Spectrofluorometric Characterization of β-Lactoglobulin B Covalently Labeled with 2-(4′-Maleimidylanilino)naphthalene-6-sulfonate

Henrik Stapelfeldt,[†] Carl E. Olsen,[‡] and Leif H. Skibsted*

Department of Dairy and Food Science and Chemistry Department, Royal Veterinary and Agricultural University, DK-1958 Frederiksberg C, Denmark

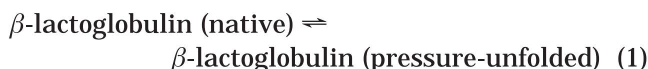
Bovine β-lactoglobulin, genetic variant B, has been labeled with 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid through covalent attachment through the Cys-121 thiol group for the study of stepwise pressure denaturation of this whey protein by fluorescence spectroscopy. The labeling was performed under nondenaturing conditions with a factor of 5 excess of the fluorophore in dimethylformamide/water (1:10) to yield the whey protein highly labeled after chromatographic separation. MALDI-TOF mass spectroscopy confirmed labeling. The emission from the fluorophore, which is sensitive to the microenvironment, has been characterized for the labeled protein (aqueous pH 7.4 solution, 25 °C) and has a $\lambda_{em,max} = 410$ nm ($\lambda_{ex,max} = 318$ nm) with a fluorescence lifetime of 6.1 ± 0.2 ns. Fluorescence anisotropy increases and fluorescence quantum yield ($\Phi_f = 0.103$ at 320 nm) decreases with increasing excitation wavelength. For increasing hydrostatic pressure, fluorescence quantum yield showed a minimum at ∼50 MPa, corresponding to the pre-denatured "pressure-melted" state in which thiol reactivity previously was found to increase prior to reversible protein unfolding.

Keywords: *β-Lactoglobulin; fluorescence labeling; 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid*

INTRODUCTION

The major whey protein of cows' milk, β-lactoglobulin, is at neutral pH a noncovalent dimer consisting of two identical monomers of molecular mass 18.3 kDa, each containing one reactive thiol, Cys121, and two disulfide bridges, Cys66−Cys160 and Cys106−Cys119 (Monaco et al., 1987). The thiol group is important in determining the properties of β-lactoglobulin especially after partial or domain-specific denaturation, when it becomes exposed to water and may act as an antioxidant (Møller et al., 1998) or initiate a sulfide/disulfide intermolecular interchange reaction eventually leading to polymerization as known for thermal gel formation (Roefs and de Kruif, 1994). The binding of molecules such as vitamin A (Papiz et al., 1986) and free fatty acids (Perez et al., 1989) in the inner calyx of β-lactoglobulin, which is important for dissolution and absorption of hydrophobic nutrients, is also sensitive to minor changes in the protein conformation.

The reactivity of Cys121 is largely increased by unfolding of the protein by heat treatment (Sawyer, 1968), which is a standard unit process in dairy technology, and by high hydrostatic pressure (Tanaka et al., 1996), which is considered to be a promising technology for the food industry in relation to inactivation of bacteria in foods and for change of functional properties of proteins. β-Lactoglobulin is among the most pressure-sensitive proteins known, having a reaction volume of -73 mL mol^{-1} (Stapelfeldt and Skibsted, 1999), corresponding to a half-denaturation pressure of 123 MPa for the process



$$\beta\text{-lactoglobulin (native)} \rightleftharpoons \beta\text{-lactoglobulin (pressure-unfolded)} \quad (1)$$

Although in situ high-pressure measurements of intrinsic fluorescence provided results in agreement with the two-stage denaturation equilibrium of eq 1 (Stapelfeldt et al., 1996), dynamic measurement of thiol reactivity using Ellman's reagent and stopped-flow absorption spectrometry clearly gave indication of a stepwise denaturation process in which the thiol is becoming increasingly reactive at lower pressures than required for pressure denaturation (Møller et al., 1998). Such pressure-melted or pre-denatured states of protein are of great theoretical interest and of practical importance for development of new dairy products (de Jong and van der Linden, 1998), and we are exploring methods to detect conformational changes in different regions of pressurized β-lactoglobulin in order to describe the thermodynamics of the individual steps in pressure (and thermal) denaturation. One such strategy is selective fluorescent labeling of amino acid side chains followed by time-resolved fluorescence spectroscopy. Covalent labeling of the thiol of bovine β-lactoglobulin also opens new ways for measuring radiationless energy transfer to bound molecules such as vitamin A and fluorescent fatty acid analogues, which further may help to understand the effects of hydrostatic pressure on the conformation of β-lactoglobulin. For development of the labeling

* Address correspondence to this author at Food Chemistry, Department of Dairy and Food Science, Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark (telephone +45 35283221; fax +45 35283344; e-mail ls@kvl.dk).

[†] Present affiliation: Institute of Food Research and Nutrition, Danish Veterinary and Food Administration, Soendervang 4, DK-4100 Ringsted, Denmark.

[‡] Chemistry Department.

10.1021/jf9812026 CCC: $18.00 © 1999 American Chemical Society
Published on Web 09/14/1999

procedure and characterization of the adduct formed, we chose the B-variant of β-lactoglobulin, which in high purity was available in our laboratory and thus allowed for initial investigations of pressure effects without complicating the interpretation of the observations by differences in aggregation of the two genetic variants (Pessen et al., 1985). Although the labeling of proteins with maleimide-containing probes has been described in the literature (Hermanson, 1996), this study presents, to the best of our knowledge, the first labeling procedure of β-lactoglobulin with 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS), in which steps are taken not to cause severe denaturation of the protein during labeling, together with a detailed photophysical characterization of the fluorescent adduct.

EXPERIMENTAL PROCEDURES

Materials. β-Lactoglobulin B (98%, as determined by capillary electrophoresis) was isolated from acid whey of fresh skim milk of homozygotous cows from the field station of the Royal Veterinary and Agricultural University in Taastrup by anion-exchange chromatography followed by ultrafiltration using extensive diafiltration to remove lactose and salts, and finally the product was lyophilized. To gain as native a protein as possible, the temperature of any purification step never exceeded 45 °C, as described by Kristensen et al. (1998).

The sodium salt of MIANS was obtained from Molecular Probes (Eugene, OR) and used without further purification. 1,4-Bis(4-methyl-5-phenyl-2-oxazolyl)benzene (dimethyl-POPOP) purissum grade was obtained from Fluka (Buchs, Switzerland). Other chemicals were of analytical grade, and water was purified through a Milli-Q purification train (Millipore, Bedford, MA).

Purification of Labeled Protein. Micropreparative chromatographic purification of labeled β-lactoglobulin B was performed at ambient temperature with a SMART system (Pharmacia, Uppsala, Sweden) mounted with a Superdex 75 PC 3.2/30 size exclusion column and a PC 3.2/10 desalting column, using a 50-mL loop and a 0.10 mL min^{-1} flow of the eluents, 50 mM TRIS buffer (pH 7.4, $I = 0.16$) and water, respectively. Concentration of the purified labeled protein was performed with ULTRAFREE-MC tubes from Millipore (Bedford, MA) in a Sorvall RMC14 centrifuge operated at 8000g and 15 °C for 6 min.

Absorption spectroscopy was carried out with an HP8452A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA).

Fluorescence Spectrometry. Measurements were carried out with an SLM 48000S multifrequency, phase modulation spectrofluorometer (SLM Instruments Inc., Urbana, IL) with a 450-W Xe arc lamp, SLM MC200 excitation, and MC320 emission monochromator mounted with Hamamatsu R928P photomultiplier tubes at a right angle to the line of illumination. Emission spectra reported are corrected for instrument sensitivity characteristics, and the excitation spectra reported are corrected for lamp spectral characteristics according to the method of ratio recording using a solution of rhodamine 101 (3 g/L ethylene glycol) as quantum counter (Eaton, 1988). Measurements were made in thermostated quartz cells with right angle observation at the center. Air-saturated solutions were used for the measurements. Fluorescence intensity at elevated pressure was measured in a thermostated SLM HPSC-3K high-pressure spectroscopy cell with a closed 0.75-mL quartz bottle with a Teflon lid. Fluorescence anisotropy measurement was carried out using motorized Glan-Thompson calcite polarizers mounted in T-format and collecting emission through the emission monochromator and a Schott KV-389 filter.

Fluorescence quantum yield (Φ_f) was determined using the optically dilute concept of Demas and Crosby (1971), using a solution of quinine sulfate in aqueous 0.50 M H_2SO_4 as fluorescence quantum yield reference with $\Phi_f = 0.546$ (Melhuish, 1961).

Emission Lifetimes. The fluorescence lifetime was determined by the multifrequency phase and modulation technique using an Omnichrome Series 2074 cw He−Cd laser (Omnichrome, Chino, CA) as a light source (Schott UG11 band-pass filter) in the SLM 48000S spectrofluorometer with the intensity of the exciting light from the laser sinusoidally modulated by means of a Pockel's cell. Phase and modulation lifetimes (t_p and t_m) were then calculated according to

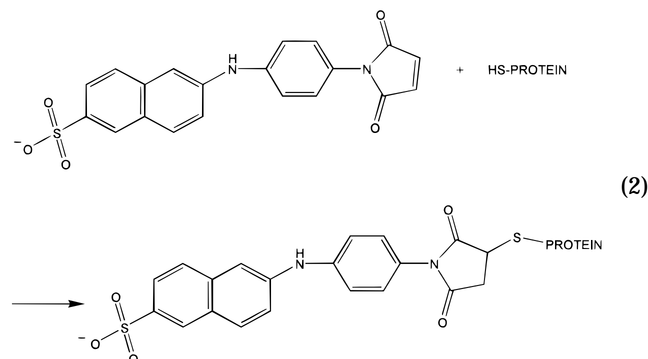
$$\tau_p = \frac{\tan \phi}{\omega} \qquad \tau_m = \frac{\sqrt{(1/m^2 - 1)}}{\omega}$$

where ϕ is the phase shift in degrees, m the relative modulation, and ω the angular modulation frequency ($\omega = 2\pi \times$ frequency). Dimethyl-POPOP dissolved in absolute ethanol was used as a fluorescence lifetime standard, with a lifetime of 1.45 ns (Lakowicz et al., 1981). For the lifetime measurements, the exciting light was polarized parallel to the vertical laboratory axis, while the emission was viewed through a Glan-Thompson calcite polarizer oriented at 54.7° ("magic angle" conditions). Emission from the samples was collected through a Schott KV 389 cut-on filter.

Mass Spectrometry. A TofSpecE MALDI-TOF mass spectrometer from Micromass (Wythenshawe, U.K.) was used for determination of molecular weights of labeled protein.

RESULTS AND DISCUSSION

High-yield labeling of the free thiol group of β-lactoglobulin with MIANS was intended according to the following addition reaction (Hermanson, 1996):



By trial and error, it was found that a molar excess of MIANS of a factor of ~5 relative to β-lactoglobulin was needed to obtain a high degree of conversion to the labeled protein at nondenaturing temperature, that is, below 45 °C. Also, MIANS had to be dissolved in a solvent other than water, in which it is only sparingly soluble, and a range of solvents were tested to ensure that addition of the nonaqueous solvent for up to 10% of the total volume did not induce other reactions in the protein. Among the solvents, methanol, ethanol, 2-propanol, dimethyl sulfoxide, and dimethylformamide, some induced polymerization of β-lactoglobulin as may be seen for 2-propanol in Figure 1.

The increase in the degree of polymerization may be attributed to lowering of the dielectricity constant and the concomitant decrease in electrostatic repulsion between proteins [cf. Walstra (1996)]. Dimethylformamide and dimethyl sulfoxide did not cause severe polymerization, in accordance with their higher dielectricity constant compared with the alcohols. However, dimethylformamide was the solvent of choice, because mixing of the aqueous protein solution with dimethyl

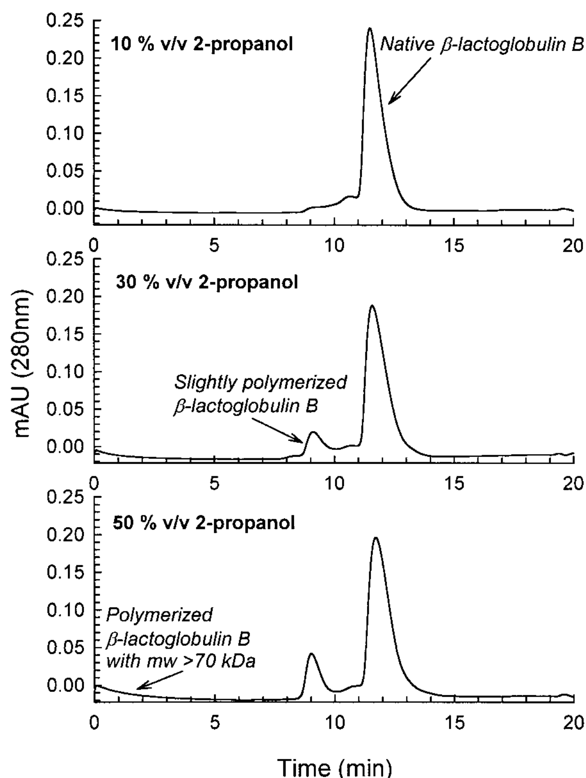


Figure 1. Formation of polymeric β-lactoglobulin B by increasing volume fraction of 2-propanol in the buffer, as observed by size exclusion HPLC using a Superdex 75 PC 3.2/30 column for the separation (for details see Experimental Procedures).

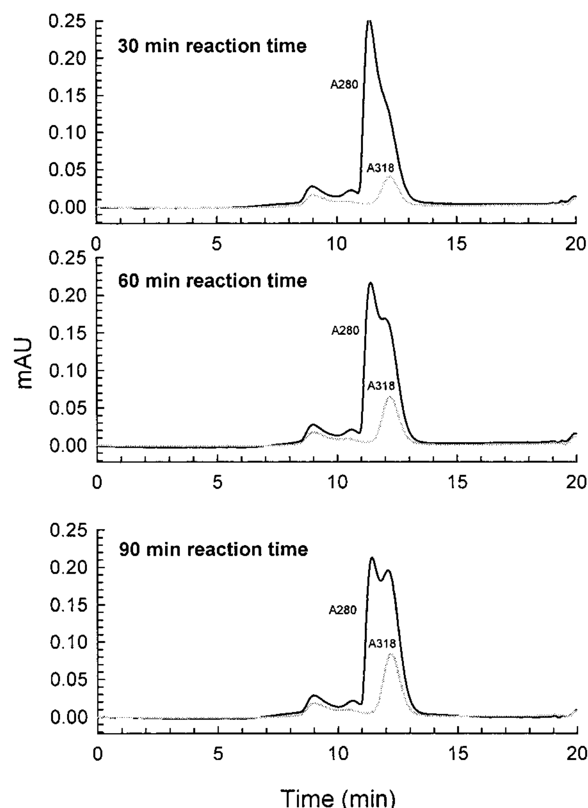


Figure 2. Time course of labeling of β-lactoglobulin B with MIANS, according to Scheme 1, as observed by size exclusion HPLC using a using a Superdex 75 PC 3.2/30 column for the separation and with simultaneous detection at 280 nm (protein) and 318 nm (label) (for details see Experimental Procedures).

sulfoxide was associated with a liberation of heat, which made cooling in ice necessary for the labeling reaction.

With MIANS in dimethylformamide, β-lactoglobulin was easily labeled, as may be seen from the time course of reaction in the size exclusion chromatograms of Figure 2. The reaction with MIANS apparently results in a more compact structure of labeled protein, as evidenced by the increased retention time of the adduct (12.2 min) compared to that of the unlabeled protein (11.5 min).

Using micropreparative size exclusion HPLC, the labeled protein was isolated from unreacted MIANS, dimethylformamide, unlabeled protein, and traces of polymerized protein. To free this labeled protein of salts prior to MALDI-TOF mass spectrometry, the sample was desalted with a Sephadex Superfine G-25 PC 3.2/10 column using water as eluent, and the fraction with labeled protein was collected. Subsequently, the somewhat diluted sample was concentrated by a factor of $\sim$20 using an ultrafiltration membrane unit for centrifuge tubes (6 min at 8000*g*). MALDI-TOF mass spectrometry confirmed the attachment of one MIANS label per β-lactoglobulin as seen in Figure 3.

It is well-known that the reaction of *N*-ethylmaleimide is not specific for cysteine and may react with lysine and histidine, especially at increased pH (Brewer and Riehm, 1967). The mass spectrogram, however, shows only the existence of protein with one label and unlabeled protein, and the labeling procedure therefore only involves one amino acid side chain. The overall labeling procedure is summarized in Scheme 1.

The labeled protein showed a bright violet fluorescence with an emission maximum at 410 nm, as may be seen from Figure 4.

The excitation spectrum, also shown in Figure 4, is due to at least two electron transitions as evidenced by the changes in fluorescence anisotropy, defined as

$$r = {}^{2}/_{5}[(3\cos^2\theta - 1)/2] \quad (3)$$

from 0.06 to 0.19 across the excitation band. θ is the angle between the emission and absorption dipoles within the fluorophore (Lakowicz, 1983). The excitation maximum at 318 nm is caused by the attached MIANS chromophore, and the peak at 275 nm is ascribed to excitation of an indole moiety, in accordance with the loss of polarization, which may be due to subsequent energy transfer to the label. From the emission intensity, the fluorescence quantum yield was calculated for four different wavelengths in the low-energy band of the excitation spectrum. The fluorescence quantum yield at 25 °C was found to decrease with excitation wavelength as seen in Table 1. This wavelength dependence cannot be explained by means of concomitant excitation of tryptophan due to overlapping absorption spectra and radiationless energy transfer from the indole moiety to the MIANS chromophore. From the fluorescence anisotropies apparent in Figure 4, using the general additivity of anisotropies (Lakowicz, 1983), and assuming only MIANS emission by energy transfer from the indole (r = 0.062) and by direct excitation (r = 0.191), <4% of the emission at 410 nm upon excitation at 320 nm can thus be ascribed for by energy transfer from the indole moiety. Moreover, the molar extinction coefficient of tryptophan is very low at wavelengths >320 nm, and no explanation for the, unexpected, wavelength depen-

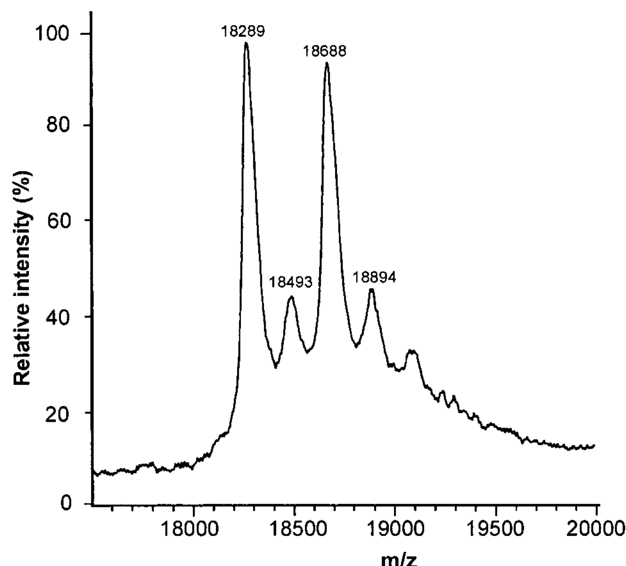


Figure 3. MALDI-TOF mass spectrogram of the MIANS−β-lactoglobulin adduct. Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) is the matrix absorbing the Nd:YAG pulse leading to final ionization of the protein. The peaks at *m/z* 18289 and 18493 are assigned to β-lactoglobulin B and (β-lactoglobulin B + sinapinic acid − H_2O), respectively, whereas peaks at *m/z* 18688 and 18894 are assigned to MIANS−β-lactoglobulin B and (MIANS−β-lactoglobulin B + sinapinic acid − H_2O), respectively. The mass difference between the two major peaks (399) corresponds within the instrument accuracy of the protein to the mass of the label (393.3 g/mol).

Scheme 1

Reaction for 30 min at room temperature:

500 ml 32 mM β-lactoglobulin (1.2 mg·ml^{-1}) in 50-mM Tris buffer, pH 7.4

+ 450 ml 50 mM Tris buffer, pH 7.4

+ 50 ml 1.6 mM MIANS in DMF (0.648 mg·ml^{-1})

↓

Micropreparative size-exclusion chromatography:

Pharmacia Superdex 75 PC 3.2/30 column, 50-ml injection loop, eluent: 50 mM Tris, pH 7.4 at 0.10 ml·min^{-1}, detection: A_{280}, and A_{318} and collection of labeled fraction.

↓

Concentration (ultrafiltration):

Millipore ULTRAFREE-MC tubes centrifuged at 8,000 g for 6 min at 15 °C.

↓

Storage at -18°C of concentrated labeled protein.

dence of the fluorescence quantum yield is obvious; elucidation of the photophysical mechanism behind it is beyond the scope of this study. The labeled β-lactoglobulin was further characterized by the fluorescence decay, which for 325 nm excitation was found to be monoexponential, in accordance with very limited energy transfer from the indole moiety and only one labeled amino acid side chain, corresponding to a lifetime of $\tau = 6.1 \pm 0.2$ ns at 25 °C, as may be seen from Figure 5.

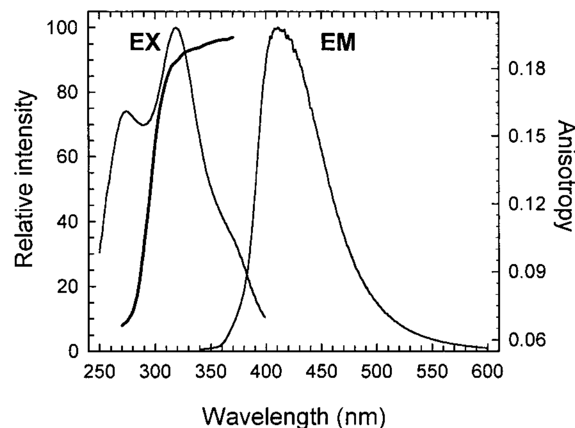


Figure 4. Excitation and emission spectra for MIANS-labeled β-lactoglobulin B in 50 mM Tris buffer (pH 7.4) at 25 °C (A_{320} = 0.02). The change in fluorescence anisotropy (bold line) indicates (at least) two transitions in the excitation spectrum.

Table 1. Fluorescence Quantum Yields, Φ_f, for MIANS-Labeled β-Lactoglobulin B in Aqueous pH 7.4 Solution at 25 °C[a]

λ_{ex} (nm)	Φ_f	λ_{ex} (nm)	Φ_f
320	0.103	330	0.071
325	0.077	335	0.061

[a] Determined relative to quinine sulfate in 0.5 M H_2SO_4, Φ_f = 0.546.

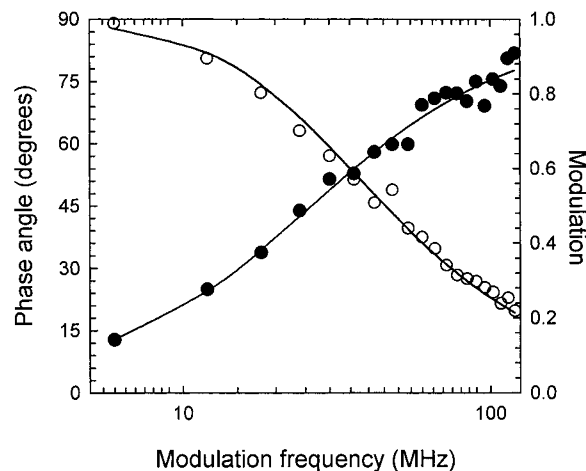


Figure 5. Multifrequency phase and modulation data for the MIANS-labeled β-lactoglobulin in 50 mM Tris buffer (pH 7.4) at 25 °C (A_{320} = 0.05). Full lines are the best fit to the phase angle data (○) and the modulation data (●), corresponding to a fluorescence lifetime of $\tau = 6.1 \pm 0.2$ ns ($\chi_R^2 = 2.74$).

The labeled β-lactoglobulin seems to be suitable for detailed denaturation studies. The fluorescence intensity thus shows a complex pressure dependence. Preliminary in situ fluorescence quantum yields for up to 125 MPa are shown in Figure 6.

When the protein is unfolding under pressure, the intensity decreases as the microenvironment of the probe is changing. However, for pressures >50 MPa, the intensity seems to increase again, observations that are clearly not in accordance with a simple two-step denaturation equilibrium. Pressure denaturation of β-lactoglobulin has previously been described as a three-step process including (i) an initial pressure-melted state for up to 50 MPa with partial collapse of the inner calyx and solvent exposure of the free thiol group, (ii) followed by a reversible denaturation with exposure of hydro-

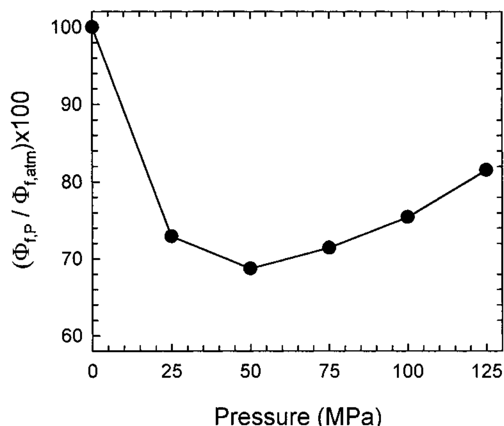


Figure 6. Pressure sensitivity of the fluorescence quantum yield of MIANS−β-lactoglobulin B adduct in 50 mM Tris buffer (pH 7.4) at 25 °C (A_{320} = 0.02), measured relative to the quantum yield of the adduct at ambient pressure (0.1 MPa).

phobic regions (half-denaturation at 123 MPa at ambient temperature and pH 7.4), and (iii) with irreversible denaturation with thiol−disulfide exchange at higher pressures (Stapelfeldt and Skibsted, 1999). The pressure-melted state was previously characterized by dynamic measurements of thiol reactivity toward Ellman's reagent using stopped-flow absorption spectrometry (Møller et al., 1998). It is encouraging that the thiol-labeled group yields exactly the same pressure profile for this initial change in protein conformation with a minimum for fluorescence quantum yield at 50 MPa. A further conclusion from the pressure dependence of the fluorescence intensity is that during the initial pressure change in conformation, the labeled thiol group is exposed to the polar solvent, resulting in a decrease in fluorescence intensity which, however, for further increase in pressure again is associated with more apolar amino acid residues of the protein observed by an intensity increase. These observations will now be studied in more detail to describe the pressure unfolding as a stepwise process also including temperature effects. Moreover, fluorescence labeling of the thiol group, inactivating this in thiol−disulfide interchange reactions, allows for the study of other molecular forces involved in aggregation processes of β-lactoglobulin, for example, interactions due to exposure of hydrophobic amino acid residues (Stapelfeldt and Skibsted, 1999) or dityrosine formation under oxidizing conditions (Østdal et al., 1996).

ACKNOWLEDGMENT

We are grateful to Dr. Kristian Rotvig Kristiansen for providing the purified β-lactoglobulin B and to Laurette Sosniecki, Astrid Bech Hansen, and Søren Dalby for excellent technical assistance.

LITERATURE CITED

Brewer, C. F.; Riehm, J. P. Evidence for possible nonspecific reactions between *N*-ethylmaleimide and proteins. *Anal. Biochem.* **1967**, *18*, 148−255.

de Jong, P.; van der Linden, H. J. L. J. Polymerization model for prediction of heat induced protein denaturation and viscosity changes in milk. *J. Agric. Food Chem.* **1998**, *46*, 2136−2142.

Demas, J. N.; Crosby, G. A. The measurement of photoluminescence quantum yields. A review. *J. Phys. Chem.* **1971**, *75*, 991−1024.

Eaton, D. F. Reference materials for fluorescence measurement. *J. Photochem. Photobiol. B: Biol.* **1988**, *2*, 523−531.

Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, CA, 1996; p 148.

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.

Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Plenum: New York, 1983; pp 111−153.

Lakowicz, J. R.; Cherek, H.; Balter, A. Correction of timing errors in photomultiplier tubes used in phase-modulation fluorometry. *J. Biochem. Biophys. Methods* **1981**, *5*, 131−146.

Melhuish, W. H. Quantum efficiencies of fluorescence of organic substances: effect of solvent and concentration of the fluorescent solute. *J. Phys. Chem.* **1961**, *65*, 229−235.

Møller, R. E.; Stapelfeldt, H.; Skibsted, L. H. Thiol reactivity in pressure-unfolded β-lactoglobulin. Antioxidative properties and thermal refolding. *J. Agric. Food Chem.* **1998**, *46*, 425−430.

Monaco, H. L.; Zanotti, G.; Spadon, P.; Bolognesi, M.; Sawyer, L.; Eliopoulos, E. E. Crystal structure of the trigonal form of bovine beta-lactoglobulin and of its complex with retinol at 2.5 Å resolution. *J. Mol. Biol.* **1987**, *197*, 695−706.

Østdal, H.; Daneshvar, B.; Skibsted, L. H. Reduction of ferrylmyoglobin by β-lactoglobulin. *Free Radical Res.* **1996**, *24*, 429−438.

Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglobulin and its similarity to palsma retinol-binding protein. *Nature* **1986**, *324*, 383−385.

Pérez, M. D.; Diaz de Villegas, C.; Sánchez, L.; Aranda, P.; Ena, J. M.; Calvo, M. Interaction of fatty acids with β-lactoglobulin and albumin from ruminant milk. *J. Biochem.* **1989**, *106*, 1094−1097.

Pessen, H.; Purcell, J. M.; Farrell, H. M., Jr. Proton relaxation rates of water in dilute solutions of β-lactoglobulin. Determination of cross relaxation and correlation with structural changes by use of two genetic variants of a self-associating globular protein. *Biochim. Biophys. Acta* **1985**, *828*, 1−12.

Roefs, S. P. F. M.; de Kruif, C. G. A model for the denaturation and aggregation of β-lactoglobulin. *Eur. J. Biochem.* **1994**, *226*, 883−889.

Sawyer, W. H. Heat denaturation of bovine β-lactoglobulins and relevance of disulfide aggregation. *J. Dairy Sci.* **1968**, *51*, 323−329.

Stapelfeldt, H.; Skibsted, L. H. Pressure denaturation of β-lactoglobulin studied by intrinsic fluorescence polarization, Rayleigh scattering, radiationless energy transfer and hydrophobic fluoroprobing. *J. Dairy Res.* **1999**, in press.

Stapelfeldt, H.; Petersen, P. H.; Kristiansen, K. R.; Qvist, K. B.; Skibsted, L. H. Effect of high hydrostatic pressure on the enzymic hydrolysis of β-lactoglobulin B by trypsin, thermolysin and pepsin. *J. Dairy Res.* **1996**, *63*, 111−118.

Tanaka, N.; Tsurui, Y.; Kobayashi, I.; Kunugi, S. Modification of the single unpaired sulfhydryl group of β-lactoglobulin under high pressure and the role of the intermolecular S−S exchange in the pressure denaturation. *Int. J. Biol. Macromol.* **1996**, *19*, 63−68.

Walstra, P. Dispersed systems: Basic Considerations. In *Food Chemistry*; Fennema, O. R., Ed.; Dekker: New York, 1996; pp 95−155.

Received for review November 5, 1998. Revised manuscript received July 12, 1999. Accepted July 19, 1999. This research was sponsored by the Danish Dairy Board and the FØTEK program through LMC−Center for Advanced Food Studies.

JF9812026