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12-Bromododecanoic acid binds inside the calyx of bovine β-lactoglobulin

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Abstract The X-ray structure of bovine β-lactoglobulin with the ligand 12-bromododecanoic acid as a model for fatty acids has been determined at a resolution of 2.23 Å in the trigonal lattice Z form. The ligand binds inside the calyx, resolving a long-standing controversy as to where fatty-acid like ligands bind. The carboxylate head group lies at the surface of the molecule, and the lid to the calyx is open at the pH of crystallization (pH 7.3), consistent with the conformation observed in ligand-free bovine β-lactoglobulin in lattice Z at pH 7.1 and pH 8.2.

Key words: Bovine β-lactoglobulin; Ligand binding; Fatty-acid complex; X-ray crystal structure

1. Introduction

Bovine β-lactoglobulin (BLG) [1–3] is a member of the lipocalin family, whose general function appears to be the solubilization and transport of hydrophobic molecules [4,5]. In a number of lipocalins, ligands have been observed to bind inside the calyx (or cup-shaped cavity) that is formed by an 8-stranded β-barrel. For example, the structures of plasma retinol-binding protein [6,7] and epididymal retinoic acid binding protein [8] revealed buried retinoid species; the mouse major urinary protein a thiazoline derivative [9]; bilin-binding protein a biliverdin molecule [10]; odorant-binding protein an unidentified ligand, possibly a pyrazine derivative [11]; and for nitrophorin 1 a heme, bound through a histidine ligand to the protein [12]. There is evidence that bovine BLG binds a wide range of ligands, including fatty acids [13–23], retinol and retinol derivatives [20–27], porphyrin species [27,28], assorted aromatic molecules [23,29–32] and alkanone species [33,34]. Despite many studies of ligand binding, there is, however, no unequivocal evidence as to where ligands, especially fatty-acid type molecules, bind. There is general consensus that retinoid species bind inside the calyx, by analogy with the behavior of other lipocalins cited above, but the location of the primary fatty-acid binding site remains uncertain. Very recently evidence was presented for two distinct independent binding sites in BLG, one inside the calyx that accommodated molecules such as retinol, and another outside the calyx that accommodated fatty-acid type molecules [22,23]. These results are in conflict with an earlier study, which suggested competitive binding [21], and a recent study, which suggested an internal location as the primary binding site for fatty acids [20].

To date, despite many attempts to crystallize ligand-BLG complexes and a growing literature on structures of the morphologically promiscuous BLG [35–41], direct structural evidence for the mode of ligand binding is essentially absent, although possible modes of binding have been reported [42,43]. In this study, we present the first direct and unequivocal evidence for the site and orientation of ligand binding by a fatty-acid derivative to variant A of bovine β-lactoglobulin (BLGA). The ligand chosen was the fatty acid molecule, 12-bromododecanoic acid: the bromo substituent being selected to allow unequivocal determination of not only the presence of the ligand in the structure but also of the orientation of the ligand in its binding site.

2. Materials and methods

A sample of bovine BLGA, prepared as previously described [44] from the milk of homozygous cows in 0.010 M phosphate buffer at pH 7, was saturated with 12-bromododecanoic acid, present in a molar ratio of 1.5:1 (excess acid present as a micellar suspension), and adjusted to a protein concentration of ~18 mg/ml. Crystals were grown from hanging drops (2 μl protein solution plus 2 μl precipitant solution). The precipitant solutions formed a 6×4 screen matrix comprising six equally spaced concentrations of ammonium sulfate from 2.2 M to 2.8 M, and four buffers at a concentration of 0.20 M with pH 6.1 (cacodylic acid-KOH), 6.9 (bis-Tris-HCl), 7.7 (Tris-HCl) and 8.5 (TAPSO-KOH). The crystal harvested for data collection was equilibrated with a well solution with measured pH of 7.3 (Tris-HCl).

Data collection was with a Rigaku RU200/RAxis IIC system; key statistics are summarized in Table 1. The structure was solved by molecular replacement (AMoRe [45]), using the structure of unliganded BLGA in lattice Z at pH 7.1 as the search model [40]. This structure was independently redetermined in orthorhombic lattice Y [39] and has identical threading to a second independently redetermined structure in triclinic lattice X [38]. The 12-bromododecanoic acid was immediately visible in the resultant difference Fourier map (Fig. 1). Several rounds of rebuilding (TURBO-FRODO [46]) and restrained least-squares refinement (X-Plor [47,48]) followed, incorporating the ligand, for which a standard all-*trans* geometry, adapted from hexadecanoic acid, was extracted from the structure of fatty-acid binding protein from human muscle [49] (2hmb in the Protein Data Bank) with a C–Br bond length of 1.913 Å. Several corrections to side-chain conformations and small adjustments to the main-chain were made. Refinement and structure validation statistics are summarized in Table 2 [50]. The PDB deposition code is 1bso.

3. Results and discussion

3.1. Structure validation

The final model of BLGA liganded with 12-bromododecanoic acid was refined with data to a resolution of 2.22 Å, which corresponded to the threshold where the mean ratio of $I/\sigma(I)$ fell to 2.0. The final model comprised all 162 resi-

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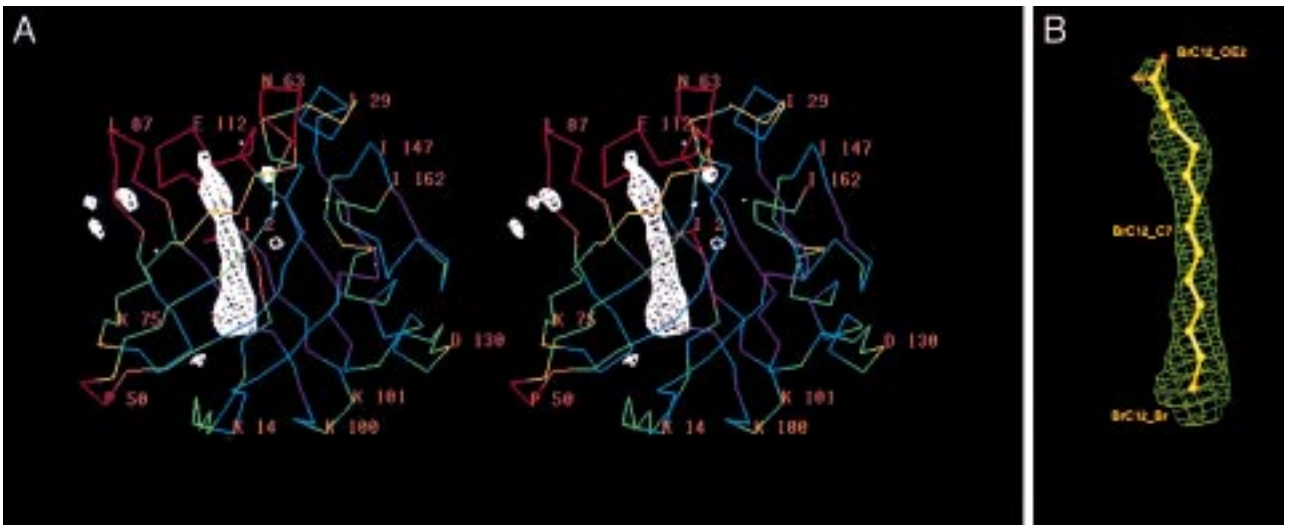

Fig. 1. The BLGA-BrC12 complex. A: Stereodiagram of the Cα trace of the BLGA-BrC12 complex at pH 7.3 in lattice Z, showing omit electron density attributed to ligand. The atoms are colour-coded by atomic displacement parameter (B factor) as follows: purple $B < 20$ Å^2; blue $20 < 30$ Å^2; green $30 < 40$ Å^2; yellow $40 < 50$ Å^2; orange $50 < 60$ Å^2; red $B > 60$ Å^2. Difference ($F_o - F_c$) electron density is drawn at a contour level of three times the RMS level. In addition to the electron density of the ligand, other electron density is related to side-chain rotations. B: Closeup of A in the vicinity of the ligand showing the final fit of 12-bromododecanoic acid to the electron density. Figure prepared with TURBO-FRODO [46].

dues; final discrepancy indices for R and R_{free} (data set aside from all refinement and Fourier map calculations immediately after solution of the structure by molecular replacement methods [48]), although relatively high (see Table 2), are typical for BLG structures, where nearly a quarter of the residues are located in rather mobile surface loops and N- and C-terminal

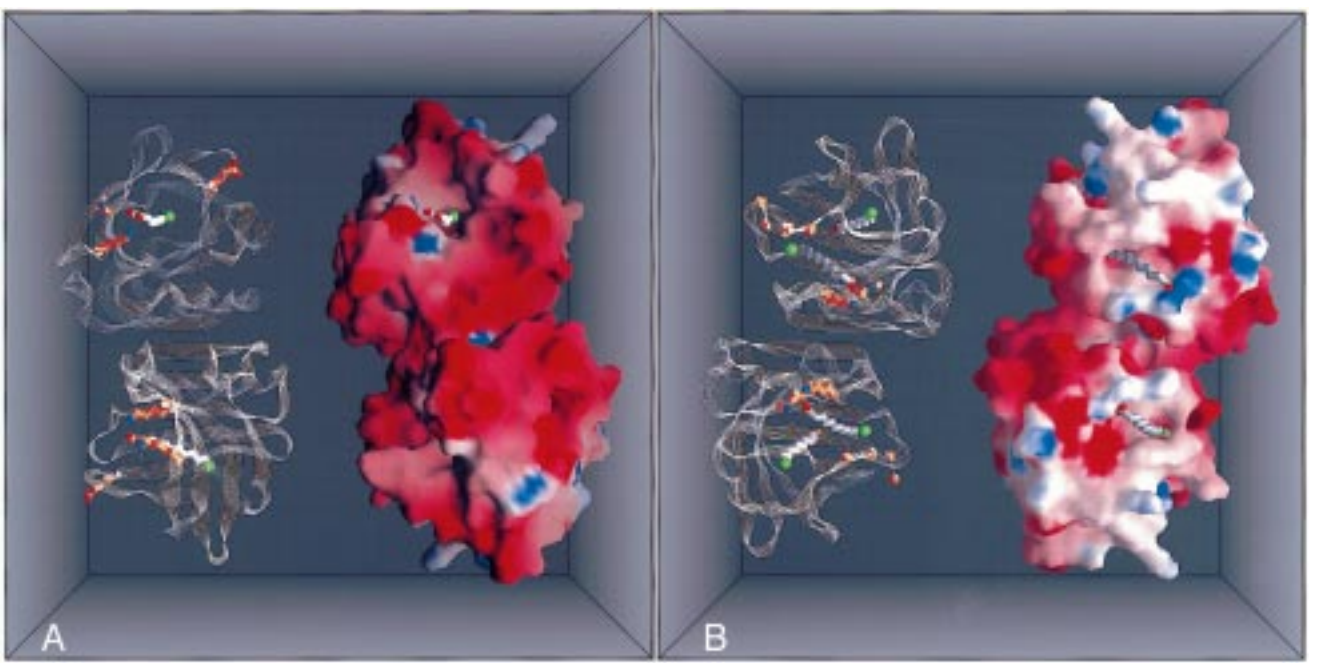

Fig. 2. GRASP [53] representations of surface-charge potential of the dimer of BLGA-BrC12 (right hand panels). The molecular surface is created with a probe atom of 1.4 Å radius. The colour intensity increases with absolute value of the electrostatic potential, where blue corresponds to positively charged surface and red to negatively charged surface. The left-hand panels show the underlying polypeptide fold in the same orientation as the surface in the right-hand panels, and key side-chains, colour-coded orange (carbon), red (oxygen) and blue (nitrogen). The bromine atom of the ligand is drawn as a green 1.6-Å radius sphere. A, left: View into the internal cavity of BLGA. The side-chains are shown for Lys^{60} and Lys^{69}, which form salt bridges with the carboxyl end of the internally bound ligand, and for the absolutely conserved Glu^{89} residue of the calyx lid. Note the generally very strongly negative electrostatic potential over this surface. B, right: Model of a possible site for external binding of 12-bromododecanoic (carbon atoms in grey). In order to create a cleft for the ligand, the N-terminus has been rotated ~15° at Gln^{5}, while preserving the salt bridge between the N-terminus and Glu^{108} – the Cα atoms of the five residues moved are drawn as orange spheres. The ligand conformation has been adjusted in order to create salt bridges with Lys^{135} and Lys^{138} from the 3-turn α-helix that runs left-right across the second β-sheet. Note the deep but charged cleft between the two monomers, and the generally hydrophobic flat surface away from the dimer interface. The internally bound ligand is also shown (carbon atoms in white).

Table 1
Data collection statistics for BrC12-BLGA in lattice Z at pH 7.3

Unit cell: a, b, c (Å); α, β, γ (°)	54.03, 54.03, 112.18; 90, 90, 120
Space group, Z (mol/cell), temperature (K)	$P3_221$, 6, 295
Redundancy, unique data, resolution range (Å)	2.62, 9695, 15–2.23
Completeness, R_{merge} (last shell)	99.5 (98.7) %, 0.048 (0.548)
$\langle I/\sigma(I)\rangle$ (last shell)	18.7 (2.0)

regions. Bond lengths and angles were tightly restrained to standard values [47], leading to small RMS deviations from the target geometry (see Table 2). The Ramachandran plot reveals one residue in a disallowed region, Tyr^{99}. This residue is the central residue of a very well-defined γ-turn, characteristic of all structures of BLG determined to date [36–41].

3.2. Overall structure

The structure of BLG is now well defined in three crystal forms, triclinic (lattice X) [38], orthorhombic (lattice Y) [39] and trigonal (lattice Z) [40,41]. The latter form appears to give a clearer definition of loops and terminal regions, despite data to lower resolution than has been achieved with lattice X and Y forms. As illustrated in Fig. 1 for the complex with 12-bromododecanoic acid in lattice Z at pH 7.3, BLGA folds into an 8-stranded β-barrel that is lined with hydrophobic residues to create a deep cavity inside which is located 12-bromododecanoic acid. As is also typical of the lipocalin family, a 3-turn α-helix is attached to the outside of the barrel or calyx. In reality, the molecule has two distinct β-sheets, an antiparallel four-stranded curved β-sheet (strands β-A1, β-B, β-C and β-D) and a flatter orthogonally packed antiparallel β-sheet (strands β-E, β-F, β-G, β-H and β-A2). The second sheet makes only a single main chain-main chain contact with the first sheet between strands D and E. The first β-strand (residues 16–27) is kinked very strongly at residue 22 to give sub-strands β-A1 and β-A2, which contribute to both β-sheets. The second β-sheet is largely covered by the three-turn α-helix (residues 129–142), and parts of the N-terminal (residues 1–7) and C-terminal (residues 153–157) regions. Strand β-A2 is linked to another strand, β-I (residues 146–152), which is critically involved in dimer formation: β-I packs antiparallel against the equivalent strand of a dyad-related molecule, such that the second β-sheet spans both molecules of the dimer. In lattices Y and Z, the dyad is a crystallographic symmetry axis. The functional form of BLG in solution over the pH range 3.5–8.5 appears to be the dimer, which has a dissociation constant in the micromolar range [2,51,52]. The overall fold and dimer are illustrated in Fig. 2 [53], along with the ligand, 12-bromododecanoic acid (BrC12), which is unambiguously bound inside the calyx. There is no evidence for binding of this ligand to any outside surface of the molecule, despite the presence of excess ligand. The side of the molecule, where the entrance to the internal cavity is located, is illustrated in Fig. 2A. The surface charges, which lead to BLG variants A, B, and C having isoelectric points, p*I*, of ∼5.3 [54], are largely concentrated on this surface. As is apparent in the right-hand panel of Fig. 2B, upon dimerization of BLGA a deep cleft is formed between the 3-turn α-helices on one side of the molecule. Parallel to the helix and on the other side of the cleft is a somewhat hydrophobic patch that has been implicated in some binding studies [22,23,42]. A model for external binding of BrC12 is illustrated in Fig. 2B.

Table 2
Refinement statistics for BrC12-BLGA in lattice Z at pH 7.3

R (# reflns), R_{free} (# reflns)	0.234 (9184), 0.276 (511)
# Water molecules	66
Ligand	12-bromododecanoic acid
Ramachandran plot[a]	
Core/allowed/disallowed	83.2/15.4/0.7%
Geometry rms deviations[a]	
Bond distances (Å), angles (°)	0.007, 1.5
Dihedral angles (°)	25.5
Average thermal parameters (B, Å^2)	
All atoms, protein atoms	42.9, 41.3
Main-chain, side chain	38.5, 44.4
Waters, ligand	72.6, 52.1

[a]Calculated by PROCHECK [50].

3.3. Ligand binding – general aspects

There are remarkably few structural adjustments made by the host molecule, BLGA, in order to accommodate the 12-bromododecanoic acid ligand. Of the 15 hydrophobic side-chains lining the cavity, 11 are in hydrophobic contact with the ligand (interatomic distances less than 4.2 Å) and for only one residue, Ile^{71}, does the side chain adopt a different rotamer compared to that found in BLGA at pH 7.1. The overall RMS difference for the superposition of the positions of Cα atoms of unliganded BLGA (pH 7.1) and liganded BLGA (pH 7.3) is only 0.169 Å for 156 out of 162 residues; only six residues had an RMS difference greater than 0.5 Å, as calculated by TURBO-FRODO [46]. Specifically, there are noticeable perturbations of residues only in the mobile CD and EF loops. On the surface of the protein only Glu^{112} and Glu^{134} change side-chain rotamer. The loop EF (residues 85–89) is found in the open conformation, with essentially the same residue-by-residue conformations as those for unliganded BLGA in lattice Z at pH 7.1 and 8.2. In contrast to these latter two structures, this loop is more clearly defined in the liganded derivative, with significantly smaller B values relative to the average B value for each structure.

The conformation of the 12-bromododecanoic acid ligand requires little perturbation from a fully extended all-*trans* conformation in order to be accommodated inside the calyx, as illustrated in Fig. 1B. About bond C3–C4, the torsional angle is 130°, a staggered conformation. The fit of the 12-bromododecanoic acid inside the calyx is illustrated in Fig. 3 [53], where the molecular surface of the ligand has been laid onto the molecular surfaces of the first β-sheet (Fig. 3A) and the second β-sheet (Fig. 3B). The closest contact of the ligand with Trp^{19} is more than 7 Å distant. The bromo group is comfortably positioned inside the calyx, making no contact shorter than 3.9 Å with the surrounding protein. As is apparent in Fig. 3, there is substantial space for the bromo group, consistent with its high B value (Table 3), relative to adjacent

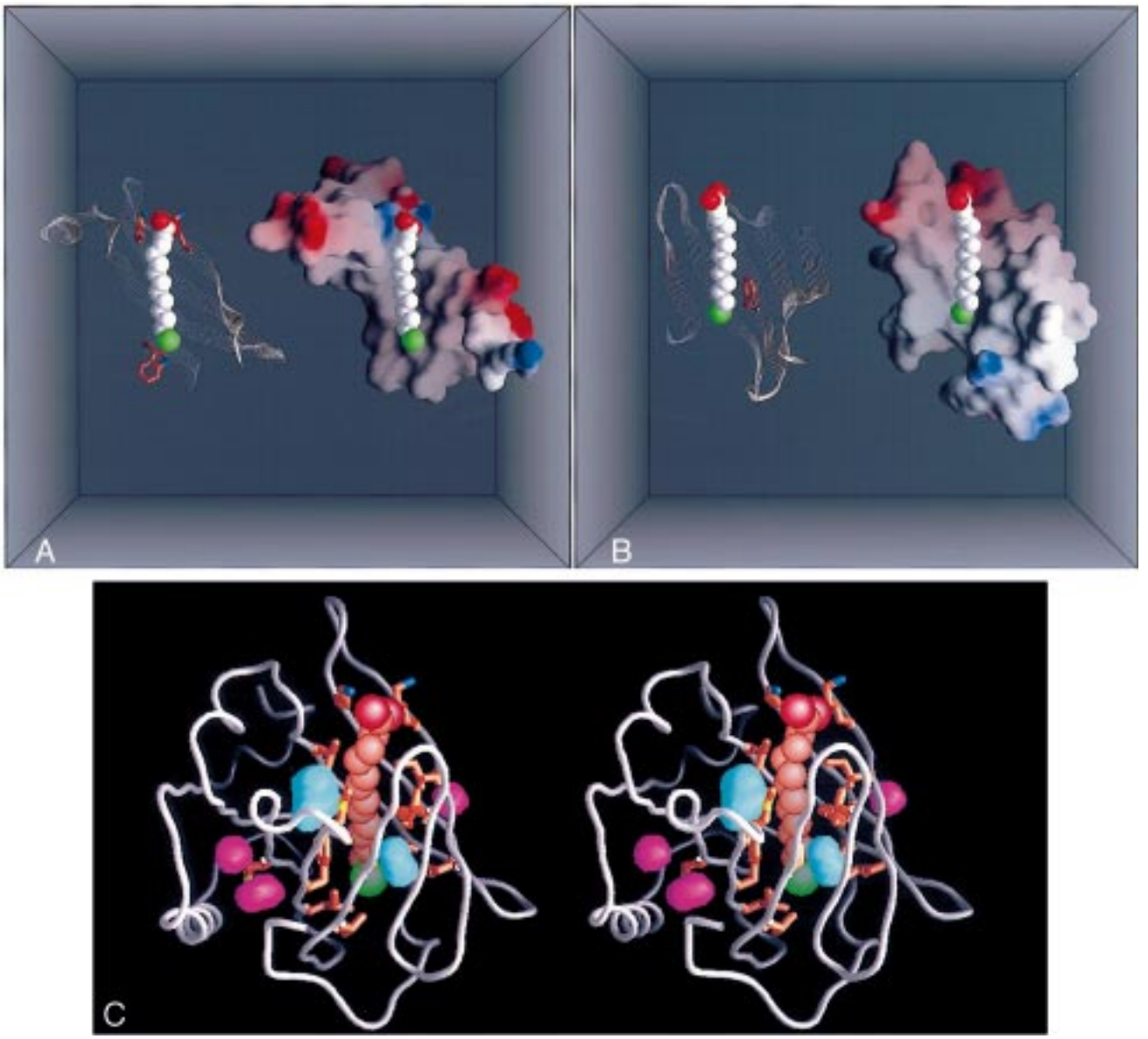

Fig. 3. GRASP [53] representation of the protein-ligand interface and cavities of the BLGA-BrC12 complex. The ligand is drawn as 1.4-Å radius spheres, except for the bromine atom, which is drawn as a 1.6-Å radius sphere. For parts A and B, the surface (right-hand panels) and the underlying polypeptide fold (left-hand panels) are drawn in the same orientation. See also the caption for Fig. 2. A, top left: The fit of 12-bromododecanoic acid into the first β-sheet (β-A1 (bottom strand), β-B, β-C, β-D (top strand)). Note the interaction of the ligand with Lys^{60} and, to a lesser extent, Lys^{69}, and the remoteness of the ligand from Trp^{19}. B, top right: The fit of 12-bromododecanoic acid into the second β-sheet (β-E (left-most strand), β-F, β-G, β-H, β-A2, (right-most strand)). Phe^{105}, which makes many contacts with the ligand is shown for reference. C, bottom: Stereodiagram showing (i) the protein backbone (white worm); (ii) the ligand (coloured spheres: red oxygen, orange carbon, green bromine); (iii) the side-chains of all residues that make contacts with the ligand of less than 4.25 Å (coloured rods: orange carbon, blue nitrogen, yellow sulfur); and (iv) cavities, calculated with a probe radius of 1.4 Å, which remain after ligand binding (coloured cyan inside the calyx and magenta outside the calyx). In addition, the side-chain of the free thiol at Cys^{121} is shown. Note that all residues in contact with the hydrocarbon portion of the ligand are hydrophobic. Note also the cavity in the vicinity of Cys^{121}, which is ∼12 Å distant from the putative external ligand depicted in Fig. 2B.

carbon atoms. As is apparent in Fig. 3A,B, the ligand-binding cavity is exclusively hydrophobic, except for two lysine residues, Lys^{60} and Lys^{69}, at the opening of the cavity. Lys^{60}NZ hydrogen bonds with the carboxyl end group of the ligand (K60NZ...OE2(BrC12) 2.9 Å); both atoms hydrogen bond to a water molecule. Lys^{69} forms an intermolecular salt bridge with Glu^{62} in a neighbouring molecule in the crystal, but reorientation in solution would enable it to interact with the carboxyl group of the ligand. There is extensive interaction of the hydrocarbon tail of the ligand with the host protein, as tabulated in Table 3 and illustrated in Fig. 3C. Including the bromo substituent, a total of 35 hydrophobic contacts are made, which lie in the range 3.5–4.2 Å. The majority of these contacts (a total of 24) involve the deeply buried half of the molecule, $-(CH_2)_5-CH_2Br$. The loose binding of the carboxyl half of 12-bromododecanoic acid is reflected in the temperature factors, which range from ∼28 $Å^2$ at the buried end to ∼90 $Å^2$ at the exposed carboxyl end of the ligand (detailed in

Table 3
Hydrophobic contacts (<4.2 Å) between 12-bromododecanoic acid and BLGA

Atom Ligand	B	Atom BLGA	Distance	Atom Ligand	B	Atom BLGA	Distance
Br	57	L54CD2	4.1	C8	29	F105CZ	4.2
		L46CD1	4.1	C7	34	I84CD1	3.8
		V94CG2	3.9			M107SD	3.7
		L103CD2	4.0	C6	43	M107SD	3.9
C12	28	L46CD1	3.9			V41CG2	3.9
		L46CD2	3.9			I71CD1	4.0
		F105CD2	3.9	C5	47	I71CD1	3.7
		F105CG	4.1			M107SD	3.7
		F105CE2	4.1			M107CE	3.8
C11	31	V92CG1	3.9	C4	53	I71CD1	4.0
		I56CD1	4.1			V41CG1	4.0
C10	28	F105CD1	4.2	C3	64	I71CD1	3.6
		F105CE1	3.6	C2	78	I71CD1	3.9
		F105CZ	3.5	C1	85	K60NZ	3.6
		F105CE2	3.9	OE2	90	K60NZ	2.9
		I56CD1	4.1			WAT187O	3.4
		I56CG2	4.2	OE1	86	K69NZ	3.9[a]
C9	29	F105CE1	4.0			K69CD	3.2
		F105CZ	4.1			K69CE	3.6

The atomic displacement parameters (B, Å^2) of the ligand atoms are provided.
[a]In the crystal, Lys^{60} is oriented to form an intermolecular salt bridge rather than an intramolecular salt bridge with the ligand.

Table 3), consistent with the relatively weak electron density for the carboxyl end of the ligand observed in the original molecular replacement solution and later omit maps (see Fig. 1). The extent of ligand-protein interaction is consistent with the observed binding constants, K_d, of $\sim$10 μM for this class of ligands [13–23].

A total of four relatively diffuse water molecules near the top of the cavity in the structure of BLGA at pH 7.1, as well as a buried water molecule near the site of the bromo substituent, are displaced on ligand binding. Two new water sites are created; one is involved in hydrogen bonding with K60NZ and OE2(BrC12). With a loose solvent structure at the entrance to the calyx and given the open conformation of loop EF, one would expect ligand binding and dissociation to be rapid. It is clear from Fig. 3C, in which the unfilled internal cavities still remaining near the ligand are shown, that there is room for substantially larger, and longer, molecules to bind in the internal cavity. The approximate volumes of these cavities are 60 Å^3, for the one in the vicinity of the bromine atom, and 28 Å^3 for the other near atom C5 of the ligand. As the bulky bromo substituent appears to define a fixed orientation for the ligand, it is possible that unsubstituted fatty acids may be insufficiently ordered to be visible in electron density maps.

Thus, at least for 12-bromododecanoic acid, and very likely for fatty acids in general, little adjustment in either the structure of the ligand or of the host is required in order to form the host-guest complex for binding inside the calyx.

3.4. Role of lysine residues in fatty acid binding

The two lysine residues at the opening of the ligand-binding cavity, Lys^{60} and Lys^{69} (Fig. 3) seem likely to play a significant role in ligand affinity. The failure of pig BLG to bind fatty acids may be due to the substitution of Lys^{69} by glutamate, as suggested [17,18]. In other non-ruminant BLGs that are reported not to bind fatty acids, such as donkey and horse [18], a basic amino acid (Lys or Arg) is maintained at position 69, but instead a glutamate is substituted for Lys^{60}. It appears then that the presence of a carboxyl residue at either position 60 or 69 can hinder the binding of fatty acids.

3.5. Do fatty acids bind inside or outside the calyx?

The crystallographic results presented herein show unequivocally that the primary site for fatty-acid binding is in the internal hydrophobic cavity of the BLG calyx, as is the case for hydrophobic ligands bound to other members of the lipocalin superfamily [4–12]. Nevertheless, a number of studies have been interpreted in terms of external binding sites [22–24,55]. Several of these involve chemical reactivity studies, which imply easy accessibility to the ligand [22,24]. Given the relatively low binding affinity of BLG for the ligands used, however, it is likely that ligand exchange occurs on a time scale similar to or faster than that of the reactions monitored, and that internal binding is not inconsistent with the results. Fluorescence measurements, which suggest that fatty acids do not bind competitively with internally bound retinol, have also been interpreted in terms of an external binding site, between the three-turn α-helix and the β-sheet [22,23], for which a possible model, which involves movement of the N-terminus by alteration of ϕ/ψ angles at Gln^5, is illustrated in Fig. 2B.

We cannot exclude the possibility that some ligands, including fatty acids, may bind externally to BLG under certain conditions. Ligand binding to BLG is sensitive to extremes of pH; however, over the pH range 5.5–8.5 changes in binding constants are observed [3], with weaker binding obtaining at higher pH. This has been variously attributed to electrostatic interactions – as is especially apparent in Fig. 2B: as pH increases, BLG becomes very negatively charged, and electrostatically a less inviting host for negatively charged fatty-acid species. In addition, over this pH range a conformational change has been identified that involves movement of loop EF: in the absence of added ligand, at low pH this loop folds over the top of the calyx, while at higher pH (greater than pH 7.1) this loop folds back to reveal the inside of the calyx [39]. Thus, steric factors are also involved in determining ligand

affinity, especially for long ligands that protrude above the surface of the protein and force loop EF to adopt the open conformation at all values of pH.

Nevertheless, several pieces of evidence support our view that the binding mode seen crystallographically is also the predominant binding mode in solution. Firstly, the binding of hydrophobic ligands inside the hydrophobic cavity, as seen, makes sense in structural and energetic terms. Secondly, competitive and reversible binding of retinol and fatty-acid species to BLG, at low salt concentrations, have been demonstrated by CD spectroscopy (unpublished observations, L.K. Creamer). Thirdly, the proposed external site, although hydrophobic (Fig. 2B), lacks some of the features expected for the binding of fatty acids with dissociation constants in the μM range: (i) to make contact with the ammonium moiety of Lys^{135} and Lys^{138} requires alteration of the ligand at several carbons from the favoured all-*trans* conformation (longer-chain fatty acids, such as palmitic or hexadecanoic acid, and stearic or octadecanoic acid and its doxyl derivatives [22] may be unable to make these salt bridges); (ii) to create a shallow hydrophobic cleft for the ligand, which still leaves much of the ligand exposed, requires an entropically unfavourable movement of the N-terminus; (iii) the patch offers limited surface complementarity to fatty acids (many fewer contacts are made compared to the internal binding of the ligand; and (iv) Cys^{121}, a possible locus of hydrogen bonding to the carboxyl head group, is buried, although there is a small cavity in the vicinity of the side-chain (Fig. 3C). In our model of external binding, SG of Cys^{121} is more than 12 Å from the ligand.

Thus, the structural result reported here appears to be representative of the location of binding of fatty-acid species to bovine β-lactoglobulin. The precise location of retinol remains to be determined, although in prior molecular modelling [37,40,56] it was located in essentially the same location as that found for the ligand BrC12. Whether longer-chained fatty acids maintain a stretched-out conformation, as observed for BrC12, or curl up remains to be determined. The latter geometry was observed in the complex of hexadecanoic acid with fatty-acid binding protein from human muscle [49], a 10-stranded protein that is a member of the calycin superfamily to which the lipocalins (and BLG) also belong [57,58].

4. Conclusions

Variant A of bovine BLG binds 12-bromododecanoic acid in a well-defined manner inside the calyx with minimal rearrangement of the protein structure. This ligand is not observed to bind to any external regions of the protein. At pH 7.3, the lid to the calyx, loop EF, remains open allowing rapid binding and release of the ligand.

5. Note added in proof

We thank Lindsay Sawyer for a manuscript describing the structure of BLG complexed with palmitate in a manner very similar to that reported here.

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