Research Article

Journal of Molecular Recognition

Received: 12 May 2010, Revised: 28 July 2010, Accepted: 29 July 2010, Published online in Wiley Online Library: 14 December 2010

(wileyonlinelibrary.com) DOI:10.1002/jmr.1084

Two modes of fatty acid binding to bovine β-lactoglobulin—crystallographic and spectroscopic studies

Joanna Loch[a], Agnieszka Polit[b], Andrzej Górecki[b], Piotr Bonarek[b], Katarzyna Kurpiewska[a], Marta Dziedzicka-Wasylewska[b] and Krzysztof Lewiński[a]*

Lactoglobulin is a natural protein present in bovine milk and common component of human diet, known for binding with high affinity wide range of hydrophobic compounds, among them fatty acids 12–20 carbon atoms long. Shorter fatty acids were reported as not binding to β-lactoglobulin. We used X-ray crystallography and fluorescence spectroscopy to show that lactoglobulin binds also 8- and 10-carbon caprylic and capric acids, however with lower affinity. The determined apparent association constant for lactoglobulin complex with caprylic acid is $10.8 \pm 1.7 \times 10^3\,M^{-1}$, while for capric acid is $6.0 \pm 0.5 \times 10^3\,M^{-1}$. In crystal structures determined with resolution 1.9 Å the caprylic acid is bound in upper part of central calyx near polar residues located at CD loop, while the capric acid is buried deeper in the calyx bottom and does not interact with polar residues at CD loop. In both structures, water molecule hydrogen-bonded to carboxyl group of fatty acid is observed. Different location of ligands in the binding site indicates that competition between polar and hydrophobic interactions is an important factor determining position of the ligand in β-barrel. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: bovine β-lactoglobulin; caprylic acid; capric acid; fatty acids; protein–ligand interactions

INTRODUCTION

The β-lactoglobulin is a protein present in the milk of many mammalian species, with human being one of the known exceptions. The concentration of bovine β-lactoglobulin (LGB) in cow's skim milk is about 2–4 g/L (Farrell *et al.*, 2004). In bovine milk lactoglobulin occurs in many isoforms, among them the most frequent are isoforms A and B (Qin *et al.*, 1998) that differ at two positions, E64G and V118A (Godovac-Zimmermann *et al.*, 1996; Oliveira *et al.*, 2001).

Despite intensive studies of biological, chemical and physical properties of LGB its biological function still remains unknown (Kontopidis *et al.*, 2004). LGB is probably involved in storage and transport through the calves gastric tract hydrophobic substances such as fatty acids and retinoids naturally present in bovine milk (Pérez and Calvo, 1995; Wang *et al.*, 1997; Kontopidis *et al.*, 2002). Is has been also observed that this protein enhances pregastric lipase activity towards triglycerides hydrolysis (Perez *et al.*, 1992).

Bovine β-lactoglobulin is a member of lipocalin family (Brownlow *et al.*, 1997; Sawyer and Kontopidis, 2000), a group of relatively small proteins with characteristic up-and-down eight-stranded β-barrel often called calyx (Åkerstrom *et al.*, 2000). Central β-barrel makes the hydrophobic scaffold of lipocalin molecule and is primary binding pocket for most of lipocalin's ligands (Skerra, 2000). Central calyx is surrounded by flexible loops that regulate access to protein β-barrel in process of reversible closing and opening induced by pH changes (Ragona *et al.*, 2000; Kontopidis *et al.*, 2004). At pH value about 6.8 and higher conformation of EF loop is changed from "close" to "open" in process called Tanford Transition enabling ligand binding inside the calyx (Tanford *et al.*, 1959; Qin *et al.*, 1998).

LGB binds chemical compounds belonging to many different chemical classes (Sawyer *et al.*, 1998), predominantly saturated and unsaturated fatty acids. Measuring radioactivity of isotopically labelled fatty acids, Spector and Fletcher (1970) demonstrated that strength of binding fatty acids to lactoglobulin decreases in array: palmitate, stearate, oleate, laurate and binding is associated with changes of tryptophan fluorescence at 333 nm (Spector and Fletcher, 1970).

Further studies indicated that lactoglobulin binds saturated fatty acids containing 12–20 carbon atoms in molecule and also wide range of unsaturated fatty acids that have hydrocarbon

* *Correspondence to: K. Lewiński, Faculty of Chemistry, Department of Crystal Physics and Crystal Chemistry, Jagiellonian University, Ingardena 3, 30-060 Krakow, Poland.*
E-mail: lewinski@chemia.uj.edu.pl

a *J. Loch, K. Kurpiewska, K. Lewiński*
Faculty of Chemistry, Department of Crystal Physics and Crystal Chemistry, Jagiellonian University, Ingardena 3, 30-060 Kraków, Poland

b *A. Polit, A. Górecki, P. Bonarek, M. Dziedzicka-Wasylewska*
Faculty of Biochemistry, Biophysics and Biotechnology, Department of Physical Biochemistry, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland

Abbreviations used: *K_a, , association constant; K'_a, , apparent association constant; LGB, bovine β-lactoglobulin.*

chain length from 14 to 20 atoms (Frapin *et al.*, 1993). The strength of binding fatty acids in descending order was determined as: palmitate, stearate, myristate, arachidate, laurate (Frapin *et al.*, 1993). Binding of short fatty acids, such as capryclic (octanoic) and capric (decanoic) acid, was excluded because no fluorescence enhancement was observed after addition of them to protein solution (Frapin *et al.*, 1993). On the base of spectroscopic and docking studies, it was suggested later that caprylic acid binds to lactoglobulin with very low specificity and affinity (Collini *et al.*, 2003).

Caprylic and capric acids are saturated medium chain fatty acids naturally present in bovine milk (Jensen *et al.*, 1990) that might have some physiological relevance. Caprylic acid could participate in bacteria cell growth inhibition (Skrivanová *et al.*, 2008) and can be used as antimicrobial food additive (Viegas and Sá-Correia, 1995; Nair *et al.*, 2005). This acid is also used in immunoglobulin purification and has antiviral activity (Dichtelmüller *et al.*, 2002). Capric acid, naturally present in bovine, human milk and in plant oils has potential pharmacological importance (Maher *et al.*, 2009). Capric acid decreases the intestinal absorption of cholesterol and slows cholesterol synthesis in the liver (Dasgupta and Bhattacharyya, 2009). Capric acid in water forms anionic vesicles that could help prolong drug release in the body or have positive effect on drug uptake from digestive system (Dew *et al.*, 2008; Maher *et al.*, 2009). It was also postulated that capric acid could have vasorelaxant activity (White *et al.*, 1991).

Papers concerning lactoglobulin interactions with fatty acids reported inconsistent dissociation or association constants for particular lactoglobulin–fatty acids complexes depending on experimental technique. For example, due to formation of protein oligomers and non-typical behaviour of hydrophobic ligands in water solutions dissociation (or association) constants for some ligands determined by fluorescence spectroscopy are higher than values from equilibrium dialysis (Muresan *et al.*, 2001). Lactoglobulin has at least two binding sites for fatty acids. Higher affinity primary binding site is usually attributed to central calyx with apparent association constants for fatty acids in range 10^5–$10^7\ M^{-1}$, secondary binding sites are probably located on protein surface and bind fatty acids with lower affinity 10^3–$10^4\ M^{-1}$ (Spector and Fletcher, 1970; Frapin *et al.*, 1993; Narayan and Berliner, 1997; Yang *et al.*, 2008). Most of the apparent association constants for fatty acids primary binding site were determined measuring tryptophan fluorescence quenching (or enhancement) at 332–334 nm and were obtained for protein concentration in the range from 1 to 100 μM (Spector and Fletcher, 1970; Frapin *et al.*, 1993; Narayan and Berliner, 1998; Yang *et al.*, 2008; Zimet and Livney, 2009). It was also found that fatty acids binding to lactoglobulin is significantly affected by protein concentration. Increase of protein concentration from 1 to 200 μM resulted in sixfold decrease of the association constant for primary palmitic acid binding site (Wang *et al.*, 1998). Authors attributed this behaviour to existence of two independent binding sites, one in monomer and second in lactoglobulin dimer. Considering these observations, binding constant studies must take into account presence of different oligomeric states of lactoglobulin.

Lactoglobulin is known as a protein with high affinity but low-binding specificity (Konuma *et al.*, 2007). LGB for many years has been considered as a potential carrier protein for different hydrophobic ligands (Muresan *et al.*, 2001; Zsila *et al.*, 2005; Mohammadi *et al.*, 2009). Recognition of binding properties of other lipocalins enabled to produce their engineered forms called anticalins that has found medical applications (Skerra, 2001, 2008). This work was undertaken as a part of systematic studies of interactions in lactoglobulin–fatty acids complexes as a first step in designing modified lactoglobulins.

MATERIALS AND METHODS

Materials

Bovine β-lactoglobulin isoform B (≥90%), caprylic acid (≥98%) and capric acid (≥98%), Tris base (≥99%) and anhydrous glycerol were purchased from Sigma–Aldrich. Ethanol (≥96%) and trisodium citrate in analytical grade were purchased from POCh (Gliwice, Poland).

Monitoring fatty acids binding by steady-state fluorescence measurements

Steady-state fluorescence was measured with Hitachi F-4500 spectrofluorimeter (Tokyo, Japan). All studies were carried out at room temperature and excitation at 295 nm. The spectra were recorded in the range from 310 to 400 nm. Both excitation and emission slits were set at 5 nm. The experiments were conducted in buffer 50 mM Tris–HCl pH 7.5. The protein concentration was 20 μM and its solution had an initial absorbance at the excitation wavelength lower than 0.1. Protein concentration was determined using molar absorption coefficient equal to 17 600 $M^{-1}\ cm^{-1}$ at 278 nm (Narayan and Berliner, 1997). LGB was present in the fatty acid solution so the protein concentration was constant during the experiment. The effect of caprylic acid and capric acid on tryptophan fluorescence was monitored by a fluorescence titration of β-lactoglobulin with 2–4 μl aliquots of concentrated caprylic and capric acids solutions. Measurements were performed in the fatty acids concentration range 4–200 μM. Stock solution of each fatty acid was made in ethanol and before measurements it was diluted in buffer up to concentration in range of 1–1.5 mM, so final ethanol concentration was in range 0.2–0.3% (v/v). The protein solutions contained identical concentration of ethanol. We did not observe the changes of the measured signal in the control measurements in which buffer was titrated by fatty acid.

For data analysis DynaFit 3.28.064 (Kuzmic, 1996) software has been used with simple reaction scheme describing ligand binding by LGB:

$$P + L \overset{K_a}{\Leftrightarrow} PL$$

where P is the free LGB, L is the fatty acid, PL is the complex of LGB with the ligand and K_a is the association constant.

Crystallization

Crystals were obtained in 20°C using vapour diffusion method in hanging drop setup. Drop containing 2 μl of 20 mg/ml protein solution in water, 0.5 μl of 10 mM caprylic acid or 1 mM capric acid solution in ethanol and 6 μl of well solution was equilibrated against 1.34 M trisodium citrate in 0.1 M Tris–HCl buffer, pH = 7.5. Crystals of protein–ligand complexes appeared after 24 h and reached their maximum size (average 0.20 mm) in 4 days. Crystals of LGB unliganded form were obtained using similar conditions with 96% ethanol instead of fatty acid solution. They appeared after 10–14 days and achieved average size 0.10 mm.

Journal of Molecular Recognition

X-ray data collection and processing

Before data collection crystals were placed for few seconds in cryoprotectant solution containing 10% glycerol in 0.1 M Tris–HCl buffer pH = 7.5 with 1.34 M trisodium citrate and transfer quickly in cryoloop to stream of low temperature gas nitrogen (Oxford Instruments). X-ray diffraction data were collected at 110 K using SuperNova diffractometer (Oxford Diffraction) with microfocus CuKα (1.54 Å) X-ray source working at 50 kV and 1 mA and 135 mm CCD Atlas detector at distance 73.2 mm. Data were processed using *CRYSALIS*^{Pro} (Oxford Diffraction) and merged using *SCALA* (Evans, 2006) from *CCP4* package (CCP4, 1994). Details of data collection statistics are summarized in Table 1.

Structure solution and refinement

Structure of unliganded lactoglobulin and structures of lactoglobulin complexes with caprylic and capric acid were solved by molecular replacement method using *MOLREP* program (Vagin and Isupov, 2001) from *CCP4* package with lactoglobulin trigonal structure 1BSY as a starting model (Qin *et al.*, 1998). Rigid body refinement performed by *REFMAC* 5.0 (Vagin *et al.*, 2004) was followed by calculation and careful examination of $2F_o - F_c$ and $1F_o - F_c$ Fourier maps using *COOT* (Emsley and Cowtan, 2004) to check overall fit of model to map. After restrained refinement of protein, ligand molecule was fitted to Fourier map and all atoms were again refined using *REFMAC*. Ligands library descriptions for restrained refinement in *REFMAC* were created using *LIBCHECK* from *CCP4* package (A.A. Vagin, *CCP4*). The occupancy of ligand molecule was initially determined by group occupancy refinement in *CNS* (Brünger, 2007) and then confirmed by comparing ligand's B-factors to B-factors of neighbouring residues. After that, *REFMAC* restrained refinement was continued. Water and ion molecules were located on Fourier maps and added to the structure. For some residues, the Fourier maps allowed us to detect alternative conformers of side chains that were also refined by *REFMAC*. Residues 111–116 located in region with poor defined electron density were initially removed from the model and then systematically added, one by one, in consecutive refinement cycles at positions indicated by difference Fourier maps. The structure was validated by *PROCHECK* (Laskowski *et al.*, 1993) and *WHATCHEK* (Hooft *et al.*, 1996), analysis of B-factors was performed by *BAVERAGE* program from *CCP4* package (CCP4, 1994). Protein secondary structure elements were determined by Kabsch and Sander algorithm implemented in *DSSP* program (Kabsch and Sander, 1983). Ligand to protein interactions were analyzed by *LIGPLOT* (Wallace *et al.*, 1995) and *PyMol* (DeLano, 2002). Statistics of structure solution, refinement and validation are summarized in Table 1.

Structures of unliganded lactoglobulin form, lactoglobulin complexes with caprylic and capric acids were deposited in Protein Data Bank as 3NPO, 3NQ3 and 3NQ9.

Results

Apparent association constants for binding medium chain fatty acids to bovine β-lactoglobulin

The fluorescence intensity change of β-lactoglobulin Trp19 and Trp61 as a function of caprylic and capric acids concentration is shown in Figure 1. The emission maximum of β-lactoglobulin was ~332 nm and was not significantly shifted upon addition of caprylic or capric acid (data not shown). The addition of both fatty acids induced monotonic decrease of fluorescence intensity reaching ~85% of initial β-lactoglobulin fluorescence (for protein

Table 1. Statistics of data collection and structure refinement

	LGB	LGB–caprylic acid	LGB–capric acid
Data collection and processing			
Temperature (K)	110	110	110
Space group	$P3_221$	$P3_221$	$P3_221$
a, *b* (Å)	52.80	53.47	53.50
c (Å)	110.64	112.06	112.14
α, β (°)	90	90	90
γ (°)	120	120	120
Resolution range (Å)	10.0–2.20	10.0–1.90	10.0–1.90
The highest resolution shell (Å)	2.32–2.20	2.00–1.90	2.00–1.90
Completeness [%] (the highest resolution shell)	96.1 (89.5)	98.3 (97.6)	99.3 (100.0)
Mean I/σI (the highest resolution shell)	11.6 (3.3)	10.5 (1.9)	23.7 (3.4)
Overall R_{merge} (the highest resolution shell)	0.050 (0.182)	0.046 (0.319)	0.060 (0.394)
Average mosaicity (°)	1.28	1.17	1.05
Structure refinement			
Number of unique reflections	8649	14,097	14,348
R (%)	21.32	23.33	23.83
R_{free} (%)	26.00	30.90	28.93
rmsd angles (°)	1.811	1.879	1.921
rmsd bonds (Å)	0.016	0.016	0.016
Average B factor (Å^2)			
Whole protein chain	27.78	29.26	29.04
Main chain protein	27.27	28.57	28.11
Ligand	—	34.68	26.62

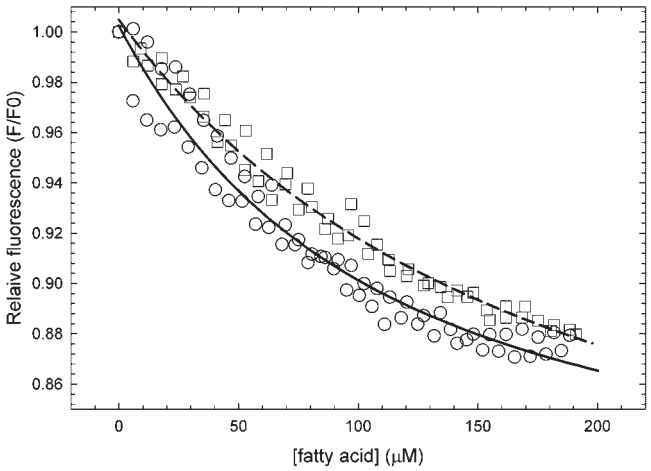

Figure 1. Fluorescence intensity changes of β-lactoglobulin as a function of caprylic (circle) and capric (square) acid concentration. *F* and F_o are the fluorescence intensities of the protein in the presence and absence of the ligand, respectively. The range of fatty acids concentrations used was from 4 to 200 μM. The lines presents best fits obtained using DynaFit 3.28.064 software.

concentration 20 μM). For lower protein concentration, lower amplitude of signal and more complicated trace of binding curves were observed (data not shown). Although it indicates that there are additional processes, such as changes of LGB dimer–monomer equilibrium ($K_d \sim 3.5–10^{-5}$ M at pH = 7.5, Wang *et al.*, 1998) or existence of two classes of binding sites dependent on oligomerization conditions, but we cannot reliably describe them. Therefore, we analyse data obtained for 20 μM BLG concentration using simplest model of interaction with apparent binding constant and fixed stoichiometry of 1:1.

The apparent association constants determined for lactoglobulin complex with caprylic acid is $10.8 \pm 1.7 \times 10^3\ M^{-1}$ while for capric acid is $6.0 \pm 0.5 \times 10^3\ M^{-1}$. These values indicate much weaker lactoglobulin affinity for caprylic and capric acid than for longer fatty acids.

Apparent association constant reported by Yang *et al.* (2008) for binding palmitic acid determined at the same protein concentration (20 μM) is $2.27 \times 10^7\ M^{-1}$. K'_a values reported by other authors for binding lauric, myristic, palmitic or stearic acid in primary binding site are also in range of $\sim 10^7–10^5\ M^{-1}$ (Frapin *et al.*, 1993; Wang *et al.*, 1998). Later papers showed on the base of crystal structures that this primary binding site was located in central calyx (Qin *et al.*, 1998; Wu *et al.*, 1999).

Overall structure of β-lactoglobulin

For unliganded lactoglobulin several different crystal forms were reported (Sawyer *et al.*, 1999), among them the most popular were triclinic lattice X (P1), orthorhombic lattice Y ($C222_1$) and trigonal lattice Z ($P3_221$). So far, all known structures of LGB–ligand complexes were determined only in space group $P3_221$ (Wu *et al.*, 1999; Kontopidis *et al.*, 2002). Reported here structures of lactoglobulin complexes with caprylic and capric acids also have this symmetry with the unit cell containing one lactoglobulin molecule in asymmetric unit. In pH range from 3.5 to 8.5 lactoglobulin exist in solution as a dimer (Qin *et al.*, 1998; Gottschalk *et al.*, 2003; Harnsilawat *et al.*, 2006). The existence of dimer with subunits related by crystallographic twofold axis was also observed in crystal structures (Qin *et al.*, 1998).

The structure of liganded and unliganded LGB is similar to other trigonal structures of lactoglobulin reported previously (Brownlow *et al.* 1997; Qin *et al.*, 1999; Yang *et al.*, 2008). Nine stranded antiparallel β-sheet (strands A–I) is a core of a protein molecule (Figure 2A). The β-strands A–H form β-barrel called central calyx, flanked by flexible loops AB, CD, EF and GH. The β-strand I is involved in dimer formation (Figure 2B). Part of a HI loop makes α-helix that is a part of β–α–β motif involving H and I β-strands. The structure of a lactoglobulin molecule is stabilized by two disulfide bridges. The first one formed by Cys106-Cys119 links strands G and H while the second bridge, Cys66-Cys160, makes connection between CD loop and C-terminus.

As the known crystal structures of β-lactoglobulin revealed, the central calyx is a primary binding site for most ligands, particularly palmitic acids. The interior of the calyx is filled with exclusively

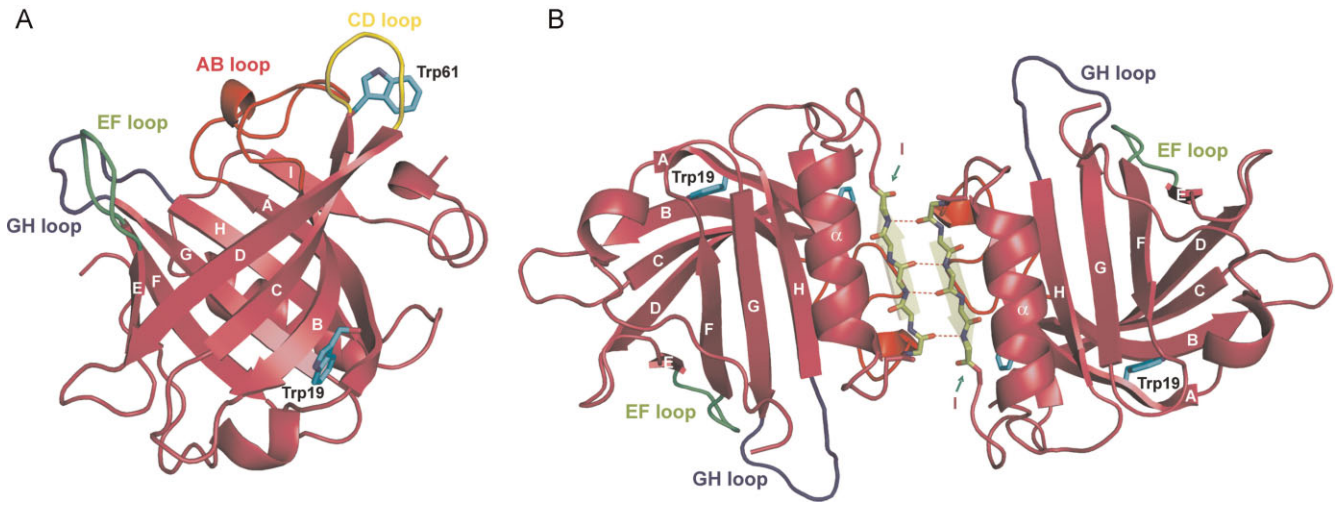

Figure 2. (A) Cartoon representation of bovine β-lactoglobulin unliganded form. Eight antiparallel β-strands (A–H) form central calyx surrounded by flexible loops: AB, CD, EF and GH. (B) Cartoon representation of β-lactoglobulin dimer. Monomers are associated by hydrogen bonds formed between residues 146–150 (Sakurai and Goto, 2002). Figures prepared using PyMol (DeLano, 2002).

Journal of Molecular Recognition

hydrophobic residues. The only polar residues Lys60, Glu62 and Lys69 are located on the CD loop at the entrance to the calyx. Similarly to other trigonal LGB structures determined at pH higher than 7.1, the flexible loop EF is in the open conformation allowing ligand to enter the central calyx (Qin *et al.*, 1999; Kontopidis *et al.*, 2002; Yang *et al.*, 2008).

The LGB molecule contains two tryptophan residues. Trp19 is located on β-strand A close to the bottom of central calyx and Trp61 is a part of flexible loop CD in vicinity of calyx entrance. Trp19 is not accessible to solvent while Trp61 is located in polar environment and has good solvent accessibility. Experiments with W19A mutant of LGB showed that this mutation results in 80% decrease of fluorescence from tryptophan compared to the wild-type protein. These data indicate that most of the fluorescence comes from Trp19 located at the calyx bottom so changes of fluorescence intensity allow to monitor presence of ligand in the central binding site (Cho *et al.*, 1994).

Structures of bovine *β*-lactoglobulin with bonded caprylic acid and capric acid

Caprylic acid is bound to protein molecule in the upper part of central β barrel. The $2F_o - F_c$ Fourier map that was weaker than for surrounding chains has indicated that binding site is not fully occupied by ligand. Refinement of the occupancy factor converged at 0.7 value for the final structure. The caprylic acid's 8-carbon chain in extended conformation is buried in the β-barrel while carboxyl group is located close to calyx entrance in close vicinity of polar residues on the CD loop (Figure 3A). The O2 atom of ligand molecule forms short hydrogen bond to water molecule A1000 that is hydrogen-bonded to OE2 atom of Glu62 (Figure 4). Occupancy of this water molecule is the same as ligand occupancy factor. The ligand carboxyl group is in position almost identical as observed for longer-chain fatty acids, e.g. palmitic acid however, the water molecule was previously never found (Wu *et al.*, 1999; Kontopidis *et al.*, 2002). The hydrocarbon chain of caprylic acid interact with hydrophobic amino acids residues in central calyx: Val92, Ile56 and Met107. Analysis of the Fourier maps revealed that side chain of Met107 exists in the structure in two alternative conformations. Refinement of the occupancy factor showed that higher-occupied conformer has an occupancy factor almost equal to the occupancy factor of the ligand. This result suggests that conformation of Met107 side chain depends on presence of ligand in the binding site. Similar observations were also reported for other LGB structures (Sawyer and Kontopidis, 2000; Kontopidis *et al.*, 2002).

Despite similarity between caprylic and capric acid, their binding in central calyx is slightly different. Comparing to caprylic acid, the end of capric acid's hydrocarbon chain is positioned deeper, close to bottom of the barrel (Figure 3B). Its position is in the same region as was found for other longer fatty acids. The hydrocarbon chain in extended conformation is surrounded by hydrophobic residues: Leu46, Ile56, Leu54, Ile84, Val94, Phe105 and Met107. Surprisingly, the carboxyl group and the water molecule that is hydrogen bonded to it with distance 2.48 Å, are also positioned in hydrophobic environment (Figure 5). In the structure with caprylic acid, this region corresponds to the middle part of caprylic acid's hydrocarbon chain. The occupancy factor for capric acid and water molecule bounded to it is 0.6, lower than was determined for caprylic acid. Analysis of Fourier maps did not reveal any additional fatty acid bonded on protein surface in the secondary binding site as was proposed on the base of the lactoglobulin complex with vitamin D_3 (Yang *et al.*, 2008).

Binding of capric and caprylic acids does not affect notably conformation of amino acids in biding site. Small positional shifts are visible for polar residues located on CD loop: Lys60, Glu62 and Lys69. In both liganded structures of lactoglobulin, the carboxyl group of Glu62 side chain is directed toward the ligand. This position allows to form water-mediated hydrogen bond to caprylic acid (Figure 5). Such a hydrogen bond to the capric acid is not possible due to large distance, however, in both structures the same conformational change of Glu62 side chain is observed.

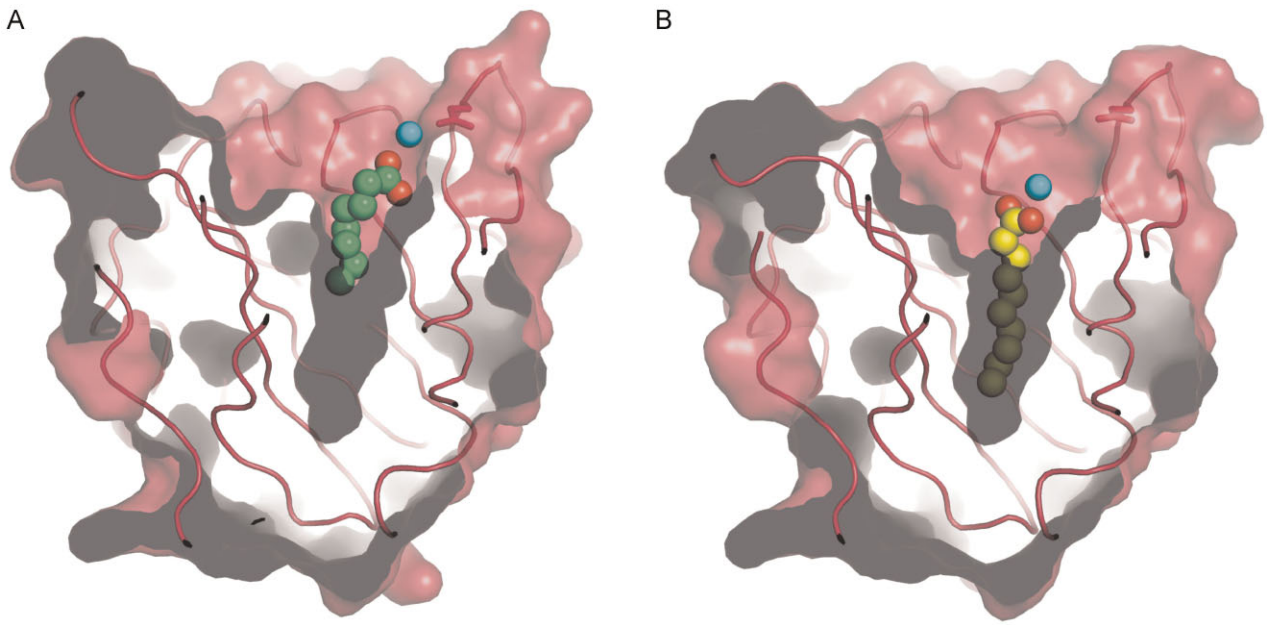

Figure 3. (A) Caprylic acid bounded to lactoglobulin occupies upper part of β-barrel. (B) Capric acid molecule bounded to lactoglobulin is buried deeper in central calyx and surrounded only by hydrophobic residues. Figures prepared using PyMol (DeLano, 2002).

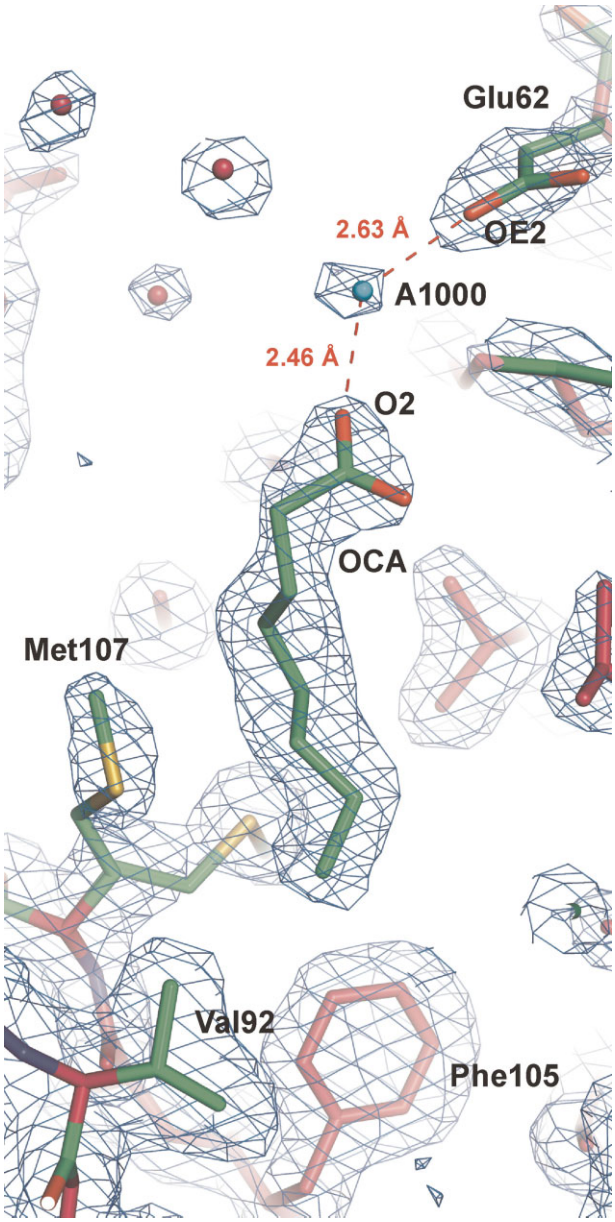

Figure 4. $2F_o - F_c$ Fourier map contoured at 1.20 σ around caprylic acid and residues involved in hydrophobic and polar interactions with ligand. Figure prepared using PyMol (DeLano, 2002).

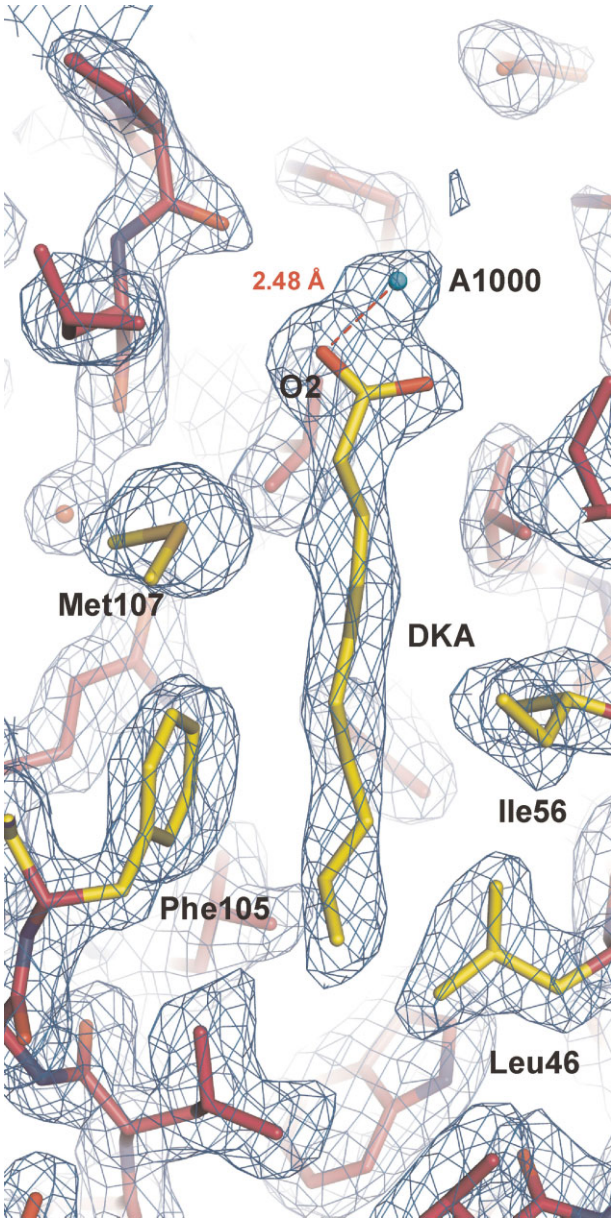

Figure 5. $2F_o - F_c$ Fourier map contoured at 1.20 σ around capric acid and residues involved in hydrophobic interactions with ligand. Figure prepared using PyMol (DeLano, 2002).

B-factor distribution for main chain atoms reveal that the most flexible parts of LGB molecule are loops CD, EF and GH for which poorly defined electron density is observed. The displacement parameters for atoms in both ligands correspond well to values determined for atoms in surrounding amino acids. Increase of B-factor is observed for atoms that are further from the bottom of the barrel.

Discussion

Bovine β-lactoglobulin is a protein present in milk, with unclear physiological function. Because of its high affinity for binding molecules with long hydrocarbon chain, transport and storage functions are attributed to this protein (Sawyer and Kontopidis, 2000; Kontopidis *et al.*, 2004). In particular, lactoglobulin prefers binding 16- and 18-carbon fatty acids like palmitic, stearic and oleic acid that are the most abundant fatty acids in milk (Frapin *et al.*, 1993; MacGibbon and Taylor 2006; Månsson, 2008). Shorter fatty acids like myristic and lauric acids are bounded with slightly smaller affinity (Spector and Fletcher, 1970; Frapin *et al.*, 1993). Fatty acids with hydrocarbon chain shorter than 12 carbons were considered as not binding to lactoglobulin (Frapin *et al.*, 1993) or binding non-specifically (Collini *et al.*, 2003).

On the other hand a number of studies show that LGB binds shorter compounds containing from 4 to 10 carbons atom. Wishnia and Pinder (1966) showed that LGB interacts with butane and pentane. Later, it was proved that lactoglobulin can bind compounds containing 7, 8 and 9 carbons what was indicated by O'Neill and Kinsella (1987) who studied lactoglobulin

Journal of Molecular Recognition

interactions with different ketones: 2-heptanone, 2-octanone, and 2-nonanone. On a base of affinity chromatography results it was shown that lactoglobulin is not able to bind very short fatty acids: acetic, propionic, and butyric acid however, can bind variety of short fatty acids esters containing from 5 to 9 carbon atoms (Pelletier *et al.*, 1998).

The crystal structures and spectroscopic studies in solution presented here have proved that such complexes exist not only for esters but also for relatively short fatty acids containing 8 or 10 carbon atoms and the ligand is bound specifically in primary binding site. It was postulated by some authors that fatty acids can be bound to other than calyx places (Spector and Fletcher, 1970; Wang *et al.*, 1998; Dong *et al.*, 2006). As a potential secondary binding sites were proposed: region between β-strand A, FG loop and loop proceeding α-helix (Wu *et al.*, 1999), cleft near N-terminus in vicinity of Gln5 (Qin[1] *et al.*, 1998) and in a surface pocket between C-terminal α-helix and β-strand I (Yang *et al.*, 2008). Only this last one was yet confirmed by the crystal structure (Yang *et al.*, 2008).

Similarly to complexes with longer fatty acids (Frapin *et al.*, 1993), changes of fluorescence intensity were also observed and made possible to determine the apparent association constants for binding capric and caprylic, 10- and 8-carbon fatty acids. The value of K'_a determined for both ligands is much smaller than for longer compounds binding in the central calyx. The large difference between apparent association constants might result from the fact that capric and caprylic acids are present in bovine milk in much smaller amounts (MacGibbon and Taylor, 2006) and the central calyx was not optimized in evolution process for binding such a short ligands.

The central calyx is a primary binding site in all known structures of lactoglobulin complexes with fatty acids. The structures show that end of ligand hydrocarbon chain is always bound deep in the hydrophobic part of β-barrel while carboxyl group is located near the calyx entrance (Wu *et al.*, 1999; Kontopidis *et al.*, 2002). This binding mode repeats for ligands that have different length. Kontopidis *et al.* (2002) noticed that 12-carbon bromododecanoic acid in extended conformation occupies almost the same space in binding site as 16-carbon palmitic acid for which some bonds have *gauche* conformation. These results suggest that for the most favourable ligands all free space in hydrophobic pocket is filled. Contrary to these observations, position of 8-carbon caprylic acid, the shortest fatty acid bounded to lactoglobulin up to date, is different. It is located in upper part of the β-barrel with polar head hydrogen bonded to Glu62 residue of flexible CD loop. End of its hydrocarbon chain is located approximately in the middle of central calyx in vicinity of Met107. This position being an alternative mode of binding is consistent with one of four possible positions proposed by Collini *et al.* (2003) on the base of docking simulation and might be the basic mode for shorter than 10-carbon fatty acids. In the crystal structure of lactoglobulin–caprylic acid complex no evidence for alternative position of ligand was found.

Comparison of lactoglobulin crystal structures with various fatty acids as a ligand seem to indicate that mode of ligand binding in the β-barrel is determined by the length of hydrocarbon chain. For fatty acids with the chain at least 10 carbons long hydrophobic interactions prevail over polar interactions and the ligand invariably has been positioned deep in the calyx. The opposite situation is observed for caprylic acid for which polar interactions outweighed hydrophobic effect. Similar binding is expected also for other shorter fatty acids.

Another unexpected feature of structures reported here is presence of strong maximum on the Fourier map in vicinity of ligand carboxyl group. This peak was interpreted as a water molecule A1000 making very short hydrogen bond to one carboxyl oxygen atom. Its occupancy factor converged to the same value as the ligand. Such a water molecule was not reported in earlier structures of lactoglobulin complexes with fatty acids, probably due to lower resolution of those structures. The short distance between the peak and carboxyl group is close to distances between oxygen and metal ion like Na^+ or Ca^{2+}; however, this interpretation was excluded because for metal ions other oxygen atoms in coordination sphere should be present. B-factors of these water molecules correspond well to surroundings atoms when their occupancies are equal to occupancy of fatty acid. The relatively short distance between water A1000 and O2 atom of caprylic and capric acid (2.46 and 2.48 Å, respectively) are not typical for hydrogen bonds length and might be result of partial saturation of binding site with ligand. On the other hand shorter than standard 2.7–3.0 Å length hydrogen bonds have been found in Relibase (Hendlich *et al.*, 2003), in some crystal structures of other proteins with fatty acids as for example structures with PDBID: 2BAB (Liavonchanka *et al.*, 2006), 2AG9 (Wright *et al.*, 2005), 1VYG (Angelucci *et al.*, 2004) and 3FYS (Nan *et al.*, 2009). Reported distances were in the range 2.30–2.60 Å. The search in the Cambridge Data Base (Taylor, 2002) revealed different distribution of distances between water oxygen and oxygen of carboxyl group depending on carboxyl group protonation. For deprotonated carboxyl group observed distances were mainly in the typical range 2.7–3.0 Å while in structures with protonated carboxyl oxygen, distances were shorter and distributed in the range from 2.50 to 2.70 Å. This observation indicates that caprylic and capric acids bound to lactoglobulin are most likely protonated. It can be explained as a result of low solubility of those fatty acids in water.

Role of water bounded to ligand carboxyl group is unclear. In the structure of lactoglobulin–caprylic acid complex this water participates in hydrogen bonds to polar residues located on the CD loop. Such interactions are impossible for capric acid because both water molecule and carboxyl group are in hydrophobic environment, too far from polar groups. This unusual position of strong polar group might be explained only by dominating role of hydrophobic interactions of other ligand's part, hydrocarbon chain located in lower region of the calyx. This observation is in agreement with earlier studies showing that ligand binding by lactoglobulin is driven mainly by hydrophobic forces (Sakurai *et al.*, 2009).

Crystals of lactoglobulin complexes with caprylic acid used in our structural studies were obtained from solutions containing ligand in molar excess in respect to the lactoglobulin. In contrast, because of limited solubility of capric acid, its concentration could not be determined reliably and this ligand was most likely in slight stoichiometric deficiency. Nevertheless, occupancy of ligand in both structures was determined as 0.6 for capric and 0.7 for caprylic acid. Also for other lactoglobulin complexes crystallographic studies revealed that ligand's occupancy in central calyx is usually fractional. In crystal structure of lactoglobulin–retinol complex (PDB: 1GX8) occupancy of the ligand located in central cavity was 0.73. This partial occupancy of the ligand was attributed to limited solubility of retinol in crystallization solution (Kontopidis *et al.*, 2002). In β-lactoglobulin structure with vitamin D3 molecules (PDB: 2GJ5) two

binding sites were localized, one in the calyx and second in a surface pocket between C-terminal α-helix and β-strand I. In both positions occupancy of the ligand was equal 0.5 (Yang *et al.*, 2008). In the lactoglobulin complex with palmitic acid (PDB: 1B0O) occupancy of palmitate in central cavity was determined to be 0.69 or 0.96 depending on crystallization procedure (Wu *et al.*, 1999). In trigonal crystal structure, lactoglobulin dimer is positioned on twofold axis. Observations that occupancies of fatty acids in central calyx are higher than 0.5 indicates that binding of ligand takes place to both dimer subunits.

Acknowledgements

This work was partially supported by Polish Ministry of Science and Higher Education, Grant number 3240/B/H03/2009/37 and Małopolski Doctoral Fellowship co-funded by the European Union in the framework of Action 2.6 of the Integrated Regional Operational Programme 2004–2006. The research was carried out with the equipment purchased thanks to the financial support of the European Regional Development Fund in the framework of the Polish Innovation Economy Operational Program (contract no. POIG.02.01.00-12-023/08).

REFERENCES

Åkerstrom B, Flower DR, Salier JP. 2000. Lipocalins: unity in diversity. *Biochim. Biophys. Acta* **1482**: 1–8. DOI:10.1016/S0167-4838(00)00137-0.

Angelucci F, Johnson KA, Baiocco P, Miele AE, Brunori M, Valle C, Vigorosi F, Troiani AR, Liberti P, Cioli D, Klinkert MQ, Bellelli A. 2004. Schistosoma mansoni fatty acid binding protein: specificity and functional control as revealed by crystallographic structure. *Biochemistry* **43**: 13000. DOI:10.1021/bi048505f.

Brownlow S, Morais Cabral JH, Cooper R, Flower DR, Yewdall SJ, Polikarpov I, North AC, Sawyer L. 1997. Bovine beta-lactoglobulin at 1.8 A resolution—still an enigmatic lipocalin. *Structure* **5**: 481–495. DOI:10.1016/S0969-2126(97)00205-0.

Brünger AT. 2007. Version 1.2 of the crystallography and NMR system. *Nat. Protoc.* **2**: 2728–2733. DOI:10.1038/nprot.2007.406.

CCP4, Collaborative Computational Project Number 4. 1994. The CCP4 suite: programs for protein crystallography. *Acta Cryst.* **D50**: 760–763. DOI:10.1107/S0907444994003112.

Cho Y, Batt CA, Sawyer L. 1994. Probing the retinol-binding site of bovine beta-lactoglobulin. *J. Biol. Chem.* **269**: 11102–11107.

Collini M, D'Alfonso L, Molinari H, Ragona L, Catalano M, Baldini G. 2003. Competitive binding of fatty acids and the fluorescent probe 1-8-anilinonaphthalene sulfonate to bovine beta-lactoglobulin. *Protein Sci.* **12**: 1596–1603. DOI:10.1110/ps.0304403.

Dasgupta S, Bhattacharyya DK. 2009. Dietary effect of capric acid containing soyphospholipids. *J. Oleo Sci.* **58**: 395–401.

DeLano WL. 2002. The PyMOL Molecular Graphics System on World Wide Web. http://www.pymol.org.

Dew N, Bramer T, Edsman K. 2008. Catanionic aggregates formed from drugs and lauric or capric acids enable prolonged release from gels. *J. Colloid Interf. Sci.* **323**: 386–394. DOI:10.1016/j.jcis.2008.04.008.

Dichtelmüller H, Rudnick D, Kloft M. 2002. Inactivation of lipid enveloped viruses by octanoic acid treatment of immunoglobulin solution. *Biologicals* **30**: 135–142. DOI:10.1006/biol.2002.0332.

Dong SY, Zhao ZW, Ma HM. 2006. Characterization of local polarity and hydrophobic binding sites of beta-lactoglobulin by using N-terminal specific fluorescence labeling. *J. Proteome Res.* **5**: 26–31. DOI:10.1021/pr0501968.

Emsley P, Cowtan K. 2004. Coot: model-building tools for molecular graphics. *Acta Cryst.* **D60**: 2126–2132. DOI:10.1107/S0907444904023510.

Evans P. 2006. Scaling and assessment of data quality. *Acta Cryst.* **D62**: 72–82. DOI:10.1107/S0907444905036693.

Farrell HM, Jr., Jimenez-Flores R, Bleck GT, Brown EM, Butler JE, Creamer LK, Hicks CL, Hollar CM, Ng-Kwai-Hang KF, Swaisgood HE. 2004. Nomenclature of the proteins of cows' milk—sixth revision. *J. Dairy Sci.* **87**: 1641–1674.

Frapin D, Dufour E, Haertle T. 1993. Probing the fatty acid binding site of beta-lactoglobulins. *J. Protein Chem.* **12**: 443–449. DOI:10.1007/BF01025044.

Godovac-Zimmermann J, Krause I, Baranyi M, Fischer-Frühholz S, Juszczak J, Erhardt G, Buchberger J, Klostermeyer H. 1996. Isolation and rapid sequence characterization of two novel bovine β-lactoglobulins I and J. *J. Protein Chem.* **15**: 743–750. DOI:10.1007/BF01887148.

Gottschalk M, Nilsson H, Roos H, Halle B. 2003. Protein self-association in solution: the bovine beta-lactoglobulin dimer and octamer. *Protein Sci.* **12**: 2404–2411. DOI:10.1110/ps.0305903.

Harnsilawat T, Pongsawatmanit R, McClements DJ. 2006. Characterization of β-lactoglobulin–sodium alginate interactions in aqueous solutions: a calorimetry light scattering, electrophoretic mobility and solubility study. *Food Hydrocolloids* **20**: 577–585. DOI:10.1016/j.foodhyd.2005.05.005.

Hendlich M, Bergner A, Günther J, Klebe G. 2003. Relibase—design and development of a database for comprehensive analysis of protein–ligand interactions. *J. Mol. Biol.* **326**: 607–620. DOI:10.1016/S0022-2836(02)01408-0.

Hooft RW, Vriend G, Sander C, Abola EE. 1996. Errors in protein structures. *Nature* **381**: 272. DOI:10.1038/381272a0.

Jensen RG, Ferris AM, Lammi-Keefe CJ, Henderson RA. 1990. Lipids of bovine and human milks: a comparison. *J. Dairy Sci.* **73**: 223–240.

Kabsch W, Sander C. 1983. Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* **22**: 2577–2637. DOI:10.1002/bip.360221211.

Kontopidis G, Holt C, Sawyer L. 2002. The ligand-binding site of bovine beta-lactoglobulin: evidence for a function? *J. Mol. Biol.* **318**: 1043–1055. DOI:10.1016/S0022-2836(02)00017-7.

Kontopidis G, Holt C, Sawyer L. 2004. Invited review: β-lactoglobulin: binding properties, structure, and function. *J. Dairy Sci.* **87**: 785–796. DOI:10.3168/jds.S0022-0302(04)73222-1.

Konuma T, Sakurai K, Goto Y. 2007. Promiscuous binding of ligands by β-lactoglobulin involves hydrophobic interactions and plasticity. *J. Mol. Biol.* **20**: 209–218. DOI:10.1016/j.jmb.2007.01.077.

Kuzmic P. 1996. Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV proteinase. *Anal. Biochem.* **237**: 260–273. DOI:10.1006/abio. 1996. 0238.

Laskowski RA, MacArthur MW, Moss DS, Thornton JM. 1993. PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**: 283–291. DOI:10.1107/S0021889892009944.

Liavonchanka A, Hornung E, Feussner I, Rudolph MG. 2006. Structure and mechanism of the *Propionibacterium acnes* polyunsaturated fatty acid isomerase. *Proc. Natl. Acad. Sci.* **103**: 2576–2581. DOI:10.1073/pnas.0510144103.

MacGibbon AKH, Taylor MW. 2006. Composition and structure of bovine milk lipids. In Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition, Fox PF, McSweeney PLH (eds). Springer: New York; 1–42. DOI:10.1007/0-387-28813-9

Maher S, Leonard TW, Jacobsen J, Brayden DJ. 2009. Safety and efficacy of sodium caprate in promoting oral drug absorption: from in vitro to the clinic. *Adv. Drug Deliv. Rev.* **61**: 1427–1449. DOI:10.1016/j.addr.2009.09.006.

Månsson HL. 2008. Fatty acids in bovine milk fat food. *Nutr. Res.* **52**: 1–3. DOI:10.3402/fnr.v52i0.1821.

Mohammadi F, Bordbar AK, Divsalar A, Mohammadi K, Saboury AA. 2009. Interaction of curcumin and diacetylcurcumin with the lipocalin member beta-lactoglobulin. *Protein J.* **28**: 117–123. DOI:10.1007/s10930-009-9171-6.

Muresan S, van der Bent A, de Wolf FA. 2001. Interaction of beta-lactoglobulin with small hydrophobic ligands as monitored by fluorometry and equilibrium dialysis: nonlinear quenching effects

related to protein–protein association. *J. Agric. Food Chem.* **49**: 2609–2618. DOI:10.1021/jf0012188.
Nair MK, Joy J, Vasudevan P, Hinckley L, Hoagland TA, Venkitanarayanan KS. 2005. Antibacterial effect of caprylic acid and monocaprylin on major bacterial mastitis pathogens. *J. Dairy Sci.* **88**: 3488–3495. DOI:10.3168/jds.S0022-0302(05)73033-2.
Nan J, Zhou YF, Yang C, Brostromer E, Kristensen O, Su X-D. 2009. Structure of a fatty-acid-binding protein from *Bacillus subtilis* determined by sulfur–SAD phasing using in-house chromium radiation. *Acta Cryst.* **D65**: 440–448. DOI:10.1107/S0907444909007756.
Narayan M, Berliner LJ. 1997. Fatty acids and retinoids bind independently and simultaneously to beta-lactoglobulin. *Biochemistry* **36**: 1906–1911. DOI:10.1021/bi9621526.
Narayan M, Berliner LJ. 1998. Mapping fatty acid binding to beta-lactoglobulin: ligand binding is restricted by modification of Cys 121. *Protein Sci.* **7**: 150–157. DOI:10.1002/pro.5560070116.
O'Neill TE, Kinsella JE. 1987. Binding of alkanone flavors to β-lactoglobulin: effects of conformational and chemical modification. *J. Agric. Food Chem.* **35**: 770–774.
Oliveira KMG, Valente-Mesquita VL, Botelho MM, Sawyer L, Ferreira ST, Polikarpov I. 2001. Crystal structures of bovine β-lactoglobulin in the orthorhombic space group $C222_1$. *Eur. J. Biochem.* **268**: 477–484. DOI:10.1046/j. 1432-1033. 2001. 0 1918. x.
Pelletier E, Sostmann K, Guichard E. 1998. Measurement of interactions between β-lactoglobulin and flavor compounds (esters, acids, and pyrazines) by affinity and exclusion size chromatography. *J. Agric. Food Chem.* **46**: 1506–1509. DOI:10.1021/jf970725v.
Pérez MD, Calvo M. 1995. Interaction of β-lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: a review. *J. Dairy Sci.* **78**: 978–988.
Perez MD, Sanchez L, Aranda P, Ena JM, Oria R, Calvo M. 1992. Effect of beta-lactoglobulin on the activity of pregastric lipase. A possible role for this protein in ruminant milk. *Biochim. Biophys. Acta* **1123**: 151–155. DOI:10.1016/0005-2760(92)90105-5.
Qin BY, Bewley MC, Creamer LK, Baker HM, Baker EN, Jameson GB. 1998. Structural basis of the tanford transition of bovine β-lactoglobulin. *Biochemistry* **37**: 14014–14023. DOI:10.1021/bi981016t.
Qin BY, Bewley MC, Creamer LK, Baker EN, Jameson GB. 1999. Functional implications of structural differences between variants A and B of bovine beta-lactoglobulin. *Protein Sci.* **8**: 75–83. DOI:10.1110/ps.8.1.75.
Qin[1] BY, Creamer LK, Baker EN, Jameson GB. 1998. 12-Bromododecanoic acid binds inside the calyx of bovine beta-lactoglobulin. *FEBS Lett.* **438**: 272–278. DOI:10.1016/S0014-5793(98)01199-5.
Ragona L, Fogolari F, Zetta L, Pérez DM, Puyol P, De Kruif K, Löhr F, Rüterjans H, Molinari H. 2000. Bovine beta-lactoglobulin: interaction studies with palmitic acid. *Protein Sci.* **9**: 1347–1356. DOI:10.1110/ps.9.7.1347.
Sakurai K, Goto Y. 2002. Manipulating monomer-dimer equilibrium of bovine Beta-lactoglobulin by amino acid substitution. *J Biol Chem.* **277**: 25735–25740. DOI: 10.1074/jbc.M203659200
Sakurai K, Konuma T, Yagi M, Goto Y. 2009. Structural dynamics and folding of beta-lactoglobulin probed by heteronuclear NMR. *Biochim. Biophys. Acta* **1790**: 527–537. DOI:10.1016/j.bbagen.2009.04.003.
Sawyer L, Kontopidis G. 2000. The core lipocalin, bovine beta-lactoglobulin. *Biochim. Biophys. Acta* **1482**: 136–148. DOI:10.1016/S0167-4838(00)00160-6.
Sawyer L, Brownolow S, Polikarpov I, Wu SY. 1998. Beta-lactoglobulin: structural studies, biological clues. *Int. Dairy J.* **8**: 65–72. DOI:10.1016/S0958-6946(98)00021-1.
Sawyer L, Kontopidis G, Wu SY. 1999. β-lactoglobulin—a three-dimensional perspective. *Int. J. Food Sci. Technol.* **34**: 409–418. DOI:10.1046/j. 1365-2621. 1999. 00320.x.
Skerra A. 2000. Lipocalins as a scaffold. *Biochim. Biophys. Acta* **1482**: 337–350. DOI:10.1016/S0167-4838(00)00145-X.
Skerra A. 2001. 'Anticalins': a new class of engineered ligand-binding proteins with antibody-like properties. *J. Biotechnol.* **74**: 257–275.
Skerra A. 2008. Alternative binding proteins: anticalins—harnessing the structural plasticity of the lipocalin ligand pocket to engineer novel binding activities. *FEBS J.* **275**: 2677–2683. DOI:10.1111/j. 1742-4658. 2008.06439.x.
Skrivanová E, Molatová Z, Marounek M. 2008. Effects of caprylic acid and triacylglycerols of both caprylic and capric acid in rabbits experimentally infected with enteropathogenic *Escherichia coli* O103. *Vet. Microbiol.* **126**: 372–376. DOI:10.1016/j.vetmic.2007.07.010.
Spector AA, Fletcher JE. 1970. Binding of long chain fatty acids to beta-lactoglobulin. *Lipids* **5**: (1970) 403–411. DOI:10.1007/BF02532106.
Tanford C, Bunville LG, Nozaki Y. 1959. The reversible transformation of β-lactoglobulin at pH 7.51. *J. Am. Chem. Soc.* **81**: 4032–4036. DOI:10.1021/ja01524a054.
Taylor R. 2002. Life science applications of the Cambridge structural database. *Acta Cryst.* **D58**: 879–888. DOI:10.1107/S090744490200358X.
Vagin AA, Isupov MN. 2001. Spherically averaged phased translation function and its application to the search for molecules and fragments in electron-density maps. *Acta Cryst.* **D57**: 1451–1456. DOI:10.1107/S0907444901012409.
Vagin AA, Steiner RA, Lebedev AA, Potterton L, McNicholas S, Long F, Murshudov GN. 2004. REFMAC5 dictionary: organization of prior chemical knowledge and guidelines for its use. *Acta Cryst.* **D60**: 2184–2195. DOI:10.1107/S0907444904023510.
Viegas CA, Sá-Correia I. 1995. Toxicity of octanoic acid in *Saccharomyces cerevisiae* at temperatures between 8.5 and 30°C. *Enzyme Microb. Technol.* **17**: 826–831. DOI:10.1016/0141-0229(94)00111-4.
Wallace AC, Laskowski RA, Thornton JM. 1995. LIGPLOT: a program to generate schematic diagrams of protein–ligand interactions. *Protein Eng.* **8**: 127–134.
Wang Q, Allen JC, Swaisgood HE. 1997. Binding of vitamin D and cholesterol to beta-lactoglobulin. *J. Dairy Sci.* **80**: 1054–1059.
Wang Q, Allen JC, Swaisgood HE. 1998. Protein concentration dependence of palmitate binding to β-lactoglobulin. *J. Dairy Sci.* **81**: 76–81. DOI:10.3168/jds.S0022-0302(98)75553-5.
White RP, Ricca GF, el-Bauomy AM, Robertson JT. 1991. Identification of capric acid as a potent vasorelaxant of human basilar arteries. *Stroke* **22**: 469–476.
Wishnia A, Pinder TW, Jr. 1966. Hydrophobic interactions in proteins. The alkane binding site of beta-lactoglobulins A and B. *Biochemistry* **5**: 1534–1542.
Wright CS, Mi LZ, Lee S, Rastinejad F. 2005. Crystal structure analysis of phosphatidylcholine-GM2-activator product complexes: evidence for hydrolase activity. *Biochemistry* **44**: 13510–13521. DOI:10.1021/bi050668w.
Wu SY, Pérez MD, Puyol P, Sawyer L. 1999. Beta-lactoglobulin binds palmitate within its central cavity. *J. Biol. Chem.* **274**: 170–174. DOI:10.1074/jbc.274.1.170.
Yang MC, Guan HH, Liu MY, Lin YH, Yang JM, Chen WL, Chen CJ, Mao SJ. 2008. Crystal structure of a secondary vitamin D3 binding site of milk beta-lactoglobulin. *Proteins* **71**: 1197–1210. DOI:10.1002/prot.21811.
Yang MC, Guan HH, Liu MY, Lin YH, Yang JM, Chen WL, Chen CJ, Mao SJ. 2008. Crystal structure of a secondary vitamin D3 binding site of milk beta-lactoglobulin. *Proteins* **71**: 1197–1210. DOI:10.1002/prot.21811.
Zimet P, Livney YD. 2009. Beta-lactoglobulin and its nanocomplexes with pectin as vehicles for ω-3 polyunsaturated fatty acids. *J. Agric. Food Chem.* **49**: 2609–2618. DOI:10.1016/j.foodhyd.2008.10.008.
Zsila F, Hazai E, Sawyer L. 2005. Binding of the pepper alkaloid piperine to bovine beta-lactoglobulin: circular dichroism spectroscopy and molecular modeling study. *J. Agric. Food Chem.* **53**: 10179–10185.

