellman's reagent and β-lactoglobulin (at pH 7.61, 0.16 M NaCl, and 25 °C) was found to change from 5.7×10^2 L mol^{-1} s^{-1} for native β-lactoglobulin to 1.6×10^3 L $\text{mol}^{-1}\text{s}^{-1}$ for pressure-denatured β-lactoglobulin. As may be seen from Figure 4, the transition in the rate by which the

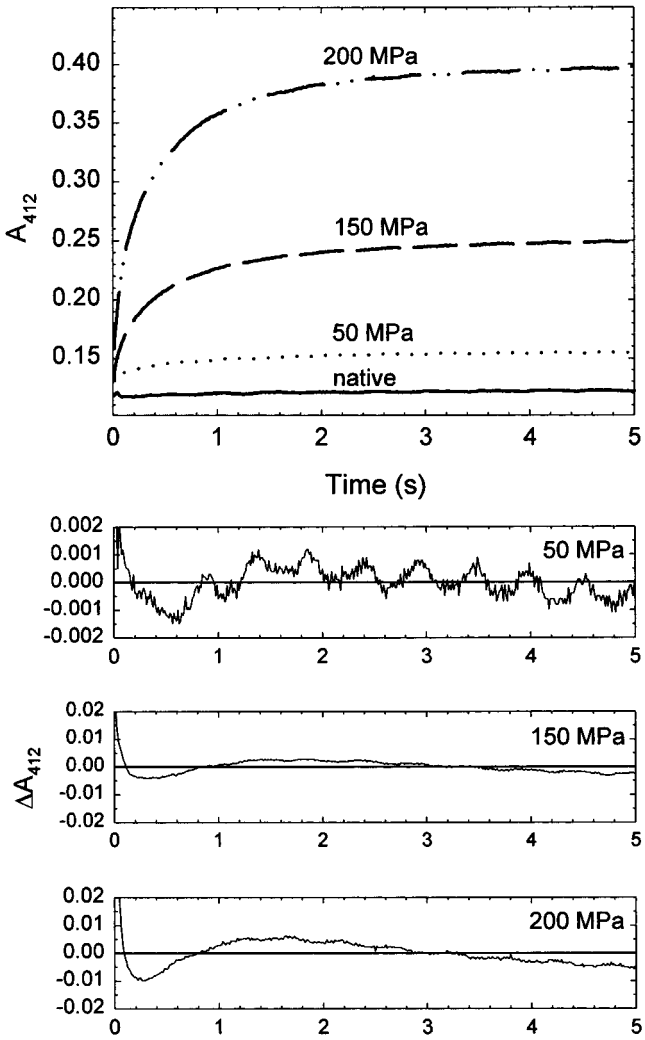

Figure 3. Absorbance at 412 nm during reaction between 0.0505 mM pressure-treated β-lactoglobulin and 1.00 mM Ellman's reagent in aqueous 0.16 M NaCl with pH 7.61, 50 mM Tris buffer at 25.0 °C using the stopped-flow technique. Pressure treatments were as indicated for 30 min at 15 °C prior to kinetic experiments. Lower panels show residuals ($\Delta A_{412} = \Delta A_{412,\text{actual}} - \Delta A_{412,\text{fit}}$) from nonlinear regression analysis: $A(t) = a + b \exp(-k'_{\text{obs}}t)$ from which pseudo-first-order rate constants (k'_{obs}) are obtained. For residual native β-lactoglobulin, see Figure 2.

thiol participates in the disulfide exchange reaction occurs ∼50 MPa, in contrast to the transition in the exposure of the thiol group, as characterized by the degree of reaction with Ellman's reagent, which occurs ∼140 MPa. The degree of exposure levels off at ∼250 MPa and can be described as a two-state equilibrium

$$\text{native } \beta\text{-lg} \rightleftarrows \text{denatured } \beta\text{-lg} \quad (4)$$

for which the equilibrium constants at different pressures (P) are calculated from

$$K_{\text{DEN}}(P) = \frac{[\text{DEN-}\beta\text{-lg}]}{[\text{NAT-}\beta\text{-lg}]} = \frac{\alpha_{\text{R-SH}}(P) - \alpha_{\text{R-SH}}(\text{NAT})}{\alpha_{\text{R-SH}}(\text{DEN}) - \alpha_{\text{R-SH}}(\text{NAT})} \quad (5)$$

where $\alpha_{\text{R-SH}}(\text{NAT})$ and $\alpha_{\text{R-SH}}(\text{DEN})$ are the fractions of the thiol group reacting with Ellman's reagent for the native and denatured β-lactoglobulin, respectively, and $\alpha_{\text{R-SH}}(P)$ is the fraction at pressure P. The inset in Figure 4 shows $K_{\text{DEN}}(P)$, as calculated according to eq

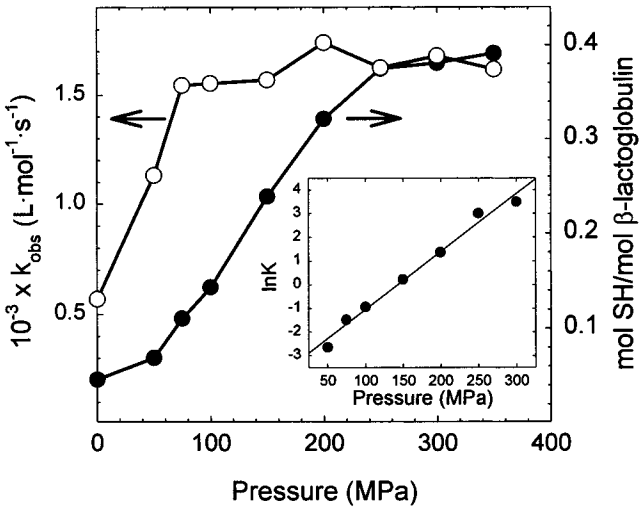

Figure 4. Thiol exposure (●), as measured relative to an L-cysteine standard, and thiol reactivity as k_{obs}, apparent second-order rate constant (○) for reaction between pressure-treated (at 15 °C for 30 min) β-lactoglobulin (0.11 mM in 0.16 M NaCl, 50 mM Tris buffer, pH 7.61) and Ellman's reagent as a function of pressure. (Inset) Equilibrium constant for pressure denaturation as calculated from degree of thiol exposure for 0.11 mM β-lactoglobulin and plotted according to $\ln K = -(\Delta V°/RT)P + \text{const}$, from which a reaction volume of $\Delta V° = -61 \pm 3$ mL/mol for β-lactoglobulin denaturation at 25 °C is calculated. Arrows indicate axis for each curve.

Table 1. Thermal Denaturation of β-Lactoglobulin As Characterized by Thiol Exposure and Thiol Reactivity[a]

temp (°C)	mol of −SH/mol of β-lg	rate (L mol^{-1} s^{-1})
25	0.048	569 ± 32
50	0.051	961 ± 17
60	0.052	916 ± 23
65	0.054	1194 ± 21
70	0.101	1288 ± 18
75	0.142	1517 ± 21
80	0.218	1425 ± 18
85	0.335	1527 ± 24
90	0.377	1417 ± 20
95	0.372	1487 ± 21

[a] Temperature applied to 0.11 mM β-lactoglobulin (in 0.16 M NaCl, pH 7.61) for 300 s followed by immediate cooling in ice. Rate measured at 25.0 °C.

5, at different pressures plotted according to the standard equation (van Eldik, 1986)

$$\ln K_{DEN}(P) = -(\Delta V°/RT)P + \text{const} \qquad (6)$$

from which a reaction volume of $\Delta V° = -61 \pm 3$ mL mol^{-1} and a half-denaturation pressure of 140 MPa are calculated. The linear dependence of $\ln K_{DEN}(P)$ as a function of pressure is in agreement with the assumption of a two-state equilibrium. However, the half-denaturation as assigned by dynamic method occurs at 50 MPa and the latter value seems to be indicative of a predenatured "melted" state in which the (small) fraction exposed to the solvent reacts more rapidly. Clearly, further characterization of this pressure-melted state will depend on combining different physical measurements, and work is in progress using fluorescence depolarization (Stapelfeldt and Skibsted, 1997).

A comparison between the static and dynamic methods for characterization of thiol exposure in denatured β-lactoglobulin was also made for thermal denaturation. As may be seen from the results presented in Table 1, the dynamic characterization using stopped-flow absorption spectrometry also for thermal denaturation indicated half-denaturation for milder conditions (lower temperature) than the static method using the degree of thiol exposure. For comparison with pressure treatment, a holding time of 5 min at the elevated temperature was chosen because of repeatability due to small effects of heating/cooling to total heat treatment and for being more readily applicable to industry than a much shorter laboratory heat treatment. As may be seen from the results in Table 1, half-denaturation occurs between 60 and 65 °C as determined by the reaction rate, but between 75 and 80 °C as determined by the degree of exposure. Notably, both the dynamic and the static methods yield comparable results for thiol exposure when fully pressure-denatured β-lactoglobulin (Figure 4) is compared with thermal-denatured β-lactoglobulin (Table 1).

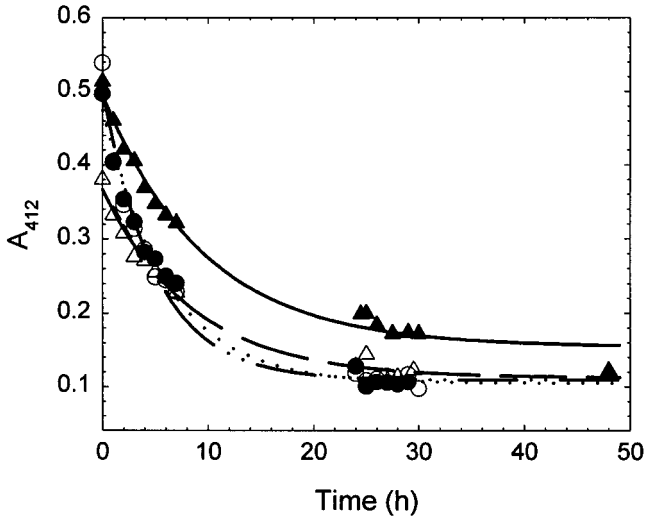

Figure 5. Exposure of thiol group in β-lactoglobulin (0.11 mM in 0.16 M NaCl, 50 mM Tris buffer, pH 7.61) pressure treated at 150 MPa and 15 °C for 30 min, followed at ambient pressure at 5 °C (▲), 10 °C (△), 15 °C (●), and 20 °C (○) for up to 2 days determined as absorbance at 412 nm using Ellman's reagent. Curves are calculated using nonlinear regression analysis: $A(t) = a + b \exp(-k_{ren}t)$ from which first-order rate constants for renaturation (k_{ren}) are obtained.

For pressure-treated β-lactoglobulin, the thiol group exposure was found to be reversible. After depressurization, the thiol group exposure decreased as shown in Figure 5 (treatment at 150 MPa for 30 min), and the rate by which the thiol exposure decreased could be described by first-order kinetics:

$$A_{412} = a + b \exp(-k_{ren}t) \qquad (7)$$

as may seen for temperature conditions of 5, 10, 15, and 20 °C in Figure 5. In a separate experiment, a solution of 0.11 mM pressure-denatured (150 MPa, 30 min, 15 °C) β-lactoglobulin was left at ambient presure for 2 days, in which period A_{412} changes from 0.38 immediately after denaturation to 0.102 after 2 days at 10 °C at ambient pressure, and subsequently re-exposed to 150 MPa for 30 min at 15 °C, which yielded a thiol exposure corresponding to an absorbance of $A_{412} = 0.34$. The decreased reactivity of the thiol group in pressure-denatured β-lactoglobulin is accordingly not the result of oxidation, but rather due to a renaturation of the denatured β-lactoglobulin. This finding confirms the results recently reported by Funtenberger et al. (1997), who concluded that pressure treatment did not induce

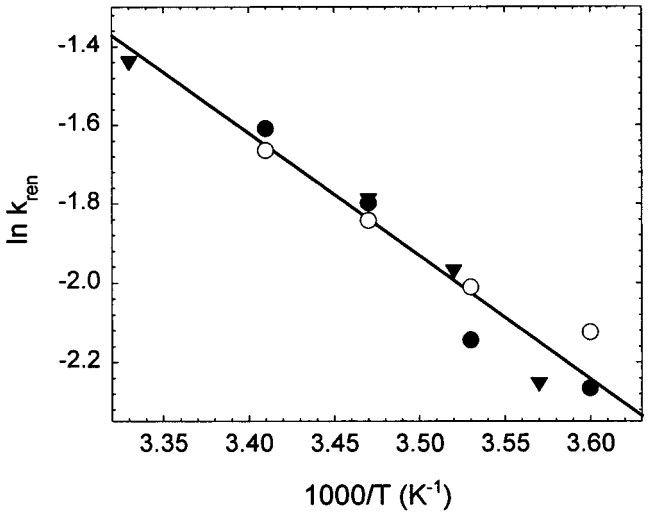

Figure 6. Arrhenius plot for thermal refolding of pressure-denatured β-lactoglobulin. Rate constants were obtained as described in the legend to Figure 5. Pressure treatment prior to refolding was 30 min at 15 °C at 150 MPa (●), 200 MPa (○), or 250 MPa (▼), respectively. Full line is common regression line: $\ln k_{ren} = (8.94 \pm 0.07) - (3105 \pm 253)T^{-1}$.

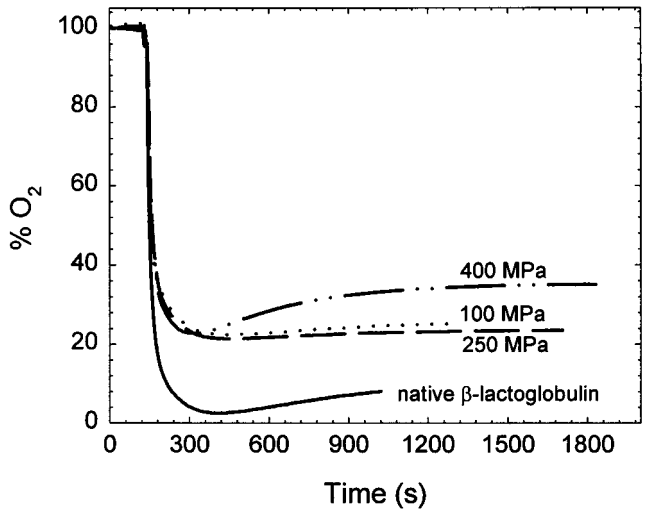

Figure 7. Progression of peroxidation in a 5.9 w/w % linoleic acid emulsion (Tween 20-stabilized oil-in-water with pH 6.8) at 25.0 °C measured electrochemically as oxygen depletion. Peroxidation was initiated after 120 s with metmyoglobin (0.2 mM) in air-saturated emulsion (air-saturated assigned as 100% O_2) with native or pressure-treated β-lactoglobulin (pressure as indicated for 30 min at 15.0 °C).

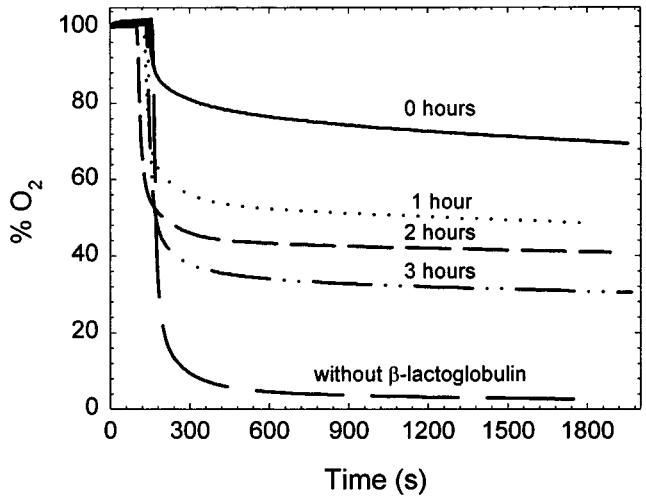

Figure 8. Progression of peroxidation in a 5.9 w/w % linoleic acid emulsion (Tween 20-stabilized oil-in-water with pH 6.8) at 25.0 °C measured electrochemically as oxygen depletion. Peroxidation was initiated after 120 s with metmyoglobin (0.2 mM) in air-saturated emulsion (air-saturated assigned as 100% O_2) with (or without) pressure-treated β-lactoglobulin (200 MPa for 30 min at 15.0 °C). Time indicated is time elapsed from end of pressure treatment during which period the pressure-treated β-lactoglobulin solution [0.11 mM in 0.16 M NaCl, pH 7.61 (Tris-buffer)] was kept at 25 °C at ambient pressure.

oxidative processes related to the thiol groups in β-lactoglobulin. We have no ready explanation of the higher thiol reactivity at 5 °C, but the slower rate may yield a different conformation or be an early sign of cold denaturation.

The rate constant obtained from the kinetic analysis presented in Figure 5 is accordingly assigned to the renaturation process. The temperature dependence for this process is rather modest and, using the Arrhenius equation and transition state theory, an activation enthalpy of $\Delta H^{\ddagger} = 23 \pm 2$ kJ mol^{-1} and an activation entropy of $\Delta S^{\ddagger} = -247 \pm 7$ J mol^{-1} K^{-1} together with a rate constant k_{ren} of 6.2×10^{-5} s^{-1} at 25 °C (corresponding to a half-life of 3.1 h) were calculated (Figure 6). These kinetic parameters apply for renaturation of β-lactoglobulin denatured at 100, 200, or 250 MPa, since a statistical analysis of the thermal renaturation of β-lactoglobulin denatured at each of these pressures provided activation parameters that were not significantly different (cf. Figure 6). The renaturation of pressure-denatured β-lactoglobulin is thus an entropy-controlled process, and the remarkable negative value for the entropy of activation reflects the high degree of order in the transition state for the refolding, which probably involves several hydrogen-bonded water molecules.

The thiol group in β-lactoglobulin is expected to function as a chain-breaking antioxidant forming rather stable thiyl radicals in the reaction with lipid peroxyl radicals:

$$ROO^{\bullet} + R{-}SH \rightarrow ROOH + R{-}S^{\bullet} \qquad (8)$$

Due to the low H abstraction reactivity of the thiol, lipid autoxidation is halted, and whey protein can be used as antioxidant (Browdy and Harris, 1997). In milk powder, lipid peroxidation is, in agreement herewith, found to depend on the preheat treatment and resulting exposure of the thiol group in the whey proteins (Stapelfeldt et al., 1997b). Using a model system based on linoleic acid peroxidation (Mikkelsen et al., 1992), pressure was clearly found to induce antioxidative properties in β-lactoglobulin, as may be seen from the oxygen depletion curves presented in Figure 7. For native β-lactoglobulin, oxygen was almost completely depleted in 6 min when oxidation was initiated by metmyoglobin. However, pressure treatment of β-lactoglobulin reduced the rate and degree of oxygen consumption. The thermal renaturation of pressure-denatured β-lactoglobulin was further confirmed when the effect of pressure-denatured β-lactoglobulin as antioxidant was compared in the antioxidant assay at 25 °C immediately after depressurizing and after 1, 2, or 3 h at ambient pressure after the pressure treatment. As may be seen from Figure 8, the decrease in antioxidative effect nicely corresponds to a half-life for renaturation of ≈3 h as determined in the kinetic analysis.

In conclusion, adjustment of the functional properties of β-lactoglobulin by pressure treatment can be described quantitatively as shown for thiol group exposure and antioxidative properties. As refolding measured by thiol reactivity (Figure 5) is slow and the thiol of β-lactoglobulin has been found to induce thiol/disulfide exchange reactions (Jegouic et al., 1997), pressurization could be used to tune cross-linking reactivity, thereby improving film formation at interfaces and protein effectivity as emulsifiers. The combination of dynamic and static methods has, moreover, shown that the reversible pressure denaturation of β-lactoglobulin ∼140 MPa seems to be preceded by a pressure-melted or predenatured state. In our current research efforts, we are extending the combined use of these methods to β-lactoglobulin subjected to higher pressures, at which denaturation gradually becomes irreversible.

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