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EF Loop Conformational Change Triggers Ligand Binding in β-Lactoglobulins*

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β-Lactoglobulins, belonging to the lipocalin family, are a widely studied group of proteins, characterized by the ability to solubilize and transport hydrophobic ligands, especially fatty acids. Despite many reports, the mechanism of ligand binding and the functional role of these proteins is still unclear, and many contradicting concepts are often encountered in the literature. In the present paper the comparative analysis of the binding properties of β-lactoglobulins has been performed using sequence-derived information, structure-based electrostatic calculations, docking simulations, and NMR experiments. Our results reveal for the first time the mechanism of β-lactoglobulin ligand binding, which is completely determined by the opening-closing of EF loop, triggered by Glu^{89} protonation. The alkaline shift observed for Glu^{89} pK_a in porcine β-lactoglobulin (pK_a 9.7) with respect to the bovine species (pK_a 5.5) depends upon the interplay of electrostatic effects of few nearby key residues. Porcine protein is therefore able to bind fatty acids provided that the appropriate pH solution conditions are met (pH > 8.6), where the EF loop conformational change can take place. The unusually high pH of binding detected for porcine β-lactoglobulin seems to be functional to lipases activity. Theoretical pK_a calculations extended to representative β-lactoglobulins allowed the identification of key residues involved in structurally and functionally important electrostatic interactions. The results presented here provide a strong indication that the described conformational change is a common feature of all β-lactoglobulins.

The physicochemical and biological characteristics of β-lactoglobulins, which belong to the lipocalin family, have been extensively studied in the last 30 years, but despite the wealth of data, the biological function of these extracellular proteins is still undefined (Ref. 1 and references therein). β-Lactoglobulins isolated from cow, goat, and sheep milk samples, under nondenaturing conditions, showed endogenously bound fatty acids (2). Many authors have suggested that bovine β-lactoglobulin (BLG)[1] has a transport and/or protective role toward bound ligands in the stomach (3). However, we have previously shown (4) that BLG, despite its high stability at acidic pH, is unable to bind fatty acids at low pH, thus indicating that it could not be employed "as is" as a transporter through the human gastric tract.

Retinoids and fatty acids have been reported to bind to BLG *in vitro* in the pH range of 6.5–8.5, with dissociation constants on the order of 65 nM and 0.6 μM, respectively (5). Specifically, titration experiments of BLG with palmitic acid (PA) (4) have clearly shown that: (i) at neutral pH the primary site for palmitic acid binding is within the protein calyx; (ii) the amount of bound PA is drastically reduced upon decreasing pH and the ligand is completely released at pH 2; (iii) in the pH range 7.3–6.4, a conformational equilibrium was observed for the bound ligand reflecting the dynamics of EF loop (region 85–90) (Fig. 1), triggered by the titration of Glu^{89} at anomalously high pK_a (~6.5) (6). On the contrary, it was shown that the highly similar porcine (PLG) (62% identity, 83% similarity) and equine (58% identity, 74% similarity) β-lactoglobulins had neither fatty acids physiologically bound nor the ability to bind them *in vitro* at neutral pH (7). We have previously reported PLG interaction studies with palmitic acid in the pH range 2–8, aimed at clarifying whether dimer formation could possibly have a role in binding (8). Indeed PLG exhibits a pH dependence of the monomer-dimer equilibrium opposite to that observed for BLG. NMR interaction studies demonstrated that PLG is unable to bind palmitic acid in this pH range. These results were rationalized by us and others (8, 9) hypothesizing that Lys^{60} and Lys^{69}, forming a superficial positively charged patch at the open end of the BLG calyx, could be responsible for electrostatic interactions with palmitic acid carboxylate, thus driving the binding. Both PLG and equine β-lactoglobulin, which did not show endogenously bound fatty acids, exhibited the K69E mutation. However, tear lipocalin, a protein closely related to BLG, was shown to bind 16-doxyl stearic acid despite the absence of any charged side chain at positions 60 (Met) and 69 (Val) (BLG numbering) (10).

The importance of clarifying the binding mechanism and, hence, the functional role of β-lactoglobulins has prompted us to extend our previous investigations to all members of this family. In the present paper the comparative analysis of β-lactoglobulin binding properties has been performed using sequence-derived information, structure-based electrostatic calculations, docking simulations, and NMR experiments. Our results reveal for the first time the mechanism of β-lactoglobulin ligand binding, which is fully determined by the conformational change involving the opening-closing of the EF loop (11).

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[1] The abbreviations used are: BLG, bovine β-lactoglobulin; PLG, porcine β-lactoglobulin; PA, palmitic acid; $^{13}C_1$ PA, singly enriched palmitic acid; ^{13}C PA, fully enriched palmitic acid; HSQC, heteronuclear single-quantum coherence; MIF, molecular interaction field; PDB, Protein Data Bank.

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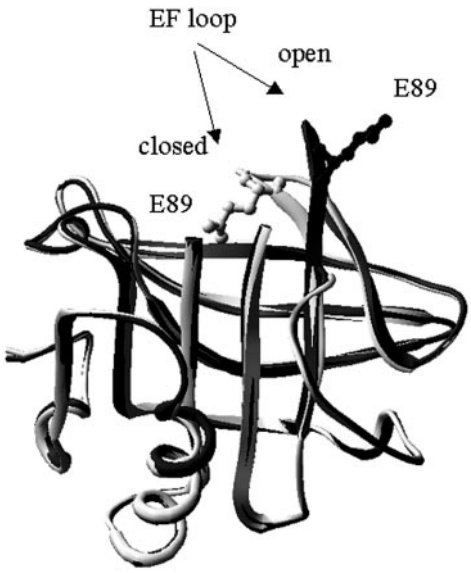

FIG. 1. **Superposition of BLG x-ray structures with EF loop in the closed (PDB code 3blg; *light gray*) and open (PDB code 2blg; *dark gray*) conformations.** Region 109–116 of both proteins has been removed from the ribbon representation in order to better visualize the EF loop region-(85–90). Asp^{89} side chain is shown.

EXPERIMENTAL PROCEDURES

Materials—PLG was purified from milk at NIZO Food Research (The Netherlands) as previously described (8). Palmitic acid was purchased from Sigma. Palmitic acid-protein complexes were prepared as described previously (4). 1.5 mM protein solutions were prepared in 10 mM Na_2HPO_4/NaH_2PO_4 solution at pH 7.0, and a molar excess of PA (4:1 molar ratio) was employed for complex preparation. NMR titrations at different pH levels were performed adding a few microliters of 0.25 N H_3PO_4 or 1 M NaOH to the PA-protein complex prepared at pH 7.0.

10 mM phosphate buffer solution, at different pH levels (7–10), was added to the tube containing PA, and NMR experiments were acquired to check the solubility of the fatty acid. A solution of 50 mM KOH at pH 12.5 was necessary to dissolve a uniformly ^{13}C-labeled PA, 5 mM, for NMR analysis.

NMR Characterization—NMR spectra were acquired on an Avance Bruker 500 MHz spectrometers at 27 °C. ^1H chemical shifts were referred to 3-trimethylsilylpropionate, and ^{13}C chemical shifts were referenced as described previously (4). ^1H one-dimensional NMR spectra of apo- and holo-PLG were run at each pH to check protein stability. One-dimensional proton decoupled ^{13}C spectra were recorded on the complex prepared with carboxyl-enriched PA ($^{13}C_1$ PA) in the pH range 7.0–10.0. The carboxyl region was acquired with a sweep width of 2,520 Hz, 2,000 time domain points, and 20,000 scans. The aliphatic PA signals were observed through one-dimensional proton decoupled ^{13}C spectra of completely enriched PA (^{13}C-PA) in complex with PLG, in the pH range 7.0–10.0 and with BLG, in the pH range 2.5–7.3. A sweep width of 5,040 Hz, 16,000 time domain points, and 3,584 scans were employed. Two-dimensional ^1H-^{13}C HSQC experiments were recorded on ^{13}C-PA and on PLG·^{13}C-PA complex with 512 and 2048 data points in the t_1 and t_2 dimensions, respectively, and a spectral width of 7002 (t_2) and 5040 (t_1) Hz. The spectra were processed and analyzed with the programs XWINNMR (Bruker) and XEASY (12).

Docking Simulations—Docking simulations were performed using the program GRID, version 21 (Molecular Discovery Ltd.) (13, 14). All available BLG structures deposited in the PDB and the PLG x-ray structure (PDB code 1exs) were employed as targets. Crystallographic water molecules were removed. The coordinates of palmitic (C16) and caprylic (C8) acids were derived from the coordinates of palmitic acid bound to BLG (PDB code 1b0o). The fatty acid carboxyl group was considered deprotonated, bearing a net negative charge of −1. The target proteins were considered rigid, and hydrogens were added with the program GRIN (part of the GRID package). The docking search was performed on the whole protein. All GRID input parameters were employed with their default values. The calculated molecular interaction fields (MIF) were inspected with Gview (part of the GRID package). The docking results were visualized with Gview, InsightII (Accelrys, San Diego, CA), and Swiss-PdbViewer (15).

Modeling and pK_a Calculations—Models for lactoglobulins have been built based on the x-ray structures of BLG with the EF loop in the open (PDB code 2blg) and closed (PDB code 3blg) conformations (11) using the Swiss-PdbViewer program (15). Gaps and insertions were shifted out of secondary structure elements, where this was possible, and built using the scan loop data-base module of Swiss-PdbViewer. The anchor residues were chosen such as to make possible the closure of the loop. Hydrogens have been added using the program Gromacs (17).

The University of Houston Brownian Dynamics (UHBD) program, version 5.1 (18), was used for the electrostatic and pK_a calculations. We employed the single-site titration model (19), which includes partial atomic charges of each ionizable group and models the ionization by adding a +1 or −1 point charge to one central atom of the ionizing group. We used a dielectric constant of 20 to represent protein interior. Partial atomic charges and radii for the protein were taken from the pK_a files provided by UHBD. The electrostatic potential computed by UHBD has been used for generating an ensemble of protonation states at different pHs using a Montecarlo procedure as described previously (20). A straightforward application of this procedure led to few but very large pK_a shifts, which were associated with atoms (and therefore with charges) too close to each other. The bumps were then removed using the program WHATIF (21), and pK_a values were computed again. The solvent was assigned a dielectric constant of 80 and ionic strength of 100 mM, and the ion exclusion radius was set to 2.0 Å. The ionic strength was chosen based on the observation that the pK_a values are fairly insensitive to ionic concentrations over 100 mM. A probe with a radius of 1.4 Å was used to define the boundary between the protein and solvent dielectric regions. Electrostatic potentials were calculated by focusing on one titrable residue at a time. The last focusing run employed a grid spacing of 0.25 Å. The temperature was set at 25 °C.

RESULTS

Docking Experiments—To evaluate the role of the EF loop conformational change on binding, different chain length fatty acids were docked within BLG and PLG employing the GRID docking program. The GRID method has been developed for determining energetically favorable binding sites for small chemical groups (probes) on a target molecule (protein) (13, 14). The probe groups are small chemical entities that are moved through a regular grid of points around the target molecule in order to calculate, at each point of the grid, an interaction energy, thus generating MIFs. The ligand molecule is represented as a collection of GRID probes, and MIFs calculated for each probe are used to define the ligand position with respect to the target and to estimate the binding energy of the ligand at its binding site.

Palmitic acid, the most abundant BLG endogenous ligand, was docked to all available BLG structures. GRID generated a docking solution for PA within the protein calyx only when the target BLG presented the EF loop in the open conformation. A very good agreement was observed for the positioning of both the aliphatic chain and the carboxyl tail of the docked and experimentally determined PA (PDB code 1b0o) (Fig. 2*A*). The inspection of MIFs, generated for hydrophobic and carboxyl oxygen probes, indicates that PA docking solution maximizes both electrostatic and hydrophobic interactions.

Docking experiments performed with the available PLG x-ray structure (PDB code 1exs), obtained at pH 3.2 with the EF loop in the closed conformation, were never successful in locating PA within the protein cavity. A model of PLG structure with the EF loop in the open conformation was obtained with the Swiss-PdbViewer program, and GRID simulations showed fatty acids located within the hydrophobic pocket of the protein (Fig. 2*B*). Interestingly the opening of the EF loop appeared to be a requisite for binding even when the shorter caprylic acid (C8) was used in docking experiments.

Electrostatics and pK_a Calculations on β-Lactoglobulins—The opening of the EF loop-(85–90) in BLG has been shown to be triggered by the titration of Glu^{89} (11). EF loop closed conformation is stabilized in BLG by a pattern of hydrogen bonds involving Asp^{88}, Glu^{89}, Asn^{90}, Asn^{109}, Ser^{110}, and Ser^{116}

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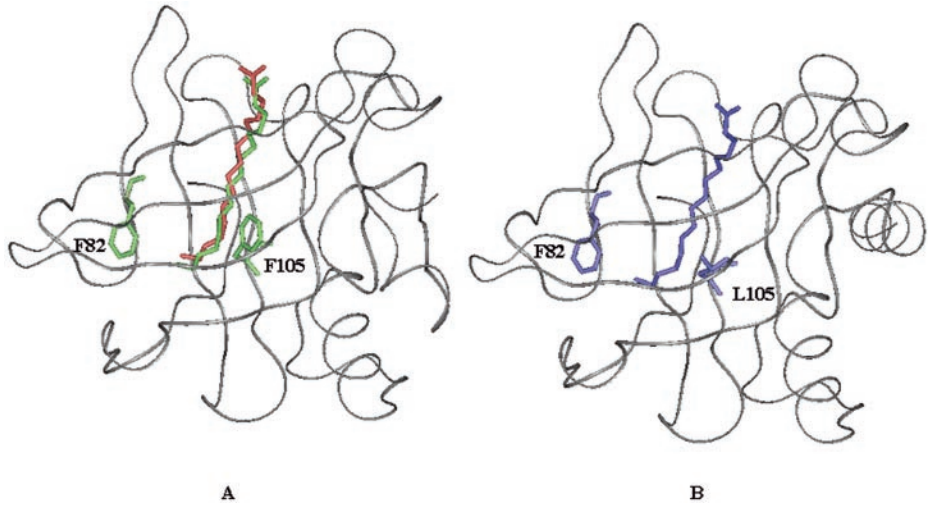

FIG. 2. **GRID docking solutions obtained for BLG (*A*) and PLG (*B*).** *A*, superposition of predicted (*green*) and experimentally observed (*red*) positions of PA. *B*, docked solution obtained for PLG (*blue*). The side chains of residues 82 and 105 are shown.

residues. The titration of the Glu^{89} side chain at unusually high pH (~6.5) is due to the loss of this H-bond pattern and causes the fold-back of the EF loop with a consequent solvent exposure of the glutamic acid side chain (Fig. 1).

If the conformational change of the EF loop determines the binding ability, it is clear that all factors affecting Glu^{89} pK_a, such as the nature and charge of close residues, will modulate the pH of binding.

A comparison of the amino acid sequences of the EF loop region of all β-lactoglobulins, (Fig. 3) revealed that although Glu^{89} is always conserved, its flanking residues (88 and 90) differ both in side chain length and charge. Charged residues close in space to Glu^{89} influence its titration as well. To study the pH dependence of the EF loop conformational change and correlate the sequence mutations with the binding ability, pK_a calculations were performed on the BLG and PLG experimentally determined structures and on the modeled structures of a few lactoglobulins (lacc_felca, laca_canfa, lacb_horse, laca_horse, lacb_macgi) selected on the basis of their clustering in the phylogenetic tree (built with the ClustalW program (22)) (Fig. 4).

Two models were built for each β-lactoglobulin, using as a template BLG structures with the EF loop in the open (PDB code 2blg) and closed conformations (PDB code 3blg), respectively. pK_a calculations were performed using the University of Houston Brownian Dynamics program, which solves the Poisson-Boltzmann equation and provides electrostatic free energies, generating a statistical ensemble of protonation states by a Montecarlo procedure (18). The obtained Glu^{89} pK_a values are reported in Table I.

The inspection of all β-lactoglobulin structures allowed the identification of those side chains close to the Glu^{89} carboxylic group. Most of these residues never display any charge in different β-lactoglobulins; however, a small subset, consisting of residues 88, 90, 107, 108, and 116, shows a charge change along the different species; lacb_macgi was excluded from this analysis because its low sequence identity with BLG (34%), localized mainly at the level of the EF loop, made its model less reliable. Moreover, residue 116 is close to an insertion making it impossible to reliably discuss any result on Glu^{89} pK_a. It is worth noting that on going from BLG to PLG, Glu^{89} pK_a increases by nearly two pH units, thus suggesting that PLG binding should indeed occur but at pH higher than 8.0.

NMR Titration Experiments—PLG interaction studies with $^{13}C_1$ and ^{13}C PA were performed in the pH range 8.0–10.0, *i.e.* at pH values higher than the calculated Glu^{89} pK_a. The increase in PA resonance intensities was used to monitor the uptake of ligand by the protein, because PA is solubilized only through protein binding. Fig. 5 shows that $^{13}C_1$ resonance becomes measurable at pH 8.6, and its intensity increases with pH, showing that PLG can bind PA upon EF loop opening. A line width increase of carboxyl resonance is observed raising the pH from 8.6 ($\Delta\nu_{1/2}$ = 11 Hz) to 9.7 ($\Delta\nu_{1/2}$ = 17 Hz). The amount of bound PA as a function of pH, as derived from the integration of C_{16} methyl resonance, is shown in Fig. 6. The transition midpoint occurs at pH 9.7, thus affording a clear indirect estimate of Glu^{89} pK_a of PLG.

We observed, from the analysis of ^1H NMR spectra, that PLG maintains its conformation up to pH 10, whereas at higher pH the protein starts to precipitate. ^1H and ^{13}C chemical shifts of PA complexed with PLG are reported in Table II.

DISCUSSION

The influence that pH exerts on protein structure is widely believed to be electrostatic in nature via changes in the protonation state of titrable groups, which in turn influence processes like ligand uptake or release, partial or global unfolding, and protein-protein association (23). Our results clearly show for the first time that the protonation state of Glu^{89} influences ligand binding in β-lactoglobulins, forcing the EF loop to act as a mobile lid, hindering the access to the protein cavity when it is in the closed conformation. NMR titration experiments have shown that PLG is able to bind PA, provided that the appropriate pH solution conditions (pH > 8.6) are met. The dependence of the amount of bound PA on pH can be fitted with a sigmoid curve, both for BLG and PLG, thus indicating that the same binding mechanism is at work for the two proteins. In PLG the observed transition midpoint is highly shifted toward alkaline pH (pH 9.7) with respect to BLG (pH 5.5) (Fig. 6), reflecting the different pK_a of Glu^{89} side chains in the two proteins. These data strongly suggests that, contrary to previous reports (24), the EF loop conformational change also occurs in PLG and is likely to be a common feature of all β-lactoglobulins. To further investigate this phenomenon, pK_a calculations were performed for representative β-lactoglobulins from different species. It has been shown by us and others that theoretically determined pK_a values are in good agreement with experimentally determined ones (25), and we feel confident that the pK_a shifts calculated for the different models reflect a reliable trend, which should be considered as such, rather than as an assessment of the exact pK_a value of each titrating group. It is generally agreed that large pK_a shifts can be ascribed to the electrostatic effects of desolvation, nearby charges, and/or to the disruption of hydrogen bonds (25). To rationalize Glu^{89} pK_a

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P02754|LACB_BOVIN   LIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR
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P33685|LACA_CANFA   IVVPRTMEDL DLQKVAGTWH SMAMAASDIS LLDSETAPLR
P33688|LACC_FELCA   ATVPLTMDGL DLQKVAGTWH SMAMAASDIS LLDSEYAPLR
P33687|LACB_FELCA   ATVPLTMDGL DLQKVAGMWH SMAMAASDIS LLDSETAPLR
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P04119|LACB_PIG     VEVTPIMTEL DTQKVAGTWH TVAMAVSDVS LLDAKSSPLK
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P19647|LACA_EQUAS   TDIPQTMQDL DLQEVAGRWH SVAMVASDIS LLDSESAPLR
P07380|LACA_HORSE   TDIPQTMQDL DLQEVAGRWH SVAMVASDIS LLDSESVPLR
P11944|LACB_MACGI   VENIRSKNDL GVEKFVGSWY LREAAK--T- -MEFSIPLFD
Q29614|LACB_MACEU   VENIRSKNDL GVEKFVGSWY LREAAK--T- -MEFSIPLFD
Q29146|LACB_TRIVU   IENIHSKEEL VVEKLIGPWY RVEEAK--A- -MEFSIPLFD

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P33688|LACC_FELCA   VYVQELRPTP RDNLEIILRK WEQKRCVQKK ILAQKTELPA
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P02756|LACB_CAPHI   VFKIDALNEN ---KVLVLDT DYKKYLLFCM ENSAEPEQS-
P02757|LACB_SHEEP   VFKIDALNEN ---KVLVLDT DYKKYLLFCM ENSAEPEQS-
P33685|LACA_CANFA   EFKINYVEEN ---QIFLLDT DYDNYLFFCE MNADAPQQS-
P33688|LACC_FELCA   EFKISYLDEN ---ELIVLDT DYENYLFFCL ENADAPGQN-
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P02758|LACB_HORSE   EFKINYLDED ---TVFALDT DYKNYLFLCM KNAATPGQS-
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Q29614|LACB_MACEU   EFEIYISSES ASYTFSVMET DYDSYFLFCL YNISDREK--
Q29146|LACB_TRIVU   EFEIYIPSES ASYTLSVMET DYDNYILGCL ENVNYREK--

P02754|LACB_BOVIN   LACQCLVRTP EVDDEALEKF DKALKALPMH IRLSFNPTQL EEQCHI
P02755|LACB_BUBBU   LACQCLVRTP EVDDEALEKF DKALKALPMH IRLSFNPTQL EEQCHV
P02756|LACB_CAPHI   LACQCLVRTP EVDKEALEKF DKALKALPMH IRLAFNPTQL EGQCHV
P02757|LACB_SHEEP   LACQCLVRTP EVDNEALEKF DKALKALPMH IRLAFNPTQL EGQCHV
P33685|LACA_CANFA   LMCQCLARTL EVDNEVMEKF NRALKTLPVH MQLLN-PTQA EEQCLI
P33688|LACC_FELCA   LVCQCLTRTL KADNEVMEKF DRALQTLPVD VRLFFDPTQV AEQCRI
P33687|LACB_FELCA   LVCQCLTRTL KADNEVMEKF DRALQTLPVH VRLFFDPTQV AEQCRI
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Q29614|LACB_MACEU   MACAHYVRRI E-ENKGMNEF KKILRTLAMP YTVIEVRTR- -DMCHV
Q29146|LACB_TRIVU   MACAHYERRI E-ENKGMEEF KKIVRTLTIP YTMIEAQTR- -EMCRV
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FIG. 3. **ClustalW (22) alignment of β-lactoglobulins.** The following species are reported: *BOVIN* (cow), *BUBBU* (domestic water buffalo), *CAPHI* (goat), *SHEEP*, *CANFA* (dog), *FELCA* (cat), *PIG*, *HORSE*, *EQUAS* (donkey), *MACGI* (eastern gray kangaroo), *MACEU* (tammar wallaby), and *TRIVU* (brush-tailed possum).

shifts in the analyzed models, contributions due to mutations at the level of residues either involved in H-bonds or close in space to the Glu^{89} side chain were therefore considered. As shown in Table I, Glu^{89} pK_a computed for lacb_pig, lacc_felca, and lacb_horse are higher than that computed for lacb_bovin, reflecting the mutation of the flanking Asn^{88} residue to a negatively charged aspartic acid. The strongest validation of these observations comes from NMR titration experiments demonstrating that PLG binding occurs at higher pH with respect to BLG. The agreement between experimental and theoretical pK_a gave us confidence in the interpretation of the pK_a trend calculated for all β-lactoglobulins. It is important to stress that

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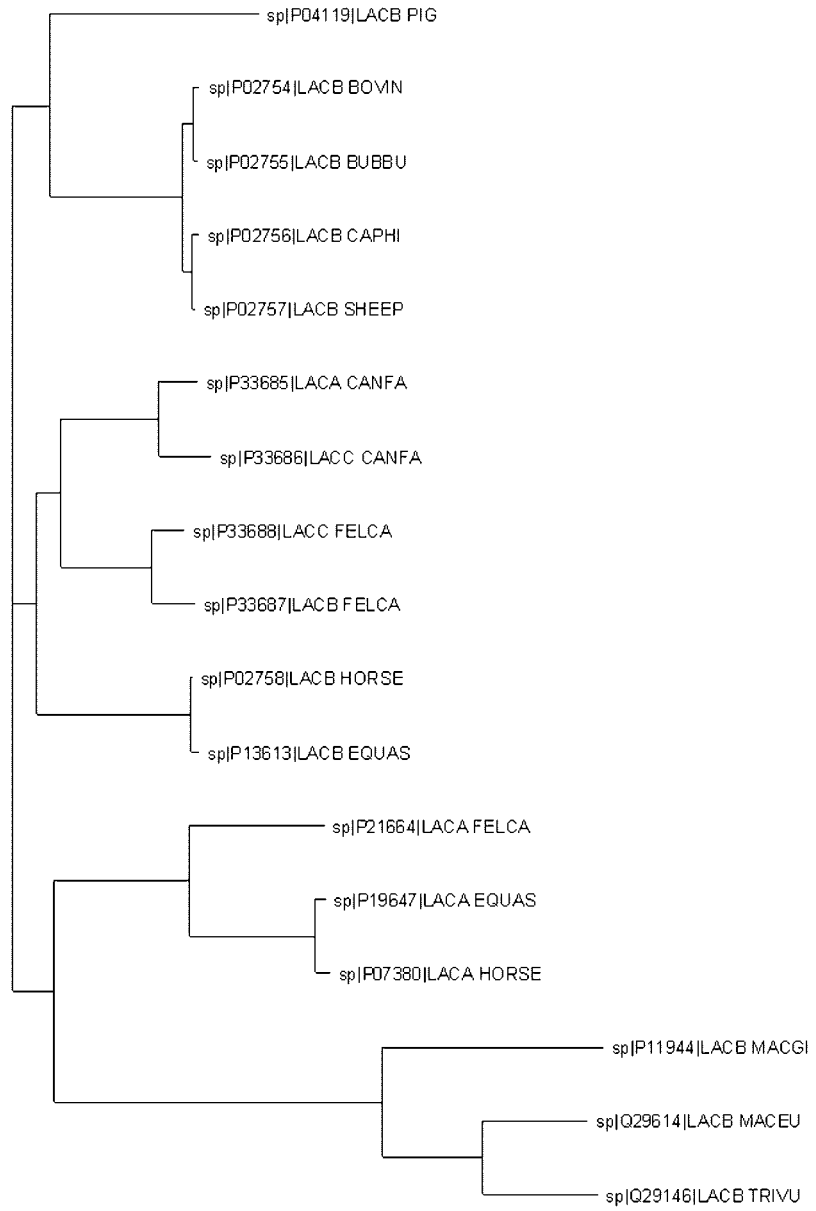

FIG. 4. **Phylogenetic tree of β-lactoglobulin family.** This diagram, produced by the program ClustalW (22), is drawn only approximately to scale, but the branch points are in correct order.

TABLE I
Calculated pK_a values of Glu_{89} in β-lactoglobulins from different species

β-Lactoglobulin	Key residues				Glu^{89} pK_a	
	88	89	90	116	Closed	Open
Lacb_bovin	Asn	Glu	Asn	Ser	5.59	3.75
Lacb_pig	Asp	Glu	Asn	Ser	7.40	4.42
Lacc_felca	Asp	Glu	Asn	Asn	6.51	4.36
Laca_canfa	Glu	Glu	Asn	Ser	5.64	3.31
Lacb_horse	Asp	Glu	Asp	Ser	6.24	3.96
Laca_horse	Gly	Glu	Arg	His	3.92	3.96
Lacb_bovin mutant 1	Gly	Glu	Arg	Ser	5.10	
Lacb_bovin mutant 2	Gly	Glu	Arg	His	3.30	

laca_canfa, showing a negative charged residue at position 88, similar to lacb_pig, lacc_felca, and lacb_horse, exhibits a calculated pK_a of 5.64, similar to that obtained for lacb_bovin (5.61). The analysis of the model showed that Glu^{88} side chain in laca_canfa points in the opposite direction with respect to Glu^{89}, with the distance between the two carboxylic groups being the highest (8.5 Å) among all the analyzed structures (in the range 3.5–6.0 Å), thus reducing the electrostatic effects.

The low pK_a value calculated for laca_horse correlates well with the presence of two close, positively charged residues (Arg^{90} and His^{116}). To single out whether the main role is played by the flanking Arg^{90} or by the close His^{116}, we modeled the following multiple mutations in BLG, N88G,N90R (mutant 1) and N88G,N90R,S116H (mutant 2), and calculated the corresponding Glu^{89} pK_a values. Interestingly a pK_a of 5.10 was calculated for mutant 1 and a pK_a of 3.30 was obtained for mutant 2, thus indicating that the close, positively charged residue 116 plays the determining role in influencing the pK_a.

It is worth noting that when Glu^{89} pK_a was calculated for all of the models with the EF loop in the open conformation, very similar values were obtained, in the range 3.75–4.36 (Table I), as expected for accessible, solvent-exposed carboxylates.

A comment could be added that further supports the reliability of pK_a calculations. The low pK_a value (1.05) estimated for Asp^{98} in lacb_bovin is in good agreement with the value of 2 that was determined experimentally by NMR (20).

Docking experiments run on BLG and PLG indicated that the opening of the EF loop plays a key role in modulating the binding of both proteins. The GRID solution obtained for the

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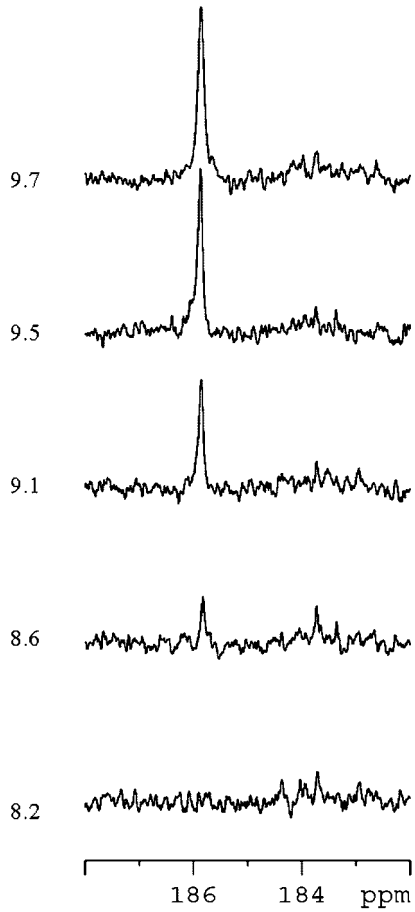

FIG. 5. **Carboxylic region of 125.7 MHz one-dimensional proton decoupled ^{13}C spectra acquired in pH range 8.2–9.7 on the complex of $^{13}C_1$ PA with PLG at 27 °C.**

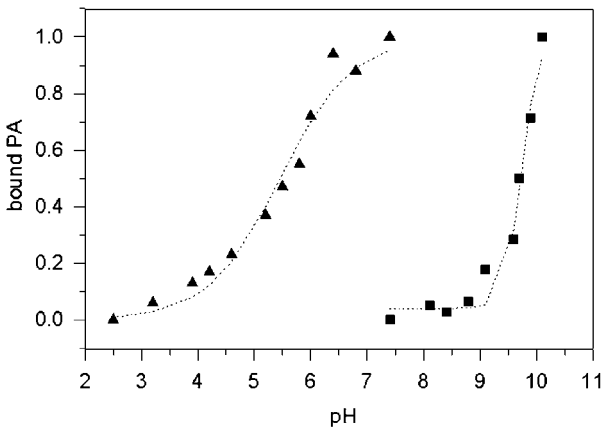

FIG. 6. **Plot of ^{13}C PA methyl resonance areas *versus* pH for BLG (*triangle*) and PLG (*square*).** Intensities are normalized according to the spectrum obtained at higher pH. The *dotted lines* represent the curve fits obtained with the program Sigma Plot.

PLG·PA complex clearly showed that fatty acid is located within the protein calyx adopting a conformation similar to that observed experimentally in BLG (Fig. 2*B*). PLG residues showing short contacts with bound PA are substantially the same as observed in holo-BLG (26). However the comparison of ^1H and ^{13}C one-dimensional and two-dimensional HSQC NMR spectra of holo-PLG and -BLG revealed that PA H-16 and H-15 resonances exhibited less marked up-field shifts when bound to PLG (δH16 = 0.69 ppm, δH15 = 1.00) rather than to BLG (δH16 = 0.20 ppm, δH15 = 0.54−0.43). This behavior can be attributed to a different distribution of aromatic residues in the binding cavity; in BLG, PA methyl protons feel the shielding effect of two aromatic residues (Phe^{82} and Phe^{105}) lying within 5 Å of its center (Fig. 2*A*), whereas in PLG the mutation F105L accounts for the reduced shielding effect (Fig. 2*B*). The minor chemical shift dispersion observed in PLG for C-4—C-12 carbons can be ascribed to the same effect. All of these NMR data confirm that PA should have the same spatial arrangement within the calyx of the two proteins. The conformational equilibrium clearly observed for PA bound to BLG, involving the carboxyl tail, at a pH value close to that of the EF loop conformational change (4), has been also observed for holo-PLG, as indicated by the ^{13}C carboxyl PA line width increase on going from pH 8.6 to 9.7.

TABLE II
^1H and ^{13}C chemical shifts of PA complexed with PLG, pH 9.7, 27 °C

Position number	^1H	^{13}C
C-1		185.85
C-2	2.08	40.99
C-3	1.43	29.29
Other	1.05	32.17
C-15	1.00	25.14
C-16	0.69	17.66

Docking simulations provided a lower binding energy for the interaction of PA with PLG (−10.6 kcal/mol) with respect to BLG (−14.81 kcal/mol), suggesting a minor affinity of the porcine protein for fatty acids. We have previously shown that binding energies provided by GRID for BLG were in good agreement with those measured through dynamic fluorescence experiments (27), and we are therefore confident of the reliability of the given energy differences. The mutation K69E, observed on going from BLG to PLG, may be held responsible for the lower interaction energy as deduced from the inspection of MIFs, showing less favorable electrostatic interactions with the ligand in PLG.

Altogether these data indicate that the pH-dependent conformational change of EF loop is a common feature of all β-lactoglobulins. The analysis presented here, while allowing the identification of structurally and functionally important electrostatic interactions in β-lactoglobulins, opens the way to the design of engineered proteins characterized by binding capability in a selected pH range.

Interestingly the binding mechanism identified for β-lactoglobulins is reminiscent of the mechanism of lipase interfacial activation associated with a conformational change, in which a lid, consisting of one α-helix, opens up by rotating around its hinge regions, thus allowing ligand binding. As it was reported that (i) β-lactoglobulins may play a role in increasing lipases activity by removing free fatty acids (28) and (ii) liver pig triacylglycerol hydrolases exhibit activity at an optimum alkaline pH of 8.5 (16), it is likely that the unusually high pH of binding detected for PLG is functional in lipases activity.

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EF Loop Conformational Change Triggers Ligand Binding in β-Lactoglobulins

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