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pH and Ionic Strength Dependence of Protein (Un)Folding and Ligand Binding to Bovine β-Lactoglobulins A and B†

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ABSTRACT: Formation of complexes between bovine β-lactoglobulins (BLG) and long-chain fatty acids (FAs), effect of complex formation on protein stability, and effects of pH and ionic strength on both complex formation and protein stability were investigated as a function of pH and ionic strength by electrophoretic techniques and NMR spectroscopy. The stability of BLG against unfolding is sharply affected by the pH of the medium: both A and B BLG variants are maximally stabilized against urea denaturation at acidic pH and against SDS denaturation at alkaline pH. The complexes of BLGB with oleic (OA) and palmitic acid (PA) appear more stable than the apoprotein at neutral pH whereas no differential behavior is observed in acidic and alkaline media. PA forms with BLG more stable complexes than OA. The difference between the denaturant concentration able to bring about protein unfolding in the holo versus the apo forms is larger for urea than for SDS treatment. This evidence disfavors the hypothesis of strong hydrophobic interactions being involved in complex formation. Conversely, a significant contribution to FA binding by ionic interactions is demonstrated by the effect of pH and of chloride ion concentration on the stoichiometry of FA·BLG complexes. At neutral pH in a low ionic strength buffer, one molecule of FA is bound per BLG monomer; this ratio decreases to ca. 0.5 per monomer in the presence of 200 mM NaCl. The polar heads of bound FA appear to be solvent accessible, and carboxyl resonances exhibit an NMR titration curve with an apparent pK_a of 4.7(1).

β-Lactoglobulin (BLG)[1] is the most abundant protein in the whey of bovine milk (*1*−*3*). At room temperature and under physiological conditions, this small protein (ca. 18 kDa) exists as a dimer, but it exhibits complex association equilibria, shifting between monomer, dimer, tetramer, octamer, and monomer again upon lowering the pH of the solution from 8 to 3.5 (*4*−*6*). Many genetic variants of bovine BLG are known. The two most common, A (BLGA) and B (BLGB), differ in two amino acids: Asp64 and Val118 in BLGA are substituted by Gly64 and Ala118 in BLGB (*7*). The available X-ray structures (*8*−*12*) show for BLG a β-barrel with eight strands of antiparallel β-sheets. This fold is common to the lipocalin superfamily, which includes, among others, plasma retinol binding protein (RBP), major urinary protein (MUP), α_{2u}-globulin (A2U), and apolipoprotein D. Despite a low degree of sequence similarity, these extracellular proteins share the common property of binding hydrophobic ligands (*13*, *14*). Indeed BLG, when isolated from cow milk, is found associated with fatty acids (FA), mainly palmitic, oleic, and myristic (*15*), while in vitro it is able to bind a number of hydrophobic and amphipathic molecules (*16*−*20*). BLG shows a remarkable stability even at very low pH, where it retains almost all of its structural features, to such an extent that its NMR characterization in solution was possible under these conditions (*21*−*24*).

Hydrophobic ligands have been found to bind into the β-barrel cavity (*19*, *25*, *26*). In addition, spectroscopic evidence using spin-labeled FA or fluorescence probes suggested the existence of multiple binding sites (*27*−*29*). Due to its binding properties and its stability in acid solution, BLG was proposed to act as a retinol carrier toward intestinal receptors (*8*) and/or to sequester the fatty acids produced by gastric lipase, thus buffering their adverse effects on mucosa cells (*15*). Despite all the structural information accumulated over the years, the physiological function of BLG is still

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[1] Abbreviations: BLG, β-lactoglobulin; BLGA, β-lactoglobulin genetic variant A; BLGB, β-lactoglobulin genetic variant B; FA, fatty acid; OA, oleic acid; PA, palmitic acid; PAA, polyacrylamide; DGGE, denaturant gradient gel electrophoresis; HPCZE, high-performance capillary zonal electrophoresis; $[U]_{0.5}$, urea concentration at half unfolding transition.

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debated and the molecular mechanisms of ligand binding and release are poorly understood.

We report here a study on the pH and ionic strength dependence of folding and ligand binding properties of bovine BLGs A and B, making use of denaturant gradient gel electrophoresis (DGGE), high-performance capillary zone electrophoresis (HPCZE), and ^{13}C NMR. The complexes with palmitic (PA·BLG) and oleic acid (OA·BLG) were investigated, their stoichiometry was established with stepwise interaction experiments, and their behavior on varying the properties of the medium was assessed. The three different approaches, spanning different concentration ranges and probing different features of the complexes, provide a uniform and coherent description of the relevant properties of this protein.

MATERIALS AND METHODS

Reagents. Palmitic (PA) and oleic acid (OA) selectively enriched in ^{13}C at C_1 were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA) and from Isotec Inc. (Miamisburg, OH), respectively, and were used without further purification. Lipidex-1000 (catalog no. 600830) was from Packard Instruments Co. (Meriden, CT). Buffer solutions were prepared with deionized Milli-Q water containing 0.02% NaN_3. pH measurements were performed with a pH meter, model 668, from Amel Instruments, equipped with a Mettler microelectrode; the reported values are uncorrected for isotopic effects.

Protein Samples. The A and B genetic variants of bovine BLG were purified from untreated, fresh cow milk according to Monaco et al. (*9*). Milk was centrifuged twice for 1 h at 8000 rpm at 4 °C in order to remove the fat. The pH of the liquid was then adjusted to 6.6, and the casein was precipitated by addition of $CaCl_2$ up to 0.12 M and removed by centrifugation. The sample was dialyzed vs 20 mM Tris, pH 7.2, and applied to a column of DEAE-cellulose equilibrated and eluted with the same buffer. The unbound material was discarded, and BLG was eluted, making the buffer 0.4 M in NaCl. The mixture of BLGs A and B was further purified by gel filtration on Sephadex G-100 equilibrated with Tris buffer, pH 7.2. The two genetic variants were separated by anion-exchange chromatography on a DEAE-cellulose column, equilibrated with 0.05 M phosphate, pH 5.8, and eluted with a linear gradient from 0 to 0.11 M NaCl (*30*). Starting with a volume of 4 L of milk, 1.8 g of BLGA and 1.6 g of BLGB were obtained. These samples will be referred to as natural BLGs. The NMR and CZE experiments were performed using the B variant while both variants were used for the other experiments.

The apo form of BLGB was prepared by stripping the natural ligand(s) on Lipidex-1000. Aliquots of the defatted protein in 10 mM phosphate buffer, pH 7.4, were washed in an Amicon ultrafiltration cell and eventually concentrated using a Centricon YM10 membrane (Amicon). The protein concentration was determined from the UV absorbance data ($\epsilon_{280} = 17460\ M^{-1}\ cm^{-1}$).

Preparation of the Complexes. Solutions containing the appropriate amount of FA in freshly distilled diethyl ether were introduced in 5 mm tubes (for NMR) or vials (for electrophoretic experiments), and the solvent was slowly evaporated in a dry nitrogen stream. BLG solutions (in 10 mM phosphate buffer, pH 7.3−7.5), at a concentration of ca. 20 mg/mL or of 1 mg/mL, respectively, were then incubated with the FA for 8−10 h at 313 K in a thermostated bath. For stepwise interaction experiments in ^{13}C NMR, the FA:BLG molar ratio was gradually increased between 0.5 and 2 or 3.

NMR Spectra. The ^{13}C NMR spectra were recorded on Bruker AC200, Bruker AC300, and Bruker DRX300 spectrometers operating at 50.3 or 75.4 MHz. All spectra were obtained at 313 K, unless otherwise specified. Different protocols were employed to optimize the signal-to-noise ratios at the two fields. Typically, at 4.7 T, after a 75° pulse with 1 s relaxation delay, 40000 transients were collected over a spectral width of 11000 Hz, using 8K data points. At 7.1 T, a 90° pulse was employed for 20000 transients over 15000 Hz (D1 = 2 s, data points 16K). A line broadening function (5−10 Hz) on 2K or 4K data points, zero-filled to 8K, was applied before Fourier transform. Spectral baseline corrections based on a fourth-order function were used, when performed over the whole spectral width, or on a linear function, when performed over a narrower spectral region (see ii below).

The amount of FA·BLG complexes was estimated through (i) the ratio of the integrated intensity of the $^{13}C_1$-FA peak with respect to the envelope of the ϵLys−βLeu carbons (δ = 39.91 ppm), (ii) the ratio of the integrated intensity of the $^{13}C_1$-FA peak with respect to the envelope of the carbonyl−carboxyl signals of the protein, or, when relevant, (iii) the integrated intensity of the $^{13}C_1$-FA peak in the difference spectra between holo- and apoprotein. All three approaches gave coherent results.

NMR Acidic Titrations. A sample of ca. 1.7 mM OA·BLGB complex was titrated from pH 7.2 to pH 2.5 with small aliquots of 0.22 M H_3PO_4 and back-titrated to pH 7.1 with 0.72 M NaOH. After each addition the solution was incubated for ca. 7 h at 313 K before a ^{13}C spectrum was recorded. The pH of the solution was checked before and after each NMR measurement. Similar experiments were repeated using a 1.4 mM solution of the complex in the presence of 2 equiv of excess of OA.

NMR Titration with Chloride Ions. PA and OA complexes were prepared in 10 mM phosphate buffer in the presence of an excess of FA (3 equiv); the sodium chloride content was then increased stepwise from 0 to 200 mM.

Bound−Unbound FA Exchange. FA·BLG complexes were prepared by incubation of a BLGB solution in 10 mM phosphate buffer with 1 equiv of $^{13}C_1$-enriched FA, and a ^{13}C NMR spectrum was recorded. The samples were transferred to NMR tubes where 3 equiv of the corresponding natural abundance FA had been previously deposited, and new ^{13}C NMR spectra were recorded to monitor the exchange between bound and unbound FA.

DGGE across Transverse Urea Gradients. Electrophoresis across transverse urea gradients (*31*, *32*) was carried out as previously described (*33*, *34*). Sample application trenches, 90 × 3 mm, were shaped in a mold (catalog no. 80-1106-89 from Pharmacia) with strips of embossing tape. A denaturant gradient, 65 mm high and corresponding to 4.5 + 4.5 mL of solution, was cast between two 2 cm high concentration plateaus. The additive was used at concentrations between 0 and 8 M. The PAA concentration in the slab was 5% T and 4% C.

Table 1: Running Conditions in Urea− and SDS−DGGE

		urea−DGGE		SDS−DGGE	
pH	buffer	time (min)/ voltage (V)	polarity	time (min)/ voltage (V)	polarity
3.8	30 mM β-alanine/20 mM lactic acid	60/400	+ → +	60/400	− → +
4.8	80 mM γ-aminobutyric acid/20 mM acetic acid	90/500	+ → -	90/500	− → +
6.1	30 mM histidine/30 mM 2-(*N*-morpholino)ethanesulfonic acid	120/300	− → +	120/300	− → +
7.4	43 mM imidazole/ 35 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid	75/300	− → +	75/300	− → +
8.7	50 mM Tris/25 mM boric acid	60/300	− → +	60/300	− → +

Sixty microliters of the sample solutions was applied per lane. Gels were subjected to electrophoresis at 288 K, in a horizontal chamber (Multiphor II, Pharmacia), with an electrode distance of 22 cm and agarose strips as anolyte and catholyte. The buffers used (*35*) and the running conditions were those listed in Table 1. Protein staining was with 0.3% w/v Coomassie Blue R250 in 30:10:60 v/v/v ethanol−acetic acid−water. The $-\Delta G_{folded}$ was estimated by extrapolating from the transition region to the position of zero urea concentration along the direction of the slanted portion of the curve, while taking equal to $|4RT|$ the distance between the bands of fully folded and fully unfolded protein (*36*).

DGGE across Transverse SDS Gradients. SDS gradients, 0−0.1% w/v, were cast as previously described (*37*). Most features of the experimental setup were similar to what has been detailed above for urea−DGGE. The PAA matrix was 10% T and 4% C. The anodic strip was made of agarose, while the cathodic strip was PAA containing a detergent gradient with the same shape as in the resolving gel (*37*). The buffers used (*35*) and the running conditions are listed in Table 1.

High-Performance Capillary Zone Electrophoresis. HPCZE of apoBLGB and of its complexes with OA and PA was performed in a bare silica capillary, 75 μm i.d. and 50 cm length, with a P/ACE2100 Beckman equipment. The separation medium was 100 mM boric acid, titrated to pH 5.6 and 7.0, added with increasing amounts of NaCl. The running temperature was 303 K, the voltage drop was 30000 V, and the detection wavelength was 214 nm.

RESULTS

NMR Spectroscopy. ^{13}C NMR Spectra of BLGB and of $^{13}C_1$-Selectively Enriched FA·BLGB Complexes. In pH 7.4 phosphate buffer, the ^{13}C NMR spectrum of delipidated BLGB shows broad peaks, as expected for a 36 kDa dimer, with the exception of the signal due to the envelope of the mobile ϵ-Lys−β-Leu carbon atoms, observed at 39.91 ppm (Figure 1a). After incubation with an excess of $^{13}C_1$-FA, a novel, sharp signal, diagnostic of the formation of a FA·BLGB complex, appears at 182.39−182.76 ppm (Figure 1b,c). When incubations are performed with increasing amounts of $^{13}C_1$-FA, at molar ratios ranging between 0.5 and 2 (for PA) or between 0.5 and 3 (for OA), the extent of FA binding to BLGB can be monitored via this peak. The ratio between its integrated intensity and the one of the envelope of the protein ϵ-Lys−β-Leu carbon signals increases until a plateau is reached at a molar ratio of 1:1 (ligand:monomer) for both tested FAs (Figure 1d). With OA, a broad peak, due to unbound, lamellar OA, is observed at δ 179.66 for

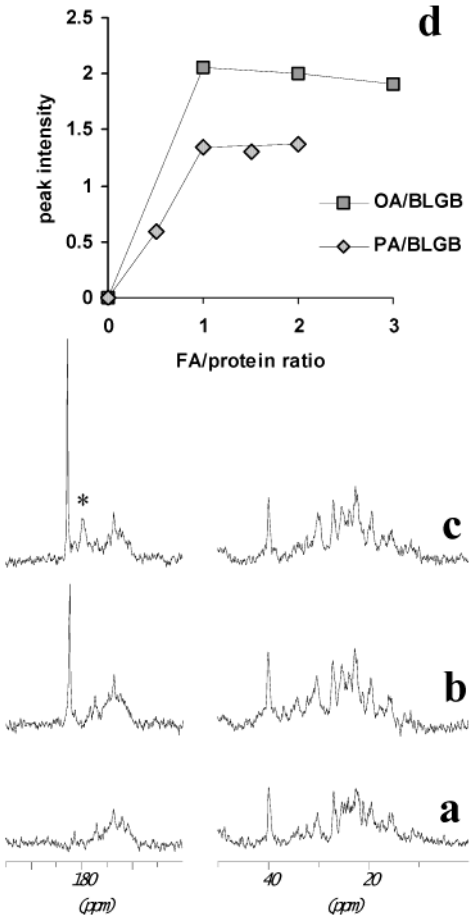

FIGURE 1: ^{13}C NMR spectra of (a) apoBLGB, (b) BLGB after incubation with ca. 1 equiv of $^{13}C_1$-enriched palmitic acid, and (c) BLGB after incubation with more than 1 equiv of $^{13}C_1$-enriched oleic acid (75 MHz, 313 K, pH 7.4). The asterisk indicates unbound enriched oleic acid. (d) Stepwise interaction of oleic (■) and palmitic acid (◆) with apoBLGB. The amount of bound FA was estimated according to procedure i, detailed in Materials and Methods (75 MHz, 313 K).

molar ratios higher than 1. The differences in the plateau values for the two acids are due to differences in their relaxation times (Beringhelli et al., in preparation).

FA·BLGB Complexes and Ionic Content of the Medium. The presence of chloride ions in the incubation medium affects both the de novo formation and stability of existing FA·BLGB complexes. The chloride concentration was increased stepwise in the solution of FA·BLG complexes prepared in 10 mM phosphate buffer; the ratio of the integrated intensity of the $^{13}C_1$-FA peak with respect to the envelope of the carbonyl−carboxyl signals of the protein was used to evaluate the changes in the amount of complexed $^{13}C_1$-FA. Figure 2 shows a decrease, down to 50%, of the

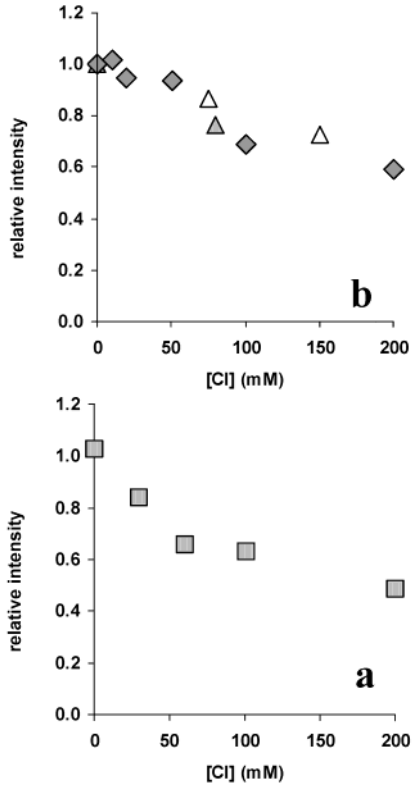

FIGURE 2: Relative decrease of the amount of bound FA during chloride titration: (a) OA·BLGB (■, stepwise increase from 0 to 200 mM); (b) PA·BLGB (◆, stepwise increase from 0 to 200 mM; △, complexes prepared in buffers containing 75 mM and 150 mM Cl^-; ▲, complexes prepared in solution with 0 mM Cl^- and then raised to 80 mM).

amount of complex in solution on increasing the chloride concentration. The solution of the $^{13}C_1$-OA·BLG complex containing 200 mM chloride was then washed in a Centricon YM10 until no chloride ion was detectable in the eluate and reconcentrated. A ^{13}C NMR spectrum showed that no further loss of $^{13}C_1$-OA had occurred. The sample was incubated with an additional 0.5 equiv of $^{13}C_1$-OA; under these conditions, 85% of the initial peak intensity could be recovered. With $^{13}C_1$-PA·BLG the results from stepwise titration (◆ in Figure 2b) were comparable with those of the samples prepared in buffers of varying chloride concentration (△ for 75 and 150 mM) and for a sample prepared in a no salt buffer, made 80 mM chloride in a single step (▲).

Stability of FA·BLGB Complexes on Varying the pH. On decreasing the pH, the signals of the carboxylic carbons of both $^{13}C_1$-FA·BLGB complexes reversibly decreased their intensity and shifted toward higher fields. The results for the OA·BLGB complex are shown in panels a (intensity ratio) and b (δ value) of Figure 3. Comparable behavior was observed in the presence of an excess of unbound ligand (data not shown).

Bound−Unbound Fatty Acid Exchange. When complexes prepared with the stoichiometric amount of $^{13}C_1$-OA and $^{13}C_1$-PA were incubated with 3 equiv of the corresponding natural abundance FA, a slow decrease in the intensity of the bound carboxylate signal occurred, indicating an exchange with the unbound FA. While in the case of the $^{13}C_1$-PA·BLGB complex a 40% decrease was observed after 200 h, for the $^{13}C_1$-OA·BLGB complex such a result was reached within 10 h (Figure S1; see Supporting Information).

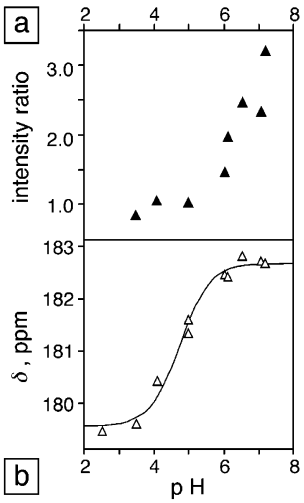

FIGURE 3: (a) pH dependence of the integrated intensity of the ^{13}C NMR signal of BLGB-bound OA. (b) pH dependence of the chemical shift of BLGB-bound OA. The curve was drawn by data fitting to the Henderson−Hasselbach equation.

Electrophoretic Techniques. Seven protein samples (natural BLGA, reduced BLGA, natural BLGB, reduced BLGB, apoBLGB, OA·BLGB, and PA·BLGB) were run in urea− and SDS−DGGE in five buffers (pH 3.8, 4.8, 6.1, 7.4, and 8.7).

Urea−DGGE. Natural BLGs. Figure 4a shows the denaturation curves across a 0−8 M urea gradient of untreated and reduced BLGA and BLGB at pH 7.4. Figure S2 compares the migration of untreated and reduced BLGB, run across 0−8 M urea gradients in buffers of increasing pH. At all tested pHs, except 4.8, the urea denaturation curves for untreated and reduced BLGs are sigmoidal, which hints to cooperative $N \leftrightarrow U_{urea}$ transitions.

In GABA−acetic acid buffer, on the contrary, a progressive change in mobility, i.e., a seemingly noncooperative transition, is observed between 0 M urea and concentrations of 7.2 M for untreated BLGA, of 6.3 M for reduced BLGA, of 6.5 M for untreated BLGB, and of 5 M for reduced BLGB. Moreover, the curves at pH 4.8 are slightly convex for BLGB and strongly concave for BLGA, and an additional protein band is observed for BLGA, both untreated and reduced, above 3.7−4 M urea and parallel to the main curve (not shown). Above 4 M urea the migration of the protein in the main curve is minimal and toward the cathode, and that of the additional band is also minimal but toward the anode. While all BLG forms bear a positive net charge at pH 3.8 and a negative net charge at pH ≥6.1, at pH 4.8 folded BLGs thus appear to dissociate as cations and fully unfolded BLGs as anions (Figure S2b vs S2a, S2c−e).

Figure 5 displays the main parameters of the denaturation curves for untreated and reduced BLGA and BLGB as a function of pH. Panel a refers to $[U]_{0.5}$, and panel b refers to the net stability of the protein in the absence of denaturant, $-\Delta G_{folded}$, expressed in RT units, as devised by Creighton (*36*). The stability of BLGs to unfolding by urea decreases as the pH increases (Figure 5a). BLGA and BLGB unfold at similar urea concentrations ($[U]_{0.5}$) at all pHs except 3.8, where BLGA slows to m_U mobility before BLGB (Figure 5a). Net stabilization ($-\Delta G_{folded}$) and cooperativity are also similar for BLGA and BLGB and decrease with increasing pH (Figure 5b).

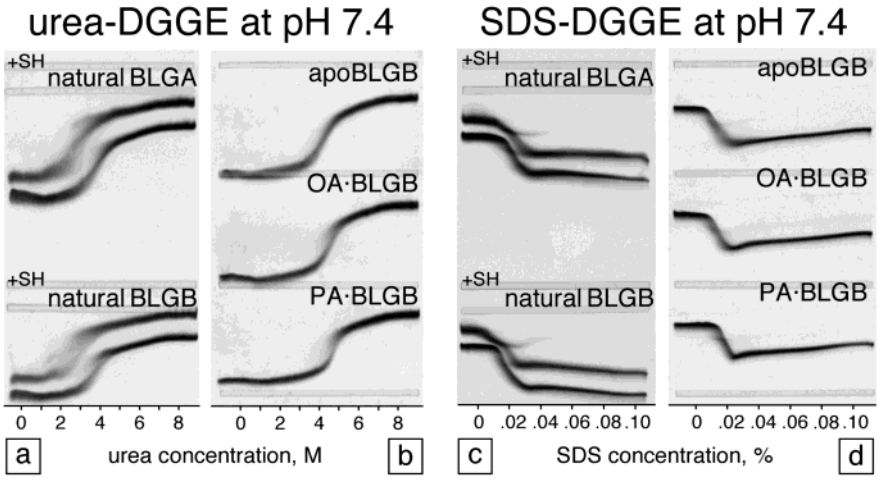

FIGURE 4: (a) Urea−DGGE of natural BLGs: top, natural BLGA; bottom, natural BLGB; both reduced (from lanes +SH) and unreduced. Run across a transverse 0−8 M urea gradient at pH 7.4. (b) Urea−DGGE of apoBLGB (top), OA·BLGB (middle), and PA·BLGB (bottom). Run across a transverse 0−8 M urea gradient at pH 7.4. (c) SDS−DGGE of natural BLGs: top, natural BLGA; bottom, natural BLGB; both reduced (from lanes +SH) and unreduced. Run across a transverse 0−0.1% SDS gradient at pH 7.4. (d) SDS−DGGE of apoBLGB (top), OA·BLGB (middle), and PA·BLGB (bottom). Run across a transverse 0−0.1% SDS gradient at pH 7.4.

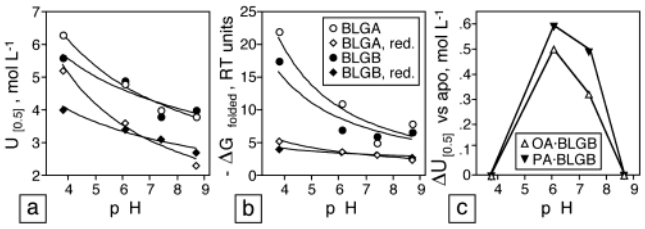

FIGURE 5: pH dependence of (a) $[U]_{0.5}$, the concentration of urea at half-transition, from the folded, fast-migrating to the unfolded, slow-migrating form of the protein: BLGA (○), reduced BLGA (◇), BLGB (●), reduced BLGB (◆). (b) $-\Delta G_{folded}$, the net stability of the protein in the absence of denaturant, expressed in RT units. (c) Increase in $[U]_{0.5}$ for OA·BLGB (△) and PA·BLGB (▼). Lines are drawn for visual aid.

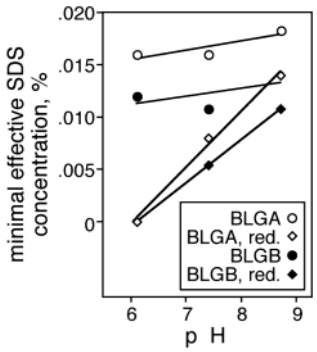

FIGURE 6: pH dependence of the minimal effective SDS concentration (i.e., able to influence protein migration) for BLGA (○), reduced BLGA (◇), BLGB (●), and reduced BLGB (◆). Lines are drawn for visual aid.

Reduced BLGs are invariably less stable to denaturation than their untreated counterparts (Figure 5a,b).

In all tested conditions, the denaturation curve of BLGA has a more even profile than that of BLGB, which suggests a faster $N \leftrightarrow U_{urea}$ transition for the former than for the latter. The reversibility of the transition decreases from pH 6.1 to pH 8.7, as the slanted portion of the curves becomes more and more blurred. A straight band of lower slope than the main curve in the transition region (a spur) pointing toward low urea concentrations [and containing molecules in metastable form, unable to refold through the duration of the electrophoretic run (*36*)] is observed at all tested pHs. This spur is more evident for BLGB than for BLGA and for reduced than for untreated BLGs.

ApoBLGB and Its Complexes with Fatty Acids. Figure 4b shows the denaturation curves in urea−DGGE at pH 7.4 of apoBLGB, OA·BLGB, and PA·BLGB. The curve for apoBLGB has an almost perfectly continuous, sigmoidal course, whereas the curves for the complexes with FA present the spurs already observed for natural BLGs (Figure 4a). Similar spurs are also present at pH 6.1 and 8.7 and in all instances appear more conspicuous for holo- than for apoBLGB (Figure S3).

Binding of the ligands results in some stabilization of BLGB against denaturation. This effect is strongly pH-dependent, being maximal at pH 6.1 and absent both at pH 3.8 and at pH 8.7 (Figure 5c).

SDS−DGGE. Natural BLGs. Figure 4c shows the denaturation patterns across a 0−0.1% SDS gradient of untreated and reduced BLGA and BLGB at pH 7.4. The tracings for untreated BLGs are close to sigmoidal, whereas those for reduced BLGs display a more complex structure, with the slanted part of the curve with a concave and then a convex course. Discontinuity and the presence of spurs in the curves of reduced vs untreated BLGs hint to a faster $N \leftrightarrow U_{SDS}$ transition for the latter than for the former and to the existence of different intermediates along the alternative denaturation paths. Similar patterns are also observed at pH 6.1, but with a very faint tracing in the slanted part of the curve, and at pH 8.7, where, on the contrary, the curve is sharp and continuous (not shown). $N \leftrightarrow U_{SDS}$ transition thus appears to become faster with increasing pH.

At pH 3.8 BLGs are precipitated in the application trenches by even the lowest concentrations of SDS, and at pH 4.8 only a small percentage of the proteins do migrate (not shown).

Figure 6 depicts the minimal concentrations of SDS able to affect protein migration (i.e., the width of the leftmost plateau, Figure 4c) at pH ≥6.1. Reduced BLGs are less stable

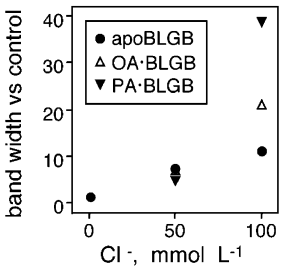

FIGURE 7: Bandwidth dependence from ionic strength in HPCZE at pH 5.6 for BLGB (●), OA·BLGB (△), and PA·BLGB (▼).

to denaturation than native BLGs, but the difference decreases with increasing pH.

ApoBLGB and Its Complexes with Fatty Acids. Figure 4d shows the denaturation curves in SDS−DGGE at pH 7.4 of apoBLGB, OA·BLGB, and PA·BLGB. At this pH the holo forms are stabilized vs apoBLGB against SDS denaturation, with the minimal effective concentration of SDS increasing by 0.003 for the complex with OA and by 0.004 for the complex with PA. No significant shift is observed on the contrary at pH 6.1 or 8.7 (not shown).

HPCZE. Figure S4 compares the electrophoretic patterns at pH 5.6 of apoBLGB, OA·BLGB, and PA·BLGB in the presence of increasing concentrations of NaCl. Raising the ionic strength in the analysis medium causes the migration time and bandwidth to increase; for apoBLGB the shift is from 5 min 30 s, and 20 s, in plain buffer (panel a) to 25 min, and 3 min 30 s, respectively, in the presence of 100 mM NaCl (panel c). Migration time differences at this pH are minimal between apoBLGB and liganded BLGB [panel d vs panel a; same finding for the complex with PA (not shown)]. A similar overall behavior is observed at pH 7.0 (not shown).

If the increase in bandwidth as a function of added chloride is computed for apoBLGB and its complexes, taking the bandwidth in the absence of NaCl as the reference value, the results plotted in Figure 7 are obtained. Upon exposure to higher ionic strength media, the polydispersity of the samples increases more extensively for liganded than for apoBLGB.

DISCUSSION

The present investigation addresses a number of related issues: (i) formation of complexes between BLGs and FAs; (ii) effect of complex formation on protein stability; (iii) effects of pH and ionic strength on both complex formation and protein stability.

The stepwise interaction experiments performed through ^{13}C NMR spectroscopy prove that in 10 mM phosphate, pH 7.4, BLG can bind one molecule of FA per monomer. The conflicting reports in the literature concerning the binding properties of BLG likely result from different conditions in protein purification or in complex preparation. Bovine milk contains ca. 30 mM chloride; indeed, after purification, only 70% of BLG monomers are found associated with lipids (*15*). The stoichiometry of a PA complex prepared in 150 mM NaCl (*20*) was determined as 1 PA per dimer. The internal cavity occupancy in a FA·BLG crystal grown in 150 mM NaCl was found to be 70% of that found in crystals grown in a medium without chloride ions (*26*). In agreement with these observations, our results show that the stability of the FA·BLG complexes decreases upon rise of the chloride concentration. The relevance of the electrostatic interactions in the FA·BLG complexes is also proved by the almost complete reversibility of the FA binding/release processes, as demonstrated by our dialysis experiments on OA/BLGB/chloride mixtures as well as by our CZE results. The bandwidth increase upon raising the ionic content of the medium indicates sample polydispersity as when the equilibrium is shifted between liganded and unliganded protein molecules.

In our experiments on the OA·BLG complex, the pH dependence of the chemical shift and the decrease in intensity of the carboxylate resonance on lowering the pH are similar to what is observed for the PA·BLG complex (*20*). When bound to BLG, the polar head of OA is therefore solvent accessible, and its protonation can reversibly modulate binding. The p*K* calculated for OA according to the Henderson−Hasselbach relationship is 4.7(1), very close to the value reported for other FA·protein complexes (*20*, *38*, *39*). The longer time required for the equilibration with natural abundance FA by PA·BLG compared with the OA·BLG complex is likely due to the different phase of the unbound molecules, solid and lamellar, respectively. This indicates an easy exchange of the ligands in the presence of an acceptor phase in solution.

Natural BLGs show a marked pH dependence in their resistance against unfolding, with higher stability at acidic than at alkaline pH. Both parameters characterizing an unfolding transition, $[U]_{0.5}$ and $-\Delta G$, show a smooth change with pH. Urea−DGGE data follow a trend similar to that found by D'Alfonso et al. (*40*) using fluorescence spectroscopy, and at all tested pHs and with both denaturing agents, DGGE gives no evidence of dissociation to monomers before unfolding or of the presence of unfolding intermediates.

After reduction, unfolding is much easier for both BLGs; this implies a major contribution to stability from the presence of two disulfide bridges. Several other proteins showed in DGGE experiments a similar decrease in stability after reduction (*33*, *41*−*43*).

The shape of the denaturation curves is almost continuous in both unreduced and reduced forms, indicating similar kinetics for the unfolding ↔ refolding process in both redox states for the bulk of protein molecules. The behavior of apoBLGB conforms to a simple two-state model, with a fast and completely reversible transition between folded and unfolded protein. Instead, spurs, corresponding to a subpopulation of metastable protein molecules that fail to refold at denaturant concentrations below $[U]_{0.5}$, are observed for both natural BLGs and FA·BLG complexes. Their width is lower for FA·BLG complexes, and this observation fits with natural BLGs being a mixture of liganded species with different FAs (*15*).

Both spectroscopic and electrophoretic data show PA interacting with BLGB more strongly than OA: indeed PA is less readily displaced by high salt concentrations and confers a higher stabilization against urea denaturation.

Binding with FA stabilizes BLGB against urea and SDS denaturations. These stabilizing effects are much less effective than observed for enzyme−inhibitor complexes (*44*) and confirm that hydrophobic interactions between FA and BLG are not very strong.

The absence of a stabilizing effect in DGGE for FA·BLGB preparations in comparison with apoBLGB at acidic pH is in agreement with the finding of ligand release in our data and literature NMR data. The same effect at alkaline pH could be due to the pH-dependent modifications in intra- and intermolecular interactions that accompany conformational transitions (*45*).

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SUPPORTING INFORMATION AVAILABLE

Time profile of the exchange between $^{13}C_1$-FA BLGB complexes and the corresponding natural abundance ligand; urea-DGGE of natural BLGB, both reduced and unreduced, in buffers from pH 3.8 to 8.7; urea-DGGE of apoBLGB, OA·BLGB and PA·BLGB from acidic to basic pH; HPCZE patterns at pH 5.6 of apoBLGB and OA·BLGB at different chloride concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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