Bovine $\beta$-lactoglobulin ($\beta$-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, $\beta$-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 $\beta$-strands that are typical of the lipocalins. We have now cocrystallized $\beta$-Lg with palmitic acid, and the refined structure ($R = 0.204, R_{\text{free}} = 0.240$ 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 $\beta$-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 $\beta$-barrel arranged to form a conical central calyx or cavity in which the hydrophobic ligand is located (2). There is an $\alpha$-helix on the outer surface of the $\beta$-barrel, and the amino acid sequence contains three structurally conserved regions (sequence motifs) together with one or more disulfide bridges (see Fig. 1). The calcin superfamily (1) extends the family to include proteins such as the fatty acid-binding proteins, which do not contain the second of these three sequence patterns but which are also antiparallel $\beta$-sheet proteins, although with 10 $\beta$-strands rather than 8. However, in common with their distant lipocalin relatives, their binding site is also within a central cavity (3).

Not only is $\beta$-lactoglobulin ($\beta$-Lg), from the milk of the domestic cow, a typical lipocalin, but it has also been studied extensively over the past 60 years (see Refs. 4–6) because of its size, convenience (~3 g/liter of milk), and stability. The ruminant protein is normally a dimer at room temperature and physiological pH, although at pH values below 3 and above 8, it dissociates into monomers, for which the structure at low pH is similar to that at pH 7 (7). A further reason for studying $\beta$-Lg is its involvement in the loss of functionality during the heat processing of milk (8). Thus, many studies on the pure protein are being directed toward unraveling the molecular mechanisms that are responsible for its thermal denaturation because this denaturation is thought to initiate the wider aggregation of milk proteins during thermal processing (9–12). The initial stage of the thermal denaturation process also involves dimer dissociation (12, 13).

The biological function of $\beta$-Lg is unknown. The amino acid composition and the quantities present in ruminant species support a nutritional role. However, the ligand binding properties that have emerged, coupled with the structural similarity of $\beta$-Lg to retinol-binding protein and possibly even to fatty acid-binding protein, have led to the suggestion that it has a transport role for ligands such as retinol or fatty acids (1, 2, 14–16). The majority of the ligands that have been examined are hydrophobic, so that a transport role, in keeping with the other lipocalins, is certainly possible, although no real proof of such a role has been published. Fatty acids have been found bound to ruminant $\beta$-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, $\beta$-Lg could facilitate the digestion of milk fat. If such a role is the true function of $\beta$-Lg, it is interesting that ruminant, but not mare and sow, $\beta$-Lgs exhibit significant fatty acid binding (18), whereas one might expect all $\beta$-Lgs to perform this function. On the other hand, $\beta$-Lg from all species appears to bind retinol, but this ligand is found associated not only with $\beta$-Lg but also with other milk proteins such as serum albumin and $\alpha$-lactalbumin (18), indicating that this interaction seems to be rather unspecific. Thus, the functional relevance of retinol binding to $\beta$-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 $\beta$-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 $\beta$-Lg co-crystallized with palmitate from sodium citrate solu-
Structurally conserved regions are at the rear of the molecule on protein dimer was dissolved in chloroform and dispensed in a glass genic variants A and B was prepared according to Puyol citrate, 0.1 M HEPES, pH 7.5. Crystals, also of the trigonal lattice Z was not directly measured. In the second method, bovine 4 solution was used to collect on a 300 mm MarResearch imaging plate system mounted at pH 7.5. The saturated protein solution was then dialyzed against dis- 37 °C. The saturated protein solution was then dialyzed against dis- 

Resolution range 20–2.5 Å 25–2.3 Å
Number of reflections measured 97,142 48,843
Number of unique reflections 6,888 8,763
Completeness 99.9% 97.8%
Multiplicity 14.1 5.57
$R_{	ext{merge}}$, (outer shell)$^a$ 7.3% (36.6%) 6.3% (37.6%)
$I/\langle I\rangle$ (outer shell)$^b$ 21.2 (3.95) 17.38 (2.57)

$^a$ $R_{	ext{merge}} = \sum_{hkl} |I_{hkl} - \langle I_{hkl}\rangle|/\sum_{hkl} I_{hkl}$, where $\langle I\rangle$ is the mean intensity of all observation of reflection. $h = hkl$.
$^b$ $\langle I\rangle$ is the S.D. of the measured intensity.

**TABLE II**

Summary of refinement statistics

|                        | First preparation | Second preparation |
|------------------------|-------------------|--------------------|
| Resolution range       | 10–2.5 Å          | 10–2.3 Å           |
| $R_{\text{final}}$ ($F > 4\sigma$) | 20.4% (19.7%) | 23.3% (21.9%) |
| $R_{\text{free}}$ ($F > 4\sigma$)  | 24.0 (23.7%)     | 28.5% (26.7%)     |
| Residues in most favored and allowed regions of Ramachandran plot | 98.6% | 98.6% |
| Mean B-factor ($\AA^2$) | 47.17            | 47.51              |

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 M 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 β-Lg solution 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 P321) 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 gel 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 concentra- tion 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 M sodium formic acid at 100 K (Cryostream, Oxford Cryosystems). Diffraction data were collected on a 300 mm MarResearch imaging plate system mounted upon an ENRAF-Nomius 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 SCALEPACK (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⁻³ in the rotation function and next highest peak (3.70 e⁻³) were used to calculate the translation function, which gave a distinct peak at a height of 9 e⁻³ for the maximum rotation peak, whereas the next highest peak was 4.5 e⁻³. The second rotation peak did not give a distinct solution in the translation function. The space group P321 was also confirmed by the translation function. The highest peak in P321 was 9 e⁻³ ($R_{factor}$ = 38.9%), whereas the highest peak in P3121 was 5.2 e⁻³ ($R_{factor}$ = 53.1%).

**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

![Diagram](image.png)
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.
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 cocrystallization 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 conformation 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. It is possible, therefore, that there is perturbation of the Trp signals from both Trp residues, although the Trp at position 19 approaches C-2 is 10.36 Å from Trp-61, and C-15 is 6.98 Å from Trp-19. It is another occupancy term for all ligand atoms was used in refinement, the B-value became markedly lower, as the occupancy was 0.285 for the 8,763 unique reflections to 2.3-Å resolution. The geometry is again acceptable, with Tyr-99 again the exception in the Ramachandran plot. It is clear that there is a bulge in the density associated with the position of Met-107. The factors for the same palmitate carbon atoms after refinement with the second data set were significantly larger than those of the adjacent side chains, but when a single occupancy term for all ligand atoms was used in refinement, the B-value became similar to those of the surrounding side chains. In the lattice Z crystal form, the two binding sites in the dimer are crystallographically identical. If the stoichiometry was one ligand per dimer, the expected occupancy would be 0.5 because, on average, each site contains half a palmitate molecule. The refined occupancy was 0.69. This is higher than 0.5, but because of the imprecise nature of the occupancy determination and the high correlation that exists between occupancy and B-factor, the result indicates a reasonable agreement with the solution study. For comparison, an identical calculation of the occupancy of palmitate in the first data set gave a value of 0.96. The bulge in the electron density at Met-107 is thus explained as the addition of two components for the Met-107 side chain, that of the native structure (Fig. 2A) and that with bound ligand (Fig. 2B). It is not clear why the preparation protocols used should produce different results and why the second one should have such a reproducibly precise palmitate to β-Lg dimer ratio. There is no obvious cooperativity between the monomers in which the binding sites, some 35 Å apart, are approached from a direction away from the dimer interface.

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), Puuy 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

as C but calculated with data from the crystals grown with prebound palmitate. The continuity of electron density between palmitate and Met-107 arises from the two possible positions of the tip of the Met residue shown in A and B.
inner binding site is reminiscent of the dynamic portal hypothesis in fatty acid-binding protein, where ligand access and binding are mediated by flexible regions of the protein backbone (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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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, E. N. and Jameson, G. B. (1998) FEBS Let. 438, 272–278). Their findings are in agreement with those presented here: the carboxylate residues in the 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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