Structure and stability of Gyuba, a β -lactoglobulin chimera

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Abstract: β -lactoglobulin (LG) contains nine β -strands (strands A–I) and one α -helix. Strands A–H form a β -barrel. At neutral pH, equine LG (ELG) is monomeric, whereas bovine LG (BLG) is dimeric, and the I-strands of its two subunits form an intermolecular β -sheet. We previously constructed a chimeric ELG in which the sequence of the I-strand was replaced with that of BLG. This chimera did not dimerize. For this study, we constructed the new chimera we call Gyuba (which means cow and horse in Japanese). The amino acid sequence of Gyuba includes the sequences of the BLG secondary structures and those of the ELG loops. The crystal structure of Gyuba is very similar to that of BLG and indicates that Gyuba dimerizes via the intermolecular β -sheet formed by the two I-strands. Thus, the entire arrangement of the secondary structural elements is important for LG dimer formation.

Keywords: designed protein; crystal structure; intermolecular β -sheet; dimer; protein stability; circular dichroism

Introduction

β -lactoglobulin (LG) is the major protein component of mammalian milk. Among the LGs, bovine LG (BLG) has been investigated most extensively.¹ BLG contains 162 amino acid residues, including five cysteines (Cys66, Cys106, Cys119, Cys121, and Cys160), four of which form two disulfide bonds (Cys66–Cys160 and Cys106–Cys119), and Cys121 retains a free thiol group. Although BLG is dimeric at neutral pH, it dissociates into monomers at low pH and very low ionic strength condition because of intermolecular electrostatic repulsions.^{2,3} However, the monomers retain their tertiary structures at acidic pH. Both crystal^{4–9} and solution^{10,11} structures of BLG show that the monomer contains nine β -strands (strands A–I), an α -helix, and three short 3_{10} helices. Eight of the β -

strands (A–H) form an up-and-down β -barrel, and the two I-strands form an intermolecular β -sheet containing four intermolecular hydrogen bonds. A mutational study has shown that salt bridges between Asp33 and Arg40 of the opposing AB-loops that are important to the stability of the dimer.³

BLG binds a variety of hydrophobic compounds including retinol, fatty acids, and vitamin D₃ at its hydrophobic cavity inside the barrel.^{12–14} The loop between the E- and F-strands (EF-loop) obstructs the entrance to the β -barrel in the crystal structure at pH = 6.2, whereas the entrance is open at pH = 7.1.⁵ This conformational transition is called the Tanford transition, and NMR studies^{15–17} have found that the deprotonation of Glu89 (pK_a = 6.9) occurs first and is followed by the unfolding of the D-strand, the EF-loop, and the GH-loop.

Equine LG (ELG) contains the same number of amino acids and the two disulfides at the same positions as BLG. The sequence identity between ELG and BLG is 57%. Although a three-dimensional structure of ELG is not available, our previous NMR study showed that the secondary and tertiary structures of

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Figure 1. (A) Amino acid sequence alignment of ELG, LPI, Gyuba, and BLG. Identical residues in ELG, LPI, Gyuba, and BLG are colored magenta. Residues in LPI, Gyuba that are identical with BLG or ELG are colored green or blue, respectively. The amino acid residues at positions 81 and 121 of Gyuba, which were chosen for technical reasons, are colored grey, and residues that do not meet the aforementioned criteria are colored black. Secondary structural elements are indicated below the sequence alignment; A–I represent β -strands. The symbols above and below the sequences indicate residues with positively or negatively charged side chains at neutral pH. (B) Gyuba subunit structure. The β -strands A–I are in yellow, the helices are blue, and the loops are grey. (C) The backbone structure of Gyuba superpositioned on those of four BLG structures (PDB IDs: 1beb,⁴ pink; 1bsq,⁶ black; 2akq,⁸ sky blue; 3blg,⁵ green).

ELG are very similar to those of BLG.¹⁸ However, there are some structural differences between BLG and ELG, including their propensity to dimerize. At neutral pH, BLG is dimeric, whereas ELG is monomeric.¹⁹ Another difference involves their structural behavior in an acidic environment. BLG maintains its tertiary structure, whereas ELG loses its tertiary structure and assumes a molten globule state with non-native α -helices, which mimics the early kinetic folding intermediate.^{19,20}

Previously, Kobayashi *et al.*²¹ constructed a chimeric LG (LPI) in which the residues that form the BLG dimer interface replaced the corresponding residues in ELG; that is, Ser34 and Glu35 in the AB-loop of ELG were replaced with Ala and Gln from BLG, respectively, and the sequence flanking (and including) the I-strand (Gly145 to Leu153) was replaced with the corresponding BLG sequence [Fig. 1(A)]. It was hypothesized that if LPI could assume the BLG conformation, then the dimer interface would be formed as a consequence of the BLG-specific AB-loop and I-strand sequences. However, LPI did not dimerize, and the authors concluded that the monomeric property of ELG cannot attribute solely to the difference in the amino acid sequence of the AB-loop and the I-strand.

For this study, we constructed a new chimeric LG, named Gyuba, which means cow and horse in Japanese. With the exceptions of positions 81 and 121, Gyuba was constructed by joining the secondary structures from BLG with those of the ELG loops. Residues 17–27 (A-strand), 42–49 (B-strand), 53–62 (C-strand), 66–73 (D-strand), 80–86 (E-strand), 89–95 (F-strand), 102–108 (G-strand), 117–124 (H-strand), 130–138 (α -helix), and 145–153 (I-strand and its flanking sequences) were regarded as the secondary structures, and the other sequences were regarded as loops [Fig. 1(A)]. Residue 81 is Val in BLG and Glu in ELG. Although this residue is in the E-strand and by the criteria described above should be a Val in Gyuba, it was replaced with Glu so that the *EcoRI* site in the gene remained intact. Residue 121 is Cys in BLG and Tyr in ELG. This residue is in the H-strand and was changed to Ala to prevent thiol-disulfide exchange. We hypothesized that all interstrand and sheet-helix interactions would be maintained in Gyuba and therefore expected it to dimerize. We thus characterized the Gyuba structure and investigated how specific residues dictate interactions between the various secondary/tertiary structures, and thus the overall conformation, of Gyuba.

Table I. Data Statistics and Refinement Statistics

<i>Gyuba</i> $P6_5$ ^a	
Data statistics	
Resolution (Å)	2.0
Unique reflections	27,566
Redundancy	10.9 (9.6)
Completeness (%)	99.8 (99.9)
R_{symm} (%) ^b	0.066 (0.395)
$I/\sigma(I)$	15.2
Model statistics	
R_{crys} (%)	23.4
R_{free} (%)	28.4
Number of water molecules	240
RMSD in bond length (Å)	0.023
RMSD in bond angle (°)	1.990

^a $P6_5$, $a = 69.6$ Å, $b = 69.6$ Å, $c = 151.3$ Å.

^b $R_{\text{symm}} = \sum h \sum i |I_h, I| - \langle I_h \rangle / \sum \sum I_h, i$.

Parentheses show the value in the highest resolution shell.

Results

Crystal structure of *Gyuba*

The *Gyuba* crystals belonged to the $P6_5$ space group, and the structure was refined to 2.0 Å (Table I). The

coordinates have been deposited in the Protein Data Bank (accession number, 3KZA). The *Gyuba* structure contains nine β -strands (A–I) and one α -helix [Fig. 1(B)]. The A–H strands form an antiparallel β -barrel, and the EF-loop covers the β -barrel entrance.

Being consistent with results from solution experiments (described later), the crystallographic data showed that *Gyuba* forms a dimer, and that the dimer interface involves the AB-loop and the I-strand, which is a structure that is similar to that found for dimeric BLG [Fig. 2(A–D)]. The amino acid sequence of *Gyuba* in the AB-loop is the same as that of ELG and differs from that of BLG at positions 34 and 35 (Ala $\rightarrow$ Ser and Gln $\rightarrow$ Glu, respectively). Although for both BLG and *Gyuba*, residue 34 interacts with residues 33 and 34 of the other subunit and residue 35 is located near the residue Asp33 of the other subunit, the mutations seem to have little effect on the AB-loop structure [Fig. 2(C,D)].

To compare the subunit structures of BLG and *Gyuba*, the backbone C α s were superposed. Because

Figure 2. (A) Superpositioned ribbon representations of the *Gyuba* dimer (blue) and the wild-type BLG dimer (yellow). The dimer interface, which is formed by the AB-loops and the I-strands, is indicated by the two dotted red circles. (B) The structures shown in (A) were rotated 90°. (C, D) Close-ups of the AB-loops of *Gyuba* (C) and BLG (D). (E) The structure of C-terminal region of *Gyuba* is fitted into the electron-density map. (F) Comparison of the C-terminal regions of *Gyuba* (blue) and BLG (1bsq, yellow).⁶

the EF-loop of Gyuba adopted the closed conformation, the BLG structures 1beb,⁴ 1bsq,⁶ 2akq,⁸ and 3blg⁵ (PDB accession numbers), for which the EF-loops also adopted the closed conformation, were used for comparison. The superpositions revealed an obvious difference between the tertiary structures of BLG and Gyuba in the C-terminal regions, that is, the distances between each of the C α s for residues 153–157 of Gyuba and the each of the BLG structures were much larger than those for the remaining sequence in these proteins (Fig. 3). The 3_{10} helix of BLG was formed by residues 153–156, whereas that of Gyuba was formed by residues 154–157 [Fig. 2(F)]. Because electron density was clearly observed for both structures [Fig. 2(E)], there was an obvious structural difference in the two C-terminal regions. The position of the Gyuba 3_{10} helix induced several new intramolecular interactions. Thr154 and Met156 of Gyuba interact with Gln126 and Tyr42, respectively, whereas Leu156 and His161 of BLG interact with Pro126 and Tyr42, respectively [Fig. 2(F)]. In addition, Met156 of Gyuba seems to be located at the position found for Cys160 in BLG, suggesting that the location of Cys66-Cys160 is different for BLG and Gyuba, although the electron density for Cys66-Cys160 in Gyuba was not found. Calculated the root mean square deviations (RMSDs) of residues 153–157 for structures obtained from triclinic (PDB ID: 1beb), orthorhombic (PDB ID: 2akq) and trigonal (PDB ID: 1bsq, 3blg) crystals revealed that residues 153–157 of four BLG structures assume a similar structure (Table II), suggesting that C-terminal regions of four BLG structures are not affected by their crystal packing. Residues 153–157 of Gyuba do not interact with other protein molecules in the crystal. Thus, differences of C-terminal regions between BLG and Gyuba are not

Figure 3. The structures of Gyuba and each BLG were superpositioned, and the calculated distances between the corresponding C α s are shown. For calculations of Gyuba and 1beb, Chain A were used. Color scheme is identical with Figure 1(C).

Table II. RMSD (C α) Calculated for Residues 5–60: 67–152 and Residues 153–157

	Gyuba	1beb	1bsq	2akq	3blg
Gyuba	—	0.44	0.53	0.62	0.65
1beb	3.74	—	0.45	0.52	0.46
1bsq	3.46	0.50	—	0.58	0.38
2akq	3.75	0.26	0.51	—	0.70
3blg	3.53	0.57	0.25	0.53	—

RMSD calculated for residues 5–60: 67–152 and residues 153–157 are presented on the right upper and the left lower half of the matrix.

likely caused by crystal packing. Except for the differences in the C-terminal region, the overall structure of Gyuba is very similar to that of BLG [Fig. 1(C)]. To evaluate the conformational differences between residues 5–60 and 67–152 of Gyuba and BLG, we calculated RMSDs for the C α s for each of the aforementioned BLG structures (PDB ID: 1beb, 1bsq, 2akq, 3blg) and Gyuba. As controls, pairwise comparisons of the RMSDs between each of the four BLG structures were made (Table II). At least for the C α positions, the structural differences for Gyuba and BLG are similar to those of the different BLG crystals.

CD spectroscopy

The near- and far-UV CD spectra of Gyuba, BLG, and ELG are shown in Figure 4. The far-UV CD spectrum of Gyuba is characteristic of a β -sheet protein. Although its spectrum differs slightly from that of BLG, the differences between the two spectra may result from contributions by the aromatic side chain transitions. BLG and Gyuba have the same number of the aromatic residues: two tryptophans (Trp19 and Trp61) and four tyrosines (Tyr20, Tyr42, Tyr99, and Tyr102). Nevertheless, the peak intensities of the near-UV CD spectrum of Gyuba are less negative than are those of BLG, which would reflect differences in the packing of the aromatic side chains or in the environment. Although aromatic rings of

Figure 4. (A) Far- and (B) near-UV CD spectra of BLG (dotted lines), ELG (thin, solid lines), and Gyuba (heavy, solid lines) at pH = 7.0.

Figure 5. (A) Far- and (B) near-UV CD spectra of Gyuba at pH = 6.9 (heavy, solid lines), pH = 1.0 (thin, solid lines), and in 8.2M urea (squares). (C, D) Acid denaturation of Gyuba was monitored by CD at (C) 222 nm and at (D) 293 nm.

Gyuba, except for Trp61, show positions similar to those of BLG in the crystal structure, the structure of residues 61–66 of Gyuba are not elucidated in this study. Therefore, the contribution of Trp61 to the near-UV CD spectrum may be different between Gyuba and BLG. Similarly, contributions by aromatic transitions may also partially underlie the differences between the far-UV CD spectra of BLG and Gyuba.

Acid denaturation of Gyuba

The dependence of the Gyuba conformation on pH was investigated by CD spectroscopy (Fig. 5). Figures 5(C) and (D), respectively, show the changes in ellipticities at 293 and 222 nm with the change in pH. The near- and far-UV CD spectra of Gyuba pH between 4 and 7 are similar, suggesting that Gyuba maintained the native state in this pH range. However, the tertiary packing of the aromatic residues is largely lost at pH below 4 as shown by the less negative $[\theta]_{293}$, suggesting that the tertiary structure of Gyuba is disrupted at pH below 4. Furthermore, the ellipticity at 222 nm for Gyuba under acidic conditions is more negative than at neutral pH, suggesting that non-native α -helices are formed at acidic pH. Both BLG and ELG form non-native α -helices during an early stage of folding.^{20,22,23} However, the acid-denaturation equilibrium profiles are dissimilar for these two proteins. At acidic pH, ELG assumes a

denatured state with non-native α -helices (i.e., like Gyuba),¹⁹ whereas BLG maintains its native structure. In this regard, Gyuba behaves more like ELG than BLG.

Thermal denaturation

To investigate the stabilities of BLG, ELG, and Gyuba, thermal denaturation was monitored using CD spectroscopy at 293 nm. The thermal denaturation of BLG was irreversible, whereas that of both ELG and Gyuba was reversible (Fig. 6). The irreversibility of the BLG denaturation is caused by an interchange of the Cys residues involved in disulfide bonds and a free Cys121. Indeed, it has been shown that thermal and urea denaturation is reversible for BLG mutants in which C121 was replaced by Ala, Ser, or Val.^{24,25}

Thermodynamic parameters were calculated using the thermal denaturation curves and assuming two-state transitions for the denaturations [Eqs. (1) and (2)]. The midpoint temperatures calculated for the wild-type ELG and BLG denaturations and for the Gyuba denaturation are 70.6°C, 74.2 and 64.8°C, respectively, and the corresponding ΔH values at these temperatures are 252 ± 3 , 228 ± 5 , and 117 ± 3 kJ/mol. Thus, the thermal stability of Gyuba is considerably lower compared with wild-type ELG and BLG, perhaps a consequence of structural mismatches between the loops and secondary structures in Gyuba (see Discussion).

Dimerization of Gyuba

The apparent molecular weights of BLG, ELG, and Gyuba were measured by gel filtration. BLG, ELG, and Gyuba each eluted as a single symmetrical peak, with elution volumes corresponding to molecular weights of 34,600, 20,400, and 37,100,

Figure 6. Thermal denaturation of BLG, ELG, and Gyuba, as monitored at pH = 7.0. (A) BLG (circles), (B) ELG (squares), (C) Gyuba (triangles). Cooling curves are shown as solid lines. (D) Heating curves for the three proteins.

Table III. Association States and Association Constants, K_a

	Calculated molecular weight	Apparent molecular weight	Association constant, K_a (M^{-1})	Quaternary state
ELG	18,500	17,000	—	Monomer
BLG	18,400	17,200	9.99×10^4	Dimer
Gyuba	18,300	16,800	9.88×10^4	Dimer

respectively. Analytical ultracentrifugation was performed under the same conditions for all three LGs, and the derived apparent molecular weights and calculated association constants (K_a ; Table III) indicated that BLG and Gyuba were dimers and had comparable K_a s, whereas ELG was monomeric. Considering that LPI, in which the amino acids in the I-strand of ELG were replaced with those of BLG, was monomeric,²¹ these results suggest that regions other than the I-strand are also important for dimerization.

Discussion

Determinants for dimer formation

For the study reported herein, the loop sequences of ELG and the secondary structure sequences of BLG were combined to create the chimeric LG, Gyuba. The Gyuba structure was characterized by X-ray crystallography, CD spectroscopy, gel filtration chromatography, and analytical ultracentrifugation, all of which indicated that Gyuba dimerized in a manner similar to that of BLG. Specifically, the RMSDs for the C α s of residues between 5–60 and 67–152 (including those of the I-strand) of Gyuba and the different crystal structures of BLG are similar (Table II). Although the loops of Gyuba contain 30 residues that differ from those found in BLG, the substitutions seem to have very little effect on its secondary structure. On the other hand, the ability of Gyuba to dimerize was achieved by altering the ELG sequence at 40 positions. Because LPI is monomeric,²¹ it is apparent that, of those 40 residues, the nine substitutions surrounding the I-strand were not sufficient for dimerization. The residues responsible for dimer formation reside instead in the 31 residues of the other secondary structures. Although a high-resolution structure of ELG is unavailable, our observations suggest that at least some of the 31 substitutions change the overall arrangement of the secondary structures in ELG, thereby inhibiting dimerization. Recently, the importance of incorporation of water molecules and increase of flexibility (entropic contribution) for dimer formation of BLG was suggested from thermodynamic and structural studies.^{26,27} The 31 differences between ELG and BLG amino acid sequences may also affect hydration or dynamic properties of the molecules.

Stability of Gyuba

Although both Gyuba and BLG dimerize, the stability of Gyuba is substantially less than that of BLG and ELG (Fig. 6). Acid denaturation was common to both Gyuba and ELG and was not observed for BLG. Generally, the pH-dependent change in protein stability can be explained by differences in the pK_a values of the native and denatured states²⁸ and may be associated with an Asp or Glu carboxyl in the ELG sequence (Asp9, Glu14, Glu34, Glu77, Glu81, and/or Asp88) that has an abnormally low pK_a in the native structure. We predicted the pK_a values of these residues using the Gyuba crystal structure and PROPKA 3.0,²⁹ but we found them to be within the expected range for Asp and Glu, that is, 3.96–5.44. Thus, we could not identify a residue(s) responsible for acid-induced denaturation.

It would be worthwhile to mention the predicted pK_a value of Glu89, which is located at the EF loop and implicated in the so-called Tanford transition, was very high (8.11). In the crystal structure of Gyuba, the side chain of Glu89 was buried like as the corresponding residue of BLG with the closed loop conformation. Although Asn88 of the EF loop of BLG was replaced with Asp in Gyuba, this substitution does not affect the conformational property of the EF loop.

Even at neutral pH, Gyuba was much less stable than BLG or ELG, possibly because of the substitutions at positions 121, because of the loop/secondary structure interactions, and/or because of the conformational change around Cys66–Cys160 disulfide in Gyuba. In previous report, it was revealed that a Cys121Ala mutation reduced the stability of BLG by 6.5 kJ/mol, although it improved the reversibility.²⁵ Because Gyuba has the same substitution, it must be at least partly responsible for destabilization of Gyuba. However, influence of Cys121Ala mutation on the cooperativity (m value) seems to be less than that of Cys121Ser or Cys121Val mutation.²⁵ An interaction between Pro126 (in the loop that connects the H-strand and α -helix) and Tyr20 (in the A-strand) is found in BLG, whereas a corresponding interaction is absent in Gyuba because residue 126 is Gln. That interactions between proline and aromatic residues contribute to protein stability have been suggested by studies on the HP domain³⁰ and exendin-4.³¹ In addition, a statistical study³² showed that 45% of all prolines in the surveyed proteins interact with aromatic residues, which suggests that prolines facilitate tertiary interactions. Thus, it is likely that the lack of a Pro at position 126 in Gyuba destabilizes its structure relative to BLG. The atoms of the disulfide-bonded pair, Cys66–Cys160, could not be fit to the electron density of the Gyuba crystal, because it was ill defined. In addition, the location of Cys66–Cys160 residues in Gyuba must differ from that in BLG, because

Met156 of Gyuba was found at the position corresponding to Cys160 of BLG. That no free thiol groups were present in Gyuba was confirmed by a 5,5'-dithiobis(2-nitrobenzoic acid) assay performed using guanidinium-HCl-denatured Gyuba (data not shown). Therefore, a potential structural polymorphism involving Cys66-Cys160 in Gyuba may be responsible for the observed smeared electron density and relative instability of its overall conformation. Although other residues may partially destabilize Gyuba, the loops must substantially contribute to the relative instability.

Notably, both the T_m and the cooperativity of the thermal denaturation curve were lower for Gyuba compared with BLG and ELG. The values of ΔH for our thermal denaturations of ELG (250 kJ/mol at 70°C) and BLG (230 kJ/mol at 74°C) are consistent with those calculated from data acquired under somewhat different conditions (270 kJ/mol at 70°C, pH = 4, and 2M urea for ELG and 290 kJ/mol at 74°C, pH = 2 for BLG).^{33,34} However, ΔH for the thermal denaturation of Gyuba (120 kJ/mol at 65°C) is $\sim 50\%$ of those values. Given that the structures of Gyuba and BLG are similar, it is unlikely that, on unfolding, the number of interactions broken (e.g., hydrogen bonds, van der Waals interactions) or the hydrophobic area exposed differs substantially between these proteins. The relatively small ΔH calculated for thermal denaturation of Gyuba may be the result of an incorrect assumption, that is, the two-state model is not an accurate description of unfolding if a metastable intermediate(s) exists during the transition. Conformational polymorphism involving Cys66-Cys160 might cause a non-two-state transition.

Finally, it is interesting to consider how evolutionary selective pressure affects protein-folding cooperativity. Baker and coworkers³⁵ used a computationally designed protein, Top7, to address this point and concluded that highly cooperative folding is likely the result of natural selection. Our results support this conclusion. Although Gyuba assumes a BLG-like conformation and dimerizes with a similar K_a , the cooperativity of its thermal denaturation is much lower than that of the naturally occurring proteins, BLG and ELG.

Materials and Methods

Materials

The sources for the enzymes, chemicals, and the kits for the molecular biological experiments have been described.¹⁸ DNA oligomers were obtained from Operon Biotechnologies (Tokyo, Japan). The purified BLG variant B was obtained from Sigma. Other chemicals were analytical grade from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise noted.

Design of the Gyuba gene and construction of its expression vector

The amino acid sequences of ELG and the BLG variant B were used to design the Gyuba gene. The gene was constructed using cassette mutagenesis.¹⁸

Expression, refolding, and purification of Gyuba

Gyuba was expressed and refolded as described for ELG.³⁶ Refolded Gyuba was purified by DEAE-Sephacrose chromatography with a linear gradient of 0–350 mM NaCl in 50 mM Tris-HCl (pH = 8.0). The fraction that contained Gyuba was further purified by gel-filtration chromatography (Sephacryl S-100 equilibrated with 5 mM NH_4HCO_3) and then by Q-Sephacrose chromatography with a linear gradient of 0–500 mM NaCl in 20 mM PIPES (pH = 7.0). Native PAGE and reverse-phase HPLC were used to assess the purity of Gyuba, which was then dialyzed against distilled water and lyophilized.

Crystallization

Purified Gyuba was dissolved in 50 mM Tris-HCl (pH = 7.0). Gyuba was crystallized by the hanging drop method with 2 μL of the protein solution and 2 μL of the reservoir solution at room temperature. Initially, Crystal Screen and Crystal Screen2 (Hampton Research, USA) were used as reservoir solutions. Crystals of Gyuba were obtained when the reservoir solutions contained 2.0M ammonium sulfate. Next, to optimize the crystallization condition, 2.0–2.5M ammonium sulfate solutions were tested. Good crystals of Gyuba were obtained with ammonium sulfate reservoir solutions between 2.0 and 2.1M.

Structural determination of Gyuba

Data were collected at AR-NW12A station at PHOTON FACTORY, Japan, with the crystal at -180°C in mixed paraffin oil/Paratone-N. Data were processed with HKL2000³⁷ and scaled using SCALA.³⁸ Data and refinement statistics are summarized in Table I.

The crystal data were processed using a $P6_5$ unit cell with cell dimensions of $a = b = 69.6 \text{ \AA}$, $c = 151.3 \text{ \AA}$, $\alpha = \beta = 90^\circ$, and $\gamma = 120^\circ$. The Matthews coefficient³⁹ was 2.9 with 57.9% solvent content, and we used the 1beb structure (a dimeric BLG) as the search model. Molecular replacement performed with Molrep⁴⁰ gave an R -factor and correlation coefficient of 0.483 and 0.518, respectively. We then replaced the bovine sequence with the Gyuba sequence. Several cycles of refinements were performed using REFMAC5⁴¹ and manual model fitting to give final R -factor and R -free of 23.4 and 28.4%, respectively, at 2- $\text{\AA}$ resolution. The final structure did not include three regions: residues 1–2, 61–66, and 158–162 as the electron density for each of these sequences was too weak to fit. The structure included 240 water molecules.

CD measurements

CD measurements were recorded using a J-720 (JASCO, Tokyo) or a Chirascan (Applied Photophysics, UK) spectropolarimeter. The solution conditions were 50 mM sodium phosphate (pH = 7.0), 25°C. Protein concentration was 10–20 μ M and was determined spectrophotometrically. The extinction coefficients used for ELG and BLG were reported values of 12,000 M⁻¹ cm⁻¹ at 280 nm¹⁹ and 17,600 M⁻¹ cm⁻¹ at 278 nm,⁴² respectively. The value for Gyuba was determined to be 17,000 M⁻¹ cm⁻¹ at 280 nm by the method of Gill and von Hippel⁴³. The cuvette path length was 1 mm for far-UV CD measurements and 10 mm for near-UV CD measurements.

A total of 10 mM citrate (ionic strengths were adjusted to 0.1M with KCl) was used for pH titrations at a pH range from 2.5 to 7.0. At pH below 2.5, 0.1M HCl/KCl was also used. A total of 20 mM PIPES (pH = 7.0) was used for the thermal denaturation experiments. Thermal denaturation curves were obtained by monitoring the changes in molar ellipticity at 293 nm. A scan rate of 2°C/min was used. The midpoint temperature, T_m , and the enthalpy change, ΔH , were calculated by fitting the following equations.

$$[\theta]_{\text{obs}} = \frac{[\theta]_N + [\theta]_D \exp(-\Delta G(T)/RT)}{1 + \exp(-\Delta G(T)/RT)} \quad (1)$$

$$\Delta G(T) = \Delta H(T_m) \left(1 - \frac{T}{T_m} \right) + \Delta C_p \left\{ T - T_m - T \ln \left(\frac{T}{T_m} \right) \right\} \quad (2)$$

where $[\theta]_{\text{obs}}$ is the observed ellipticity, and $[\theta]_N$ and $[\theta]_D$ are the extrapolated ellipticities for the native and denatured states, respectively. The Gibbs free energy change of denaturation, $\Delta G(T)$, is a function of temperature, and ΔC_p is the heat capacity change of denaturation.

Gel-filtration chromatography

Purified protein samples were dissolved in 20 mM PIPES (pH = 7.0) containing 200 mM NaCl and then loaded onto a column of Superdex 75 (GE Healthcare) at a flow rate of 0.5 mL/min. The eluates were monitored at 280 nm. Bovine serum albumin (67,000), ovalbumin (43,000), bovine carbonic anhydrase (30,000), chymotrypsinogen A (25,000), and ribonuclease A (14,000) served as molecular weight standards and were used with the elution volumes to construct a calibration curve.

Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed using a Beckman XL-A analytical ultracentrifuge at 27,000 rpm, 20°C. An An-60 Ti rotor and an aluminum-filled Epon centerpieces were used.

Before ultracentrifugation, proteins were dissolved in 20 mM PIPES (pH = 7.0) containing 200 mM NaCl and dialyzed against the same buffer. Radial distributions were analyzed as described in Ref. 21.

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