Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

A novel pH-dependent dimerization motif in β-lactoglobulin from pig (*Sus scrofa*)

Flip J. Hoedemaeker,[a,b]* Ronald W. Visschers,[c] Arno C. Alting,[c] Kees G. de Kruif,[c] Maxim E. Kuil[a] and Jan Pieter Abrahams[a]

[a]Department of Biophysical and Structural Chemistry, Leiden Institute for Chemistry, Leiden University, Einsteinweg 55, PO Box 9502, 2300 RA Leiden, The Netherlands, [b]Key Drug Prototyping BV, Einsteinweg 55, PO Box 9502, 2300 RA Leiden, The Netherlands, and [c]NIZO Food Research and Wageningen Centre for Food Sciences, Kernhemseweg 2, PO Box 20, 6710 BA Ede, The Netherlands

Correspondence e-mail: hoedemae@chem.leidenuniv.nl

Received 18 October 2001
Accepted 11 January 2002

PDB Reference: porcine β-lactoglobulin, 1exs, r1exssf.

β-Lactoglobulin (BLG) is a lipocalin and is the major protein in the whey of the milk of cows and other ruminants, but not in all mammalian species. The biological function of BLG is not clear, but a potential role in carrying fatty acids through the digestive tract has been proposed. The capability of BLG to aggregate and form gels is often used to thicken foodstuffs. The structure of the porcine form is sufficiently different from other known BLG structures that SIRAS phases had to be measured in order to solve the crystal structure to 2.4 Å resolution. The r.m.s. deviation of C^{α} atoms is 2.8 Å between porcine and bovine BLG. Nevertheless, the typical lipocalin fold is conserved. Compared with bovine BLG, the tilted α-helix alters the arrangement of surface residues of the porcine form, completely changing the dimerization behaviour. Through a unique pH-dependent domain-swapping mechanism involving the first ten residues, a novel dimer interface is formed at the N-terminus of porcine BLG. The existence of this novel dimer at low pH is supported by gel-filtration experiments. These results provide a rationale for the difference in physicochemical behaviour between bovine and porcine BLG and point the way towards engineering such dimerization motifs into other members of the lipocalin family.

1. Introduction

β-Lactoglobulin (BLG) is a major whey protein in the milk of many mammalian species (Flower *et al.*, 2000; Sawyer & Kontopidis, 2000). In the food industry, bovine BLG is used as a thickening agent because of its excellent gelation properties (Renard & Lefebvre, 1992). BLG belongs to the large and diverse lipocalin family, which has representatives in higher organisms and prokaryotes (Ganfornina *et al.*, 2000). Lipocalins have an eight-stranded antiparallel β-barrel that forms around a central cavity (the calyx), a short 3_{10}-helix near the N-terminus at the 'closed end' of the barrel, a single peripheral α-helix and one or two disulfide bridges. Lipocalins can occur as monomers or dimers; sometimes the dimers are formed by a domain-swapping mechanism (Spinelli *et al.*, 1998). The lipocalins are in turn related to two other structural superfamilies, the fatty-acid-binding proteins (FABPs) and the avidin family (Flower *et al.*, 2000). Lipocalins are mostly known for their potential to bind small hydrophobic compounds within their central cavity. Some members of this family show other traits, such as the ability to bind to cell-surface receptors or to form complexes with other soluble proteins (Flower *et al.*, 2000). The binding pocket of the related bilin-binding protein (BBP) from *Pieris brassicae* was successfully re-engineered to bind fluorescein, demonstrating the versatility of the lipocalin fold in ligand recognition (Beste *et al.*, 1999).

1.1. Ligand binding

The main function of BLG seems to be nutritional since it occurs at relatively high concentrations in milk. However, like other lipocalins, some BLGs can bind hydrophobic ligands like retinol and fatty acids *in vitro* (Cho *et al.*, 1994; Katakura *et al.*, 1994; Lange *et al.*, 1998; Glasgow *et al.*, 1999). Bovine BLG can be isolated from milk with bound fatty acids (Perez *et al.*, 1989) and has been cocrystallized with palmitic acid bound tightly in the central cavity of the calyx (Wu *et al.*, 1999). Other binding sites that are located more on the outside of the protein have also been suggested on the basis of binding studies (Lange *et al.*, 1998; Narayan & Berliner, 1996). Furthermore, bovine BLG is relatively stable at acidic pH and resists pepsin hydrolysis, suggesting it may pass through the stomach in an intact form. However, BLGs from different species differ in their ability to bind lipids (Perez *et al.*, 1993), challenging a general conclusion that these proteins have a possible transport role.

1.2. Enzymatic activity

In general, lipocalins do not exhibit enzymatic activity, but plant lipocalins are known to catalyse the interconversion of carotenoids (Hieber *et al.*, 2000) and bovine BLG has been shown to have a weak non-specific Mg^{2+}-dependent endonuclease activity (Yusifov *et al.*, 2000). It remains unclear if the latter activity is relevant for the function of BLGs in milk. The main residue implicated in the nuclease activity is Glu134, which is pointing outwards from the only helix in the structure. Judging from the amino-acid homology, the nuclease activity may well also be present in porcine BLG.

1.3. Lipocalin dimerization motifs

In a recent review, it was pointed out that lipocalins can occur in many oligomeric states (Flower *et al.*, 2000) and that lipocalins can dimerize in a number of different ways. Lipocalin dimerization motifs can also involve domain swapping: most notable is the example of bovine odorant-binding protein, where the complete C-termini including the helices are swapped (see Fig. 4*a*; Tegoni *et al.*, 1996). Bovine BLG, which has 66% sequence identity to porcine BLG, also forms dimers. Both in the triclinic (PDB entry 1beb; Brownlow *et al.*, 1997) and in the trigonal crystals (PDB entry 1b0o; Wu *et al.*, 1999), the β-I strands of each monomer join to form a β-sheet and both *AB* loops form a second interaction.

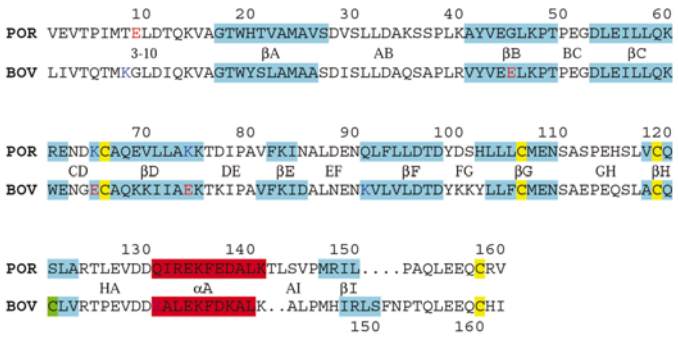

Figure 1
Structural alignment of porcine and bovine BLG. Secondary-structure elements are highlighted and numbered. Cysteines involved in disulfide bridges are highlighted in yellow; the single free cysteine in bovine BLG is indicated in green.

1.4. Tanford transition

It has long been recognized that bovine BLG undergoes a conformational change around pH 7.0. This phenomenon is known as the Tanford transition, based on the work of Tanford *et al.* (1959). Structural analysis by Qin *et al.* (1998) shows that this transition mainly involves loop *EF* (residues 85–90). Below pH 7.0 this loop forms a lid closing the calyx. Recent results indicate that the environment of Tyr42 also changes significantly during this transition (Oliveira *et al.*, 2001). It is possible that the binding properties of BLG are regulated in this fashion. It is not known at present whether this transition also occurs in porcine BLG. Similar titration experiments with porcine BLG did not yield conclusive results (Hambling *et al.*, 1992).

1.5. Comparison between bovine and porcine BLG

Porcine BLG is found in the milk of pigs in two isoforms: A and C. These isoforms differ in amino-acid sequence at two positions: Glu27 and Gln86 in isoform A are changed to Asp and His, respectively, in isoform C (Kessler & Brew, 1970; Bell *et al.*, 1981). Despite the fact that porcine BLG shares about 66% amino-acid identity with bovine BLG (see Fig. 1; Conti *et al.*, 1984), it has distinct properties. Firstly, porcine BLG does not form gels upon aggregation as does bovine BLG (Roefs & de Kruif, 1994). This difference has been attributed to the lack of a free sulfhydryl group in porcine BLG. The free cysteine at position 121 in bovine BLG is replaced by a serine residue in porcine BLG (Burova *et al.*, 1998). Secondly, whereas binding of fatty acids *in vivo* and *in vitro* can be demonstrated readily for bovine BLG, porcine BLG does not seem to be able to bind hydrophobic ligands at all (Perez *et al.*, 1993). Finally, under ambient conditions (above pH 3.5) bovine BLG exists mainly as a dimer; below pH 3.5 bovine BLG is found to be monomeric only (Fogolari *et al.*, 1998; Verheul *et al.*, 1999; Uhrínová *et al.*, 2000). In contrast, porcine BLG has been reported to be monomeric at neutral pH (Kessler & Brew, 1970).

2. Experimental procedures

2.1. Protein purification

Porcine BLG was purified using a large-scale purification method described elsewhere (Ugolini *et al.*, 2001). Briefly, 3 l of porcine milk were collected from different sows. The milk was defatted by a mild heat treatment and centrifugation. Subsequently, the defatted milk was brought to pH 4.6 and the precipitated caseins were removed by centrifugation. Cation-exchange chromatography on a 400 ml S-Sepharose FF column (Pharmacia Biotech, Sweden) was used to purify the porcine BLG from the remaining whey. BLG was eluted as a single peak. Protein purity was checked by mass spectrometry,

revealing the presence of variants A (70%) and C (30%), and gel electrophoresis. The overall purity exceeds 95%. The preparation yielded a total of 3.3 g protein. Purified BLG was lyophilized prior to crystallization experiments.

2.2. Crystallization and data collection

The best crystals were obtained from microbatch solutions (1 µl) containing 12.5 mg ml^{-1} protein, 1.3 *M* NaCl and 100 m*M* formate buffer pH 3.2 at 293 K under paraffin oil. Crystals typically appeared within 24 h and grew to maximum dimensions of 0.4 × 0.4 × 0.4 mm. Crystals were soaked for at least 48 h in a solution containing 1.5 *M* NaCl, 100 m*M* formate buffer pH 3.2, 30% glycerol before data collection. To obtain a lead derivative, a crystal was soaked for 10 d in a solution containing 10 m*M* lead (II) acetate, 1.5 *M* NaCl, 100 m*M* formate buffer pH 3.2, 30% glycerol.

Data were collected on an FR591 rotating-anode generator (Enraf–Nonius, Delft, The Netherlands) equipped with a MAR345 image-plate detector (MAR Research, Norderstedt, Germany) using Cu $K\alpha$ radiation at 100 K.

2.3. Data refinement and model building

Data were indexed with *MOSFLM* (Leslie, 1992) and scaled with *SCALA* (Evans, 1997). An initial lead site was found with a Patterson search with *SHELXL* (Sheldrick & Schneider, 1997) as implemented in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). This site was refined with *SHARP* (de La Fortelle *et al.*, 1997), after which a second minor lead site was found. After density modification with *SOLOMON* (Abrahams, 1997), assuming a solvent content of 68%, 145 of 160 residues could be traced in the electron density. Finally, the structure was refined with *REFMAC* and *ARP*/*wARP* (Murshudov *et al.*, 1997; Perrakis *et al.*, 1999), using *O* (Jones *et al.*, 1991) for model building.

Surface-area calculations were obtained from the PQS server (http://pqs.ebi.ac.uk) and verified with the program *SURFACE* (Collaborative Computational Project, Number 4, 1994).

2.4. Gel-filtration analyses

For the analysis of the aggregation state of the porcine and bovine BLG, protein solutions of 1 mg ml^{-1} in 50 m*M* phosphate buffer pH 3, pH 5 or pH 7 were prepared and stirred for 45 min or 3 h prior to analysis. For the analysis, 160 µl of sample was applied to a TSK450 column (Tosoh-Haas). The eluate was monitored in a SEC-MALLS (size-exclusion chromatography multi-angle laser light scattering) detector that determines molecular weight and radius on the basis of light-scattering intensities (Wyatt, 1993).

3. Results

3.1. X-ray diffraction

Porcine BLG crystallizes readily, but only under acidic conditions (pH 3.0–4.0, formate buffer) and with sodium chloride as precipitant. The best crystals diffract to up to 2.3 Å resolution on a rotating anode. The space group of the crystals as deduced from the diffraction pattern was $P3_221$ or $P3_121$. The overall *B* factor derived from the Wilson plot was rather high (55 Å^2).

3.2. Structure determination

All attempts to solve the structure with molecular replacement using existing models of bovine BLG were unsuccessful, even though the two proteins show very high homology (66% amino-acid identity; see Fig. 1). Even brute-force molecular replacement/rigid-body refinement (Hoedemaeker *et al.*, 1999), previously successful in a very difficult case, did not produce a solution. We eventually solved the phases using SIRAS (single isomorphous replacement with anomalous scattering) with a single lead (II) acetate derivative, even though the lead absorption edges are not near the Cu $K\alpha$ radiation wavelength. Apparently, the anomalous signal of Pb at 1.54 Å (f'' = 8.505) is sufficient for SIRAS phasing. Using *SHELXL* (Sheldrick & Schneider, 1997), we found one Pb atom in the asymmetric unit (ASU) (occupancy 0.4). Upon refining this lead site with *SHARP* (de La Fortelle *et al.*, 1997), a second (minor) site became apparent. Subsequent density modification with *SOLOMON* (Abrahams, 1997), assuming a solvent content of 68% (one monomer per ASU), solved the phase ambiguity and produced a high-quality electron-density map in space group $P3_221$ in which virtually the whole backbone could be traced. The model was refined to an *R* value of 21.8% ($R_{\rm free}$ = 28.2%), the final model contains 1248 protein atoms (160 residues), 154 water molecules, one glycerol molecule and one sodium ion. The rationale behind this presumed sodium ion lies in the coordination geometry and the reasonable *B* value on the introduction of the sodium scattering factor. The final solvent content is 69.8%.

3.3. The structure of porcine BLG

As expected, porcine BLG adopts a typical lipocalin fold with a central eight-stranded antiparallel β-barrel (βA-H), a single α-helix and an extra C-terminal β-strand (βI). The central cavity, which in other lipocalins is shown to be a binding site for small hydrophobic ligands, is empty. The *EF* loop, which rearranges in the Tanford transition, is in the closed conformation covering the entrance to this cavity (see Fig. 2). No density was found for the residues corresponding to the C variant; therefore, the model is based upon the A variant (Kessler & Brew, 1970).

3.4. The dimer interface

In the crystal structure, every BLG monomer makes contacts with three neighbouring molecules. The major interaction is formed by a reciprocal insertion of the N-terminal residues (1–12) of two monomers. The N-termini are essentially swapped between two monomers, tightly linking the monomers and burying 1758 Å^2 of accessible surface area per monomer (over 19% of the total accessible surface area per

Table 1
Reciprocal contacts in the dimer interface.

First monomer	Second monomer	Distance (Å)
2 O	12 N	2.99
4 O	10 N	2.53
6 O	8 N	2.79
7 O	96 N	2.80
9 $O^{\varepsilon 2}$	142 $O^{\gamma 1}$	2.86
12 $O^{\gamma 1}$	1 O	3.16

monomer). Apart from main-chain hydrogen bonds, one (reciprocal) side-chain interaction is present, namely between Glu9 of one monomer and Thr142 of the other (see Table 1).

3.5. Comparison with the bovine structure

We measured the aggregation state of porcine BLG using SEC-MALLS gel-filtration analysis at various pH values. The use of size-exclusion chromatography coupled with multi-angle light scattering makes it possible to obtain a much more reliable estimate of the molecular mass (distribution) of proteins and protein complexes under a large variety of solvent conditions, without resorting to 'universal' calibrations that may not be applicable to the system under study. The results are listed in Table 2. Porcine BLG has an apparent molecular weight of approximately 19 kDa, corresponding to the monomer, at pH 5.0 and 7.0, but is dimeric (MW = 31 kDa) at pH 3.0. In contrast to porcine BLG, the bovine form is monomeric at pH 3.0, but mostly dimeric at pH 5.0 and 7.0. The dimer interface of bovine BLG, as observed in the triclinic lattice (lattice X, PDB entry 1beb; Brownlow *et al.*, 1997) consists mainly of interactions in the C-terminal ends of the monomers. The βI strands of each monomer join to form a β-sheet and both *AB* loops form a second interaction. In this interface, 572 Å^2 per monomer is buried. Essentially the same dimer interface is observed in trigonal (lattice Z) crystals, where the interface lies on the crystallographic twofold axis (484.1 Å^2; PDB entry 1b0o; Wu *et al.*, 1999). This interface is not present in crystals of porcine BLG. Instead, the βI strands form hydrogen bonds with a network of surrounding waters and the side chain of Arg133.

Table 2
Gel-filtration analyses of porcine and bovine BLG.

pH	3.0	5.0	7.0
Porcine BLG (kDa)	31	19	19
Bovine BLG (kDa)	23	34	33

4. Discussion

4.1. Porcine BLG does not bind hydrophobic ligands

Porcine BLG was isolated and purified from pig's milk using a relatively mild extraction procedure. Nevertheless, no electron density is found for any kind of naturally bound ligand. The *EF* loop, which functions as a lid on the entry to the central calyx, is in the closed position. The *EF* loop is responsible for the Tanford transition in bovine BLG, where it changes to the open position above pH 7.5 (Tanford *et al.*, 1959; Qin *et al.*, 1998). Glu89, the major residue involved in the Tanford transition, has virtually the same environment as in the corresponding bovine structures, but the reversible pH-dependent change in conformation of this loop has not been demonstrated (Hambling *et al.*, 1992). It remains to be demonstrated whether opening of the loop is necessary for binding of hydrophobic compounds such as fatty acids in the central calyx. If this is the case, then bovine BLG would only bind ligands at neutral or higher pH. The putative role of BLGs as carriers of hydrophobic ligands is still debatable. The observation that bovine BLG is resistant to proteolysis by pepsin has played an important role in the hypothesis that BLGs can carry important lipids in milk through the stomach of the infant to the gut (Reddy *et al.*, 1988). The fact that porcine BLG has never been shown to bind any ligands (Perez *et al.*, 1993) and the absence of BLG in the milk of other mammals,

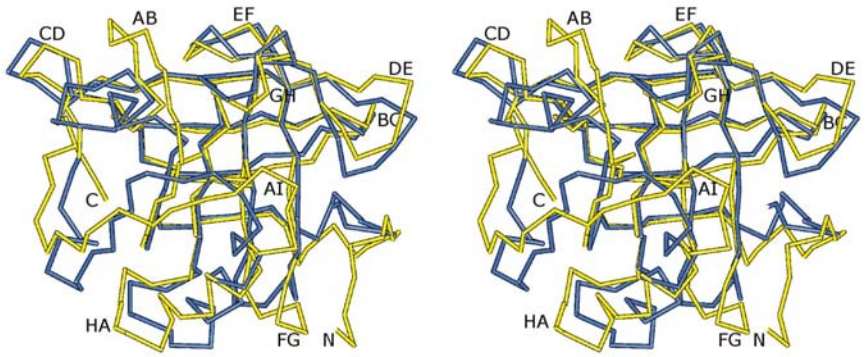

Figure 2
(Divergent) stereo line plot of porcine BLG (yellow) aligned with bovine BLG (1beb, blue). The loops are indicated according to the general lipocalin nomenclature (see also Fig. 1). This figure was produced with *SETOR* (Evans, 1993)

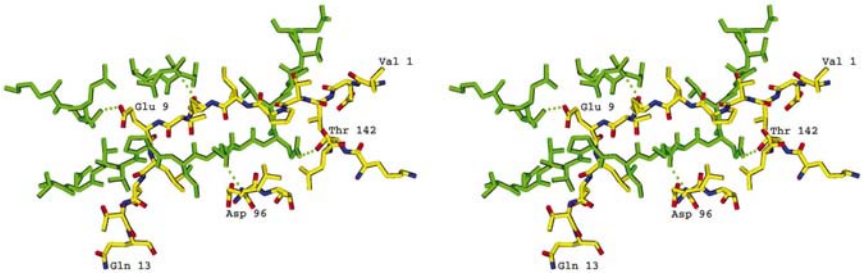

Figure 3
(Divergent) stereo image of the dimer interface. Residues forming side-chain interactions, as well as residues 1 and 13, are labelled. This figure was produced with *SETOR* (Evans, 1993)

such as humans (Brignon *et al.*, 1985), has cast further doubt on this hypothesis.

4.2. A novel dimerization motif in porcine BLG

In the porcine BLG dimer, the N-terminal strands of both monomers link both molecules like folding arms (see Fig. 3), which is unlike any other dimerization motifs found in lipocalins. The resulting dimer interface is completely different from that observed in bovine BLG (Fig. 4*f*; Qin *et al.*, 1998). In comparison with other lipocalin dimers (see Fig. 4) this interface buries a large amount of surface area, over 1700 Å^2 per monomer in comparison to 572 Å^2 in bovine BLG. In the monomeric form (at pH 5.0 and higher), porcine BLG is most likely to reduce its surface area through a rearrangement and internalization of its N-termini, comparable to bovine BLG. Proper understanding of the dimer formation of these whey proteins is essential for understanding their stability, aggregation and gelation properties.

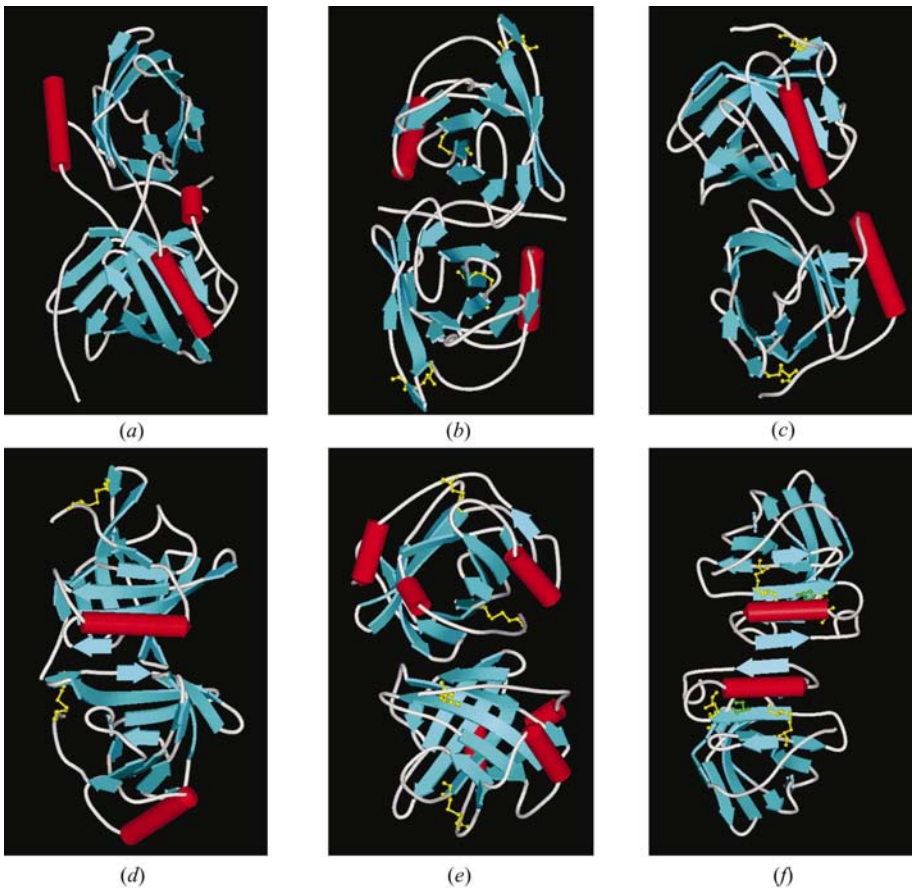

Figure 4
Different (putative) dimeric forms of lipocalins. Disulfide bridges are indicated in yellow; free cysteines are indicated in green. The dimers were automatically generated from the original PDB entries by the PQS server (http://pqs.ebi.ac.uk). (*a*) Bovine odorant-binding protein (1obp; Tegoni *et al.*, 1996), buried surface area 2399.7 Å^2. (*b*) Porcine BLG (1exs; this paper), buried surface area 1757.7 Å^2. (*c*) Major horse allergen (1ew3; Lascombe *et al.*, 2000), buried surface area 1023.3 Å^2. (*d*) Porcine odorant-binding protein (1e06; Spinelli *et al.*, 1998), buried surface area 848.2 Å^2. (*e*) Nitrophorin 4 (1eqd; Weichsel *et al.*, 2000), buried surface area 789.1 Å^2. (*f*) Bovine BLG (1b0o; Wu *et al.*, 1999), buried surface area 484.1 Å^2. The figure was produced with *Weblab Viewer Lite* (MSI).

4.3. Dimer formation of porcine BLG happens only at low pH

In contrast to bovine BLG, dimer formation in porcine BLG occurs at low pH: gel-filtration experiments confirm that porcine BLG is mainly dimeric at pH 3.0 and monomeric at pH 5.0 and pH 7.0, whilst bovine BLG is essentially monomeric at pH 3.0 but dimeric at higher pH. The dimerization of porcine BLG might be physiologically relevant, since acidic conditions prevail in the stomach. The interaction between Glu9 of one monomer and Thr142 of the other is interesting in this respect. At pH 3.2, these residues presumably form a hydrogen bond. An amino-acid alignment based on the structural superposition of porcine and bovine BLG (Fig. 1) reveals that this glutamate is absent in bovine BLG, where a lysine is present at position 8. This lysine, if present at this position in porcine BLG, would most probably prevent the N-terminal dimerization (see Fig. 3). Therefore, the formation of this particular dimer interface may be impossible in bovine BLG. The electrostatic potential of the residues involved also provides a rationale for the observed pH dependence of dimerization. Interestingly, Lys8 in bovine BLG is involved in a 'lock-and-key' crystal contact in lattice Z crystals (Qin *et al.*, 1998), but not in lattice X crystals (Brownlow *et al.*, 1997).

Likewise, the absence of a bovine-like C-terminal dimer interface in porcine BLG can be explained by sequence differences (see Fig. 1). Porcine BLG has an insertion of two amino acids directly after the α-helix and a deletion of four residues directly after βI. Because the disufide bond between residues 66 and 158 (porcine numbering) is conserved, the deletion in the porcine sequence relative to bovine BLG has to be accommodated by a conformational change. As a result, the α-helix of porcine BLG is rotated by approximately 25° relative to the β-barrel and βI shifts not only in space but also in the sequence, altering the C-terminal interface of porcine BLG.

4.4. Domain swapping and local unfolding

The propensity of BLGs to form dimers seems to be inversely related to protein stability (Burova *et al.*, manuscript in preparation). It is possible that local destabilization of the monomers could initiate dimer formation in some of these lipocalins and that the exact nature of the dimer formed is dependent on the local destabilization. This is an attractive hypothesis, since it would

Table 3
Data collection and refinement statistics.

Values in parentheses refer to the outermost resolution shells.

	Native data set	Pb derivative
Symmetry	$P3_221$	$P3_221$
Unit-cell parameters (Å, °)	$a = b = 80.6$, $c = 78.5$, $\gamma = 120$	$a = b = 80.8$, $c = 79.6$, $\gamma = 120$
Molecules per ASU	1	
Matthews coefficient (Å^3 Da^{-1})	4.04	
Solvent content (%)	69.8	
Resolution range (Å)	100–2.39 (2.44–2.39)	100–2.95 (3.03–2.95)
No. of measurements	124487 (8192)	70377 (5297)
No. of unique reflections	12225 (870)	6624 (486)
Completeness (%)	99.8 (97.3)	99.8 (99.8)
Multiplicity	10.2 (9.4)	10.6 (10.9)
R_{meas}†	0.052 (0.250)	0.054 (0.327)
$I/\sigma(I)$	9.5 (2.7)	10.8 (2.6)
Phasing power (*SHARP*)		
Isomorphous		1.27
Anomalous		1.95
Refinement statistics		
Resolution range (Å)	40–2.38 (2.51–2.38)	
R_{work}†	0.218 (0.258)	
Reflections used	11450 (1497)	
R_{free}†	0.282 (0.318)	
Reflections used	573 (83)	
ESU (free R) (Å)	0.241	
R.m.s. angles (°)	3.049	
R.m.s. bonds (Å)	0.013	
Average B (protein) (Å^2)‡	42	
Average B (waters) (Å^2)	58	

† The definition of R_{meas} is described by Evans (1997). The definitions of R_{work} (and R_{free}) are described by Murshudov *et al.* (1997). ‡ The average B value of protein atoms was calculated excluding poorly defined loops with B values over 100 Å^2.

account for our finding that the dimer interfaces occur at the terminal parts of the protein, where pH-induced local destabilization is most likely to occur. This seems to be a general feature of domain-swapped proteins, but is particularly true for lipocalins because of the high stability of the central calyx.

The overall B value of the structure is high, as was expected from the Wilson plot (see Table 3). Some of the loops have very high B values of more than 100 Å^2, indicating positional insecurity (loops *CD*, *FG* and part of the loop *AB* in particular). Most of the significant deviations of ideal bond angles occur in these loops, resulting in an overall r.m.s. deviation in bond angles of about 3° (see Table 3). Nevertheless, we decided to include these loops in the final model. These loops do not contain residues involved in the dimer interface. The observed high B values might in part be explained by pH-induced local instability and flexibility.

Dimerization by domain swapping under conditions favouring partial denaturation has been described in detail for RNase A recently (Liu *et al.*, 2001). RNase A is able to form both an N-terminally swapped and a C-terminally swapped dimer under similar conditions and the coexistence of both dimer forms can apparently lead to oligomer formation. Domain swapping has also been implicated in oligomer formation of human prion protein (Knaus *et al.*, 2001). This is probably not the case with porcine and bovine BLG, where only one dimer form is favoured based on small differences in sequence. Oligomer formation does not seem to occur for porcine BLG and gelation of bovine BLG is attributed to the free cysteine becoming available during (partial) denaturation. More studies will have to be performed in order to establish a clearer relationship between dimer formation and the stability of these lipocalins, especially in the light of the pH dependence.

The authors are indebted to E. Silletti and E. Floris for skilful assistance in the purification of porcine BLG and to Dr T. V. Burova for helpful discussions.

References

Abrahams, J. P. (1997). *Acta Cryst.* D**53**, 371–376.
Bell, K., McKensie, H. A. & Shaw, D. C. (1981). *Mol. Cell Biochem.* **35**, 103–111.
Beste, G., Schmidt, F. S., Stibora, T. & Skerra, A. (1999). *Proc. Natl Acad. Sci. USA*, **96**, 1898–1903.
Brignon, G., Chtourou, A. & Ribadeau-Dumas, B. (1985). *J. Dairy Res.* **52**, 249–254.
Brownlow, S., Morais Cabral, J. H., Cooper, R., Flower, D. R., Yewdall, S. J., Polikarpov, I., North, A. C. & Sawyer, L. (1997). *Structure*, **5**, 481–495.
Burova, T. V., Grinberg, V. Y., Tran, V. & Haertle, T. (1998). *Int. Dairy J.* **8**, 83–86.
Cho, Y., Batt, C. A. & Sawyer, L. (1994). *J. Biol. Chem.* **269**, 11102–11107.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Conti, A., Godovac-Zimmerman, J., Pirchner, F., Liberatori, L. & Braunitzer, G. (1984). *J. Biol. Chem. Hoppe Seyler*, **367**, 871–878.
Evans, P. R. (1997). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **33**, 22–24.
Evans, S. V. (1993). *J. Mol. Graph.* **11**, 134–138.
Flower, D. R., North, A. C. & Sansom, C. E. (2000). *Biochim. Biophys. Acta*, **1482**, 9–24.
Fogolari, F., Ragona, L., Zetta, L., Romagnoli, S., De Kruif, K. G. & Molinari, H. (1998). *FEBS Lett.* **436**, 149–154.
Ganfornina, M. D., Gutierrez, G., Bastiani, M. & Sanchez, D. (2000). *Mol. Biol. Evol.* **17**, 114–126.
Glasgow, B. J., Marshall, G., Gasymov, O. K., Abduragimov, A. R., Yusifov, T. N. & Knobler, C. M. (1999). *Invest. Ophthalmol. Vis. Sci.* **40**, 3100–3107.
Hambling, S. G., MacAlpine, A. S. & Sawyer, L. (1992). *Advanced Dairy Chemistry*, edited by P. F. Fox, pp. 141–190. Amsterdam: Elsevier.
Hieber, A. D., Bugos, R. C. & Yamamoto, H. Y. (2000). *Biochem. Biophys. Acta*, **1482**, 84–91.
Hoedemaeker, F. J., Siegal, G., Roe, S. M., Driscoll, P. C. & Abrahams, J. P. (1999). *J. Mol. Biol.* **292**, 763–770.
Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991). *Acta Cryst.* A**47**, 110–119.
Katakura, Y., Totsuka, M., Ametani, A. & Kaminogawa, S. (1994). *Biochim. Biophys. Acta*, **1207**, 58–67.
Kessler, E. & Brew, K. (1970). *Biochim. Biophys. Acta*, **200**, 449–458.
Knaus, K. J., Morillas, M., Swietnicki, W., Malone, M., Surewicz, W. K. & Yee, V. C. (2001). *Nature Struct. Biol.* **8**, 770–774.
La Fortelle, E. de, Irwin, J. J. & Bricogne, G. (1997). *Crystallographic Computing 7*, edited by P. E. Bourne & K. D. Watenpaugh. Oxford University Press.
Lange, D. C., Kothari, R., Patel, R. C. & Patel, S. C. (1998). *Biophys. Chem.* **74**, 45–51.
Lascombe, M. B., Gregoire, C., Poncet, P., Tavares, G. A., Rosinski-Chupin, I., Rabillon, J., Goubran-Botros, H., Mazie, J. C., David, B. & Alzari, P. M. (2000). *J. Biol. Chem.* **275**, 21572–21577.

Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EAMCB Newsl. Protein Crystallogr.* **26**.
Liu, Y., Gotte, G., Libonati, M. & Eisenberg, D. (2001). *Nature Struct. Biol.* **8**, 211–214.
Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). *Acta Cryst.* D**53**, 240–255.
Narayan, M. & Berliner, L. J. (1996). *Biochemistry*, **7**, 1906–1911.
Oliveira, K. M., Valente-Mesquita, V. L., Botelho, M. M., Sawyer, L. & Ferreira, S. T. (2001). *Eur. J. Biochem.* **268**, 477–483.
Perez, M. D., Diaz de Villegas, C., Sanchez, L., Aranda, P., Ena, J. M. & Calvo, M. (1989). *J. Biochem. (Tokyo)*, **106**, 1094–1097.
Perez, M. D., Puyol, P., Ena, J. M. & Calvo, M. (1993). *J. Dairy Res.* **60**, 55–63.
Perrakis, A., Morris, M. & Lamzin, V. S. (1999). *Nature Struct. Biol.* **6**, 458–463.
Qin, B. Y., Bewley, M. C., Creamer, L. K., Baker, H. M., Baker, E. N. & Jameson, G. B. (1998). *Biochemistry*, **37**, 14014–14023.
Reddy, I. M., Kella, N. K. D. & Kinsella, J. E. (1988). *J. Agric. Food Chem.* **36**, 737–741.
Renard, D. & Lefebvre, J. (1992). *Int. J. Biol. Macromol.* **14**, 287–291.
Roefs, S. P. F. M. & de Kruif, K. G. (1994). *Eur. J. Biochem.* **226**, 883–889.
Sawyer, L. & Kontopidis, G (2000). *Biochim. Biophys. Acta*, **1482**, 136–148.
Sheldrick, G. M. & Schneider, T. R. (1997). *Methods Enzymol.* **277**, 319–343.
Spinelli, S., Ramoni, R., Grolli, S., Bonicel, J., Cambillau, C. & Tegoni, M. (1998). *Biochemistry*, **37**, 7913–7918.
Tanford, C., Bunville, L. G. & Nozaki, Y. (1959). *J. Am. Chem. Soc.* **81**, 4032–4035.
Tegoni, M., Ramoni, R., Bignetti, E., Spinelli, S. & Cambillau, C. (1996). *Nature Struct. Biol.* **3**, 863–867.
Ugolini, R., Ragona, L., Silletti, E., Fogolari, F., Visschers, R. W., Alting, A. C. & Molinari, H. (2001). *Eur. J. Biochem.* **268**, 4477–4488.
Uhrínová, S., Smith, M. H., Jameson, G. B., Uhrin, D., Sawyer, L. & Barlow, P. N. (2000). *Biochemistry*, **39**, 3565–3574.
Verheul, M., Pedersen, J. S., Roefs, S. P. F. M. & de Kruif, K. G. (1999). *Biopolymers*, **49**, 11–20.
Weichsel, A., Andersen, J. F., Roberts, S. A. & Montfort, W. R. (2000). *Nature Struct. Biol.* **7**, 551–554.
Wu, S. Y., Perez, M. D., Puyol, P. & Sawyer, L. (1999). *J. Biol. Chem.* **274**, 170–174.
Wyatt, P. J. (1993). *Anal. Chim. Acta*, **272**, 1–40.
Yusifov, T. N., Abduragimov, A. R., Gasymov, O. K. & Glasgow, B. J. (2000). *Biochem. J.* **347**, 815–819.