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β-Lactoglobulin Structure and Retinol Binding Changes in Presence of Anionic and Neutral Detergents

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Bovine β-lactoglobulin (β-LG) in vivo (in milks) has been found in complexes with lipids such as butyric and oleic acids. To elucidate the still unknown structure−function relationship in this protein, the structural changes of β-lactoglobulin variant A (β-LG A) in the presence of anionic surfactant such as sodium *n*-dodecyl sulfate (SDS) and in the presence of nonionic surfactant such as Triton X-100 have been investigated. Subsequently, the retinol binding by β-LG has been investigated in the presence of various amounts of these surfactants as its binding indicator. The results of UV−vis and fluorescence studies show a higher denaturating effect of SDS at acid pH that can be due to greater positive charges of β-LG at this pH indicating also the nonspecific hydrophobic interactions of Triton X-100 with β-LG at all studied pHs. Isothermal titration calorimetry (ITC) measurements indicate the endothermic nature of β-LG/SDS interactions and the exothermic nature of Triton X-100/β-LG interactions. The analysis of the binding data demonstrates the absence of considerable changes in retinol binding properties of β-LG in the presence of various amounts of these surfactants. This implies that surfactant binding does not change the conformation of β-LG in the regions defining the retinol-binding site.

KEYWORDS: β-Lactoglobulin; sodium *n*-dodecyl sulfate; Triton X-100; retinol; isothermal titration calorimetry; fluorimetry

INTRODUCTION

The interactions of proteins with surfactants have been studied extensively, since they are of great importance in a wide variety of industrial, biological, pharmaceutical, and cosmetic systems (*1*−*5*). The interactions between biopolymers and surfactants depend strongly on the type of biopolymer and surfactant as well as on medium and its physicochemical properties such as pH, ionic strength, and temperature (*6*−*14*). Protein−surfactant interactions often alter the stabilities of many proteins. An understanding of the mechanisms involved in protein−surfactant interactions provides a basis for the evaluation of protein stability and for rational strategies to optimize the applications of surfactants. In these studies, the globular protein β-lactoglobulin (β-LG), which is the major protein in the whey of ruminant milk (*15*) and binds retinol and its derivatives, was used. Its interactions with amphiphilic and hydrophobic ligands such as retinoids, long chain fatty acids, and surfactants were investigated by different methods. Although there was no evidence for sequential loosening of the protein structure (e.g., the α-helix displacement or unfolding exposing the hydrophobic, external face of the β-barrel), all the parameters examined in these studies were unlikely to be linked with such structural changes. Thus, partial structural unfolding of β-LG could not be excluded (*16*−*23*).

Bovine β-lactoglobulin (β-LG) is a major whey protein of bovine milk with known primary, secondary, and three-dimensional structures but with still unknown biological function(s) (*24*). All the structural data concerning β-LG suggest that this protein shall be classified in the superfamily of hydrophobic molecule transporters termed lipocalins (*25, 26*). Its polypeptide chain is composed of 162 amino acid residues including two disulfide bonds (Cys66−Cys160 and Cys109−Cys119) and one free cysteine (Cys121) (*24*). The molecule of β-LG is constituted by nine antiparallel β-strands and one α-helix (*25, 26*). The core of the β-LG molecule includes a structural motif similar to that found in retinol binding protein

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(RBP) (*26*). Like RBP, β-LG is able to bind a wide variety of hydrophobic molecules (*24, 27*).

When the retinol binding by β-lactoglobulin is considered, there is some circumstantial evidence of protrusion of a retinol hydroxyl group out of the binding site deduced from its susceptibility to an attack by dehydrogenase (*28*). This could also indicate the external placement of the retinol binding site on the β-LG molecule (*25*). β-LG is known to bind tightly, in vitro, one retinol molecule per monomer (*28*). Developments in structural studies of β-LG (*25, 26, 29*), retinol binding protein (*30*), and bilin binding protein (*31*) show that these hydrophobic molecule transporters share a three-dimensional structural pattern termed β-barrel. Unambiguous crystallographic data analysis (*30, 31*) indicates that the ligands of retinol binding protein and bilin binding protein are bound inside the calyx formed by the β-barrel. The exact placement of the binding sites in other proteins from this super family is less clear.

It has been suggested that in the case of β-LG and some other proteins close to their isoelectric points, cooperative binding with sodium *n*-dodecyl sulfate (SDS) can induce an association of the protein to form *n*-dodecyl sulfate-complexed aggregates (*32*). Electrophoretic measurements indicate that the binding of *n*-octylbenzene-*p*-sulfonate anions to β-LG occurs in three stages (*33*). Additional studies show that, at low concentrations of SDS, the β-LG complexes with amphiphilic ligands aggregate in solution, while this does not occur at higher SDS concentrations. Calorimetric measurements at pH below isoelectric pH represent an exothermic process. However, the extent of exothermicity decreased with increasing of pH (*34*).

It has been concluded from an unfolding study of β-LG by urea in the presence of SDS that stability of the β-LG increased in the presence of SDS suggesting that SDS may occupy the cavity of the β-barrel (binding pocket). However, this claim has not been proven by binding measurement of retinol (*23*).

The binding studies on a homologous series of *n*-alkylsulfonate ligands with β-LG were performed by Busti et al.; they conclude that these surfactants stabilize the monomeric structure of β-LG (*22*).

It has been shown that the interactions of anionic phospholipids with β-LG cause a structural reorganization of the elements of its structure accompanied by an increase in α-helical content and a loosening of the protein tertiary structure (*35*).

A study of the interactions of the mixtures of cationic−anionic surfactants with β-LG was also reported recently by Lu et al. (*36*). They concluded that the extent of interaction is dependent on the ratio of surfactants in the mixture. Despite these reports, there are no comprehensive studies on the structure−function relationship of β-LG in the presence of surfactants.

In the present study, the structural changes of β-LG have been investigated initially in the presence of SDS and Triton X-100 using various experimental techniques such as UV−vis, fluorescence, and isothermal titration calorimetry (ITC). Subsequently, the retinol binding properties of β-LG as its functional indicator was investigated in the presence of the various amounts of these surfactants using the spectrofluorimeter titration method. Comparison of the results allows for the determination of some aspects of the structure−function relationship of β-LG in the presence of these surfactants.

MATERIALS AND METHODS

Chemicals. β-Lactoglobulin variant A (β-LG A) was isolated from the milk of a homozygous cow by a salting out/in method according to Maillart and Ribadeau-Dumas precipitating the majority of whey protein when conserving uniquely β-LG in solution (*37*). Homogeneity of the protein preparation was assessed by high-performance gel permeation chromatography and SDS gel electrophoresis. The obtained preparations of β-lactoglobulin were over 98% pure. Sodium *n*-dodecyl sulfate (SDS), *N*-acetyl-L-tryptophanamide (NATA) and *trans*-retinol palmitate were purchased from Sigma Chemical Co. Triton X-100, glycine, ethanol, Na_2HPO_4, and NaH_2PO_4 were obtained from Merck Chemical Co. All the used reagents were of the highest degree of purity. All of the solutions were prepared using double-distilled water. The 50 mM glycine pH 2.0 and the 50 mM phosphate pHs 6.7 and 8.0 were used as buffers. All of solutions were used fresh after preparation. The concentrations of β-LG and retinol were determined from the optical density of prepared solutions using the extinction coefficients of 17 600 M^{-1} cm^{-1} at 280 nm and of 48 000 M^{-1} cm^{-1} at 330 nm, respectively (*18*).

Apparatus. Fluorescence measurements were performed using a RF-5000 Shimadzu spectrofluorimeter with a thermostatted cell compartment at 298 K. Isothermal titration calorimetry (ITC) was performed at 298 K on a thermal activity monitor calorimeter (Thermometrics AB, Järfälla, Sweden) equipped with a high performance titration unit and a nanowatt amplifier. The absorbance measurements were carried out using a Carry-500 Scan UV−vis-NIR double beam spectrophotometer, which is well equipped with a thermostatted cell compartment at 298 K.

Fluorescence Spectroscopy Measurements. Fluorescence spectroscopy is used to study the binding and the conformational changes of proteins exploiting the intrinsic fluorescence of tryptophan (Trp) residues, which are particularly sensitive to the changes of their microenvironments (*38*). During fluorescence measurements, the samples were put in quartz cuvettes of 1 cm optical path length. In typical experiments, 3.0 mL of β-LG solution was placed into the cuvette. Emission spectra were recorded after each addition of SDS and Triton X-100 stock solutions (10 mM) at 298 K. The excitation was performed at 280 nm, and the emitted light was recorded between 300 and 400 nm for SDS and between 285 and 385 nm in the case of Triton X-100 binding studies. The observed fluorescence intensities were corrected for dilution. The band slits for excitation and emission were 3 and 5 nm for SDS and 1.5 and 3 nm for Triton X-100, respectively.

During binding experiments, fluorescence spectra were recorded at 298 K and between 300 and 400 nm (excitation: 280 nm). The binding of retinol was measured following the decrease of protein tryptophan fluorescence at 330 nm.

The following procedure was used for titration of β-LG solutions or various [surfactant]/[β-LG] solutions, with retinol: 3 mL of β-LG solutions or various [surfactant]/[β-LG] solutions were placed in a cuvette, and small increments (1−5 μL) of the ligand solution were injected in the cuvette with a Hamilton syringe. The experiments were performed in 50 mM glycine at pH 2.0 and in 50 mM phosphate buffer at pHs 6.7 and 8.0. In order to eliminate the dilution of β-LG solution by the added ligand solution and tryptophan fluorescence changes induced by alcohol, a blank containing *N*-acetyl-L-tryptophan amide, NATA, solution titrated with ligand was monitored as described above. The fluorescence intensity changes of the blank were subtracted from fluorescence intensity measurements of the ligand/protein complexes for every considered titration point. The β-LG solutions were freshly prepared just before the measurements, and their absorbencies at 280 nm did not exceeded 0.1; to avoid inner filter effect, β-LG concentration was around 3 μM. The concentration of NATA has been selected in the way that it had the same absorbance at 280 nm as the studied β-LG solution.

Isothermal Titration Calorimetric Measurements. The nanowatt isothermal titration microcalorimeter was a calorimeter supported by Thermometric 2277, thermal activity monitor (Thermometrics AB, Järfälla, Sweden), controlled by Digitam 4.1 software. The instrument had an electrical calibration with a precision better than ± 1%. The reaction and reference cell of the calorimeter were made from stainless steel. This isothermal titration calorimeter was used to measure enthalpies of mixing at 298 K for the interaction of SDS at pH 6.7 and Triton X-100 at pHs 6.7 and 8.0 with β-LG. Aliquots (250 μL) of SDS or Triton X-100 solution (10 mM) were injected sequentially (10 μL in each injection) by a 250 μL Hamilton syringe controlled by a Thermometric 612 Lund pump into a 2600 μL reaction cell containing initially either buffer solution or β-LG solution. The concentration of

β-LG in the cell was around 3 μM. Each injection took 5 min, and there was an interval of 20 min between every successive injection. The solution in the reaction cell was stirred at a speed of 60 rpm. All the solutions were degassed before the measurements. All the experiments were carried out at least twice using freshly prepared samples, and the results are reported as the averages. The reproducibility of the enthalpy changes measured on a particular sample by ITC was acceptable with less than 10% of error. Data were analyzed using the proprietary software Digitam 4.1 supplied by Thermometric AB (Järfälla, Sweden).

UV−Vis Spectroscopy Measurements. During UV−vis spectroscopy measurements, 800 μL of β-LG solution was placed into the 1 cm optical path quartz cuvettes. The absorbance spectra were recorded between 250 and 350 nm after each addition of SDS and Triton X-100 stock solutions at 298 K. The observed absorbances were corrected for dilution. The β-LG solutions were freshly prepared just before the measurements.

Determination of the Apparent Dissociation Constants. The following procedure was used for the titration of β-LG solutions or various [surfactant]/[β-LG] solutions with retinol: 3 mL of protein solution or various [surfactant]/[β-LG] solution was placed in a cuvette and small increments of retinol solution were injected with a micropipette. The ethanol added with retinol during titration never exceeded 3% (v/v). Differences in fluorescence intensity at 330 nm (excitation at 280 nm) were monitored in order to measure apparent dissociation constants of β-LG and various [surfactant]/[β-LG] molar ratios with retinol. It was assumed that the change in the fluorescence depends on the amount of protein/ligand complex. The apparent dissociation constants were determined according to Cogan et al. using the following equation (*39*):

$$P_0\alpha = (L_0/n)(\alpha/(1-\alpha)) - K_d/n \quad (1)$$

where α is the fraction of free binding sites, L_0 is the total ligand concentration, and P_0 is the total protein concentration.

By plotting $P_0\alpha$ versus $\alpha/(1-\alpha)$, a straight line is obtained with an intercept of K_d/n and a slope of L_0/n, where K_d is the apparent dissociation constant and n is the apparent molar ratio of β-LG/retinol at saturation. The α is defined as the fraction of unoccupied binding sites on the protein molecules. The value of α was calculated for every desired point on the titration curve of fluorescence quenching intensity using the relationship

$$\alpha = (F - F_{min})/(F_0 - F_{min}) \quad (2)$$

where F represents the fluorescence intensity (corrected for blank) at a certain L_0, F_{min} represents the fluorescence intensity upon saturation of β-LG molecules, and F_0 is the initial fluorescence intensity without retinol.

RESULTS AND DISCUSSION

Influence of Various Surfactant Concentrations on the Structure of β-LG. The addition of SDS and Triton X-100 resulted in the enhancement of the β-LG fluorescence emission maximum. Particularly, in the case of studies of the Triton X-100/β-LG complex, the slightly blue shift of the emission maximum was observed. When ionic surfactant are added into β-LG solution, the ionic surfactant monomers first bind electrostatically to charged residues at the β-LG surface, and this binding induces an expansion of the β-LG structure. This expansion opens for interactions of the surfactant hydrophobic tails with the β-LG nonpolar globulin interior (nonspecific, cooperative binding). This leads to β-LG unfolding or aggregation and loss of its secondary structure. It has been reported that nonionic surfactants interact with proteins weaker than ionic surfactants do (*1*). The nonionic surfactants interact with the β-LG via hydrophobic interactions.

The β-LG molecule contains 3 Arg residues, 14 Lys, and 1 His, and these residues could be the binding sites of anionic surfactants. When SDS (anionic surfactant) was added to β-LG solution at pH 2.0, they could form precipitates between 30 and 35 of [SDS]/[β-LG] molar ratios because β-LG is positively charged at pH 2.0 (*15*). Such a behavior was observed in β-LG/SDS mixed solution at pH 6.7 at about 10-fold higher molar ratios [SDS]/[β-LG] between 300 and 310 because both SDS and β-LG are negatively charged at pH 6.7. These results are similar to what was already observed for other positively charged protein−surfactant systems (*36, 40*). At pH 2.0, β-LG is positively charged (the isoelectric point of β-LG is 5.2 (*15*)), so it can form precipitates with negatively charged SDS because of the formation of electrostatically neutral complexes of β-LG/SDS. At pH 6.7, when anionic surfactant SDS binds to β-LG, the β-LG/SDS complex remains negatively charged, so it precipitates at higher molar ratios due to unfolding and aggregation (the precipitation has been observed).

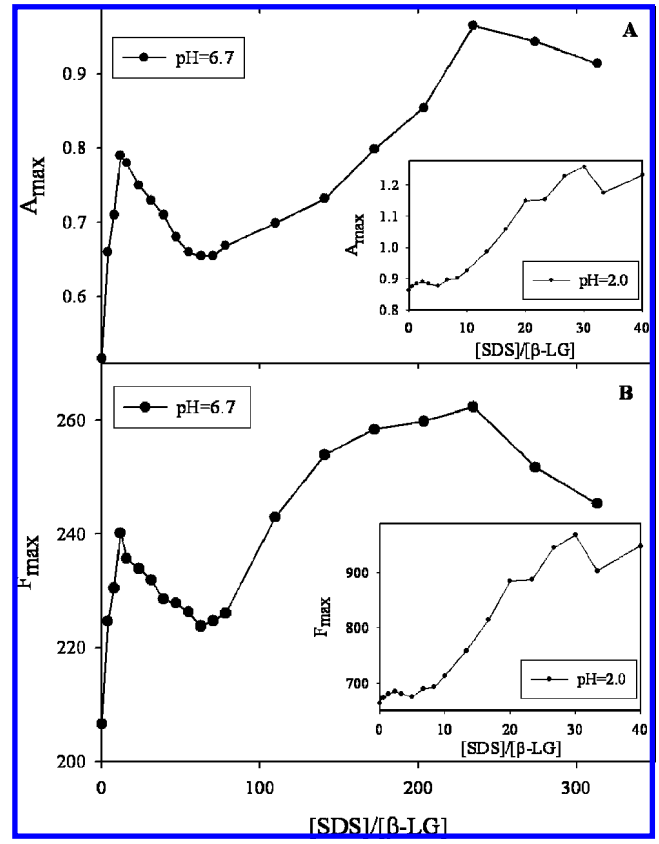

Figure 1. Variation in absorbance of β-LG at 280 nm (**A**) and changes in fluorescence emission maximum intensity of β-LG (**B**) vs molar ratio of [SDS]/[β-LG] in 50 mM glycine pH 2.0 and 50 mM phosphate buffer pH 6.7 at temperature of 298 K.

The nonionic surfactant Triton X-100 can bind to β-LG and change β-LG structure, with the effect weaker than anionic surfactant SDS do.

UV−Vis Absorption Spectra. UV−visible spectroscopy was also used to analyze the binding of SDS and Triton X-100 to β-LG. The absorption maximum of β-LG depends upon the microenvironment in which the probe is located albeit this dependence is significantly smaller than in the case of fluorescence measurements. The study of the absorbance of β-LG during its interactions with surfactants allows the determination of the micropolarity of the local environment surrounding the probe. **Figure 1A** shows the plot of the maximum of absorbance of β-LG versus molar ratio of [SDS]/[β-LG] in pHs 2.0 and 6.7 at 298 K. As shown in this figure, the overall structural changes are similar at both pHs. Two distinct conformational changes can be distinguished. First, transition is observed at

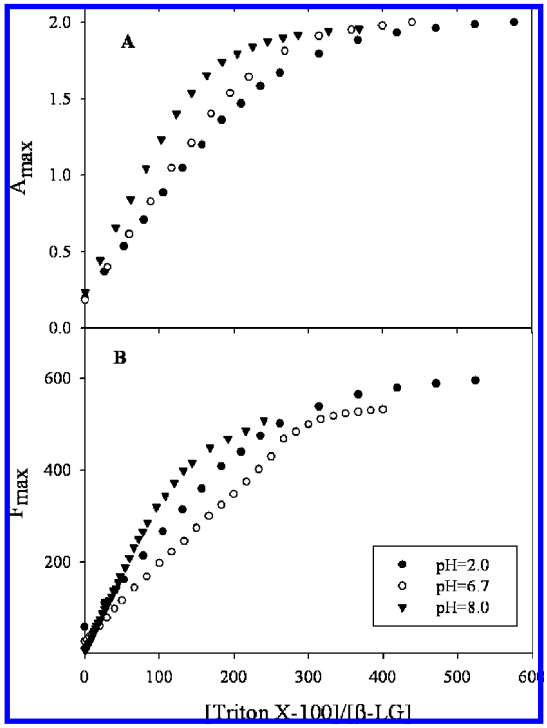

Figure 2. Changes in absorbance of β-LG at 280 nm (**A**) and changes in fluorescence emission maximum intensity of β-LG (**B**) vs molar ratio of [Triton X-100]/[β-LG] in 50 mM glycine pH 2.0 and 50 mM phosphate buffer pHs 6.7 and 8.0 at temperature of 298 K.

molar ratios of 4 and 10 at pHs 2.0 and 6.7, respectively. These points are corresponding to the first maximum in **Figure 1A**. The following conformational changes are beginning at molar ratios of 8 and 60 at pHs 2.0 and 6.7, respectively. It appears that the binding of initial surfactant ions to negatively charged sites on the surface of β-LG induces a conformational transition, which may be related to two different folding states. However, the second transition at higher molar ratio of SDS corresponds certainly to β-LG unfolding. Ultimately, the unfolded structures aggregate and precipitate. The following mechanism can be proposed for binding of SDS to β-LG:

$$F_1 \rightarrow F_2 \rightarrow U$$

where F_1 and F_2 are corresponding to two different folded states and U corresponds to the unfolded state.

Figure 2A shows the changes in maximum of absorbency of β-LG solution versus [Triton X-100]/[β-LG] molar ratios at various pH and at temperature of 298 K. No precipitation of β-LG was observed at any of the studied pHs. The cooperative character of binding is obvious at all studied pH. Analysis of the location of these transition curves shows that the binding strength decreases with increase of the pH. This fact can be related to the larger hydrophobic surface area of the β-LG/Triton X-100 complex at lower pH due to the predominant nature of hydrophobic interactions in the case of Triton X-100 binding. However, the two-step binding process that has been observed in the case of SDS binding was absent in the case of Triton X-100.

Fluorescence Spectra. The β-LG fluorescence depends on the microenvironment of the Trp residues of β-LG (*33, 41*). β-LG contains two Trp residues, Trp 19 and Trp 61 (*15*). **Figure 3** shows a ribbon diagram of a single unit of bovine β-LG. As it shown in this figure, Trp 19 is in an apolar environment in the main cavity of β-LG, whereas Trp 61 protrudes beyond the surface of the molecule and is quite close to the Cys 66−Cys

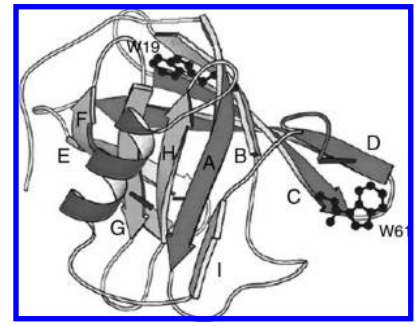

Figure 3. Ribbon diagram of a single unit of bovine β-LG. The locations of Trp19 and Trp61 are indicated.

160 disulfide bridge (*42, 43*). Since the disulfide bridges are effective Trp fluorescence quenchers, the intrinsic fluorescence of β-LG is almost exclusively attributed to Trp 19 (*21, 44*). The surfactant ions would cluster around the oppositely charged side chains of proteins; that is, anionic surfactants cluster around protonated Arg and Lys side chains, and cationic surfactants cluster around anionic Glu and Asp side chains (*1*). Analysis of the 3D structure of β-LG shows that, when SDS molecules bind to the residues of Arg and Lys, they are far away from Trp 61 and have little effect on Trp 61, so the fluorescence intensity changes are comparatively lower than in the case of Triton X-100 placed more evenly around the β-LG molecule.

Figure 1B shows the changes in maximum fluorescence intensity versus [SDS]/[β-LG] molar ratio at pHs 2.0 and 6.7 and 298 K. As shown in this figure, the variations of F_{max} versus the [SDS]/[β-LG] mole ratio are very similar to what is seen in case of absorbency measurements A_{max}. The maxima were observed at [SDS]/[β-LG] molar ratios of 3 and 9 at pHs 2.0 and 6.7, respectively. Nearly the same wavelength of maxima were observed for A_{max}. The precipitation of the complex was observed at molar ratios of 30 and 280 at pHs 2.0 and 6.7, respectively. These results agree well with UV−vis results of study of β-LG/SDS interactions.

It is known that the transfer of Trp from an aqueous to a hydrophobic environment leads to a blue shift in wavelength and to an increase in intensity of the emission maximum. This is the case of the results described here. An exposure to increasing concentrations of Triton X-100 in pH 2.0 induces a slight blue shift in the fluorescence emission maximum of Trp and a substantial increase in the fluorescence intensity (graphs are not shown). Similar behavior was observed also in the case of UV−vis absorbance in presence of this surfactant. The increase in the absorbance intensity indicates the perturbation of tertiary interactions quenching the otherwise β-LG fluorescence in the native conformation. As seen in the 3D structure of native β-LG, Trp 19, situated at the bottom of the calyx formed by eight antiparallel β-strands, contributes about 80% to the total fluorescence (*17*) and a disulfide bond quenches Trp 61 emission (*45*). Therefore, smaller quenching of Trp 61 by a disulfide bond, when β-LG binds to Triton X-100, leads probably to an increase in the fluorescence intensity of β-LG. It indicates also that the protein unfolds partially during its interaction with Triton X-100. A blue shift of the emission maxima indicates that at least part of the Trp residues is transferred/moved into a more hydrophobic environment during the interaction of β-LG with the Triton X-100. The same effect can be observed in other pHs. **Figure 2B** shows the changes in the maximum of the fluorescence intensity versus the [Triton X-100]/[β-LG] molar ratio at various pHs and 298 K. No precipitation of β-LG is observed at any of the studied pHs. Similarly to UV−vis measurements, the cooperative binding

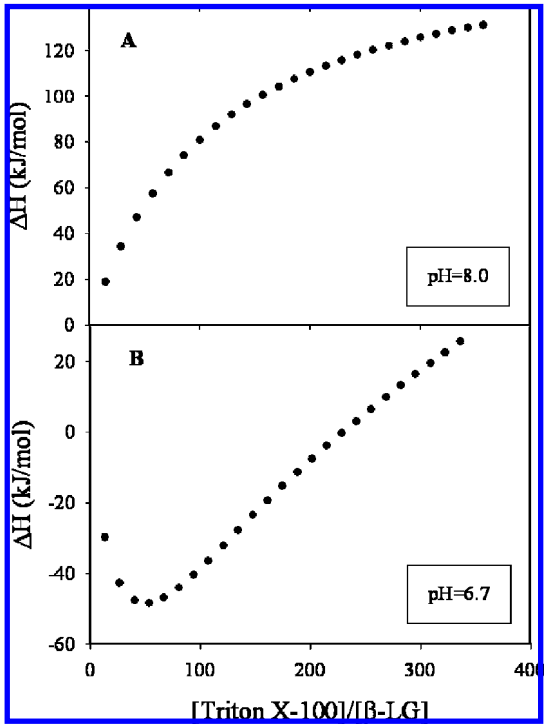

Figure 4. Variation of enthalpy of interaction vs [Triton X-100]/[β-LG] in 50 mM phosphate buffer at pHs 8.0 (**A**) and 6.7 (**B**) and at 298 K.

was observed that indicates the decrease of the strength of the binding with the increase of pH. Consequently, similar interpretation can be advanced in the case of the interpretation of obtained fluorescence data. Hence, all of these spectrophotometric results are coherent.

Isothermal Titration Calorimetric Study. One of the major factors influencing the electrostatic interactions of charged biopolymers in aqueous solutions is the pH, since it affects both the sign of the charge and its magnitude. Therefore, the effect of pH and surfactant type on the structure of β-LG was examined systematically in this study. The influence of surfactant concentrations at various pHs on the enthalpy changes associated with protein−surfactant interactions was studied using ITC when SDS or Triton X-100 solutions were added into β-LG solution (298 K, 50 mM phosphate buffer, pHs 6.7 and 8.0). We have investigated the changes in the enthalpy of the interactions of SDS with β-LG at pH 6.7 and at the temperature of 298 K (data not shown). The results indicate that the β-LG/SDS interaction is endothermic and shows a linear increase with increasing SDS concentrations starting about 10 kJ/mol at molar ratio 0 until about 100 kJ/mol at molar ratio of 350. This indicates the hydrophobic nature of interactions at this pH. β-LG at pH 6.7 is negatively charged, so its bulk electrostatic interactions are repulsive. In such a case, the hydrophobic interactions between the nonpolar tail of SDS and hydrophobic patches at the surface of β-LG should prevail and should be the major driving force of complex formation.

The dependence of ΔH_{int} versus the mole ratio of [Triton X-100]/[β-LG] at pH 6.7 is complex. As we can see in **Figure 4B**, a relatively high exothermic enthalpy change was observed below 50 molar ratio of [Triton X-100]/[β-LG]. Its exothermicity grows up and falls rapidly to about zero and mutates into endothermic enthalpy change at higher surfactant concentrations. Such behavior is not observed at pH 8.0. The curve of ΔH_{int} versus [Triton X-100]/[β-LG] (**Figure 4A**) shows an increase

Table 1. Apparent Dissociation Constants (K_d) and Apparent Molar Ratio (n) of Retinol−β-LG Complexes in Molar Ratio of [Surfactant]/[β-LG] at Various pH and 298 K[a]

pH	surfactant	mole ratio of [surfactant]/[β-LG]	K_d ($\times 10^{-8}$ M)	n
2.0	SDS	0.0	7.24	0.79
		5.0	6.32	0.74
		10.0	6.04	0.69
		20.0	5.88	0.65
		30.0	4.95	0.63
	Triton X-100	4.6	9.73	0.81
		9.2	10.78	0.89
		13.7	11.61	0.93
		18.3	11.26	0.97
6.7	SDS	0.0	5.94	0.83
		5.0	6.28	0.85
		10.0	6.95	0.88
		20.0	7.20	0.92
		30.0	7.51	0.95
	Triton X-100	3.9	6.54	0.91
		7.9	7.21	0.94
		11.8	8.09	0.97
		15.7	8.94	0.99
8.0	Triton X-100	0.0	5.42	0.74
		3.6	6.98	0.77
		7.3	8.13	0.91
		17.2	9.86	0.96
		22.9	11.37	1.07

[a] Results expressed per protein monomer.

in endothermicity with increasing surfactant concentrations, and the saturation of complex formation at the surface of β-LG is obvious.

Although, it is usually difficult to assign precise molecular events to enthalpy changes measured in thermodynamic experiments because of the multiplicity of different physicochemical phenomena contributing to the measured signal (e.g., various kinds of association−disassociation processes and conformational changes), but the fact that the enthalpy changes observed during β-LG/Triton X-100 interactions in the pH range 6.7−8.0 were endothermic under some circumstances but exothermic under other circumstances suggests that at least two different physicochemical phenomena could occur in this pH range: (1) The single well-known conformational transition of β-LG at this pH range is a Tanford transition (*46*). (2) The dissociation of β-LG dimer to monomer at this pH range (β-LG is predominately dimeric at pH 6.7 and in monomer form at pH 8.0 (*47*).

Influence of Surfactant Concentration on Retinol Binding Properties of β-LG. The apparent binding constants and the apparent molar ratios of retinol/β-LG complexes are reported in **Table 1**. The β-LG/retinol complex at pH 8.0 displayed the smallest apparent dissociation constant ($K_d = 5.42 \times 10^{-8}$ M), and the association of retinol with β-LG was slightly weaker at pH 2.0 ($K_d = 7.24 \times 10^{-8}$ M) and pH 6.7 ($K_d = 5.94 \times 10^{-8}$ M).

The obtained results show that β-LG tryptophan fluorescence intensity decreases significantly when retinol is bound to β-LG. This agrees well with the previous results of Futterman and Heller (*28*) and Georghiou and Churchich (*48*). The apparent β-LG/retinol dissociation constants decrease between pHs 2.0 and 8.0. This observation does not support the suggestions of Fugate and Song (*16*) who claimed that the binding of retinol to β-LG is pH-independent in the pH range 2.0−7.5 but agrees with the results reported by Dufour et al. (*18*). In the case of this study, the obtained results show that the β-LG/retinol binding is pH-dependent. Bigger quenching by retinol of

tryptophan fluorescence at pHs 6.7 and 8.0 as compared with what is observed at acidic pH could indicate (if tryptophan−retinol excitation energy transfer contributes to the quenching phenomenon) that distances between tryptophan and retinol become shorter at neutral pH. All the obtained data suggest that β-LG conformation changes in the studied pH range and that its binding properties change with its ionization status (*18*).

The appearance of precipitation at a [SDS]/[β-LG] molar ratio of about 30 can be explained taking in to account the total net charge on β-LG at pH 2.0, which should be around +20. Hence, about 20 SDS anions would bind to β-LG at a molar ratio of 30. Such binding stoichiometry would cause the neutralization of charges on the protein and its precipitation. The analysis of retinol binding constants (n, K_d) as a function of SDS concentrations (**Table 1**) shows only a small increase in binding affinity. This can be related to the increase of the hydrophobic area of β-LG/SDS complexes and insignificant changes in the conformation of β-LG, especially around the calyx formed by the β-barrel. In such a case, the significant variation of the fluorescence emission spectra of β-LG due to interaction with SDS could be related to changes of the polarity of the microenvironment around the indol moiety of Trp 61 protruding on the surface of the protein. The binding of SDS to Arg^+ and Lys^+ residues has a greater influence on fluorescence quantum yield of this Trp. However, when taking in to account the results presented in **Figure 1B**, it seems that this fluorescence enhancement effect occurs in two distinct steps. Consequently, the occurrence of two local conformational changes due to binding of SDS is proposed. These local changes do not have great influence on the retinol binding site and consequently on retinol binding properties. The results of retinol binding studies in the presence of various SDS concentrations at pH 6.7 show slight increase in both n and K_d with the increase in SDS concentration. β-LG is dimeric at pH 6.7 while it is in predominately monomeric form at pH 2.0. Additionally, the binding affinity of SDS decreases with the rise of pH because of the increase of negative charge density and the decrease of electrostatic attraction between SDS anions and β-LG. These phenomena are probably the main causes of these observations.

The binding of Triton X-100 to hydrophobic patches on the β-LG surface is nonspecific. The hydrophobic interactions are the major driving forces of these interactions. Consequently, the increase of surfactant binding affinity with growth of pH can be related to increase of the area of hydrophobic clusters on β-LG at higher pH. The retinol binding affinity increases slightly with [Triton X-100]/[β-LG] molar ratio. This documents the inefficency of this surfactant as denaturant of β-LG. Additionally, it is clear that Triton X-100 cannot compete with retinol for binding site, due to its big size.

In the case of Triton X-100, at pH 2.0, both n and K_d increase with the increase in concentration of Triton X-100. The same is observed at other pHs in the case of β-LG retinol binding in the presence of various concentrations of this surfactant (**Table 1**). While Triton X-100 concentrations increase in solution, the hydrophobicity of β-LG medium increases, and the induced folding changes of β-LG in the presence of this surfactant increase the values of n and K_d. Comparison between n and K_d in the presence of SDS and Triton X-100 at various pHs shows that the number of observed independent binding sites for retinol (n) in the present of Triton X-100 is greater than in the presence of SDS. This may due to the increase of hydrophobicity of medium with rise of Triton X-100 molar ratio affecting hydrophobic interactions, which may cause the expansion of the β-LG molecule. The study of interactions of SDS and Triton X-100 with β-LG at various pHs shows the significant changes in tertiary structure of β-LG due to its interactions with SDS, which follows a two-step mechanism. Surprisingly, the retinol binding studies do not show considerable changes in the binding properties of β-LG during its simultaneous interactions with SDS. This could be explained by the stability of the conformation of the calyx or by the stability of the retinol binding site. Also, as shown by spectrometric studies, secondary structure of β-LG remains stable during these interactions.

The obtained results attest smaller denaturing activity of Triton X-100 in respect to tertiary structure of β-LG. However, despite all that, high affinity for Triton X-100 of β-LG reduces with pH increase. Insignificant effect of Triton X-100 on retinol binding of β-LG attests the noncompetitor rule of this surfactant with retinol for occupation of hydrophobic calyx. The obtained information about the binding of these popular surfactants can be useful in their application in the dairy food industry and in the formulation of functional surfactant complexes with other proteins of interest. This may also give some guidance as to what could be expected during interactions of charged and neutral lipids with β-LG and other food proteins.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; SDS, sodium *n*-dodecyl sulfate; ITC, isothermal titration calorimetry; Trp, tryptophan; Cys, cysteine; Arg, arginine; Lys, lysine; Glu, glutamic acid; Asp, aspartic acid.

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