Modification of Bovine β-Lactoglobulin by Glycation in a Powdered State or in an Aqueous Solution: Effect on Association Behavior and Protein Conformation

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The effect of glycation with lactose on the association behavior and conformational state of bovine β-lactoglobulin (β-LG) was studied, using size exclusion chromatography, polyacrylamide gel electrophoresis, proteolytic susceptibility, and binding of a fluorescent probe. Two modification treatments were used, i.e., aqueous solution glycation and dry-way glycation. The results showed that the latter treatment did not significantly alter the nativelike behavior of the protein while the former treatment led to important structural changes. These changes resulted in a specific denatured β-LG monomer, which covalently associated via the free thiol group. The homodimers thus formed and the expanded monomers underwent subsequent aggregation into a high molecular weight species, via noncovalent interactions. The association behavior of glycated β-LG is discussed with respect to the known multistep denaturation/aggregation process of nonmodified β-LG.

Keywords: *β-Lactoglobulin; glycation; denaturation; aggregation; conformation*

INTRODUCTION

Bovine β-LG is widely used as a functional ingredient in whey-containing food formulations by the food industry (De Wit, 1989). The thermal behavior of β-LG has been extensively investigated because heating, the most commonly applied process in food technology, affects its functional properties. Several recent studies, carried out in water or in low-salt concentrations at relatively low temperatures (60−75 °C) and near neutral pH, have described the process of denaturation and aggregation of β-LG as a multistep mechanism (Roefs and De Kruif, 1994; Iametti et al., 1995, 1996; Hoffmann and Van Mil, 1997; Hoffmann et al., 1997a,b; Prabakaran and Damodaran, 1997; Qi et al., 1997). The overall sequential model involves the dissociation of the natural dimer, conformational changes, and subsequent aggregation of unfolded denatured protein monomers via hydrophobic interactions and thiol−disulfide interchange reactions, the latter being predominant.

The heat-induced denaturation and aggregation process may be affected by several modifications of native β-LG. Chemical modification of the protein, including acylation, phosphorylation, esterification, and glycosylation, were reported to enhance the protein functionality by improving its heat stability (Kester and Richardson, 1984). Protein modification can also be obtained by glycation, via the early Maillard reaction which occurs naturally in food products. Recently we, and other researchers, have shown that mild heat treatment of milk leads to the covalent linkage of lactose on β-LG via the Maillard reaction (Maubois et al., 1995; Burr et al., 1996; Léonil et al., 1997). In subsequent studies, we have identified the glycation sites after a mild heat treatment in an aqueous solution and under a restricted water environment, i.e., in a powdered state (Morgan et al., 1997, 1998). In fact, temperature and water activity are among the most important parameters that affect the Maillard reaction (O'Brien, 1995).

The work described in this paper was designed to investigate the effect of the glycation conditions (dry and aqueous systems) on both the association behavior and the conformational changes of the glycated β-LG formed. The extent of the glycation reaction in both systems was monitored by measuring free amino groups and by electrospray ionization mass spectrometry (ESI-MS). The molecular species were characterized by proteolytic susceptibility, binding of the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS), sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and size exclusion chromatography (SEC). The results are discussed with respect to the known β-LG denaturation/aggregation process.

MATERIALS AND METHODS

Materials. Fresh bovine raw milk was obtained from the experimental dairy farm (INRA, Rennes, France). β-LG B was prepared from the milk of homozygous cows by a modified method of Fauquant et al. (1988), as previously described (Léonil et al., 1997), in which membrane processes and low temperatures (below 56 °C) are involved. The freeze-dried β-LG B powder contained 93.2% total nitrogen, 2% nonproteic nitrogen, 0.3% ash, 2.2% citrate, 0.6% lactose, and 2% water. β-LG represented 98% of the protein and α-lactalbumin less than 2%, based on reverse-phase HPLC analysis.

Monohydrated lactose (ref 107660) was obtained from Merck (Darmstadt, Germany). Tosyl phenylalanine−chloromethyl ketone treated trypsin (EC 3.4.21.4, ref 37257) was obtained from Serva (Heidelberg, Germany). *N*-Acetyl-L-cysteine (NAC, ref 01039) was obtained from Fluka (Buchs, Switzerland). Porcine pepsin (EC 3.4.23.1, ref P-6887), soybean trypsin inhibitor (ref T-9003), *o*-phthalaldehyde (OPA, ref P-1378), 5,5′-dithiobis(2-nitrobenzoic acid) (DNTB, ref D-8130), ANS (ref A-1028), Triton X-100 (T-9284), and standard proteins used

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for size exclusion column calibration [aldolase (ref A-7145), human serum albumin (ref A-9511), ovalbumine (ref 5503), β-lactoglobulin (ref L-3908), ribonuclease A (ref R-5375)] were obtained from Sigma Chemical Co. (St. Louis, MO).

Glycation Experiments. Glycation was performed with lactose in both dry and aqueous systems.

For the dry-way glycation experiments (DW), β-LG B (0.15 mM) was dissolved in a 15 mM lactose solution and the pH was adjusted to 7.2 with 0.5 M NH_4OH. After being freeze-dried, the protein−sugar powders were kept under 65% relative humidity (saturated KI solution) and 50 °C for various periods (2, 6, 12, 24, and 48 h). The lactose/β-LG molar ratio was 100 (10 times lower than in raw milk) to avoid hygroscopic effects due to high lactose content. The temperature was set to 50 °C to accelerate the Maillard reaction. After heat-treatment, the powders were dissolved in cooled distilled water to obtain a 0.15 mM β-LG solution, and free lactose was removed by extensive dialysis (50-mL samples in 5 L of distilled water containing 0.02% sodium azide, 4 water batches in 72 h) in Spectra/Por 1 cellulose membranes (molecular mass cutoff 6−8 kDa, Spectrum, Laguna Hills, CA) against distilled water at 4 °C.

For solution glycation experiments (SOL), solutions containing β-LG B (0.15 mM) and lactose (150 mM) were adjusted to pH 7.2 with 0.5 M NH_4OH, heat treated at 60 °C for various periods (6, 12, 24, 60, and 130 h), and then cooled in an ice−water bath. Dialysis was performed as above. The lactose/β-LG molar ratio for solution glycation was the same as in raw mi1k (i.e., 1000). A mild heating (60 °C) was chosen to limit thermal denaturation and aggregation effects. Consequently, prolonged heating times were necessary to obtain a significant linkage of lactose.

For both procedures, control experiments were carried out with no added lactose.

Dialyzed samples of control β-LG B (Ct) and glycated β-LG B (Lac) were precipitated at pH 4.6 with 1 M HCl at 23 °C for 1 h. The pH 4.6 precipitation step was integrated in the preparation process of glycated proteins to avoid the presence of any insoluble material that could interfere in the subsequent biochemical characterizations. The acidified solutions were filtered on a cellulose acetate 0.45 μm Nalgene filter (Polylabo, Strasbourg, France) and pH was adjusted to 7.2 with 2 M NH_4OH. Samples aliquots, taken before and after acid precipitation, were analyzed by ESI−MS, SEC, and SDS−PAGE (see below).

After the filtrates were freeze-dried, powders were kept at 4 °C before further characterizations.

Characterization of the Native and Glycated β-LG. *Protein Concentration*. Protein concentration was measured by the absorbance of samples at 278 nm, using a specific absorption coefficient $A^{1\%}_{1cm}$ of 9.6 (Eigel et al., 1984), and by the micro-Kjeldhal method (Ogg, 1960). No significant differences were observed between the two methods ($R^2 = 0.98$, $n = 24$).

Determination of the Extent of Glycation. Free amino groups were measured using the OPA/NAC reagent with 1% sodium dodecyl sulfate, essentially as described by Garcia Alvarez-Coque et al. (1989). The average number of lactose linked per β-LG monomer and the distribution of glycoforms were assessed by ESI−MS, as described previously (Morgan et al., 1997). An API-III$^+$ triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Ontario, Canada), fitted with an articulated pneumatically assisted nebulization probe and an atmospheric pressure ionization source, was used.

Determination of the Free Thiol Content. The free thiol content was determined according to the method of Ellman (1959). β-LG (0.8 mM) was dissolved in a 50 mM phosphate buffer (pH 7.2) containing 8 M urea (buffer A). Fifty microliters of this β-LG solution was added to 950 μL of buffer A containing 210 μM 5,5′-dithiobisnitrobenzoic acid (DNTB) in a 10-mm quartz cuvette. For the blank, 1 mL of buffer A containing 200 μM DTNB was used. Absorbance was read at 412 nm, in an Uvikon 922 spectrophotometer (Kontron Instruments S. A., Montigny-le-Bretonneux, France) thermostated at 25 °C after 3 min. The free thiol content was calculated from three averaged measurements using a molar extinction coefficient of 13 600 M^{-1} cm^{-1}. The variation coefficients of the analysis were lower than 4%.

Size Exclusion Chromatography. The separation of monomers, dimers, and polymers was achieved by SEC in control and glycated β-LG before and after acid precipitation. The chromatographic analysis was carried out as decribed by Law et al. (1993). A Superdex 75 HR column (10 i.d. × 300 mm, Pharmacia, Uppsala, Sweden) was equilibrated with 0.1 M Tris/HCl buffer containing 0.5 M NaCl, pH 6.8. Samples were diluted with 1 volume of the elution buffer and 100 μL were injected and eluted at a flow rate of 1 mL/min. The absorbance was monitored at 280 nm. The standard proteins used for calibration were aldolase (158 kDa), human serum albumine (66 kDa), ovalbumin (43 kDa), β-lactoglobulin (36 kDa), and ribonuclease A (13.7 kDa). The void volume was determined with Blue Dextran 2000. The percentage of soluble protein was calculated using the peaks area ratio of the soluble and unprecipitated β-LG.

Gel Electrophoresis. SDS−PAGE of native and modified β-LG, before and after precipitation at pH 4.6, was performed as described by Laemmli (1970) using 15% acrylamide gels under reducing and nonreducing conditions. Disulfide bonds were reduced by an overnight incubation in the electrophoresis sample buffer containing 170 mM 2-mercaptoethanol (2-ME) at 23 °C. Samples containing approximatively 10 μg of protein were analyzed. Gels were stained with Coomassie Brilliant Blue R250. A low molecular weight markers kit (14.4−94 kDa) from Pharmacia (Upsala, Sweden) was used for calibration.

Limited Proteolysis. Tryptic hydrolysis of native and modified β-LG (1 mg/mL) was performed in a 50 mM Tris/HCl buffer pH 8.2 at an enzyme/substrate molar ratio of 1/150. For peptic hydrolysis, native and modified β-LG (1 mg/mL) were dissolved in a 20 mM citric acid/HCl buffer pH 2.0 and pepsin was used at an enzyme/substrate molar ratio of 1/10. Digestions were performed at 37 °C. Aliquots were taken at different time intervals for analysis. Tryptic hydrolysis was stopped by cooling and by adding Soybean Trypsin Inhibitor at a enzyme/inhibitor molar ratio of 1/5. Peptic hydrolysis was stopped by cooling and by adding 0.2 volume of a 1 M Tris/HCl buffer pH 8.2. Both hydrolysis reactions were monitored by SEC (0.1 M Tris/HCl pH 6.8 + 0.5 M NaCl, at a flow rate of 1 mL/min) with the same column and equipment as above. The volume injected was 100 μL, and the detection was carried out at 214 nm.

Binding of 8-Anilino-1-naphthalenesulfonic Acid. Surface hydrophobicity of native and modified β-LG was evaluated using ANS as a fluorescent probe. Spectrofluorometric measurements were taken on a Perkin-Elmer LS 50B fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, U.K.) at neutral and acidic pH. At neutral pH, β-LG samples ($P_0 = 17.5$ μM) were dissolved in 3 mL of a 50 mM phosphate buffer pH 6.8, and at acidic pH the β-LG samples ($P_0 = 3.5$ μM) were dissolved in 3 mL of a 20 mM citric acid/HCl buffer pH 2. Small amounts of a 1 or 3 mM aqueous solution of ANS at 23 °C were added for the titration (the maximum volume added was 125 μL). The fluorescence intensity (FI) of bound ANS was recorded at $\lambda_{ex} = 390$ nm and $\lambda_{em} = 480$ nm using 5-nm emission and excitation slit widths.

The binding parameters (number of sites n and dissociation constant K_D) were obtained graphically using the Scatchard equation (eq 1) (Scatchard, 1949):

$$\frac{\nu}{L} = \frac{n}{K_D} - \frac{\nu}{K_D} \quad (1)$$

where ν (moles of ligand bound per mole of total protein) is equal to $(L_0/P_0)(FI/FI_{max})$ and L (free ligand concentration) is equal to $L_0(1 - FI/FI_{max})$. L_0 is the total ligand concentration (ranging from 0 to 120 μM), P_0 is the total protein concentration, FI_{max} is the fluorescence intensity obtained when all the ligand molecules are bound. Hence, a plot of $(FI/(P_0(FI_{max} -$

Table 1. Experimental Conditions for the Preparation of the Different Samples of β-LG and Corresponding Average Number of Lactose Bound per Monomer (ANLBM), Distribution of Glycoforms (Total and pH 4.6 Material), Percentage of Soluble Protein (Mean of Three to Four Repeated Measurements ± Standard Deviation), and Samples Codes

treatment	reaction time (h)	lactose	ANLBM	distribution of glycoforms	soluble protein (%)	sample code
no treatment	0	−	0.03	0 and 1 lactose adducts	90.2 ± 5.5	native
dry-way treatment (DW)	12	−	0.03	0 and 1 lactose adducts	94.2 ± 3.4	DW-Ct1
	12	+	3.81	from 1 to 7 lactose adducts	96.7 ± 5.1	DW-Lac1
	48	−	0.03	0 and 1 lactose adducts	92.7± 5.8	DW-Ct2
	48	+	7.67	from 3 to 12 lactose adducts	97.2 ± 2.8	DW-Lac2
solution treatment (SOL)	60	−	0.03	0 and 1 lactose adducts	86.7 ± 6.6	SOL-Ct1
	60	+	1.31	from 0 to 3 lactose adducts	69.0 ± 3.6	SOL-Lac1
	130	−	0.03	0 and 1 lactose adducts	67.6 ± 4.0	SOL-Ct2
	130	+	2.21	from 0 to 4 lactose adducts	52.3 ± 6.5	SOL-Lac2

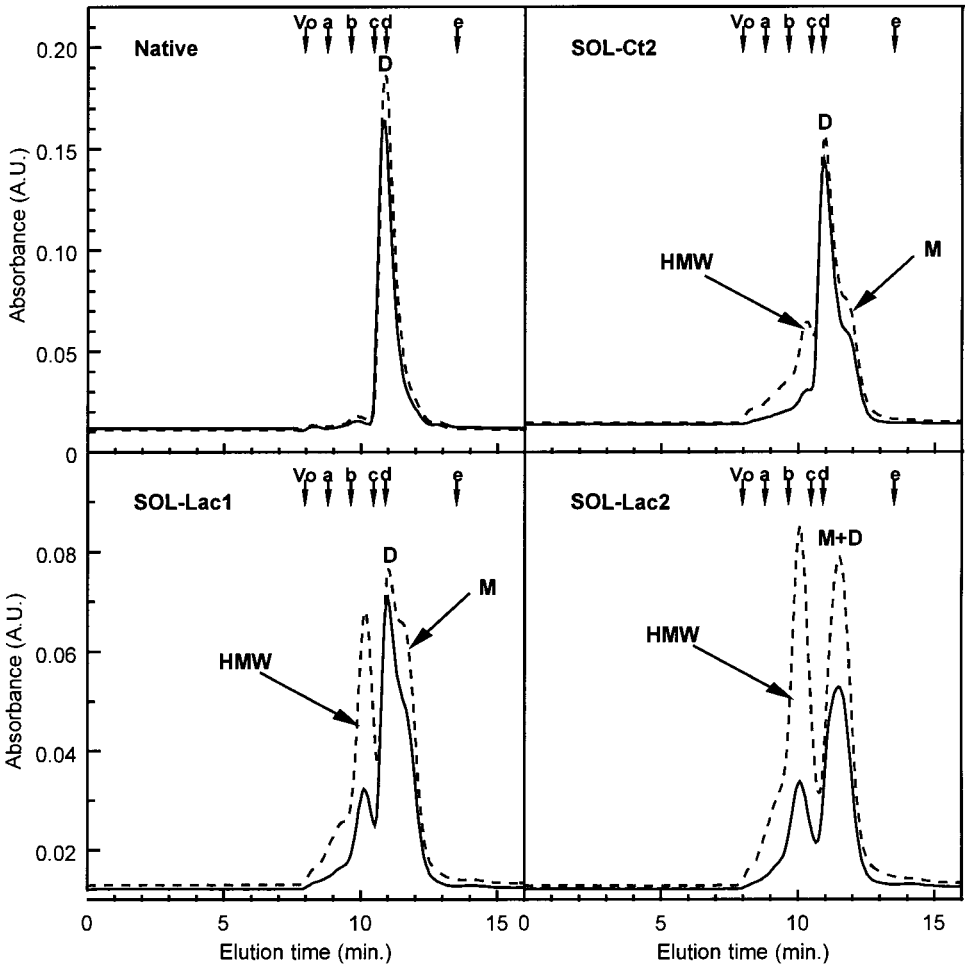


Figure 1. SEC profiles of native and solution-treated samples before (dashed line profiles) and after pH 4.6 treatment (solid line profiles). Abbreviations: M, monomers; D, dimers; HMW, high molecular weight species. Void volume (Vo) and elution times of markers (a, 158 kDa; b, 66 kDa; c, 43 kDa; d, 36 kDa; e, 13.7 kDa) are indicated by arrows.

FI)) versus (L_0/P_0).(FI/FI_{max}) gives a slope equal to $(-1/K_D)$ and an intercept equal to n/K_D. FI_{max} was evaluated from the total binding of ANS (1 and 5 μM) to Triton X-100 (5% (v/v) aqueous solution), as described by Cairoli et al. (1994).

RESULTS AND DISCUSSION

The same extent of glycation and distribution of glycoforms, as measured by ESI−MS, was found for the total and pH 4.6 soluble material (Table 1), indicating that the cause of the solubility losses was not glycation by itself. The average number of lactose linked per monomer reached 7.7 after 48 h in the dry-way glycated β-LG, whereas the solution glycated β-LG possessed 2.2 sugar molecules linked per monomer after 130 h. As already demonstrated in our previous study (Morgan et al., 1998), the restricted water environment promoted the rate of the early Maillard reaction, i.e., formation of the Amadori compound, despite a lactose/β-LG molar ratio 10 times higher in the aqueous preparation procedure. The ESI−MS spectra of the different glycated species revealed an important heterogeneity of the resulting glycoforms, as previously described (Morgan et al., 1997, 1998). This heterogeneity is expressed at two distinct levels. First, each sample is a mixture of several glycated β-LGs that differ by the number of lactose molecules linked per monomer (intermolecular heterogeneity). Second, inside one population of β-LG glycoform (with the same number of lactose molecules

linked per monomer), binding of lactose occurs at different sites (intramolecular heterogeneity).

Effect of the Glycation Treatments on the Association State of the Modified β-LG. *Solubility of the modified β-LG.* The susceptibility of the modified β-LG to the pH 4.6 precipitation step of the preparation procedures has been used as an element of the glycated β-LG structural characterization. The percentage of pH 4.6 soluble protein, determined by SEC, is reported in Table 1. Solubilty remained unchanged for the dry-way-treated proteins. On the contrary, a significant decrease of the solubility was observed after heating for 60 h for the solution-treated proteins. This decrease was more pronounced when heating was carried out in the presence of lactose. The loss of soluble β-LG followed 1.5 reaction order kinetics in both cases (fittings were carried out using linear correlation which gave $R^2 = 0.94$ and 0.99 for control and glycated β-LG, respectively). The calculated rate constants ($k_{1.5}$) were 1.1×10^{-6} s^{-1} and 0.4×10^{-6} s^{-1} for the glycated and control β-LG respectively (with $C_o = 2.74$ g L^{-1}). It is well-known that the loss of solubility of β-LG upon heating is due to denaturation and/or disulfide-linked polymerization, occurring at temperatures above 60 °C (Roefs and De Kruif, 1994; Iametti et al., 1995, 1996; Hoffmann and Van Mil, 1997; Hoffmann et al., 1997a,b; Prabakaran and Damodaran, 1997; Qi et al., 1997). Accordingly, for the control β-LG treated at 60 °C in solution, prolonged heat treatment (more than 60 h) was needed to detect a significant solubility loss.

Association Tendency. The SEC analysis of unprecipitated and pH 4.6 soluble protein in various samples is shown in Figure 1. As far as dry-way glycated β-LG is concerned, neither qualitative nor quantitative modifications were observed on the chromatograms. On the contrary, for samples modified in solution, a modification of the association state was observed. First, high molecular weight molecules (HMW) were detected in the solution-modified β-LG, more abundant in the glycated species (SOL-Lac2) than in the control ones (SOL-Ct2). The relative abundance of these oligomers and polymers increased with the heating period. The solubility losses were mainly due to the precipitation of the HMW but a fraction remained soluble at pH 4.6. Second, the equilibrium at neutral pH between the dimeric and the monomeric forms of β-LG was shifted during the aqueous glycation reaction in favor of the monomeric forms, which became predominant in the sample heated for 130 h with lactose (SOL-Lac2). The same analysis was carried out after freeze-drying of the modified samples and some reversibility of the monomerization was observed for the SOL-Lac2 sample (data not shown).

Hence, heating β-LG in an aqueous solution in the presence of lactose led to an increased proportion of monomeric and polymeric forms, the formation of the latter being probably promoted by the presence of the former. It is interesting to note that some high molecular weight molecules remained soluble at pH 4.6, whereas a weak percentage of the monomeric form precipitated out (in the SOL-Lac2 sample), high-lighting the difficulty of evaluating "denaturation" as previously noted by Prabakaran and Damodaran (1997).

Characterization of the Intermolecular Interactions Involved in the Formation of the High Molecular Weight Species in the Solution-Treated β-LG. To determine the nature of the interactions involved in the formation of the high molecular weight species, observed in the samples heated in solution at 60 °C with lactose, SDS−PAGE analysis of samples were performed with and without disulfide bonds reduction with 2-ME. Results concerning the pH 4.6 soluble protein are presented in Figure 2.

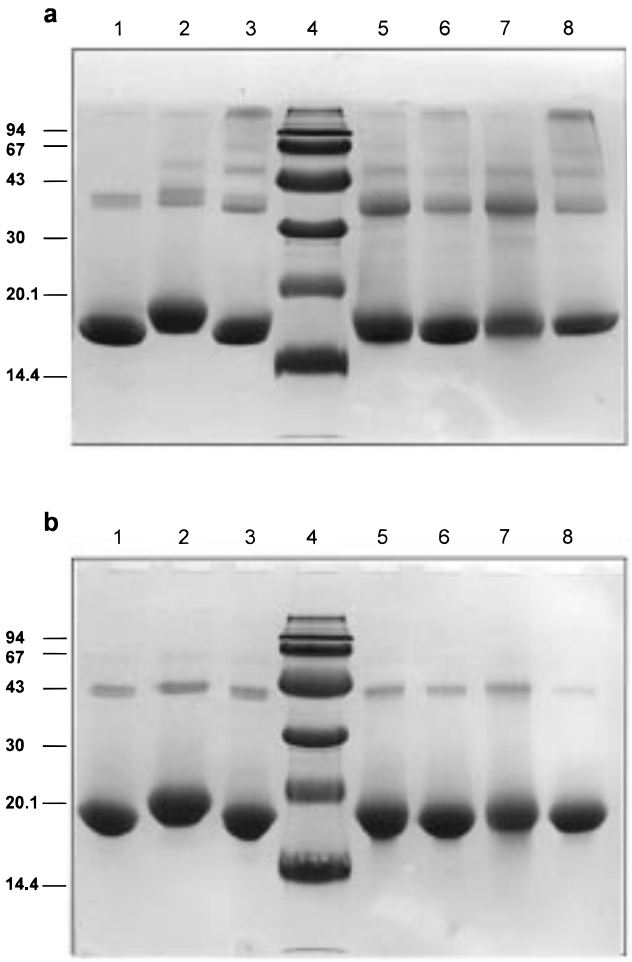


Figure 2. SDS−PAGE of native and modified β-LG before (a) and after (b) disulfide bond reduction by 2-ME. Samples: native (line 1), DW-Lac2 (line 2), DW-Ct2 (line 3), markers (line 4), SOL-Lac1 (line 5), SOL-Ct1 (line 6), SOL-Lac2 (line 7), and SOL-Ct2 (line 8).

Under nonreducing conditions (Figure 2a), the stained bands corresponding to the monomeric and dimeric protein were detected in all the samples. The relative intensities of the dimer bands were low in the native and control samples. A faint band which corresponded probably to the trimeric protein was also detected in all samples except the native one.

The lower electrophoretic mobility of the monomeric protein, observed for the DW-Lac2 sample, was explained by the higher molecular mass of this sample that contained 7.7 lactose linked per monomer. Except that, the electrophoretic pattern of the DW-Lac2 sample was not significantly different from the native one. However, some polymeric material were detected in the DW-Ct2 sample.

The treatment of β-LG in solution has led to important modifications of the electrophoretic patterns. The relative intensities of the dimer bands in the SOL-Lac samples were higher than those in the SOL-Ct samples. For example, in the SOL-Lac2 sample, the dimer band intensity was approximatively equal to that of the monomer. Some disulfide-linked polymers that could not

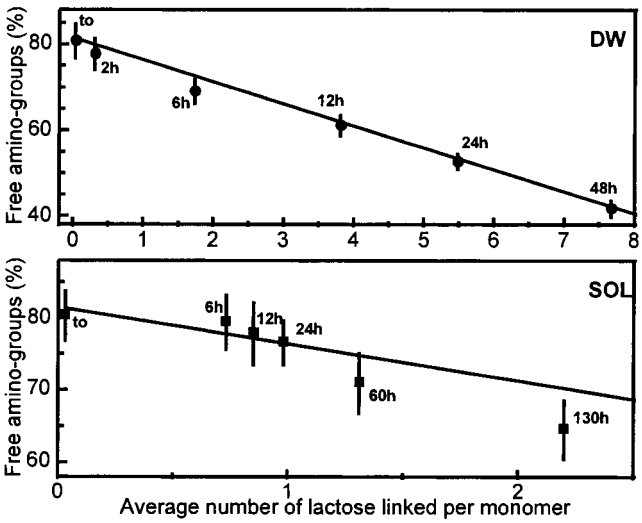


Figure 3. Relationship between free amino groups (mean of four repeated measurements ± standard deviation) and the average number of lactose molecules linked per monomer in the modified samples. Abbreviation: DW, dry-way glycation experiment; SOL, solution glycation experiments. The drawn line represents the theoretical relationship obtained in the early Maillard reaction.

penetrate the gel were weakly detected in the control samples but not in the glycated ones. These results suggested that glycation of β-LG in solution led to the formation of specific covalent dimers without any production of disulfide-linked polymers. The free thiol content, determined by the Elmann's method (Elmann, 1959), of native and SOL-Lac2 samples, were respectively 0.98 and 0.28 free thiol group per monomer. These values confirmed the importance of the covalent dimers formation in SOL-Lac samples.

After disulfide bonds reduction with 2-ME (Figure 2a), monomer bands were mainly observed. Trace amounts of dimers were still detected, even in the native β-LG. These covalent dimers might arise from an incomplete reduction of disulfide bonds by 2-ME. However, the presence of covalent linkages different from disulfide bonds was also suspected in solution glycated samples, as it will be discussed later.

No differences of the electrophoretic patterns were observed for unprecipitated and pH 4.6 soluble protein (data not shown). This was also the case for the characterization of the glycation by ESI−MS, indicating that the acid precipitation did not concern a specific β-LG glycoform. These data suggested that both species (monomers and covalent dimers) were concerned by the acid precipitation after heating with lactose at 60 °C. The solubility losses were likely related to the formation of these structurally modified monomers and dimers that underwent noncovalent polymerization near neutral pH and that precipitated at pH 4.6.

Recent studies have shown that the aggregation of β-LG heated in the 58−65 °C range involved thiol−disulfide exchange reactions, leading to the formation of linear disulfide-linked aggregates of β-LG monomers (Roefs and De Kruif, 1994; Iametti et al., 1996; Hoffmann and Van Mil, 1997; Hoffmann et al., 1997a,b; Prabakaran and Damodaran, 1997). Since the temperatures used in this study were low (50 and 60 °C), such high molecular weight aggregates were weakly represented in control samples. However, heating in the presence of lactose at 60 °C in solution led to the formation of monomers and covalent dimers that exhibited a high tendency to self-associate via noncovalent interactions into polymers at neutral pH.

The early Maillard reaction leads to a sugar adduct, and subsequent secondary reactions may lead to reactive intermediates that produce nitrogenous oligomers and polymers by cross-linking reactions with amino acids in proteins (O'Brien, 1995). As far as the early Maillard reaction was concerned, a linear relationship between the percentage of the residual free amino groups and the average number of lactose molecules linked per monomer was expected. This was the case for the β-LG samples glycated in a powdered state (Figure 3). On the contrary, the percentage of residual free amino groups was slightly lower than expected for β-LG glycated for 130 h in solution (SOL-Lac2). In this sample, the decrease of the free NH_2 percentage might arise, at least partly, from some secondary reactions. The isomerization of lactose during heating, even at low temperatures, led to lactulose (O'Brien, 1995) which is a known cross-linking agent (Matsuda et al., 1991). Therefore, some NH_2 groups could be involved in such protein cross-linkings.

The above results showed that glycation of β-LG in solution at 60 °C promoted the formation of disulfide-linked homodimers, probably by reaction between the two free thiol groups of two different monomers (Cys_{121}). Thiol/disulfide interchange reactions were not observed and, consequently, the covalent aggregation of β-LG was blocked at the dimer step. However, noncovalent aggregation occurred by association of the monomers and the covalent homodimers, leading to dramatic losses of solubility. On the other hand, when glycation was conducted in a powdered state the native behavior was not significantly altered, with respect to solubility and association tendency.

Effect of the Glycation Treatments on the Conformational State of the Modified β-LG. *ANS Binding.* Having demonstrated the presence of intermolecular associations following glycation of β-LG in solution and the absence of them following dry-way glycation, we then evaluated the conformational state of the modified protein by ANS binding and limited proteolysis.

The results of ANS titration experiments at pH 6.8 and pH 2 are displayed in Figure 4. The binding parameters, obtained graphically using the Scatchard equation are given in Table 2. At both pH values, K_D values were in the range $(1-5) \times 10^{-5}$ M and n values were lower than 0.4, in agreement with the results reported by Laligant et al. (1991). The number of binding sites was always higher at pH 2 than at pH 6.8. For native and dry-way glycated β-LG, the increase in the number of binding sites (n) could be interpreted as a consequence of the transition from the dimer (pH 6.8) to the monomer (pH 2), since hydrophobic residues are newly exposed (Iametti et al., 1996). For the dry-way modified β-LG, a slight increase in the strength of ANS binding was observed, as shown by the values of n/K_D. This effect could be attributed to the dry treatment of the powders, since there were no significant differences between the control and glycated samples. The tighter binding of ANS was much more striking for the samples treated in solution, which exhibited a higher number of binding sites. Such changes indicated that β-LG had swollen during the solution treatment and possessed newly exposed hydrophobic patches on the protein surface. An increase in ANS binding sites upon heating

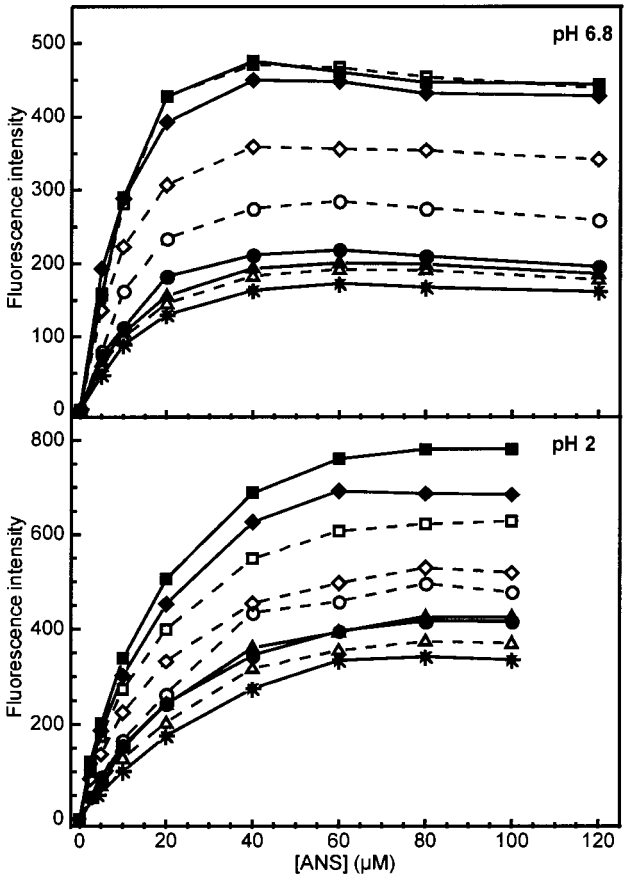


Figure 4. ANS titration curves for native, glycated (solid lines and closed symbol) and control β-LG (dashed lines and open symbols) at pH 6.8 and pH 2. Symbols: *, native; ▲ △, DW-Lac1 and DW-Ct1; ● ○, DW-Lac2 and DW-Ct2; ◆ ◇, SOL-Lac1 and SOL-Ct1; ■ □, SOL-Lac2 and SOL-Ct2.

Table 2. Binding Parameters Obtained from ANS Titration Experiments for Native and Modified β-LG at pH 6.8 and pH 2

		dry-way treatment		solution treatment			
	native	DW-Lac2	DW-Ct2	SOL-Lac1	SOL-Ct1	SOL-Lac2	SOL-Ct2
			pH 6.8				
K_D ($M \times 10^{-5}$)	2.54	1.62	2.81	0.65	1.02	1.66	1.74
n	0.13	0.14	0.26	0.23	0.21	0.37	0.37
n/K_D[a]	1.0	1.7	1.8	6.7	3.9	4.2	4.1
			pH 2				
K_D ($M \times 10^{-5}$)	4.22	2.10	3.57	1.11	1.27	1.17	0.97
n	0.24	0.22	0.34	0.32	0.24	0.37	0.26
n/K_D[a]	1.0	1.9	1.7	5.0	3.3	5.5	4.8

[a] Values are relative to that of the native sample.

of β-LG (3.8 mg/mL) in the 20−80 °C range has been shown by Iametti et al. (1995). The increase in the tightness of ANS binding was more pronounced when heating was carried out in the presence of lactose. When ANS titration was performed at pH 2, the n/K_D ratio increased upon heating for both control and glycated samples. The same tendency was observed at pH 6.8 for SOL-Ct samples. However, for SOL-Lac samples, the accessibility of ANS for binding sites first increased and then decreased after a prolonged heating period (130 h). This behavior could be correlated with the presence of HMW species at neutral pH, more abundant in SOL-Lac2 (see Figure 1), which exhibited higher n values (unfolding) and a lower apparent dissociation constant (polymerization via hydrophobic interactions). Laligant et al. (1991) have also shown that retinol binding sites were masked during the dimerization of β-LG through hydrophobic interactions.

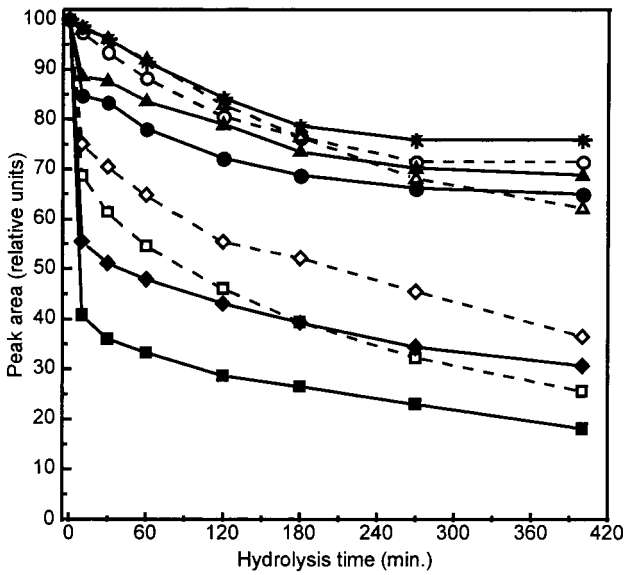


Figure 5. Time-course peptic hydrolysis of native, glycated (solid lines and closed symbol) and control β-LG (dashed lines and open symbols): *, native; ▲ △, DW-Lac1 and DW-Ct1; ● ○, DW-Lac2 and DW-Ct2; ◆ ◇, SOL-Lac1 and SOL-Ct1; ■ □, SOL-Lac2 and SOL-Ct2.

Proteolytic Susceptibility. The time-course hydrolysis of native and glycated β-LG by trypsin and pepsin was determined from SEC profiles. No significant difference in the rate of disappearance of the whole protein was observed between samples when proteolysis was performed with trypsin (data not shown). Several sensitive cleavage sites were accessible regardless of the association and conformational states of the modified β-LG samples. In contrast, the rate and extent of peptic digestion differed from one sample to another (Figure 5). Due to its remarkable acid stability (Kella and Kinsella, 1988), only 25% of native β-LG was hydrolyzed after 400 min under the experimental conditions used. This relative resistance was also observed for the DW modified samples, suggesting that this treatment, performed with or without lactose, did not significantly affect the conformational state of β-LG. On the contrary, heat treatment of β-LG in an aqueous solution increased its susceptibility to pepsin. Both the rate and the extent of hydrolysis were greatly increased, via a combined effect involving heat denaturation and lactose binding. It is well-known that heating or chemical denaturation of β-LG leads to an unfolded state highly susceptible to proteolytic enzymes (Mohan Reddy et al., 1988). However, thermal treatment in the presence of lactose in solution, leading to Maillard adducts, resulted in specific structural effects. These specific effects could be partially related to the unique association behavior exhibited by the solution glycated β-LG, as reported above. The relative susceptibility of the various neoformed species (i.e., monomers, dimers, and high molecular weight species) during the early stage of peptic hydrolysis is shown in Figure 6. For simplicity the time scale of the SEC profiles have been reduced to 7−15 min, i.e.,

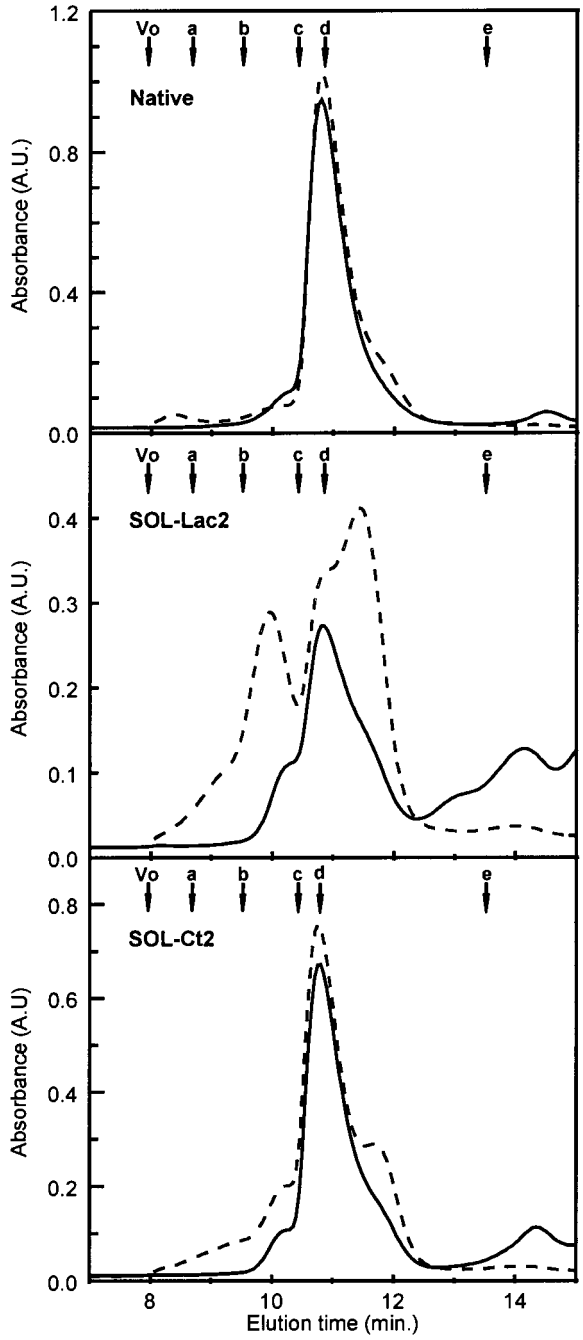


Figure 6. Evolution of the SEC profiles in the first 10 min of peptic hydrolysis of native and solution-treated β-LG (SOL-Lac2 and SOL-Ct2). The dashed lines represent the chromatographic profiles before hydrolysis and the solid lines after 10 min of hydrolysis. Only the protein elution period is presented. Void volume (V_o) and elution times of markers (a, 158 kDa; b, 66 kDa; c, 43 kDa; d, 36 kDa; e, 13.7 kDa) are indicated by arrows.

around the protein elution period (8−12.5 min). The HMW peak, important in SOL-Lac2, nearly disappeared after 10 min of hydrolysis. This result suggested that at the hydrolysis pH (pH 2) the dissociation of noncovalent interactions in the HMW species led to expanded monomers and dimers (covalent), that were readily hydrolyzed by pepsin. The dimeric peak was almost unchanged after 10 min of hydrolysis. The disappearance of the monomeric forms upon digestion suggests that the modified monomer is highly expanded at an acidic pH.

GENERAL DISCUSSION

This study revealed that the process carried out for β-LG glycation exerts a major influence on the formed molecular architectures.

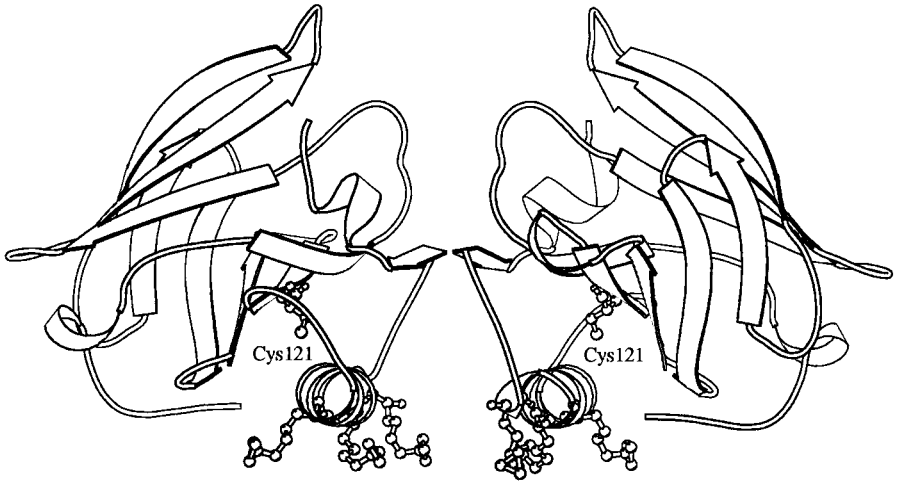
Glycation of β-LG in a restricted water environment preserved, to a great extent, the nativelike association and conformational state and produced a highly glycated protein, with potentially interesting functional applications. Among them, an increase of the thermal stability has been reported by Bouhallab et al. (1998).

Glycation of β-LG in solution at 60 °C near neutral pH affected the early steps of the heat-induced denaturation/aggregation process of the protein. The consequence of such a modification may be described by the following reaction scheme:

$$N_2 \overset{(1)}{\leftrightarrow} N \overset{(2)}{\rightarrow} N^* \overset{(3)}{\rightarrow} [N\text{-}N]^*$$
$$\downarrow \qquad\qquad \downarrow$$
$$\text{Polymerization}$$
$$(4)$$

The first step involves the well-described dissociation of the natural dimer (N_2) of β-LG into monomers (N). At the temperature used in this study, and without lactose added during thermal treatment, subsequent reactions lead to denaturation of the monomer but further aggregation is not thermodynamically promoted (only a weak quantity of polymers are formed). When heating is performed in the presence of lactose, covalent linkage of sugar molecules on the protein induces specific conformational changes, leading in a second step to a swollen monomer N*, highly susceptible to peptic hydrolysis. The reactivity of the free thiol group of this swollen monomer seems to be enhanced, leading to the formation of a stable covalent homodimer (step 3). Brownlow et al. (1997) have shown, using crystallographic measurements, that the dimer dissociation is not sufficient to increase the exposition of the free thiol group (Cys_{121}), buried in a β-structured hydrophobic region of the molecule and sheltered by the α-helix (Scheme 1). Qi et al. (1997) have demonstrated that both the dissociation of the dimer and the destabilization of some secondary structure elements near the dimer interface are needed to permit a reaction of the free thiol group. It is therefore reasonable to postulate that the covalent linkage of lactose allows for the reaction between the two free cysteine residues of two swollen monomers. Using tandem mass spectrometry in the neutral loss scanning mode, as previously described (Mollé et al., 1998), we have identified the glycated lysine residues in the β-LG sample heated with lactose for 130 h in an aqueous solution. Three of these glycation sites are located in the α-helix ($Lys_{135,138,141}$ shown in Scheme 1) and the linkage of lactose in this domain could result in a local unfolding facilitating the reaction between the free thiol groups. The swollen β-LG monomer, formed by heating in the presence of lactose, must be distinguished from the one described by either Iametti et al. (1996) or Qi et al. (1997). In these studies, performed almost in the same heating conditions but without sugar, the observed monomeric intermediate form was described as a molten globule-like state. This particular state has been notably characterized by an

Scheme 1. 3D Structure of β-LG Dimer Showing the Dimer Interface[a]



[a] The figure is based on the X-ray crystallographic data of Brownlow et al. (1997) and was produced using MOLSCRIPT (Kraulis, 1991). The free thiol group (Cys_{121}) and the α-helix lysine residues ($Lys_{135,138,141}$) are indicated.

increased exposure of the free thiol group due to conformational modifications at the dimer interface, but changes in the secondary structure also permitted thiol/disulfide interchange reactions. Hoffmann and Van Mill (1997) and Iametti et al. (1996) have shown that the free thiol group plays a primary role in the heat-induced aggregation of β-LG at neutral pH by promoting disulfide interchange reactions. During aqueous glycation, thiol/disulfide interchange reactions seemed to be depressed and the homodimers formed ([N−N]*) were therefore stabilized against further covalent aggregation. A subsequent step (step 4) involves noncovalent polymerization, probably through hydrophobic interactions, of unfolded homodimers and swollen monomers.

This reaction scheme differs from the nonmodified β-LG polymerization models, proposed by Roefs and De Kruif (1994) and by Griffin et al. (1993), where the polymerization unit is an activated β-LG monomer. Consequently, on the basis of the results obtained in this work, we think that β-LG glycation studies would significantly advance the understanding of the complicated relationship between the structural changes and the aggregation mechanism. In this context, the characterization of local conformationnal changes in solution and dry-way glycatyed β-LG in relation to the glycation sites involved is in progress using monoclonal antibodies as structural probes.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; ANS, 8-anilino-1-naphthalenesulfonic acid; Ct, control sample; DTNB, 5,5′-dithiobisnitrobenzoic acid; DW, dry-way glycation; ESI−MS, electrospray ionization mass pectrometry; FI, fluorescence intensity; HMW, high molecular weight species; Lac, glycated sample; NAC, *N*-acetyl-L-cysteine; OPA, *o*-phthalaldehyde; SDS−PAGE, sodium dodecyl sulfate−gel electrophoresis; SEC, size exclusion chromatography; SOL, solution glycation; 2-ME, 2-mercaptoethanol.

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