**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 protonation. The alkaline shift observed for Glu in porcine β-lactoglobulin with respect to the bovine species 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 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 nonde-naturing conditions, showed endogenously bound fatty acids. Many authors have suggested that bovine β-lactoglobulin (BLG) has a transport and/or protective role toward bound ligands in the stomach. However, we have previously shown 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 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) 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 at anomalously high pH (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 and Lys, 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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‡ The abbreviations used are: BLG, bovine β-lactoglobulin; PLG, porcine β-lactoglobulin; PA, palmitic acid; 13CPA, fully enriched palmitic acid; HSQC, heteronuclear single-quantum coherence; MIF, molecular interaction field; PDB, Protein Data Bank.
**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 Na5HPO4/NaH2PO4 solution at pH 7.0, and a molar excess of PA (4:1 molar ratio) was used for complex preparation. NMR titrations at different pH levels were performed adding a few microliters of 0.25 N H3PO4 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 13C-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 13C 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 13C spectra of completely enriched PA (13C1 PA) in complex with PLG, 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 13C spectra of completely enriched PA (13C1 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-13C HSQC experiments were recorded on 13C-PA and on PLG-13C-PA complex with 512 and 2048 data points in the t1 and t2 dimensions, respectively, and a spectral width of 7002 (t1) and 5040 (t2) Hz. The spectra were processed and analyzed with the programs XWINNMR (Bruker) and KEASY (12).

**Docking Simulations**—Docking simulations were performed using the program GRID, version 21 (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).

**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 (proteins) (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. 2A). 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. 2B). 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 pKa Calculations on β-Lactoglobulins**—The opening of the EF loop (85–90) in BLG has been shown to be triggered by the titration of Glu89 (11). EF loop closed conformation is stabilized in BLG by a pattern of hydrogen bonds involving Asp88, Glu89, Asn109, Ser110, and Ser116.
residues. The titration of the Glu89 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 Glu89 pKₐ, 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 Glu89 is always conserved, its flanking residues (88 and 90) differ both in side chain length and charge. Charged residues close in space to Glu89 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, pKₐ 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_maeci) 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 3b1g), respectively. pKₐ 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 Monte Carlo procedure (18). The obtained Glu89 pKₐ values are reported in Table I.

The inspection of all β-lactoglobulin structures allowed the identification of those side chains close to the Glu89 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_maeci 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 Glu89 pKₐ. It is worth noting that on going from BLG to PLG, Glu89 pKₐ 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 13C₁ and 13C PA were performed in the pH range 8.0–10.0, i.e., at pH values higher than the calculated Glu89 pKₐ. 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 13C₁ 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 (Δν₁/₂ = 11 Hz) to 9.7 (Δν₁/₂ = 17 Hz). The amount of bound PA as a function of pH, as derived from the integration of C₁₆ methyl resonance, is shown in Fig. 6. The transition midpoint occurs at pH 9.7, thus affording a clear indirect estimate of Glu89 pKₐ 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 13C 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 titratable 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 Glu89 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 pKₐ of Glu89 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, pKₐ calculations were performed for representative β-lactoglobulins from different species. It has been shown by us and others that theoretically determined pKₐ values are in good agreement with experimentally determined ones (25), and we feel confident that the pKₐ shifts calculated for the different models reflect a reliable trend, which should be considered as such, rather than as an assessment of the exact pKₐ value of each titrating group. It is generally agreed that large pKₐ shifts can be ascribed to the electrostatic effects of desolvation, nearby charges, and/or to the disruption of hydrogen bonds (25). To rationalize Glu89 pKₐ
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 Glu89 side chain were therefore considered. As shown in Table I, Glu 89 pKa 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 pKa gave us confidence in the interpretation of the pKa trend calculated for all β-lactoglobulins. It is important to stress that

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), HORSE, EQUAS (donkey), MACGU (eastern gray kangaroo), MACEU (tammar wallaby), and TRIVU (brush-tailed possum).

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lac\(c\)a\(_{\text{canfa}}\), showing a negative charged residue at position 88, similar to lac\(b\)\(_{\text{pig}}\), lacc\(_{\text{felca}}\), and lac\(b\)\(_{\text{horse}}\), exhibits a calculated \(pK_a\) of 5.64, similar to that obtained for lac\(b\)\(_{\text{bovin}}\) (5.61). The analysis of the model showed that Glu 88 side chain in lac\(c\)a\(_{\text{canfa}}\) points in the opposite direction with respect to Glu89, 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 lac\(c\)a\(_{\text{horse}}\) correlates well with the presence of two close, positively charged residues (Arg90 and His116). To single out whether the main role is played by the flanking Arg90 or by the close His116, we modeled the following multiple mutations in BLG, N88G,N90R (mutant 1) and N88G,N90R,S116H (mutant 2), and calculated the corresponding \(pK_a\) 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 Glu89 \(pK_a\) 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 calculated for lac\(c\)a\(_{\text{horse}}\) 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

![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.](image)

**Table I**

| β-Lactoglobulin | Key residues | Glu\(_{88}\) \(pK_a\) |
|------------------|--------------|---------------------|
|                  | 88 89 90 116 | Closed Open         |
| lac\(b\)\(_{\text{bovin}}\) | Asn Glu Asn Ser | 5.59 3.75           |
| lac\(b\)\(_{\text{pig}}\) | Asp Glu Asn Ser | 7.40 4.42           |
| lacc\(_{\text{felca}}\) | Asp Glu Asn Asn | 6.51 4.36           |
| laca\(_{\text{canfa}}\) | Glu Glu Asn Ser | 5.64 3.31           |
| lacc\(_{\text{horse}}\) | Asp Glu Asp Ser | 6.24 3.96           |
| laca\(_{\text{horse}}\) | Gly Glu Arg His | 3.92 3.96           |
| lac\(b\)\(_{\text{bovin}}\) | Gly Glu Arg Ser | 5.10                |
| mutant 1         | Gly Glu Arg His | 3.30                |
| mutant 2         | Gly Glu Arg His |                     |
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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. 2B). PLG residues showing short contacts with bound PA are substantially the same as observed in holo-BLG (26). However the comparison of $^1$H 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 ($\delta H16 = 0.69$ ppm, $\delta H15 = 1.00$) rather than to BLG ($\delta H16 = 0.20$ ppm, $\delta H15 = 0.54 - 0.43$). 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. 2A), whereas in PLG the mutation F105L accounts for the reduced shielding effect (Fig. 2B). 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.

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 a-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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PLG-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.

![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, PA with PLG at 27 °C.](image)

**Fig. 5.**

**Fig. 6.**

| Position number | $^1$H | $^{13}$C |
|-----------------|-------|---------|
| C-1             | 185.85| 40.99   |
| 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   |

**TABLE II**

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

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