β-Lactoglobulin Binds Palmitate within Its Central Cavity*

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Bovine β-lactoglobulin (β-Lg) has been studied extensively in both the isolated and the naturally occurring states. It is a commercially important whey protein of obvious nutritional value but, so far, one that has no clearly identified biological function. In common with many of the other members of the lipocalin family to which it belongs, β-Lg binds hydrophobic ligands, and it appears possible that there are at least two distinct binding sites per monomer for a variety of ligands. By comparison with other members of the family, there is a probable binding site in the central cavity of the molecule that is formed by the eight antiparallel β-strands which are typical of the lipocalins. We have now co-crystallized β-Lg with palmitic acid, and refined the structure (R = 0.204, Rfree = 0.240 for 6,888 reflections to 2.5-Å resolution) reveals that the ligand binds in the central cavity in a manner similar to the binding of retinol to the related lipocalin, serum retinol-binding protein. The carboxyl group binds to both Lys-60 and Lys-69 at the entrance to the cavity. The hydrophobic tail stretches in an almost fully extended conformation into the center of the protein. This is the first direct observation of a ligand binding to β-Lg.

The lipocalin family is a large and diverse family of proteins with functions varying from insect camouflage to small hydrophobic molecule transport typified by the serum retinol-binding protein (1). The crystal structures so far determined reveal the typical lipocalin to be an eight-stranded antiparallel β-barrel arranged to form a conical central caclyx or cavity in which the hydrophobic ligand is located (2). There is an α-helix on the outer surface of the β-barrel, and the amino acid sequence contains three structurally conserved regions (sequence motifs) together with one or more disulfide bridges (see Fig. 1). The calycin superfamily (1) extends the family to include proteins such as the fatty acid-binding proteins, which do not contain such a role has been published. Fatty acids have been found bound to ruminant β-Lg that has been freshly isolated from milk, and Perez et al. (17) have suggested that by removing free fatty acids as they are formed by pregastric lipases, β-Lg could facilitate the digestion of milk fat. If such a role is the true function of β-Lg, it is interesting that ruminant, but not mare and sow, β-Lgs exhibit significant fatty acid binding (18), whereas one might expect all β-Lgs to perform this function. On the other hand, β-Lg from all species appears to bind retinol, but this ligand is found associated not only with β-Lg but also with other milk proteins such as serum albumin and α-lactalbumin (18), indicating that this interaction seems to be rather unspecific. Thus, the functional relevance of retinol binding to β-Lg is also open to question.

As part of our continuing study of the relationship between the structure of the protein and the thermally induced aggregation, we sought convincing evidence of the binding of hydrophobic ligands to the molecule. Monaco et al. (19) reported a possible binding site for retinol on the outer surface of the molecule in a groove formed between the helix and the β-sheet. This result, which has been questioned (16, 20, 21), is based upon an unrefined difference electron density map. Apart from the location by analogy and modeling (21, 22), some experimental evidence for an internal binding site comes from the fluorescence and site-directed mutagenesis work of Cho et al. (23). The existence of two independent ligand binding sites as proposed by Narayan and Berliner (20) adds further support to the possible existence of a central site (see Fig. 1). However, to provide unequivocal evidence, we have now refined the crystal structure of β-Lg co-crystallized with palmitate from sodium citrate solu-
Complex of Palmitate with β-Lactoglobulin

EXPERIMENTAL PROCEDURES

The crystalline complex was prepared in two distinct ways. In the first method, bovine β-Lg (B variant, Sigma) was dissolved in H2O to a concentration of 40 mg/ml and crystallized at 20 °C by the sitting drop method (24) using 1.34 mM sodium citrate, 0.1 M HEPES, pH 7.5, as the precipitant. Typically, a microbridge was placed in 1.0 ml of precipitant solution (the reservoir) in a 24-well Linbro tissue culture plate. For a sitting drop, 4 μl of precipitant solution (the reservoir) in a 24-well Linbro tissue culture plate. For a sitting drop, 4 μl of precipitant was added to 12 μl of reservoir solution. Then, 0.4 μl of 100 mM palmitic acid in ethanol was added to the drop (a molar ratio of 10/protein dimer) and mixed by pipette before the well was sealed with a glass coverslip. Because the palmitic acid was supersaturated with respect to the aqueous phase, a white precipitate appeared in the drop. After ~4–5 days, the white precipitate had disappeared, and lattice Z crystals (space group P3_21) grew from the clear drops. The pH of the crystals was assumed to be 7.5, although it was not directly measured. In the second method, bovine β-Lg of mixed genetic variants A and B was prepared according to Puyol et al. (25) and delipidated by charcoal treatment at pH 3 as described by Chen (26). Palmitic acid at a molar concentration ratio of 2:1 with respect to the protein dimer was dissolved in chloroform and dispensed in a glass tube. After the organic solvent was evaporated under nitrogen, a solution of delipidated β-Lg was dissolved in 0.29 M NaCl, 2.5 mM KH2PO4, 16 mM K2HPO4, pH 7.4, and the mixture was incubated overnight at 37 °C. The saturated protein solution was then dialyzed against distilled water and freeze-dried. Analysis by gas chromatography showed that the complex had about 1 mol of palmitate bound to 1 mol of dimeric protein. The freeze-dried material was dissolved in H2O to a concentration of 40 mg/ml and crystallized as a sitting drop (4 μl of protein solution + 12 μl of well solution) over a well solution of 1.4 mM sodium citrate, 0.1 M HEPES, pH 7.5. Crystals, also of the trigonal lattice Z form, grew in a few days and appeared identical to the crystals from the first preparation (see Table I).

A crystal of about 0.3–0.4 mm in length was collected in a 0.5-mm Cryoloop (Hampton Research, Inc.), dipped briefly in immersion oil (Type B, Cargille), and frozen by plunging into liquid N2. The frozen crystal was then transferred to a magnetic goniometer head in a stream of N2 at 100 K (Cryostream; Oxford Cryosystems). Diffraction data were collected on a 300 mm MarResearch imaging plate system mounted upon an ENRAF-Nonius FR571 rotating anode generator operating at 40 kV and 80 mA and producing Cu-Kα radiation from a graphite crystal monochromator.

At least 90° of data were collected in 1.5° oscillations (i.e. >60 images), each of a 20-min duration. The data were processed by DENZO (27) and reduced with SCALPACK (27). The statistics are given in Table I. The structure was solved by molecular replacement using AMORE (28) with the refined β-Lg lattice X monomer (space group P1: a = 37.5 Å, b = 49.6 Å, c = 56.6 Å, α = 123.4°, β = 97.3°, γ = 103.7°) as the search model. Data within the resolution range of 10–4 Å and a Patterson radius of 18 Å were used to calculate the rotation and translation functions. The maximum peak (4.86 e−/Å3) in the rotation function and next highest peak (3.70 e−/Å3) were used to calculate the translation function, which gave a distinct peak at a height of 9σ for the maximum rotation peak, whereas the next highest peak was 4.5σ. The second rotation peak did not give a distinct solution in the translation function.

The space group P3_21 was also confirmed by the translation function. The highest peak in P3_21 was 9σ (R-factor = 38.9%), whereas the highest peak in P3_21 was 5.2σ (R-factor = 53.1%). Rigid body refinement of the correct solution reduced the R-factor slightly from 38.9 to 38.0%. Positional, occupancy, and temperature factor refinement was performed using SHELXTL (29). The refinement calculation was interleaved with several rounds of model-building with the program O (30). Water molecules were added using the program SHELX97 (29). The final refinement statistics are summarized in Table II.

RESULTS AND DISCUSSION

A monomer of the crystal structure of β-Lg refined at 1.8-Å resolution in triclinic lattice X (16) was used as the search model in the structure determination of the lattice Z crystal form containing the palmitate. Although the 3.0-Å lattice Z structure (16) could have been used, the higher resolution lattice X structure was preferred as the better starting model. Baker and co-workers (31) have refined the structure of the lattice Z form at three distinct pH values (pH 6.2, 7.1, and 8.2) showing that there is a distinct movement of a loop as the pH value is raised. This movement uncovers a buried carboxyl group, observed during titration by Tanford et al. (32), probably identified as Glu-89 by Brownlow et al. (16) but confirmed convincingly by Qin et al. (31). Glu-89 is part of the EF loop

### Table I

<table>
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<tr>
<th>Data collection statistics</th>
<th>First preparation</th>
<th>Second preparation</th>
</tr>
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<tbody>
<tr>
<td>Space group, P3_21</td>
<td>a = b = 53.48 Å</td>
<td>a = b = 53.52 Å</td>
</tr>
<tr>
<td></td>
<td>c = 111.64 Å</td>
<td>c = 111.37 Å</td>
</tr>
<tr>
<td></td>
<td>α = β = 90°</td>
<td>α = β = 90°</td>
</tr>
<tr>
<td></td>
<td>γ = 120°</td>
<td>γ = 120°</td>
</tr>
<tr>
<td>Resolution range</td>
<td>20–2.5 Å</td>
<td>20–2.3 Å</td>
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<tr>
<td>Number of reflections measured</td>
<td>97,142</td>
<td>48,843</td>
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<tr>
<td>Number of unique reflections</td>
<td>6,888</td>
<td>8,763</td>
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<tr>
<td>Completeness</td>
<td>99.9%</td>
<td>97.8%</td>
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<tr>
<td>Multiplicity</td>
<td>14.1</td>
<td>5.57</td>
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<tr>
<td>Rmerge (outer shell)</td>
<td>7.3% (36.6%)</td>
<td>8.3% (37.6%)</td>
</tr>
<tr>
<td>Rmerge (outer shell)</td>
<td>21.3 (3.95)</td>
<td>17.38 (2.57)</td>
</tr>
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</table>

\* Rmerge = ΣhkI − |I|/ΣhkI, where <I> is the mean intensity of all observation of reflection hkl.

\* a (I) is the S.D. of the measured intensity.

### Table II

<table>
<thead>
<tr>
<th>Summary of refinement statistics</th>
<th>First preparation</th>
<th>Second preparation</th>
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<tr>
<td>Bond lengths (Å)</td>
<td>0.004</td>
<td>0.006</td>
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<tr>
<td>Angle distance (Å)</td>
<td>0.018</td>
<td>0.02</td>
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<td>Residues in most favored and allowed regions of Ramachandran plot</td>
<td>98.6%</td>
<td>98.6%</td>
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Fig. 2, A, diagram of the native structure at pH 6.5 (lattice X) showing the position of Met-107 when palmitate is absent. B, diagram of the structure at pH 7.5 (lattice Z) showing the movements of the side chain of Met-107 when palmitate binds. The movement of the EF loop can also be seen. There are no other significant movements between the bound and free forms of the protein. C, stereodiagram, produced by the program O (30), of a section of the electron density map around the bound palmitate with the refined structure superimposed. This is the structure of the protein cocrystallized with palmitate. Note the discrete density for Met-107 and the palmitate. D, stereodiagram showing essentially the same view of the complex of palmitate with β-lactoglobulin.
whose movement allows access for the binding of palmitate in that it is the movement of the loop as the pH is raised from pH 6 to 7.5 that opens up the entrance to the calyx (Fig. 2, A and B). The occlusion of, or at least hindrance to, the binding site at low pH may provide an explanation for the failure of our soaking experiments with the lattice X crystal form at pH 6.5. We also noted that co-crystallization appears to favor the lattice Z crystal form. In a control experiment without palmitate added, only crystals of the orthorhombic lattice Y grew at pH 7.5, whereas in drops with palmitate added also at pH 7.5, we never obtained this form but only obtained lattice Z.

Fig. 2C shows the electron density map with the final refined structure superimposed. The final R-factor was 0.204, and R_{free} was 0.240 for the 6,888 unique reflections to 2.5-Å resolution. The geometry is acceptable, and the Ramachandran plot shows that essentially all residues are in the allowed regions, with Tyr-99 the notable exception, adopting a classic γ-turn conformational common to nearly all of the lipocalin structures. The density for the palmitate is clear and shows a kink at C-6 associated with the movement of the side chain of Met-107. To allow access to the calyx, the EF loop, associated with the Tanford transition (16, 31), is also repositioned. The local environment of the palmitate within the pocket is shown in Fig. 3, which indicates the distances between side chains and the fatty acid. The binding site in β-Lg is rather fully extended, but there is space for longer fatty acid molecules such as stearate and oleate to be accommodated within the calyx, with the carboxyl group making the same interactions with Lys-60 and Lys-69. The association constants for both acids are similar (33, 34). In the fatty acid-binding protein family, palmitate is also observed in an extended form, although alternative conformations of bound fatty acids have been observed (35, 36).

As perturbation of the Trp fluorescence can be used to monitor binding of hydrophobic ligands to β-Lg, the distances to the two Trp residues were determined directly. The closest approach of C-2 is 10.36 Å from Trp-61, and C-15 is 6.98 Å from Trp-19. It is possible, therefore, that there is perturbation of the Trp fluorescence can be used to monitor binding of hydrophobic ligands to β-Lg, the distances to the two Trp residues were determined directly. The closest approach of C-2 is 10.36 Å from Trp-61, and C-15 is 6.98 Å from Trp-19. It is possible, therefore, that there is perturbation of

At this stage, little can be said about the existence of a second binding site. We were surprised to find the palmitate in the calyx, because the report of Narayan and Berliner (20) establishes fatty acid binding at a site that is not perturbed by retinol, and the report of Cho et al. (23) connects Lys-69 with retinol binding. Further, contrary to the findings of Narayan and Berliner (20), Puyol et al. (18) find that palmitate and retinol compete on binding to β-Lg, the former displacing the latter. Thus, the groove identified by Monaco et al. (19) on the outer surface of the protein (see Fig. 1) has yet to be confirmed as a binding site for any ligand, despite several strands of circumstantial evidence that point to its existence (see Ref. 21). Many of the reported ligand binding studies have been made at pH values at or above 7, the pH at which the inner binding site is known to be more accessible. It is tempting to speculate that the inner site becomes accessible at high pH, whereas at lower pH only the putative outer site is available. This gating of the

FIG. 3. Schematic diagram showing the distances less than 4 Å of the palmitate atoms from the side chain atoms of the protein. At pH 7.5 the carboxyl group will be deprotonated, and the lysine residues will be protonated. The side chain atom names (CD2, CZ, etc.) are those used by the Brookhaven Protein Data Bank and refer to the IUPAC names (C2, C3, etc.).
inner binding site is reminiscent of the dynamic portal hypo-
thesis in fatty acid-binding protein, where ligand access and
binding are mediated by flexible regions of the protein back-
bone (Ref. 36 and references therein). Whereas in the trigonal
lattice Z form β-Lg, discrete arrangements of the "portal" loop
EF exist, in the triclinic lattice X and the orthorhombic lattice
Y apo forms, these loops have weak electron density. To clarify
this point, binding studies need to be performed at lower pH
values, both in the crystal structure and in solution.

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Maria Bewley, Lawrie Creamer, Linda Gilmore, Tomasz Haertlé, Carl
Holt, Geoff Jameson, Bin Qin, and Thales Rocha for stimulating
discussions.

Note Added in Proof—Since this paper was accepted, an independent
study on 12-bromododecanoic acid binding to the lattice Z form of bovine
β-Lg has been published by Qin et al. (Qin, B. Y., Creamer, L. K., Baker,
findings are in agreement with those presented here: the carboxylate
values, both in the crystal structure and in solution.

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