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# Structure, Oligomerisation and Interactions of $\beta$ -Lactoglobulin

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Additional information is available at the end of the chapter

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## Abstract

$\beta$ -Lactoglobulin ( $\beta$ Lg), as the most abundant whey protein in ruminant milk and as a useful model protein, is the subject of countless biophysical studies in the literature, yet its physiological role is hitherto unknown. This chapter deals with studies that focus on the structure of  $\beta$ Lg, its oligomeric behaviour and the interactions that this protein participates in. These and further studies are necessary to understand how the protein's physicochemical properties may influence the processing, digestion and immunogenicity of ruminant milks and their products. However, there is also a need for research into the interactions that occur naturally between  $\beta$ Lg and other components in milk, as this may give us insight into the physiological role of the protein.

**Keywords:**  $\beta$ -lactoglobulin, milk protein, lipocalin, oligomeric state, structure, Tanford transition

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## 1. Introduction

$\beta$ -Lactoglobulin ( $\beta$ Lg) is found in the milk of many mammals and, although it is the most abundant protein present in the whey fraction of ruminant milk [1], it is completely absent in human milk. Due to its abundance and relative ease of purification, bovine  $\beta$ Lg has served as a model protein for countless biophysical studies of folding, stability and self-association. Although this has created an extensive literature on the nature of  $\beta$ Lg, its physiological function is yet to be determined.

$\beta$ Lg belongs to the lipocalin family of proteins, most of which have roles that involve ligand-binding [2]. Its ability to bind hydrophobic molecules *in vitro* has prompted speculation that  $\beta$ Lg is involved in the transport of insoluble and/or chemically sensitive molecules between mother and offspring. However, it is necessary to draw the distinction between demonstrating binding *in vitro* and identifying an endogenous ligand that translates to a physiological role of  $\beta$ Lg *in vivo*.

Understanding the behaviour of this protein is of particular interest to the dairy industry, given the potential of  $\beta$ Lg to affect the processing and manufacture of milk products; for example,  $\beta$ Lg aggregation upon heat treatment is known to contribute to the fouling of heat exchangers during the processing of milk [3].  $\beta$ Lg has also been identified as one of the main immunogenic proteins in cow milk and thus contributes to cow milk allergies [4]. It is, therefore, of value to understand this protein's physicochemical properties and how they may influence the processing, digestion and immunogenicity of ruminant milk and their products.

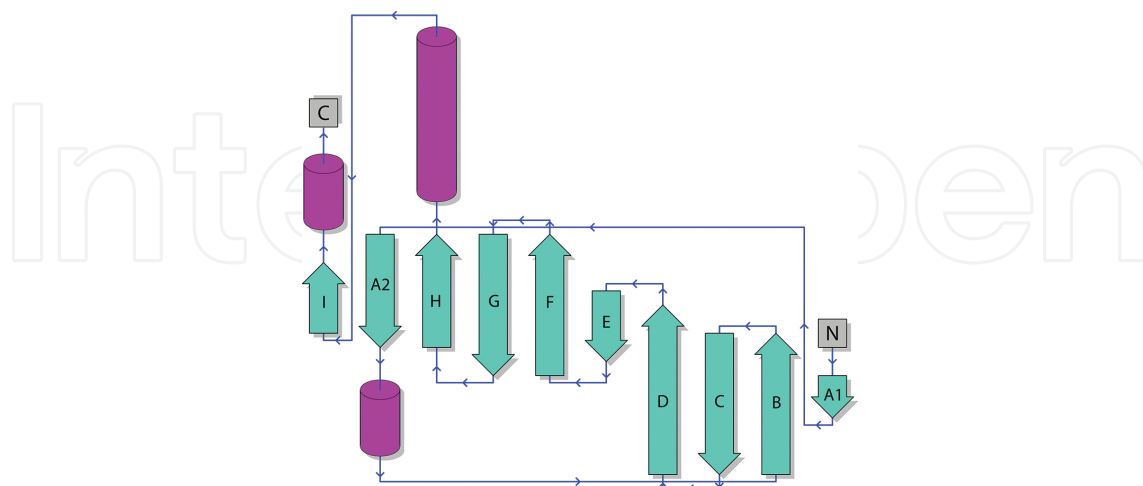
The purpose of this chapter is to review the knowledge that has been gathered for a range of  $\beta$ Lg orthologues from various species with regard to structure, oligomerisation and interaction behaviour under predominantly physiological conditions and to consider the current gaps in our knowledge. The thermal denaturation behaviour of  $\beta$ Lg, including heat-induced interactions and fibril formation, has been dealt with in detail elsewhere [5].

## 2. Structure of $\beta$ Lg

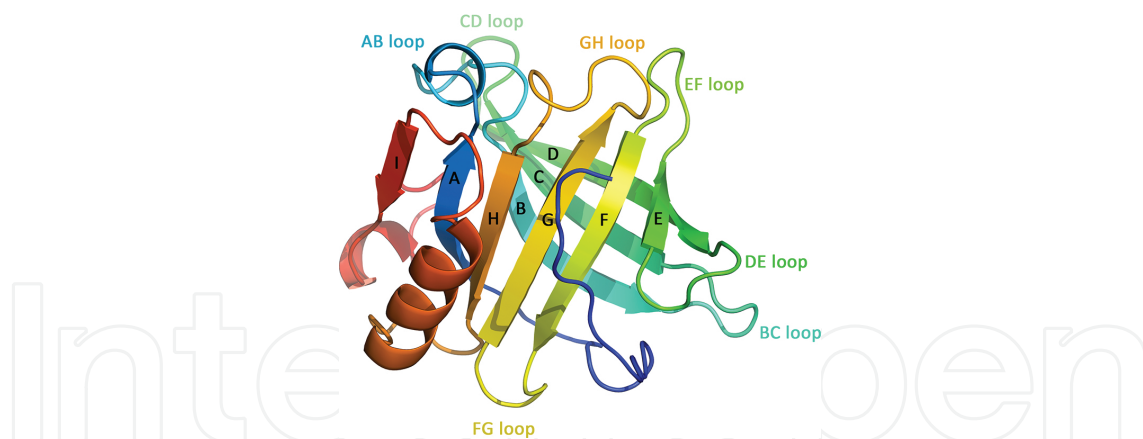
The first reported atomic level resolution structure of  $\beta$ Lg, solved by X-ray crystallography for bovine  $\beta$ Lg [6], showed remarkable similarity to retinol-binding protein and led to the classification of  $\beta$ Lg as a lipocalin. Lipocalins are a family of proteins that share a similar structure despite great diversity at the sequence level. The conserved lipocalin fold comprises an eight-stranded anti-parallel  $\beta$ -sheet (strands A–H) that is folded back upon itself to enclose an internal cavity, often termed a calyx, together with a three-turn  $\alpha$ -helix calyx (cup) handle that lies approximately above strand H (**Figure 1**) [7]. This fold allows lipocalins to bind a wide range of hydrophobic ligands, with the cavity size and loop scaffold at the cavity entrance determining selectivity. While they were once simply classified as transport proteins, lipocalins are now known to exhibit vast functional diversity, yet most involve some form of ligand binding [2].

Numerous high-resolution atomic structures now exist for bovine  $\beta$ Lg, along with structures of orthologues from sheep, goat and reindeer and the more distantly related pig. Like other lipocalins, bovine  $\beta$ Lg is a small protein, in this case of 162 amino acids with a monomeric mass of ~18,300 Da. As shown in a three-dimensional cartoon form in **Figure 2**, each subunit consists predominantly of an antiparallel  $\beta$ -sheet formed by eight  $\beta$ -strands, A–H, wrapped around to form a flattened calyx [9]. The calyx is flanked on its outer surface by a three-turn alpha helix. The dimer interface, at least for  $\beta$ Lg from ruminants, is formed by the ninth  $\beta$ -strand (I) along with the loop connecting strands A and B. The loops at the closed end of the

calyx (BC, DE and FG) are quite short, whereas those at the open end (AB, CD, EF and GH) are longer and more flexible [10].



**Figure 1.** Topology diagram showing characteristic features of lipocalin proteins. The eight  $\beta$ -strands A–H form the antiparallel  $\beta$ -barrel. Strand A is kinked (giving rise to strands A1 and A2) and connects the  $\beta$ -sheet comprising strands A1, B, C and D to the  $\beta$ -sheet comprising strands E, F, G, H and A2. The commonly occurring N-terminal  $3_{10}$  helix and the ubiquitous 3-turn  $\alpha$ -helix following strand H are shown. Generally, a ninth  $\beta$ -strand follows the 3-turn  $\alpha$ -helix and is packed against strand A2. Figure generated by PDBsum [8] using the structure of bovine  $\beta$ Lg, PDB ID: 1BSO.



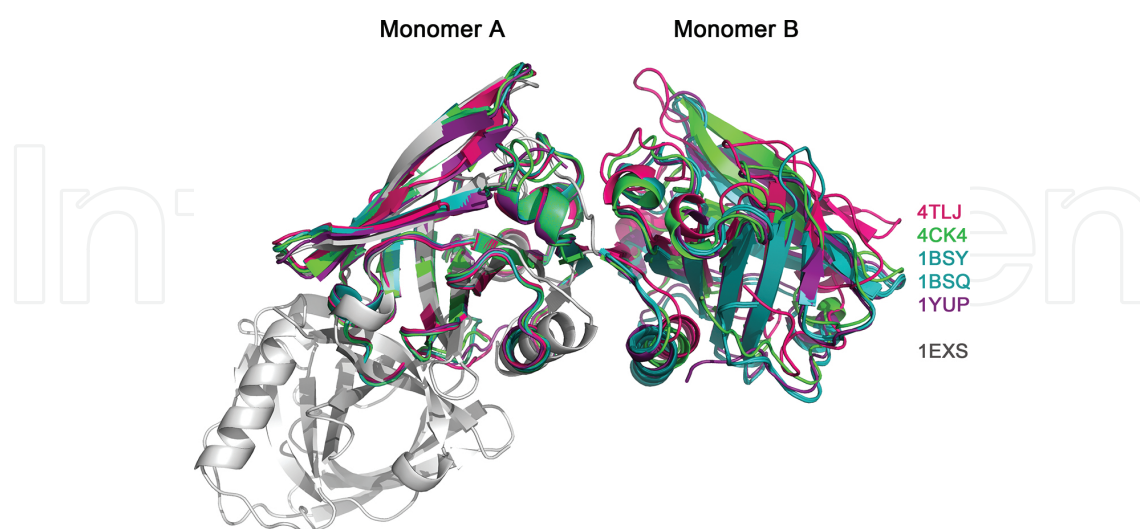
**Figure 2.** Crystal structure of one monomer of bovine  $\beta$ Lg (PDB ID: 1BSO). Eight  $\beta$ -strands (A–H) form the central antiparallel  $\beta$ -sheet calyx. The calyx is flanked on its outer surface by a three-turn  $\alpha$ -helix. The ninth  $\beta$ -strand, I, and the AB loop are involved in dimer formation. The polypeptide chain is shown in rainbow colours, beginning in blue at the N-terminus and ending in red at the C-terminus.

Each monomer of  $\beta$ Lg contains five cysteine residues. One exists as a free thiol on strand G and is buried beneath the  $\alpha$ -helix that lies alongside the  $\beta$ -barrel, whereas the other four form two disulfide bridges. The first (Cys66–Cys160) links the C-terminus to the CD loop, while the second (Cys106–Cys119) links  $\beta$ -strands G and H [11]. The presence and correct arrangement of these disulfide bonds are crucial for the correct folding of  $\beta$ Lg. Recombinant expression of

this protein in a soluble form in bacteria requires the simultaneous coexpression of a disulfide bond isomerase along with the use of modified *Escherichia coli* Origami (DE3) cells [12]. These cells, which carry mutations in the thioredoxin reductase and glutathione reductase genes, provide an oxidising environment and, together with the disulfide bond isomerase, allow the proper formation of disulfide bonds in the cytoplasm [13].

At least eleven protein sequence variants of bovine  $\beta$ Lg have been described, with A and B the most common variants [14]. Variant B differs from A by two amino acid substitutions: Asp64Gly in the mobile surface loop (CD) and Val118Ala in the hydrophobic core [15]. Crystal structures of both variants A and B at pH 7.1 have allowed observation of the structural consequences of these sequence differences [11]. Only minor differences can be seen in the calyx, while small changes occur in the main chain conformations in the vicinity of the Asp64Gly mutation. The substitution of Val118 for Ala causes changes in the local structure creating a void volume that weakens several hydrophobic interactions. This may be responsible for the slight decrease in thermal stability of variant B relative to variant A [11]. The conformation of the EF loop is slightly different, but this may be due to differences in crystallisation or imprecision in definition due to the high mobility of this loop.

Crystal structures of ovine (sheep) [16, 17], caprine (goat) [18, 19] and reindeer  $\beta$ Lg [20] indicate that these orthologues share a high degree of structural similarity with bovine  $\beta$ Lg, at both the tertiary and quaternary level (**Figure 3**), with minimal root-mean-square deviations when aligning the C- $\alpha$  atoms of these structures (**Table 1**). However, there are significant differences between the structures of these orthologues and that of porcine  $\beta$ Lg, which in the crystal structure features a completely different quaternary association [21]. This is not unreasonable considering the lower level of sequence identity (63–65%) between porcine  $\beta$ Lg and bovine, ovine, caprine and reindeer  $\beta$ Lg, where the latter four share sequence identity in pairwise comparisons of 93–99% (**Table 1**).



**Figure 3.** Crystal structure of caprine  $\beta$ Lg (PDB: 4TLJ, pink) overlaid with bovine  $\beta$ Lg structures (PDB: 1BSY, 1BSQ, blue), ovine  $\beta$ Lg (PDB: 4CK4, green), reindeer  $\beta$ Lg (PDB: 1YUP, purple) and porcine  $\beta$ Lg (PDB: 1EXS, grey). Monomer A of each structure is superposed to highlight the variation in orientation of the second monomer.

	Bovine A	Bovine B	Ovine	Caprine	Reindeer	Porcine
<b>Bovine A</b>						
% sequence identity	100	98.77	95.06	95.06	93.21	65.00
rmsd monomer	-	0.208	0.463	0.397	0.429	1.849
rmsd dimer	-	0.221	1.023	1.400	0.569	19.36
<b>Bovine B</b>						
% sequence identity	98.77	100	96.30	96.30	94.44	63.75
rmsd monomer	0.208	-	0.409	0.386	0.394	1.792
rmsd dimer	0.221	-	0.964	1.318	0.496	19.13
<b>Ovine</b>						
% sequence identity	95.06	96.30	100	99.38	93.21	62.50
rmsd monomer	0.463	0.409	-	0.324	0.310	1.920
rmsd dimer	1.023	0.964	-	1.076	0.739	19.49
<b>Caprine</b>						
% sequence identity	95.06	96.30	99.38	100	93.21	62.50
rmsd monomer	0.397	0.386	0.324	-	0.404	1.398
rmsd dimer	1.400	1.318	1.076	-	1.242	19.69
<b>Reindeer</b>						
% sequence identity	93.21	94.44	93.21	93.21	100	65.00
rmsd monomer	0.429	0.394	0.310	0.404	-	1.723
rmsd dimer	0.569	0.496	0.739	1.242	-	19.06
<b>Porcine</b>						
% sequence identity	65.00	63.75	62.50	62.50	65.00	100
rmsd monomer	1.849	1.792	1.920	1.398	1.723	-
rmsd dimer	19.36	19.13	19.49	19.69	19.06	-

Source: PDB IDs for structures used for alignment, performed in PyMol: Bovine A: 1BSY, Bovine B: 1BSQ, Ovine: 4CK4, Caprine: 4TLJ, Reindeer: 1YUP and Porcine: 1EXS.

**Table 1.** The percentage sequence identity of  $\beta$ Lg orthologue protein sequences, the rmsd when aligning the C $\alpha$  atoms of monomers and the rmsd when aligning the C $\alpha$  atoms of dimers.

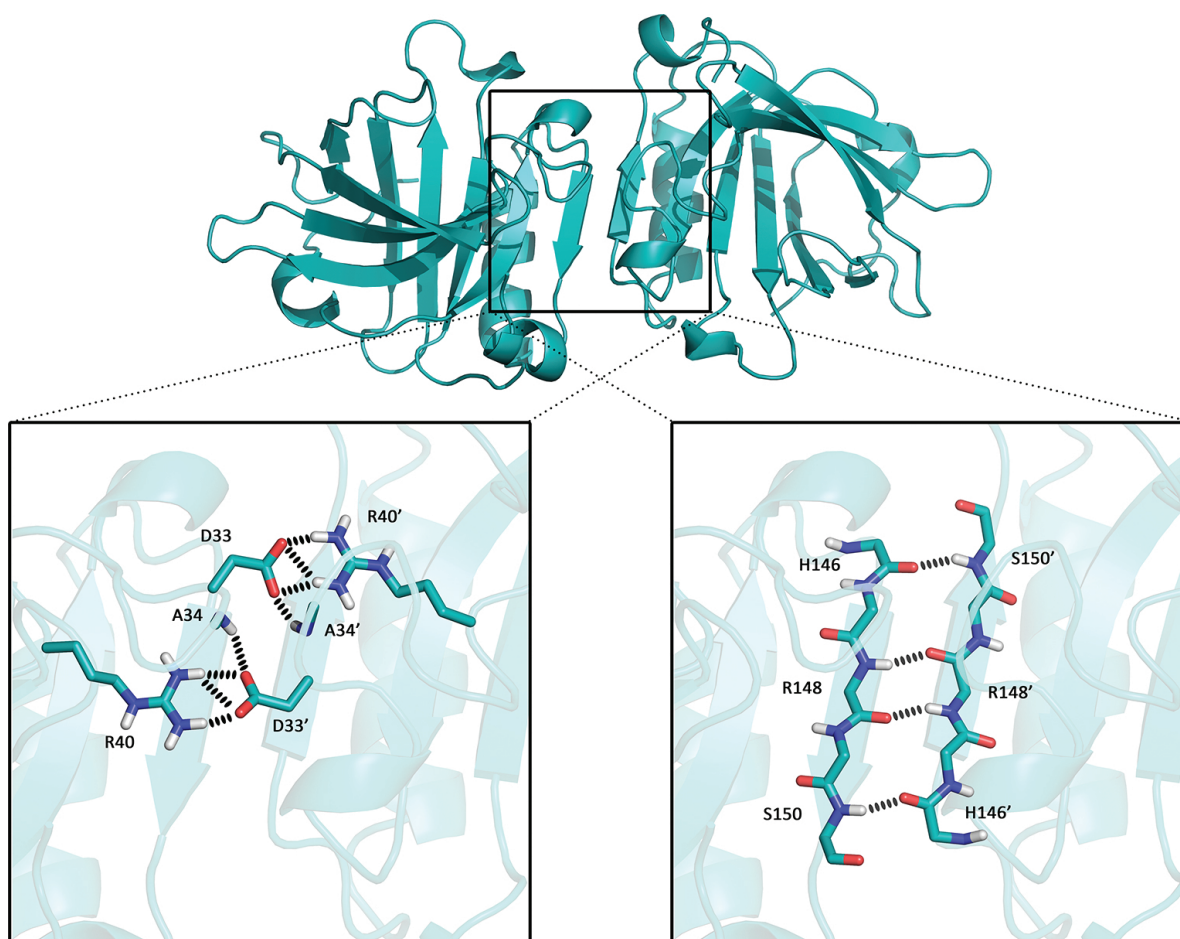
Although there is a high level of structural similarity among the bovine, caprine, ovine and reindeer orthologues, there are subtle differences between them. When dimers are selected for alignment the root-mean-square deviations for the superposition of these structures are higher than when a single monomer is used for the alignment (**Table 1**). This indicates that while the tertiary fold of these orthologues is similar, there is flexibility in the orientation of the monomers relative to each other (**Figure 3**), with different crystal forms, including those of bovine  $\beta$ Lg, sampling different conformations.

The ultrahigh resolution crystal structures of caprine and ovine  $\beta$ Lg [17, 18] make it possible to clearly define features that in lower resolution bovine and reindeer  $\beta$ Lg structures are



obscured by disorder and conformational promiscuity. These features include the long flexible CD and GH loops, the C-terminal region, and the AB loops at the dimer interface. However, the more mobile regions of bovine and reindeer  $\beta$ Lg structure loops are also the more mobile regions of the caprine and ovine  $\beta$ Lg structures. The dimer interface in these ruminant  $\beta$ Lg structures is formed by the antiparallel association of the I  $\beta$ -strands and electrostatic interactions between Asp33 and Arg40 residues located within the AB loop of each monomer (**Figure 4**). Hydrogen bonding between the main chain of Ala34 and the side chains of the Asp/Arg pair holds this residue (Ala34) in an unfavourable conformation. The only other residue found in a less favourable region of the Ramachandran plot is the highly conserved Tyr99, which forms part of a  $\gamma$ -turn [22].

Bovine  $\beta$ Lg undergoes several conformational changes with pH. Several studies have used nuclear magnetic resonance (NMR) spectroscopy to determine the three-dimensional structure of bovine  $\beta$ Lg at pH 2.6 [23–25], where at very low ionic strength the protein becomes monomeric (see the next section for more details on the oligomeric behaviour of  $\beta$ Lg). While monomer–dimer exchange makes it difficult to obtain NMR structures at neutral pH [26], this



**Figure 4.** Dimer interface of bovine  $\beta$ Lg [PDB ID: 1BEB]. Close-up views of AB-loops and I-strands are shown where inter subunit hydrogen bonds and electrostatic interactions between side chains and main chains can be seen.

low pH NMR structure can be compared to crystal structures of bovine  $\beta$ Lg solved at higher pH values. The structure of this low pH form is very similar to a subunit of the dimer at pH 6.2 [23]. There are slight deviations in the orientation of the loops and of the three-turn helix flanking the calyx, but overall the tertiary fold and, in particular, the hydrophobic cavity are well maintained at low pH.

Bovine  $\beta$ Lg undergoes another reversible pH-induced conformational change, termed the Tanford transition [26, 27], at close to physiological pH that may be of functional significance. This transition involves movement of the EF loop, which is located at the mouth of the calyx. The EF loop is in a closed conformation at pH  $\sim$ 7.1 and below [11], burying Glu89 as glutamic acid, whereas it adopts an open conformation at pH values above 7, exposing Glu89 as a glutamate. The structures of ovine, caprine and reindeer  $\beta$ Lg solved at pH 6.5–7, 6.8 and 6.9, respectively, all show the EF loop in the closed position, in agreement with bovine structures below pH 7. A recent structure of caprine  $\beta$ Lg [19] shows an asymmetric dimer with the EF loop of one subunit in the closed position and the other in the open position, suggesting that goat  $\beta$ Lg also undergoes the Tanford transition. It is possible that the Tanford transition plays a role in regulating the ligand-binding properties of  $\beta$ Lg.

### 3. Oligomerisation

The oligomerisation of  $\beta$ Lg has been studied intensively using various techniques including analytical ultracentrifugation, isothermal titration calorimetry and small angle X-ray scattering [18, 28–31]. Utilising both sedimentation velocity and sedimentation equilibrium analytical ultracentrifugation, Mercadante et al. [31] investigated the oligomerisation of bovine  $\beta$ Lg over a pH range of 2.5–7.5. Interestingly, at pH 2.5, 3.5, 6.5 and 7.5, the weight-averaged sedimentation coefficient increased with increasing protein concentration, suggesting a monomer–dimer equilibrium. However, at pH 4.5 and 5.5, the weight-averaged sedimentation coefficient stayed the same, consistent with a single species (a dimer) predominating across the concentrations used in the experiment. This suggests that the dimer is more strongly associated at pH 4.5 and 5.5, values which lie near the isoelectric point of the protein, than at pH 2.5, 3.5, 6.5 and 7.5.

Mercadante et al. [31] went on to characterise the binding energy of dimer formation as a function of ionic strength. They demonstrated that an increase in ionic strength strongly favours formation of the dimer. At low pH, dissociation of the dimer is extremely sensitive to ionic strength. This is due to the fact that at low pH, ionic strength stabilises the dimer by the association of anions near the dimer interface which mitigate charge repulsion of the positively charged subunits. On the other hand, at neutral pH, a relatively low density of cations in the region of the AB and GH loops can also help to stabilise the dimer, but the effect of ionic strength is less pronounced due to the smaller magnitude of the nominal charge on the protein ( $-9$  at pH  $\sim$ 7.5 and  $+20$  at pH  $\sim$ 2.5).

A recent paper [32], featuring the use of synchrotron FT-IR techniques to study the dimer–monomer equilibrium at pH  $\sim$ 7 of bovine  $\beta$ Lg at high salt and high protein concentrations, reported dimer dissociation constants orders of magnitude greater than the micro molar values



reported by a host of others (see Supplementary Table in reference [31]). However, inspection of the FT-IR data would support an interpretation that at the higher concentrations aggregation and denaturation of originally dimeric  $\beta$ Lg were occurring.

Importantly, the study by Mercadante et al. [31] indicates that under physiological conditions bovine  $\beta$ Lg self-associates into a dimer with a dissociation constant within the range of the concentrations studied (5–45  $\mu$ M). Bovine  $\beta$ Lg is, therefore, likely to be dimeric at the concentration and pH typically associated with milk ( $\sim 3 \text{ mg mL}^{-1}$  or  $\sim 165 \mu\text{M}$  and pH 6.5 [33]). A study of caprine  $\beta$ Lg suggests a comparable dissociation constant as for bovine  $\beta$ Lg under similar conditions [18]. Given the high level of sequence identity between caprine and ovine  $\beta$ Lg [17], it is likely that ovine  $\beta$ Lg exhibits similar oligomerisation behaviour. The nonruminant equine and porcine  $\beta$ Lg orthologues, however, are monomeric at physiological pH [21, 34]. In contrast to bovine  $\beta$ Lg, through a domain-swapping mechanism, porcine  $\beta$ Lg forms a dimer at low pH [21].

The dimer interface of bovine, caprine, ovine and reindeer  $\beta$ Lg orthologues is composed of an intermolecular  $\beta$ -sheet formed between the I-strands of each monomer along with electrostatic interactions and hydrogen bonds between residues located on the AB loops (see **Figure 4**). Sakurai and Goto [28] investigated the impact of these elements on the monomer–dimer equilibrium of bovine  $\beta$ Lg at neutral pH using analytical ultracentrifugation. They engineered mutants that either disrupted the intermolecular  $\beta$ -sheet or the electrostatic interactions between the AB loops. All of the mutants that introduced a proline residue within the I-strand led to dissociation of the dimer, due to disruption of the intermolecular  $\beta$ -sheet. Substitution of the Asp33 or Arg40 residues of the AB loop with oppositely charged residues was also unfavourable for dimer formation, due to the electrostatic repulsion introduced. When these charge mutants were mixed they were able to form heterodimers, suggesting that the electrostatic interactions between these aspartate and arginine residues contribute to stabilisation of the dimer.

Some orthologues, however, such as equine  $\beta$ Lg, exist as monomers at neutral pH despite the presence of the I-strands and AB loop residues [35]. Sakurai and Goto [28] created another set of bovine  $\beta$ Lg mutants in which they substituted the remaining residues at the dimer interface with those found in the monomeric equine and porcine  $\beta$ Lg sequences. These sequence-based mutations did not largely affect the association constant, indicating that dimer stabilisation cannot be ascribed simply to the interface residues of bovine  $\beta$ Lg that differ in the monomeric  $\beta$ Lg variants.

In a similar, but opposite, experiment Kobayashi et al. [36] aimed to convert the monomeric equine  $\beta$ Lg to a dimeric form by substituting I-strand and AB loop residues with those found in bovine  $\beta$ Lg. That is, Ser34 and Glu35 in the AB loop of equine  $\beta$ Lg were replaced with Ala and Gln, and the sequence comprising the I-strand was replaced with the corresponding bovine  $\beta$ Lg sequence. Interestingly these mutants did not form a dimer, further suggesting that the difference in oligomerisation behaviour between the bovine and equine orthologues cannot be explained simply by the sequence differences at the dimer interface. They hypothesised that structural differences must exist between equine and bovine  $\beta$ Lg that prevent the same interactions occurring at the dimer interface.

To assess this, Ohtomo et al. [35] constructed a chimera named Gyuba, which means cow and horse in Japanese. Gyuba was made by joining the secondary structural elements of bovine  $\beta$ Lg with the loops of equine  $\beta$ Lg. The chimera was able to form dimers, and its crystal structure showed that it had a very similar dimer interface as seen for bovine  $\beta$ Lg (PDB ID: 3KZA). Taken together, these studies suggest that the entire arrangement of the secondary structural elements and loops of  $\beta$ Lg, including hydrophobic interactions, hydrogen bonds between I-strands, and electrostatic interactions and hydrogen bonds at the AB loops, is necessary for dimerisation. Further, it is tempting to speculate that protein dynamics may also play a role in dimer formation.

## 4. Interactions

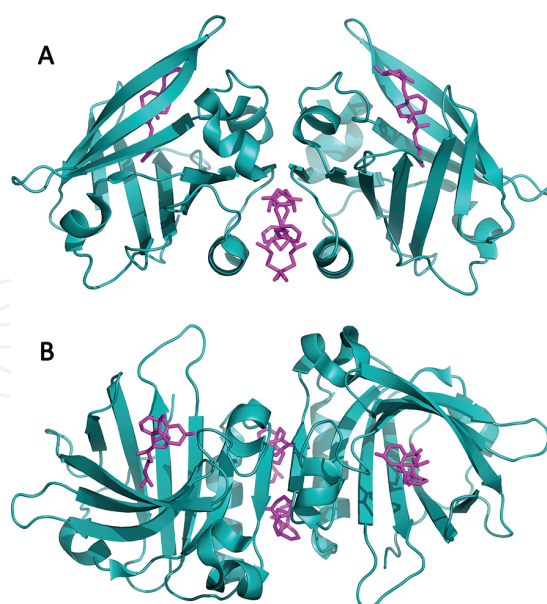
Due to its similarity to retinol-binding protein, the ability of  $\beta$ Lg to bind retinol was examined by fluorescence spectroscopy [34]. The fact that retinol was able to bind to  $\beta$ Lg, and that it could be modelled into the crystal structure of  $\beta$ Lg in a similar position as seen bound in retinol-binding protein, led to speculation that the biological function of  $\beta$ Lg is to transport vitamin A in milk [6]. However, since then bovine  $\beta$ Lg has been shown to be capable of binding a range of small hydrophobic molecules, as demonstrated in various ligand-bound crystal structures. These bound ligands include vitamin D [33, 37], vitamin A [38], cholesterol [33], a range of fatty acids [39–43] and the fatty-acid derivative 12-bromododecanoic acid [9], as well as more diverse molecules including SDS [44] and various anaesthetic drugs [45]. Ligand binding has also been investigated by a variety of other methods, including equilibrium dialysis, affinity chromatography, electron spin resonance spectroscopy, spectrophotometry and perturbation of intrinsic tryptophan fluorescence [33, 46]. Provided that there is a chromophore on the ligand, induced circular dichroism provides, along with X-ray crystallography, the most definitive method for characterising the binding of ligands. These studies have revealed a broad range of ligands that  $\beta$ Lg is capable of binding to.

Ligand-bound crystal structures serve as definitive proof that small hydrophobic ligands are accommodated within the hydrophobic calyx of  $\beta$ Lg. Ligand binding, therefore, is dependent on the opening of the EF loop at the mouth of the calyx which, for bovine  $\beta$ Lg, occurs near pH 7 as Glu89 becomes deprotonated and is exposed as a glutamate. The pKa of Glu89 in porcine  $\beta$ Lg is higher than in bovine  $\beta$ Lg (calculated 9.7 compared to observed 7.3 (see reference [11] and references therein to Tanford's original work)), and thus porcine  $\beta$ Lg is only able to bind fatty acids above pH 8.6 [47]. In ligand-bound structures the density is often quite poor for the extremity of the ligand and can be ambiguous as to which orientation the molecule faces. However, most can be interpreted by taking into account their chemistry, for instance cholesterol and vitamin D were built into their electron density placing their hydroxyl groups facing out of the calyx as opposed to being buried in the centre of the protein [33]. Qin et al. [9] used a fatty acid derivative, 12-bromododecanoic acid, to unequivocally determine the orientation of the ligand in the binding site, with the carboxylate head group lying at the surface of the molecule.

The lining of the hydrophobic cavity is exclusively hydrophobic, except for two lysine residues (Lys60 and Lys69) at the entrance to the calyx. It is generally agreed that there are two main interactions between  $\beta$ Lg and fatty acid ligands; one is the hydrophobic interaction between the hydrocarbon tail of the fatty acid and the interior of the hydrophobic calyx, and the other is the electrostatic attraction between the carboxyl group of the fatty acid and the amines of Lys60 and Lys69. The strength of the interaction between  $\beta$ Lg and fatty acids generally increases as the length of the hydrocarbon chain increases, due to an increase in van der Waals' forces. There is, however, an exception to this rule; the eight-carbon caprylic acid has a greater binding affinity than the ten-carbon capric acid [43]. Until recently, there was no satisfactory explanation for this result. Yi and Wambo [48] have used molecular dynamics simulations to accurately compute the binding free energies between  $\beta$ Lg and five saturated fatty acids of 8 to 16 carbon atoms. Their results agree well with experimental results; the binding free energy increases with the number of carbon atoms of the fatty acid, with the exception of caprylic acid, which has a higher binding free energy than the 2 carbon longer capric acid. The van der Waals' forces between the fatty acid tails and the interior of the  $\beta$ Lg calyx increase as the chain length increases; however, for caprylic acid the electrostatic interaction between the carboxyl group and the amines of Lys60 and Lys69 is stronger than these van der Waals' forces which pulls the caprylic acid closer to the top of the barrel. This allows the hydrophobic tail of caprylic acid to fluctuate more easily, increasing the entropy of this complex, resulting in a greater overall binding free energy.

A small number of studies suggests there may be a second, lower affinity, external binding site for hydrophobic molecules [37, 49, 50]. The lower affinity of this binding site may explain the difficulty in obtaining crystallographic evidence of this interaction. However, a crystal structure was recently solved of bovine  $\beta$ Lg that identifies two molecules of vitamin D<sub>3</sub> bound, one bound within the calyx and the second possibly bound at an exosite between the  $\beta$ -barrel and the  $\alpha$ -helix that lies alongside the barrel (**Figure 5**) [37]. The free thiol of  $\beta$ Lg (Cys121) is buried beneath this  $\alpha$ -helix and methylation of this thiol appears to reduce the affinity for palmitic acid compared to native  $\beta$ Lg, whereas the binding of retinol is not affected [49]. A second binding site may explain how  $\beta$ Lg is capable of binding such a wide diversity of shapes of ligands, yet more evidence, such as more convincing electron density, is required before the existence of this site can be conclusively proven.

A definitive role for  $\beta$ Lg is yet to be ascribed, although several predictions have been made. Most suggestions are for a role in molecular transport between mother and offspring, due to its demonstrated ability to bind a range of ligands. However, the specific identity of the ligand being transported is not clear. Fatty acids have been seen bound to  $\beta$ Lg isolated from milk under nondenaturing conditions, but are present in quantities reflecting the fatty acid composition of milk [51]. The apparent lack of selectivity makes it less likely that  $\beta$ Lg is a specific fatty acid or vitamin transporter.  $\beta$ Lg may still be involved in fat metabolism; there is evidence that  $\beta$ Lg can stimulate a pregastric lipase, potentially by binding the fatty acid products and thereby reducing their inhibitory effect on the enzyme [52].



**Figure 5.** Front view (A) and top view (B) of the crystal structure of bovine  $\beta$ Lg showing two molecules of vitamin D<sub>3</sub> (magenta) bound, indicating the locations of the putative primary ligand-binding site within the central hydrophobic calyx and the second proposed exosite between the  $\beta$ -barrel and the  $\alpha$ -helix that lies alongside the barrel. (PDB ID: 2GJ5 [37]).

The closest homologue to  $\beta$ Lg in humans is glycodelin (pregnancy protein 14). Inter alia, glycodelin has an immunosuppressive activity in the uterus and is involved in protecting products of the reproductive organs from the immune system [53]. Unlike  $\beta$ Lg, glycodelin is a glycoprotein, a property essential for its function. It has been hypothesised that  $\beta$ Lg may have diverged from glycodelin following a gene duplication event and has since lost all glycodelin-related function [33].  $\beta$ Lg may now exist primarily as an important source of amino acids for the offspring of the animals that produce it. However, the resistance of this protein to proteolysis by pepsin [54] along with the high level of sequence conservation seen among  $\beta$ Lg orthologues, including the highly conserved Glu89 within the EF loop, argue against a simple nutritive function.

Another enticing proposal is the notion that  $\beta$ Lg may possess antimicrobial activity [55–57]. The intact protein appears to be capable of inhibiting the growth of *Staphylococcus aureus*, *Streptococcus uberis* and *E. coli* bacteria largely responsible for the prevalent and costly disease, bovine mastitis [55].  $\beta$ Lg also appears to augment the antimicrobial activity of lactoferrin, a protein with a known role in the defence against mammary gland infections [56]. Pan et al. [58], however, reported a lack of antimicrobial activity of native  $\beta$ Lg, yet this may be due to the use of a commercial preparation of  $\beta$ Lg, as compared to the mild, nondenaturing isolation from milk employed in the aforementioned studies. Pellegrini et al. [57] have also described the antimicrobial activity of four peptides derived from  $\beta$ Lg following trypsin digestion. This may point to a protective physiological role in new born calves, perhaps in addition to a protective role in the secretory tissue of the mother. Further studies are needed to provide a comprehensive understanding of the relevance of these findings and to address the mechanisms underlying these antimicrobial observations.



Upon heating,  $\beta$ Lg (both bovine and caprine) associates with casein micelles through formation of a  $\beta$ Lg/casein complex [59], with covalent intermolecular disulfide bonds forming above 75 °C [60]. The pH at which the milk is heated is important in determining how much complex is formed as well as the extent of dissociation of casein from the micelles, which is implicated in the heat stability of the milk [59, 60]. Further studies such as these are necessary for understanding how the processing of both bovine and nonbovine milk may affect the properties of milk constituents and how these may then influence the digestion and immunogenicity of ruminant milk and their products.

Much research has been devoted to understanding the functionality of  $\beta$ Lg in milk. There remain large gaps in our knowledge of the interactions of proteins in milk under physiological conditions. There is, therefore, a critical need for research into the interactions that occur between  $\beta$ Lg and protein components (other than caseins) in milk under physiological/untreated conditions, as this may finally give us insight into the actual physiological role of this protein and to identify factors that distinguish human neonate responses to milk products sourced from different ruminants. In this regard, the structure of bovine  $\beta$ Lg with human immunoglobulin fragments is highly significant [61].

## 5. Conclusion

$\beta$ Lg has served as the focus of an extensive range of studies for well over half a century, creating a wealth of knowledge about this enigmatic protein. We now have a clear view of the native structure of  $\beta$ Lg. This small globular protein is characterised by a central  $\beta$ -barrel composed of eight antiparallel  $\beta$ -strands, creating a calyx that is well suited to binding hydrophobic ligands. An  $\alpha$ -helix lies alongside the barrel potentially creating a second, lower-affinity, binding site for ligands. A ninth  $\beta$ -strand, along with the loop connecting the A and B strands, forms the dimer interface of ruminant  $\beta$ Lg.

Understanding the structure of  $\beta$ Lg has given considerable insight into its behaviour in solution. Under physiological conditions, ruminant  $\beta$ Lg orthologues are predominantly dimeric. The dimer interface involves 12 intermolecular hydrogen bonds and 2 ion pairs, interactions that have been shown to be critical for dimer stability. At low pH the protein is positively charged and thus under low-salt conditions it is monomeric. Increasing the ionic strength screens these electrostatic repulsions and stabilises dimer formation.

Many questions regarding  $\beta$ Lg remain and, in particular, the physiological function of the protein is still a mystery. The proven ability of  $\beta$ Lg to bind hydrophobic molecules along with its stability at low pH, and resistance to proteolytic enzymes are strongly suggestive of a role in fatty acid transport between mother and child. Alternatively, these qualities may enable  $\beta$ Lg to enhance milk fat metabolism through the promotion of pregastric lipase activity. However, the absence of  $\beta$ Lg in other species, most notably human, needs to be remembered when considering the role of  $\beta$ Lg.

The physicochemical properties of bovine  $\beta$ Lg will, undoubtedly, continue to be investigated. What is required now is a detailed understanding of these properties in closely related



orthologues, in order to understand the underlying processes occurring during the processing and digestion of different ruminant milks. There is also a significant need for exploration into the interactions with  $\beta$ Lg that are occurring naturally in milk. This may provide the necessary insight into the function of this protein that is of physiological significance to the mother and/or her offspring, and into the functionality of this protein in milk products from different ruminants destined for human consumption.

## Author details

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