Protein Science (1999), *8*:75–83. Cambridge University Press. Printed in the USA.

Functional implications of structural differences between variants A and B of bovine β-lactoglobulin

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(RECEIVED August 5, 1998; ACCEPTED September 25, 1998)

Abstract

The structure of the trigonal crystal form of bovine β-lactoglobulin variant B at pH 7.1 has been determined by X-ray diffraction methods at a resolution of 2.22 Å and refined to values for R and R_{free} of 0.239 and 0.286, respectively. By comparison with the structure of the trigonal crystal form of bovine β-lactoglobulin variant A at pH 7.1, which was determined previously [Qin BY et al., 1998, *Biochemistry 37*:14014–14023], the structural consequences of the sequence differences D64G and V118A of variants A and B, respectively, have been investigated. Only minor differences in the core calyx structure occur. In the vicinity of the mutation site D64G on loop CD (residues 61–67), there are small changes in main-chain conformation, whereas the substitution V118A on β-strand H is unaccompanied by changes in the surrounding structure, thereby creating a void volume and weakened hydrophobic interactions with a consequent loss of thermal stability relative to variant A. A conformational difference is found for the loop EF, implicated in the pH-dependent conformational change known as the Tanford transition, but it is not clear whether this reflects differences intrinsic to the variants in solution or differences in crystallization.

Keywords: bovine β-lactoglobulin; crystal structure; genetic variants; hydrophobic stabilization

Bovine β-lactoglobulin (BLG), the major whey protein of cow's milk at a concentration of 0.3 g/100 mL (Bell & McKenzie, 1964), was first isolated by Palmer (1934). Mature bovine β-lactoglobulin has 162 residues, and is a member of the lipocalin protein superfamily (Flower, 1994, 1996). Members of this family have a distinctive eight-stranded β-barrel structure, the central cavity of which binds a variety of hydrophobic molecules (Banaszak et al., 1994). In the case of BLG, the primary site for fatty-acid binding has recently been established crystallographically to be inside this cavity (Qin et al., 1998b; Wu et al., 1999). The structure of BLG is now reliably known following two independent redeterminations of the structure in the triclinic (lattice X) (Brownlow et al., 1997) and orthorhombic (lattice Y) forms (Bewley et al., 1997). The latter structure has led to our redetermination of the structure in the trigonal (lattice Z) form (Qin et al., 1998a). In lattice Z, in contrast to lattices X and Y, the entire molecule is well defined from electron density maps.

Three variants of BLG, labeled as A, B, and C, commonly occur in cow's milk. Variants A and B (BLGA and BLGB) differ at two sites: Asp64 in A is changed to Gly in B, and Val118 in A is changed to Ala in B. Variants B and C differ at one site: Gln59 in B is changed to His in C. Thus, the isoelectric points for variants A, B, and C differ slightly: pI = 5.26, 5.34, and 5.33, respectively, in 0.1 M KCl at room temperature (McKenzie, 1971). Of technological significance, bovine BLG variants have different effects on the industrial processing of milk and on the characteristics of milk products (Hill et al., 1996). Thermal stability is in the order B < A < C, where B is most susceptible to thermal denaturation (Manderson et al., 1995; Hill et al., 1996). These variants of bovine BLG have different self-association properties (Hill et al., 1996). Variant A forms dimers and then octamers under increasingly acidic conditions, whereas variants B and C do not form octamers (Timasheff & Townend, 1961; Timasheff, 1964; Pessen et al., 1985), but do form dimers, with dissociation constants in the micromolar range at pH ~ 7 and stability constants in the order

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Abbreviations: BLG, β-lactoglobulin; BLGA, bovine β-lactoglobulin variant A; BLGB, bovine β-lactoglobulin variant B; BLGC, bovine β-lactoglobulin variant C; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); RMS, root-mean-square; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TAPSO, 3-[*N*-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid.

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C ≫ B > A (Timasheff & Townend, 1961; McKenzie, 1971; McKenzie & Sawyer, 1972; Thresher et al., 1994; Hill et al., 1996; Thresher & Hill, 1997).

Both bovine BLG variants A and B undergo pH-dependent conformational changes in the range pH 6.5 to pH 7.5, the so called N ⇔ R or Tanford transition (Tanford et al., 1959; Hambling et al., 1994). These changes are associated with an anomalous carboxylate residue, which has a pK_a of 7.3 (instead of 4.5) (Tanford et al., 1959; Tanford & Taggart, 1961), and which was tentatively identified as Glu89 (Brownlow et al., 1997). Detailed insight into the structural basis of the Tanford transition has come from the structures of bovine BLG variant A at pH values spanning the Tanford transition (pH 6.2, 7.1, and 8.2), in a single crystal form (trigonal lattice Z) (Qin et al., 1998a). The anomalous carboxylate, Glu89, is located on a flexible loop EF. At pH above 7.1 this loop is folded back to reveal the interior of the calyx. In this paper the structure of variant B at pH 7.1 is presented and compared with the structures of variant A at pH 6.2, pH 7.1, and pH 8.2 for additional insight into the structural basis of the Tanford transition and for insight into altered functionality of variants A and B of bovine BLG (Hill et al., 1996; Manderson et al., 1998). Although preliminary results have been reported on the structures of BLGA, BLGB, and BLGC in lattice Y (Bewley et al., 1997), the N- and C-terminal regions and several critical loops are very poorly defined. Moreover, the structures presented and compared here all pertain to the trigonal lattice Z, in which all regions are clearly defined, providing a coherent framework for this structure–function analysis.

Methods

A sample of BLGB was isolated from the milk of a cow homozygous in the BLG gene, as described previously (Manderson et al., 1995, 1998). The purity of the sample was confirmed by SDS-PAGE electrophoresis. BLGB samples at a protein concentration 25–30 mg/mL in 0.010 M HEPES at pH 7.4 were subjected to an ammonium sulfate crystallization screen. The screen matrix was 4 × 6, with six equally spaced concentrations of ammonium sulfate from 2.2 to 2.8 M; and four buffers at a concentration of 0.18 M with pH 5.0 (acetic acid/KOH), 6.1 (cacodylic acid/KOH), 7.4 (HEPES/KOH), and 8.7 (TAPSO/KOH). The well solution with nominal pH 7.4 that produced the crystal of BLGB suitable for data collection has its pH shifted to 7.1 by the ammonium sulfate. In general, bovine BLGB crystallizes in the lattice Z form in 2–4 days over an ammonium sulfate concentration range of 2.2 to 2.8 M. X-ray diffraction data were collected at room temperature with a Rigaku RAxis IIC image-plate detector and a Rigaku RU200 rotating-anode generator. The data were processed and indexed by program DENZO (Otwinowski, 1996–1997), and merged by program SCALEPACK (Otwinowski, 1996–1997). Data collection statistics are summarized in Table 1. The structure of bovine BLGB at pH 7.1 in lattice Z was solved by molecular replacement methods, using the program AMoRe (Navaza, 1994). The search model was the entire monomer of bovine BLGA in the lattice Z at pH 7.1, minus water molecules (Qin et al., 1998a). The initial *R*-factor was 0.35. Negative density at residues 118 and 64 confirmed the sequence differences Val118 to Ala and Asp64 to Gly for variant B, and positive density in the vicinity of loop EF indicated an alternative conformation. The program X-PLOR (Brünger, 1990) was used to refine the initial model, and the molecular graphics program TURBO (Cambillau et al., 1996) was used for rebuilding the model. After the model for the protein could not be improved further, water molecules were added using the following criteria: well-shaped electron density that appeared in both $2F_o - F_c$ and $F_o - F_c$ maps (at one and three times the RMS levels of the respective maps), and that was within hydrogen bonding distance (2.9 ± 0.4 Å) of a polar group with appropriate geometry. The resultant structure has acceptable quality statistics, as summarized in Table 2. The structure has been deposited at the Protein Data Bank with access code 1bsq.

Table 1. *Crystal and data collection parameters of BLGB lattice Z at pH 7.1*

	BLGB
Space group	$P3_221$
Unit cell *a*, *b*, *c* (Å)[a]	54.23 54.23 113.02
Alpha, beta, gamma (°)	90, 90, 120
Unit cell volume (Å^3)	287,857
Lattice type code	Z
Unique reflections	9,182
Resolution (Å)	15–2.22
R_{merge} (last shell)[b]	0.086 (0.35)
Redundancy[b]	2.68
Completeness (last shell)	91.2% (71.8%)
$I/\sigma(I)$ (last shell)	10.2 (2.13)
χ^2 (last shell)	1.05 (1.06)

[a]For BLGA at pH 7.1 and at pH 6.2, *a* and *c* are, respectively, 53.75 and 111.56 Å, and 53.96 and 112.41 Å; volume 279,111 and 283,429 Å^3. Taken from Qin et al. (1998a).

[b]$R_{merge} = \Sigma^{equivalent\ hkl}(|F_o - \langle|F_o|\rangle|)/\langle|F_o|\rangle$; redundancy = (# refln processed)/(# unique refln).

Results and discussion

General aspects of molecular conformation

The core structure and also the association of monomers into dimers for BLGB in lattice Z at pH 7.1 is very similar to other BLG structures: BLGA in lattice Z at pH 7.1 (Qin et al., 1998a), BLG (mixture of variants A and B) in lattice X at pH 6.5 (Brownlow et al., 1997), and BLGA, BLGB, and BLGC in lattice Y (Bewley et al., 1997). As illustrated in Figure 1, the core structure comprises nine strands, labeled β-A to β-I and arranged in an up-down-up-down topology to form a barrel or calyx. The calyx is comprised of two distinct sheets: sheet 1 is formed from strands β-A1 (residues 16–21), β-B (40–50), β-C (52–61), and β-D (67–76); and sheet 2 is formed from β-E (residues 80–85), β-F (91–98), β-G (101–108), β-H (117–124), and β-A2 (22–27). Strand β-I (146–152) is linked into the second β sheet via strand β-A2. Long flexible loops AB (linking strands β-A and β-B), CD, EF, and GH form the top or the open end of the calyx; short relatively rigid loops BC, DE, and FG form the bottom or closed end of the calyx. Access into the internal cavity appears to be controlled by loop EF (residues 85–90), which is able to adopt alternative open and closed conformations (Qin et al., 1998a). The second β-sheet is substantially covered by the C and N termini, and by a three-turn α-helix (129–142) that lies parallel to and approximately above strand β-H (117–124), burying the cysteine residue, Cys121. The other four cysteine residues are paired in disulfide bonds: the first is between the C terminus and flexible loop CD (Cys66–Cys160), and the second links strands β-G and β-H (Cys106–Cys119), making the

Table 2. *Comparison of refinement results for BLGA and BLGB in lattice Z at pH 7.1*

	BLGA	BLGB
Resolution limit (Å)	15.0–2.24	15.0–2.22
R_{free}[a] (# reflections)	0.279 (557)	0.286 (576)
R[a]	0.234	0.239
Number of protein atoms	1,286	1,280
Number of water molecules	62	109
RMS (bond distances in Å)	0.008	0.009
RMS (bond angles in °)	1.5	1.7
Ramachandran plot (as determined by PROCHECK)[b]		
Core	83.9%	83.1%
Allowed	14.8%	14.9%
Generously allowed	0.7%	1.4%
Disallowed	0.7%	0.7%
Average B-factor (Å^2)		
Polypeptide	48	50
Side chain	51	53
N-terminal (1–8)	59	69
Loop CD (61–66)	84	82
Loop EF (85–90)	86	77
Loop GH (109–116)	81	66
β-Sheet 1 (strands B, C, D)	47	57
β-Sheet 2 (strands E, F, G, H)	35	42
α-Helix (129–142)	44	47
β-Strand I (146–152)	36	38
Waters	71	75
Accessible surface area (Å^2)[a]		
Monomer	8,458	8,269
Dimer	15,944	15,597

[a] R (or R_{free}) $= \Sigma^{all\ hkl}(|F_o| - |F_c|)/\Sigma(|F_o|)$.
[b] CCP4 (1994) program suite.

region comprising residues 117–124 the most rigid in the structure, as evidenced by low B-values.

As also shown in Figure 1, the secondary and much of the tertiary structure of BLGA and BLGB are very similar. In the superposition of monomers of BLGA and BLGB, the RMS displacement is 0.24 Å, for the Cα atoms of 155 out of a total 162 residues. For only seven residues, mostly in loop EF (residues 85–90), are the displacements of Cα positions greater than 0.7 Å. The pairwise differences in Cα positions of BLGA and BLGB are detailed in Figure 2, which also summarizes the secondary structure of BLG. Figure 3 is a modified Ramachandran plot, which shows not only the satisfactory location of all residues into allowed regions of conformational space (except for Tyr99, which is the central residue of a well-defined γ-turn), but also shows the small differences that occur in main-chain conformation between BLGA and BLGB.

The dimer of BLGB (and also BLGA) is formed about a dyad, in the case of lattice Z, a crystallographic dyad, such that strands β-I (residues 146–152) are hydrogen bonded into an antiparallel β-sheet spanning the two monomers. For the superposition of the BLGB and BLGA dimers at pH 7.1, the comparable RMS displacement is 0.27 Å, a value only slightly greater than that for the superposition of just the monomers. The corresponding value for the superposition of BLGB at pH 7.1 and BLGA at pH 6.2 is 0.30 Å.

The most notable difference between BLGA and BLGB at pH 7.1 occurs in the EF loop (residues 85–90). In contrast to BLGA in lattice Z at pH 7.1, where this loop is in the open conformation (Qin et al., 1998a), for BLGB under ostensibly the same conditions this loop is in the closed conformation, characteristic of the structure of BLGA in lattice Z at pH 6.2 (Qin et al., 1998a). The side chain of Met107 moves to the conformation found for BLGA at pH 6.2. An explanation for the dissimilar conformations of loop EF for the structures of BLGA and BLGB at the same pH of 7.1 is offered below. A second region of difference between BLGA and BLGB is in loop CD (residues 61–67), where at residue 64 the point substitution Asp (BLGA) → Gly (BLGB) occurs.

The conformational change of loop EF, and in particular of Glu89, also alters the main-chain–main-chain hydrogen bonds between strands β-F and β-G. As is apparent in Figures 2 and 3, strands β-F and β-G maintain a similar conformation in BLGB and BLGA both at pH 7.1. For BLGA at pH 7.1 and pH 8.2 (open conformation of loop EF), there is a main-chain–main-chain hydrogen bond between Ser110 and Glu89 (Ser110_N…O_Glu89). For BLGB at pH 7.1 and BLGA at pH 6.2 (closed conformation of loop EF), however, this hydrogen bond is replaced by two main-chain–side-chain hydrogen bonds (Ser110_N…OD1_Asn90 and Asn88_O…ND2_Asn109), and by a different main-chain–main-chain hydrogen bond (Glu108_O..N_Asn90) (Qin et al., 1998a). A number of small differences between BLGB and BLGA at pH 7.1 also occur in the conformation of loop GH, but because the atomic displacement parameters (temperature factors) are relatively high and indicative of flexibility of this loop, little significance should be placed on the reliability of these differences. In any event, the origin of the different denaturation properties of BLGA and BLGB does not appear to be associated, directly or indirectly, with the conformation of loop EF or GH.

Structural consequences of sequence differences of BLGA and BLGB

The substitution Val118 (BLGA) → Ala (BLGB) occurs in strand β-H. Residue 118, which is well buried under the side chains of Leu31, Leu39, and Glu120 with an accessible surface area of 5 Å^2 for BLGA and 8 Å^2 for BLGB, lies in the most rigid part of the structure, as evidenced by the much lower than average B-values for atoms at residue 118 (35 Å^2 for BLGA and 31 Å^2 for BLGB; see also Table 2). The smaller Ala side chain of BLGB causes no detectable structural changes in the main chain (see Figs. 1, 2), and, with the exception of a change in the rotamer of Leu39 to maintain a hydrophobic contact, causes no significant movements of neighboring side chains (Figs. 4, 5). The effectiveness of hydrophobic packing around 118 is, therefore, significantly diminished for the Ala side chain of BLGB. This absence of change has important functional consequences, discussed below. Figure 4A shows the electron density in the vicinity of the substitution, superimposed on the structures of bovine BLGA and BLGB.

The second site of variation, Asp64 (BLGA) → Gly (BLGB), is located in the flexible loop between strands β-C and β-D. As is apparent from Figures 1 and 2, there are significant differences in main-chain conformation in this region, which leaves the Cα atom of residue 64 of bovine BLGB more than 1.0 Å displaced from that of BLGA. These differences are propagated and amplified into the side chains, especially those of Glu65, Glu62, and Asn63, as illustrated in Figure 4B, which also shows the electron density in this region, a region in which the structure of BLG in lattices X (triclinic) (Brownlow et al., 1997) and Y (orthorhombic) (Bewley et al., 1997) is poorly defined. As this loop CD is rather flexible

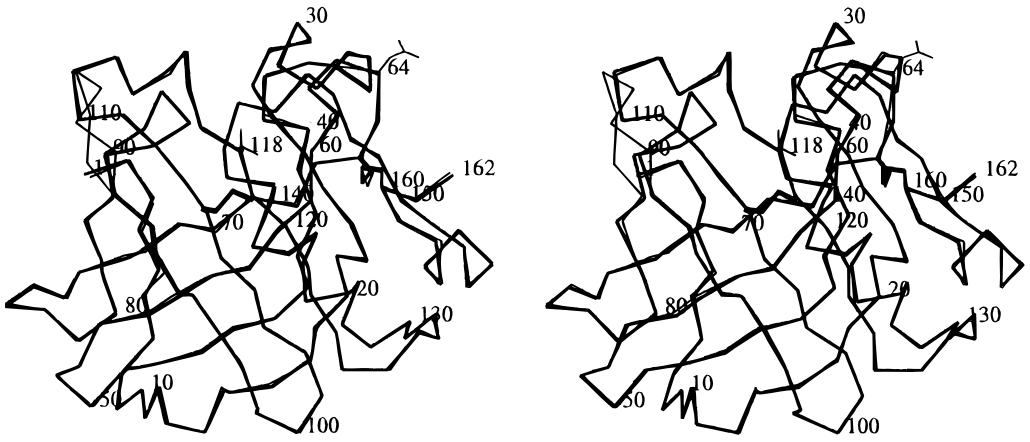

Fig. 1. Superposition of BLGA (thin line) and BLGB (thick line) in lattice Z at pH 7.1. β-Sheet 1 [front of molecule, strands A1 (16–21), B, C, D] and β-sheet 2 [middle of molecule, strands E, F, G, H, A2 (22–27)] are perpendicularly oriented. β-Sheet 2 is covered by the N and C termini, the three-turn α-helix, and β-strand I (back of molecule).

and exposed to solvent, we cannot be certain whether the differences observed are a consequence of the point mutation or are the result of crystal packing effects. However, as the negatively charged residue Asp64 of BLGA is situated close to other negatively charged residues (Glu62, Glu65), and as the mutation is to a conformationally less restricted residue, glycine, it is not unexpected that conformational changes have occurred. The changes occurring in this loop are propagated into movements of the C-terminal region through the disulfide bond, Cys66–Cys160. The substitution D64G is located on the surface of the molecule and is, therefore, a prime suspect in the reported ability of BLGA dimers (but not BLGB or BLGC dimers) to oligomerize further into octamers in the pH range 3.5 to 6.5 (Timasheff, 1964).

The crystal structures of BLGB and BLGA in lattice Z provide few clues as to the possible nature of the octameric association of BLGA. Bovine BLGB is packed in the lattice Z in the same way as BLGA. The BLGB monomers form a linear zig-zag chain, as a result of the "lock and key" interface, where a cavity near Glu44 accepts the side chain of Lys8 of a neighboring molecule (Qin et al., 1998a). The dimers alternate up or down along each chain. In each layer, these chains run parallel to each other and are in weak contact through interactions of the flexible loops, CD, EF, and GH. Asp64 of BLGA is not involved in any intermolecular contacts, at least in lattice Z. Both BLGB at pH 7.1 and BLGA at pH 6.2, which have loop EF in the closed conformation, form a pair of weak intermolecular contacts around the crystallographic twofold axis between the asparagine side chain of residue 88 and the loop EF, an interaction absent for BLGA at pH 7.1 and 8.2. Conversely, BLGA at pH 7.1 and pH 8.2, which have loop EF in the open conformation, have a pair of salt bridges between the carboxyl group of residue 62 in loop CD and the ammonium group of Lys69 at the start of strand D. However, it should be noted that the angle between the twofold axes forming the dimer interface and this interface between the loops is 60°, not an orientation conducive to octamers formed as tetramers of dimers.

Functional consequences of sequence differences of BLGA and BLGB

There is a vast and somewhat confusing literature on the different chemical and physical properties of BLG, such as chemical reactivity of the free cysteine at position 121, thermal denaturation,

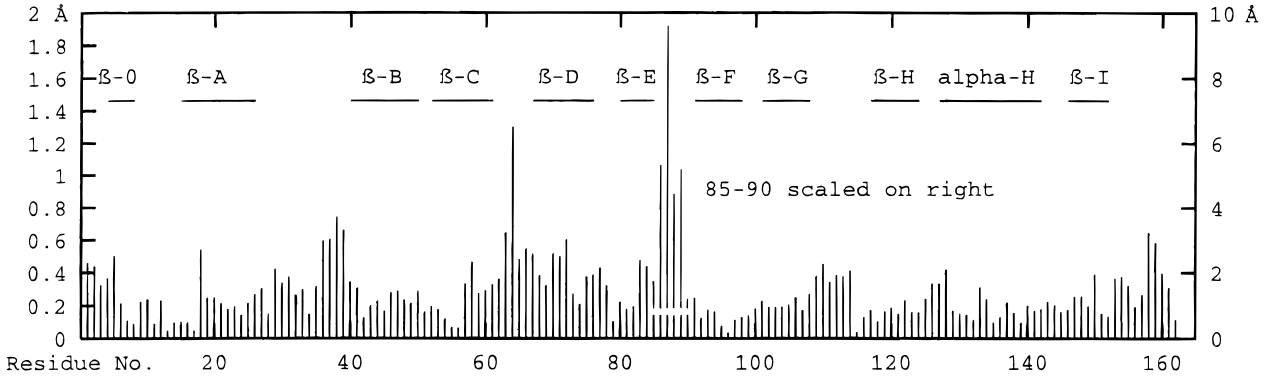

Fig. 2. The displacement (in Å) of Cα atoms of BLGA and BLGB at pH 7.1, after superposition. Note the change in scale in the region of loop EF (residues 85–90). The secondary structure is included.

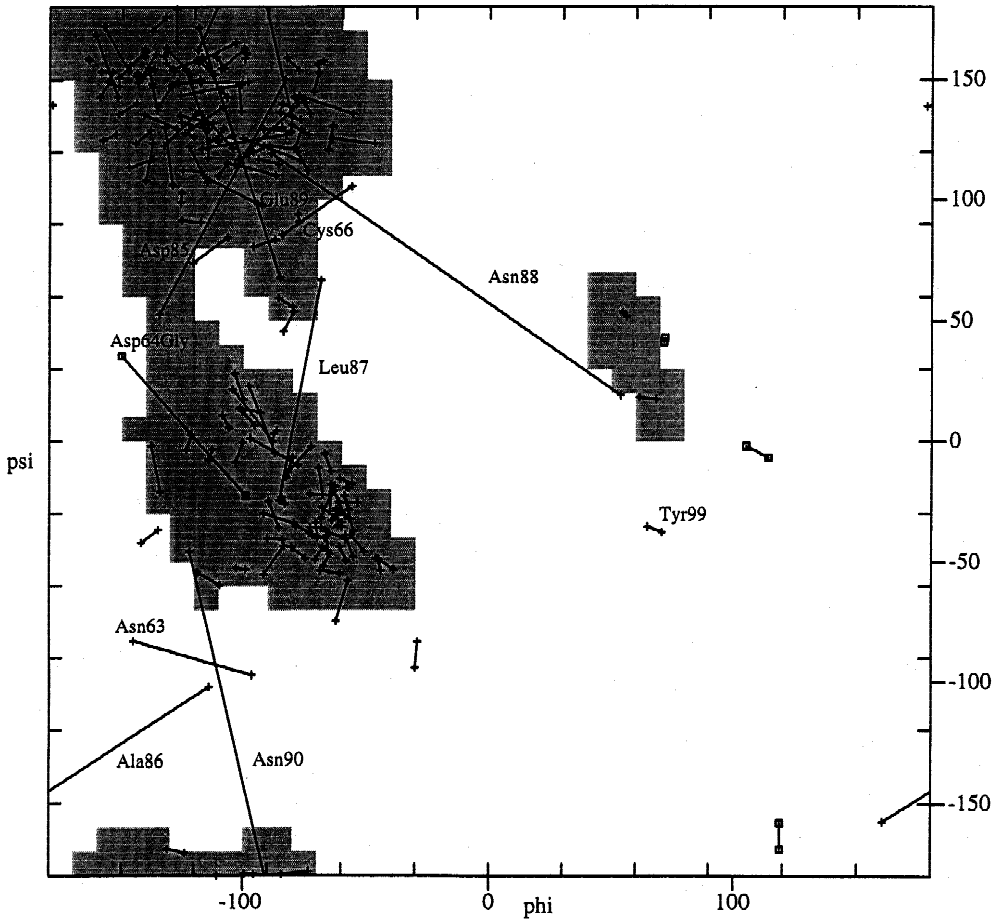

Fig. 3. Ramachandran plot of bovine BLGA and BLGB at pH 7.1. The difference in ϕ/ψ values of equivalent residues is represented by a line. Glycine residues are drawn as squares. The shaded area corresponds to that within which 90% of nonglycine residues should reside. The γ turn at Tyr99 and those residues for which there are substantial differences in ϕ/ψ values are labeled.

gelation, and thermally induced coagulation (Creamer & MacGibbon, 1996; Hill et al., 1996). In general, relative to variant A and both in vitro and in milk, variant B has greater reactivity of the free thiol (Gough & Jenness, 1962; Phillips et al., 1967), suffers a greater extent of irreversible denaturation on incubation at a fixed temperature (70–80 °C) (Alexander & Pace, 1971; Dannenberg & Kessler, 1988; Anema & McKenna, 1996; Hill et al., 1996), and induces more readily the coagulation of milk at 140 °C (Rose, 1962; Feagan et al., 1972; McLean et al., 1987; van den Berg et al., 1992; Robitaille, 1995; Hill et al., 1996)—all undesirable properties for various heat treatments involved in standard milk processing (Hill et al., 1996). Variant C is noticeably more stable than variant B to both thermal (Manderson et al., 1995, 1998; Hill et al., 1996) and urea denaturation (Y.H. Cho, H. Singh, L.K. Creamer, unpubl. obs.). Variant A, however, resembles variant B in stability at low temperature and low urea concentration, but at higher temperature and higher urea concentration it resembles variant C (see Fig. 6).

The conformational differences observed in the structures of BLGA and BLGB at pH 7.1 do not result in changes of exposure of charged residues in this or other regions of the molecule (except loop EF, discussed below). Nor is there an increase in solvent accessibility to Cys121 as a result of the sequence differences of BLGB compared to BLGA. This is true, irrespective of whether loop EF is open or closed. However, inspection of the temperature factors of various regions of the molecule of BLGB, with respect to the average temperature factor for the entire protein molecule, reveals a number of interesting differences when compared with the corresponding numbers for BLGA, as summarized in Table 2. The β-sheet formed by strands β-E, β-F, β-G, and β-H, which contains Cys121 on strand β-H, and the N-terminal region, which covers part of this β-sheet, have for BLGB relatively higher *B*-values than the corresponding regions in BLGA. In contrast, the loops EF (residues 85–90) and GH (residues 109–116) are relatively less mobile for BLGB, while the three-turn helix α-H (residues 129–142) has an average *B*-value essentially the same as the overall *B*-value. So, although the *equilibrium* solvent accessibility has not changed in the region around Cys121, the greater mobility of this region may indicate greater transient accessibility to Cys121 and, hence, greater reactivity.

The loss of two methyl groups accompanying the V118A substitution, coupled with the inability of the surrounding structure to adjust to fill this void, means that there is a decreased degree of complementarity of internal hydrophobic surface for BLGB compared to BLGA. This is consistent with the lessened rigidity in this region. Five hydrophobic contacts, for which the interatomic sep-

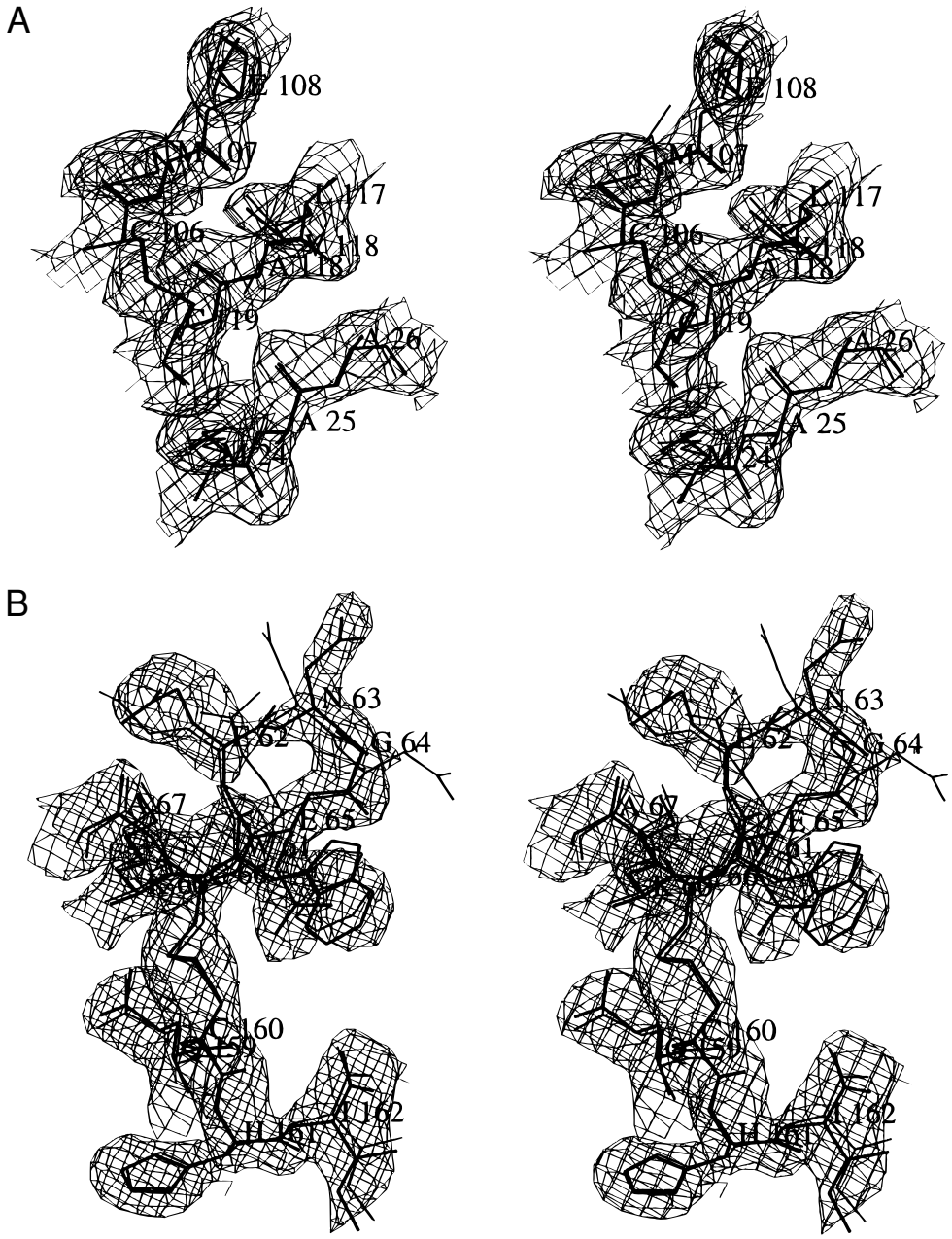

Fig. 4. Electron density ($2F_o - F_c$) omit maps. **A:** Residue 118 omitted and contoured at the 1σ level. The final coordinates are superimposed: BLGA (V118, thin line) and BLGB (A118, thick line). **B:** Residues 61–65 omitted and contoured at the 0.7σ level. The final coordinates are superimposed for loop CD and the C-terminal region of BLGA (D64, thin line) and BLGB (G64, thick line).

arations lie in the range 3.7 to 4.2 Å, are lost: V118CG2…A26CB, V118CG2…L31CD1, V118CG1…A25O, V118CG1…OH2_166, and V118CB…L39CD2. Contacts V118CG2…L39CD1 and V118CG2…L39CB in BLGA are compensated in BLGB by contacts A118CB…L39CD1 and A118CB…L39CB as a result of Leu39 adopting a different rotamer and of a small movement of the loop containing this residue, as illustrated in Figure 5. A well-defined external water, OH2_166, which is hydrogen bonded to Q120OE1 and makes contact with the very small portion of residue 118 that is exposed in variant A (V118CG1, 5 Å^2), is absent in BLGB, providing a small entropic compensation to the otherwise unfavorable entropic changes arising from the substitution V118A. Met107 adopts a similar conformation in BLGB at pH 7.1 and BLGA at pH 6.2, as a result of the closed conformation of loop EF. The relationship between enhanced complementarity of hydrophobic surfaces and increased thermostability is well-documented—although often the full consequences of loss of complementarity are masked by structural rearrangement to accommodate the change in side-chain shape (Alber et al., 1987; Eriksson et al., 1992). Moreover, the extent of (de)stabilization is greatest when the substitution occurs in the most rigid part of the structure (Alber et al., 1987), as is the case here for bovine BLGA and BLGB. Thus, the

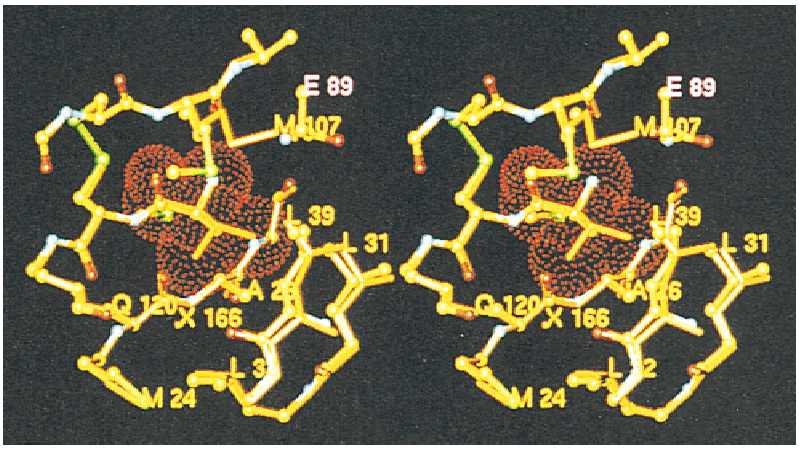

Fig. 5. Superposition of BLGB (ball-and-stick, loop EF closed) and BLGA at pH 7.1 (orange sticks, loop EF open) in the region of the residue 118. Atoms for BLGB are color-coded: red, oxygen; blue, nitrogen; green, sulfur; gold, carbon. All residues that have atoms within 4.5 Å of atom CB at this site are shown. The van der Waals surface of BLGA (Val118) is shown as red dots. Note the movement of the side chain of Met107 for BLGB as a consequence of the closed conformation of loop EF. Note also the movement of Leu39 to occupy part of the volume created by the presence of Ala118 in BLGB (in place of Val for BLGA).

decreased thermal stability of BLGB at high temperatures and low protein concentrations compared to BLGA (Manderson et al., 1998) arises from the reduced hydrophobic contacts that result from V118A substitution. Treating each contact lost as approximately equivalent to a Lennard–Jones pairwise interaction between two CH_4 moieties, for which the attraction is $\sim$1.1 kJ mol^{-1} at a separation of 4.0 Å, leads to a crude estimate of the enthalpic contribution to lowered stability of $\sim$5–6 kJ mol^{-1}, an amount similar to other comparable estimates (Shortle et al., 1990) and consistent with the relatively small but significant decrease in thermal stability of BLGB compared to BLGA. Alternatively, relative to the unfolded structure in water, the removal of the larger hydrophobic valine moiety from water, compared to the smaller alanine moiety, is entropically more favorable.

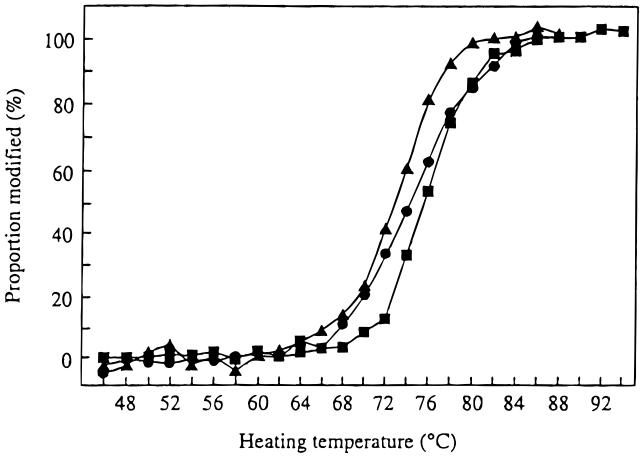

Fig. 6. Thermal denaturation of bovine BLG variants A (filled circle), B (filled up triangle), C (filled square) at pH 7 in 0.10 M phosphate buffer. The % denaturation that occurs for a fixed period (12.5 min) of incubation is plotted as a function of temperature. The distinctive curve for variant A is discussed in the text. Taken from Manderson et al. (1995) and Hill et al. (1996).

The conclusions drawn here for comparison of variants A and B are, in fact, reinforced when variant C is included in the comparison. Variant C has at position 59 at the end of strand β-C a histidine in place of the glutamine found for variants A and B. In general, variant C has enhanced thermal stability compared to variants A and B (Manderson et al., 1995, 1998; Hill et al., 1996). For BLGA and BLGB a weak hydrogen bond between the side chains of Glu44 and Gln59 is probably replaced by a salt bridge (at neutral pH) between the side chains of Glu44 and His59, leading to enhanced rigidity in this region (Bewley et al., 1997). Variant B, which lacks the salt bridge Glu44…His59, is less stable than variant C under all conditions—an enthalpic and approximately temperature-independent contribution to the free energy of stability. Variant A, which also lacks the salt bridge, is less stable than variant C at low temperatures, but at higher temperatures enjoys enhanced stability as a result of better hydrophobic packing around the Val118 residue of variant A compared to the alanine of variants B and C—the hydrophobic packing being a primarily entropic contribution (varying with temperature as $T\Delta S$) to the free energy of stability (Alber et al., 1987; Eriksson et al., 1992). Furthermore, the response of variants A, B, and C to denaturation by the chaotrope urea is the same as that to thermal denaturation (Manderson et al., 1998; Y.H. Cho, H. Singh, L.K. Creamer, unpubl. obs.).

Conformation of loop EF and the Tanford transition

The difference in the conformation of the EF loop (residues 85–90) represents the largest structural difference between the two structures compared here. The BLGA structure at pH 7.1 has this loop in its open conformation, whereas BLGB at pH 7.1 has it in the closed conformation. These open and closed conformations represent the high pH and low pH states of the Tanford transition (Qin et al., 1998a). Why then do the two genetic variants show different conformations of this loop, ostensibly at the same pH?

It is difficult to see a connection between the conformation of the EF loop and the two sites of genetic variation. The V118A substitution is internal and produces little perturbation in the equilibrium structure. The D64G substitution on loop CD is external

but distant from the EF loop: the closest contact of residue 64 with Cα atoms of the EF loop is ~25 Å for the open conformation of BLGA at pH 7.1 and ~19 Å for the closed conformation of BLGB at pH 7.1; the closest contact of any atom on loop CD with any atom on loop EF is ~9 Å for BLGA at pH 7.1 (L87CB…OE1E62) and ~12 Å for BLGB at pH 7.1 (N88ND2…OE2E62). The observed differences in conformation of the EF loop may arise from a number of factors including: (1) batches of crystals containing stochastic mixtures of crystals with loop EF in open or closed or both conformations; (2) small uncertainties in the pH of crystallization; (3) small differences in the midpoint of the Tanford transition [which is ~7.3 for both BLGB and BLGA and which lies close to the pH of crystallization (Tanford & Nozaki, 1959; Tanford & Taggart, 1961; Timasheff et al., 1966; Basch & Timasheff, 1967)]; and (4) accentuated effects of the additional negative charge of BLGA on intermolecular contacts in the crystalline state relative to the solution state. Germane to this last factor are (a) the fivefold lower solubility of BLGA compared to BLGB (Treece et al., 1964); (b) the larger unit cell volume for BLGB than for BLGA at both pH 7.1 and 6.2 (Qin et al., 1998a); and (c) the packing of molecules in lattice Z, which places loops AB, CD (the site of the D64G substitution), and EF in loose intermolecular contact. The question of possible differences between BLGA and BLGB with respect to this conformational transition are being further addressed by studies of ligand binding as a function of pH (L.K. Creamer, unpubl. obs.).

Conclusions

Bovine BLGA and BLGB differ at two sites. One is in an exposed flexible loop (D64G), which leads to observable conformational changes and is probably responsible for lowered solubility and enhanced oligomerization and gelation propensity of variant A. The other is in a buried situation on a β-strand (V118A); this sequence difference causes minimal structural change to the equilibrium structure but alters the dynamic properties of the molecule (as seen in the *B*-factors), and is probably responsible for the lowered thermal stability of variant B by disrupting internal hydrophobic packing. The EF loop is in a closed conformation, covering access to the central cavity. As the pH of crystallization (pH 7.1) lies near the midpoint of the Tanford transition (the conformational change that above pH ~7.3 exposes a buried glutamate residue in loop EF), the closed conformation of loop EF observed for BLGB at pH 7.1, representing one of two conformations present in solution, is not in contradiction with published spectroscopic and chemical data.

Acknowledgments

The first two authors contributed equally to this work. This work was supported in part by a grant from the New Zealand Dairy Research Institute, through contract DRI 403 from FoRST of New Zealand. We thank Heather Baker for advice on crystallization, Gavin Manderson for providing purified BLGB, Lindsay Sawyer, Mike Boland, Jeremy Hill, and Wayne Thresher for many helpful discussions, and "referee 2" for insightful comments on the conformation of loop EF. In particular, we thank Lindsay Sawyer for a manuscript describing the structure of BLG complexed with palmitate.

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