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Manipulating Monomer-Dimer Equilibrium of Bovine β -Lactoglobulin by Amino Acid Substitution*

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Bovine β -lactoglobulin, a major protein in cow's milk composed of nine β -strands (β A– β I) and one α -helix, exists as a dimer at neutral pH while it dissociates to a native monomer below pH 3.0. It is assumed that the intermolecular β -sheet formed between I-strands and salt bridges at AB-loops play important roles in dimer formation. Several site-directed mutants in which intermolecular interactions stabilizing the dimer would be removed were expressed in the methylotrophic yeast *Pichia pastoris*, and their monomer-dimer equilibria were studied by analytical ultracentrifugation. Various I-strand mutants showed decreases in K_a , suggesting that the intermolecular β -sheet is essential for dimer formation. By substituting either Asp³³ or Arg⁴⁰ on the AB-loop to oppositely charged residues (*i.e.* R40D, R40E, and D33R), a large decrease in K_a was observed probably because of the charge repulsion, which is consistent with the role of electrostatic attraction between Arg⁴⁰ on one monomer and Asp³³ on the other monomer in the wild-type dimer. However, when two of these mutants, R40D and D33R, were mixed, a heterodimer was formed by the electrostatic attraction between Arg³³ and Asp⁴⁰ of different molecules. These results suggested that protein-protein interactions of bovine β -lactoglobulin can be manipulated by redesigning the residues on the interface without affecting global folding.

Various proteins function in their complex form, and it is necessary to understand the mechanism of protein-protein interaction to clarify the functions of these proteins. Moreover, understanding protein-protein interactions would provide new insights to facilitate *de novo* design or novel approaches for the treatment of conformational diseases such as prion or Alzheimer's diseases in which protein-protein interactions play a key role.

We have been studying the monomer-dimer equilibrium of β -lactoglobulin (β -LG)¹ as a model of protein-protein interaction (1, 2). Bovine β -lactoglobulin is one of the major whey proteins that is abundant in cow's milk (3–5), although its function is unknown. β -LG consists of 162 amino acid residues and has a molecular weight of 18,400. It is a predominantly β -sheet protein consisting of nine β -strands and one major helix (6–11) containing two disulfide bonds (Cys⁶⁶-Cys¹⁶⁰ and

Cys¹⁰⁶-Cys¹¹⁹) and one free thiol group (Cys¹²¹). β -LG exists as a dimer at neutral pH while it dissociates into native monomers at acidic pH. However, this property is not common to all β -LG variants. Many β -LG variants have been found in the milk of other mammals with the exception of humans, some of which (for example, equine and porcine β -LG) are known to exist as monomers at neutral pH even though their amino acid sequences and secondary structure topologies are highly conserved.

There are several advantages to use of bovine β -LG as a model protein to study protein-protein interactions. (i) There have been many reports regarding the conformation of β -LG under various conditions. (ii) The range of association constant (K_a) observed for β -LG monomer-dimer equilibrium corresponds well with the measurable range for analytical ultracentrifugation (12). (iii) The crystal structure of β -LG dimer has been reported previously (6, 7), thus the detailed structure of the dimer interface is available (Fig. 1). With the x-ray dimer structure, we can calculate the thermodynamic parameters of monomer-dimer systems as reported previously (2) or can predict the residues that are important for dimer formation.

In this study, we investigated the monomer-dimer equilibrium of β -LG at neutral pH by preparing various mutants, which were classified as "structure-based mutants" and "sequence-based mutants." The structure-based mutants were designed on the basis of the dimer interface seen in the crystal structure, consisting of two I-strands and two AB-loops. At the interface, four hydrogen bonds are formed among I-strands of each monomer (Fig. 1D). In addition, eight hydrogen bonds are formed among AB-loops where it is assumed that the electrostatic attractions (*i.e.* salt bridges) between the positively charged Arg⁴⁰ and negatively charged Asp³³ play an important role (Fig. 1C). The substitution of these residues responsible for hydrogen bonds or electrostatic interactions would lead to changes in the monomer-dimer behavior. On the other hand, the sequence-based mutants were designed by the sequence alignment of various β -LG species. Monomeric β -LG variants have been detected in other species such as equine (14) or porcine β -LG (15). A comparison of the amino acid sequences between bovine β -LG and those of these other species indicated some mismatches, several of which are positioned on or near the dimer interface of bovine β -LG (3, 6). Therefore, it was expected that the substitution of the mismatched residues to those of equine or porcine β -LG might cause the dissociation of the dimer. We prepared various structure-based and sequence-based β -LG mutants and measured the effects of mutations on the monomer-dimer equilibrium monitored by analytical ultracentrifugation.

As expected, some mutants showed marked decreases in association constant (K_a) with no changes in the global fold. The relationship between the mutation site and its effect on K_a suggested that although I-strands contribute to dimer forma-

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¹ The abbreviations used are: β -LG, β -lactoglobulin; HPLC, high pressure liquid chromatography; hGH, human growth hormone.

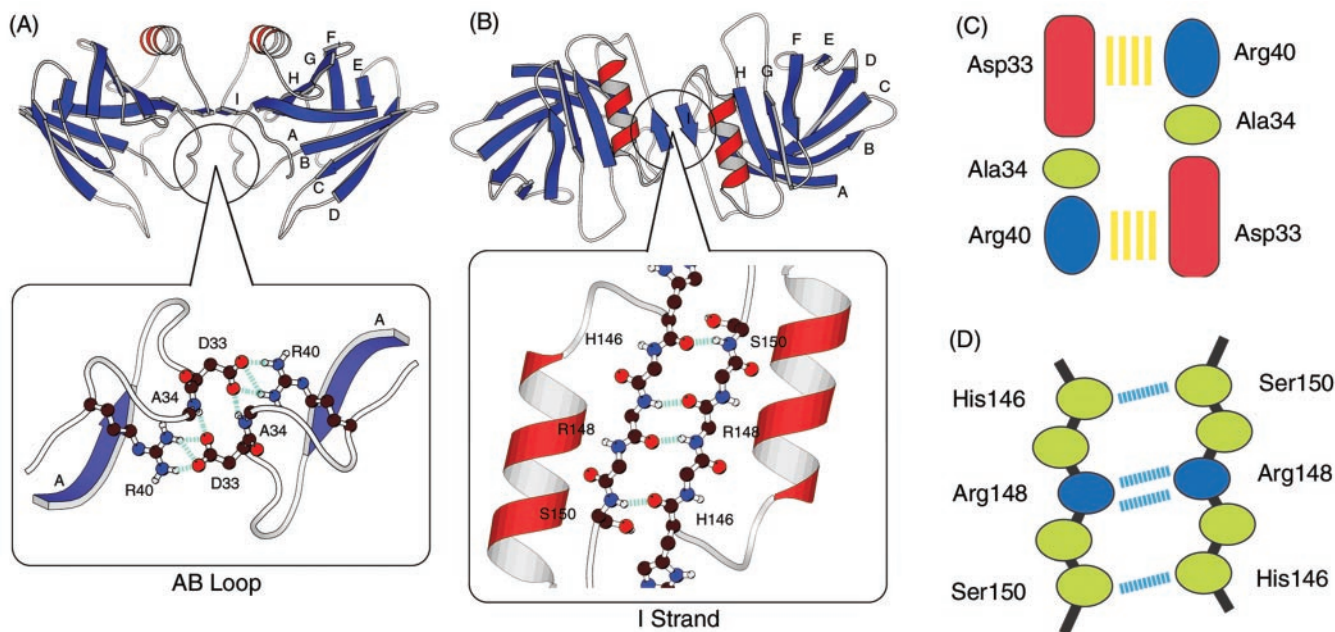


FIG. 1. **Dimer interface of β -LG determined from the x-ray structure.** Side view (A) and top view (B) with respect to β I-strands. Close views of AB-loops (A) and β I-strands (B) are also shown where intersubunit hydrogen bonds between side chains and main chains can be seen. Schematic representation of the β -LG dimer interface showing salt bridges between side chains of Arg⁴⁰ and Asp³³ at the AB-loops (C) and four hydrogen bonds between I-strand main chains (D). The diagrams in A and B were created by Molscript (13) with the Protein Data Bank code 1BEB (6).

tion by intermolecular β -sheet formation, AB-loops do so by electrostatic attraction between Arg⁴⁰ and Asp³³. Consistent with this finding, the AB-loop mutants R40D, R40E, and D33R, which had lost the ability to form homodimers because of the charge repulsion at the dimer interface, formed heterodimers by interacting with monomers with the opposite charges, i.e. R40D and D33R or R40E and D33R.

EXPERIMENTAL PROCEDURES

Strains and Vectors—*Escherichia coli* XL-I Blue was used as the host strain to construct vectors. The expression vector used was pPIC11, which is almost the same as pPIC9 with the exception of the presence of the *ori* region of the pUC plasmid. Plasmid pPIC11 containing the β -LG sequence (β -LG/pPIC11), prepared previously by the method of Kim *et al.* (16), was used as the source of wild-type β -LG gene for site-directed mutagenesis. *Pichia pastoris* GS115(*his4*) (Invitrogen) was used as the host strain for protein expression (17). All of the media used for *E. coli* and *P. pastoris* were prepared as described by Kim *et al.* (16).

DNA Manipulations and Mutagenesis—The extraction of plasmid DNA from *E. coli* cells was performed using a Wizard Plus Miniprep kit (Promega, Madison, WI). Digested plasmid DNA or PCR products were purified as described previously (16).

Mutations were introduced into plasmid β -LG/pPIC11 by two methods. First, we attempted site-directed mutagenesis using the QuikChange protocol (Stratagene, La Jolla, CA). In cases in which we could not introduce the mutations into the plasmid, we used the overlap extension method (18). To confirm introduction of the mutation, we checked the entire sequence of the β -LG gene on the plasmid using an Applied Biosystems Model 310 sequencer (Foster City, CA).

***P. pastoris* Manipulation and Protein Expression**—The plasmid obtained by gene manipulation described above was nicked using the restriction enzyme *Aat*I (Toyobo, Osaka, Japan) for transformation of *P. pastoris* strain GS115. After electroporation using a *E. coli* pulser (Bio-Rad) at a voltage of 1.7 kV, the cells were spread on RD plate medium containing no His to select transformants.

The obtained transformants were cultured on a small scale to check their protein expression. The expression quantity was checked by SDS-PAGE, and the strain with the highest level of protein expression was used for mass culture in a KMJ-2C jar fermentor (Mitsuiwa, Osaka, Japan). Cell and culture media were separated by centrifugation, and the supernatant (1 liter) was filtered using Millex-SV with a pore diameter of 5 μ m (Millipore, Bedford, MA) to remove dust or small particles. The filtered supernatant was diluted to 5 liters, and the pH was adjusted to 2.5–4.0 by adding concentrated hydrochloric acid. The

supernatant then was passed through a CM-Sepharose column equilibrated with 50 mM Gly-HCl buffer (pH 2.5–3.5) or 50 mM sodium acetate buffer (pH 4.0) to adsorb expressed β -LG mutants. For some mutants, the appropriate pH conditions were determined by a batch method. β -LG mutants were eluted with a gradient of 0–1 M NaCl. The β -LG mutant fraction was collected and dialyzed against 4 mM HCl. The dialyzed β -LG mutant solution was lyophilized. If the sample contained heterogeneous materials, the CM-Sepharose process was repeated. Purified β -LG mutants were checked by SDS-PAGE and matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy. All samples gave a single band on SDS-PAGE, and the molecular weight was within 0.1% error of the value calculated from the sequence.

CD Measurements—Far-UV and near-UV CD spectra of the β -LG mutants were measured with a Jasco J-720WI spectropolarimeter at 20 °C controlled with a Peltier thermostat (Jasco PTC-348WI). The buffer used was 20 mM sodium phosphate (pH 6.5) containing 20 mM NaCl. Near UV CD spectra were obtained at a protein concentration of \sim 1.8 mg/ml using a 5-mm quartz cell, whereas far-UV spectra were obtained at a protein concentration of \sim 0.11 mg/ml using a 2-mm cell. The CD spectra shown are averages of four measurements. The extinction coefficients of the mutants were calculated from amino acid sequences by the method of Gill and von Hippel (19).

Analytical Ultracentrifugation—Sedimentation equilibrium experiments were performed with a Beckman Optima XL-A analytical ultracentrifuge to investigate the K_a of β -LG mutants under the same conditions as the CD measurements. Sample preparation and data acquisition were performed by the same way as described in a previous study (2). The protein concentration (C_T) versus radius (r) plots were analyzed to determine K_a according to the theoretical equation for monomer-dimer equilibrium in Equation 1

$$C_T = C_M(r_0) \exp[M\phi(r^2 - r_0^2)] + (2K_a/M)[C_M(r_0)]^2 \exp[2M\phi(r^2 - r_0^2)] \quad (\text{Eq. 1})$$

where r_0 is arbitrary radius point, and $C_M(r_0)$ is the monomer concentration at r_0 , M is the molecular weight of monomer, $\phi = \omega^2(1 - \nu\rho)/(2RT)$, and R , T , and ω are the gas constant, absolute temperature, and rotor speed, respectively (20). The measurements at three different protein concentrations under the same buffer conditions were carried out using a rotor with three cells, and the results at different concentrations were analyzed by global fitting using Equation 1. Least squares curve fitting was performed using the Igor software program (WaveMetrics, Lake Oswego, OR). Sedimentation equilibrium data of a solution containing two β -LG mutants were analyzed assuming homodimer and heterodimer formation in Equation 2

TABLE I
Association constants of mutants determined by ultracentrifugation

	K_a	State	Yield
	M^{-1}		mg/liter
Wild-type	2.03×10^5	Dimer	190
Sequence-based mutant			
A34S	2.31×10^5	Dimer	72
A34T	8.25×10^4	Dimer	181
R40K	6.00×10^4	Dimer	65
W61G	1.35×10^4	In between	37
W61R	3.76×10^5	Dimer	125
Structure-based mutant			
AB loop			
D33A	2.31×10^5	Dimer	50
D28A/D33A	5.08×10^5	Dimer	125
D33R	4.21×10^3	Monomer	81
R40M	2.04×10^3	Monomer	129
R40D	3.65×10^3	Monomer	402
R40E	7.70×10^3	Monomer	75
I-strand			
H146P	8.42×10^2	Monomer	1020
R148A	9.20×10^4	Dimer	91
R148P	ND ^a	Monomer	86
S150P	1.76×10^3	Monomer	143
Heterodimerization			
D33R + R40D	1.88×10^5	Dimer	
D33R + R40E	3.86×10^5	Dimer	

^a ND, not determined because of the limits of sedimentation equilibrium analysis.

$$C_T = [C_A(r_0) + C_B(r_0)] \exp[M\phi(r^2 - r_0^2)] +$$

$$(2/M) \{K_{AA}[C_A(r_0)]^2 + K_{BB}[C_B(r_0)]^2 + K_{AB}[C_A(r_0)C_B(r_0)]\} \exp[2M\phi(r^2 - r_0^2)]$$

(Eq. 2)

where subscripts A and B refer to respective mutants, K_{AA} and K_{BB} are the association constants of homodimers, and K_{AB} is the association constant of the heterodimer.

HPLC Analysis—Gel-filtration HPLC analysis was performed with a TSK-Gel S3000W_{XL} column (Tosoh, Tokyo, Japan) at room temperature. The buffer used was 20 mM sodium phosphate (pH 6.5) containing 100 mM NaCl, and the flow rate was 0.4 ml/min. The aliquots of 20–40 μ l of sample solutions at 100 μ M (1.86 mg/ml) dissolved in the buffer were applied.

RESULTS

Conformation of Mutants—Mutant proteins, their expression yields, and results of ultracentrifugation measurements are summarized in Table I. Mutant proteins were classified into sequence-based and structure-based mutants. Sequence-based mutants were designed based on the sequence alignment of dimeric bovine and monomeric equine (14) and porcine (15) β -LG species. On the other hand, structure-based mutants were designed by examining the dimer interface of the bovine β -LG x-ray structure (3, 6). The structure-based mutants were further classified into AB-loop mutants and β I-strand mutants. For all of the mutants designed in this study, fairly good expression yields were obtained.

The far-UV CD spectrum of wild-type β -LG was characteristic of a β -sheet protein, consistent with a predominantly β -sheet structure as shown by x-ray and NMR analyses (Fig. 2). The near-UV CD spectrum of wild-type β -LG had two characteristic negative peaks of ~ 290 nm, representing a unique tertiary structure. Far- and near-UV CD spectra of each mutant were similar to those of wild-type β -LG with the exception of some mutants for which slight differences were seen in peak wavelength or intensity. From these spectra, we assumed that all of the mutants had almost the same native structure as wild-type β -LG.

Association Constants of Mutants—The results of ultracentrifugation measurements for each mutant are shown in Fig. 3 and Table I. The K_a of wild-type β -LG ($2.0 \times 10^5 M^{-1}$) is not

very high as a value for monomer-dimer equilibrium of protein-protein interaction, and the fractions of dimer at protein concentrations of 1 and 0.1 mg/ml are 80 and 51%, respectively. This relatively low K_a is related to the small area of the dimer interface (see “Discussion”). Therefore, subtle changes in the dimer interface appeared to affect critically the monomer-dimer equilibrium by changing the K_a .

Among the three types of mutants, sequence-based mutants showed only slight decreases in K_a , indicating that dimer stabilization cannot be ascribed to those interface residues of bovine β -LG that differed from the monomeric β -LG species. In contrast, some of the AB-loop and I-strand mutants showed significant decreases in K_a .

All Pro-introduced I-strand mutants (H146P, R148P, and S150P) showed a marked decrease in K_a , leading to the dissociation of the dimer. Because Pro is known to be a breaker of β -sheets, these results indicated that the introduced Pro residues disrupted the intermolecular β -sheet. In contrast, Ala-introduced mutant (R148A) showed little change in K_a , suggesting that the charge repulsion of Arg¹⁴⁸ on both I-strands does not have a functional role.

As electrostatic interactions (*i.e.* salt bridges) between Asp³³ and Arg⁴⁰ are assumed to be important for dimer formation, we substituted these residues with neutral (D33A and R40M) or oppositely charged residues (D33R, R40D, and R40E). The conversion of Asp³³ or Arg⁴⁰ to the oppositely charged residue decreased K_a . These results indicated that electrostatic repulsion at residues 33 and 40 is unfavorable for dimer formation, further suggesting that the electrostatic attraction between Asp³³ and Arg⁴⁰ contributes to the stabilization of the wild-type dimer.

Provided that there are favorable electrostatic attractions between negatively charged Asp³³ and positively charged Arg⁴⁰, the mutation of either of these two residues to a neutral residue would destabilize the dimer. In fact, D33A and R40M showed contrasting results. Whereas R40M showed a decrease in K_a , D33A did not. Because Asp²⁸ is located close to Asp³³ in the x-ray crystal structure, it was assumed that D33A mutant could form a dimer via Arg⁴⁰-Asp²⁸ interaction instead of Arg⁴⁰-Asp³³ interaction. However, this explanation was not validated, because even the D28A/D33A mutant did not show a decrease in K_a (see “Discussion”).

Heterodimer Formation—If the electrostatic interactions between Asp³³ and Arg⁴⁰ are important for the stabilization of the β -LG dimer, it was anticipated that D33R and R40D, which could not form a homodimer, would form a heterodimer in which the AB-loops with opposite charges would interact attractively.

First, ultracentrifugation was carried out to detect heterodimer formation. Sedimentation equilibrium data for a mixture of D33R and R40D indicated that the apparent molecular weight without assuming oligomer formation was close to that of the dimer. Because neither of these mutants could form dimers by themselves, it was likely that the high molecular weight component was a heterodimer of the two mutants. To estimate the K_a value of the heterodimer, we analyzed the sedimentation equilibrium data with Equation 2 in which three types of monomer-dimer equilibria were considered. Assuming that the homodimeric K_a values of D33R and R40D were the same as those measured independently for these mutants, we estimated the heterodimeric K_a to be $1.9 \times 10^5 M^{-1}$ (Table I). Similar heterodimerization was also observed for a mixture of D33R and R40E with the heterodimeric K_a value of $3.9 \times 10^5 M^{-1}$. These K_a values were comparable with that of wild-type β -LG ($2.0 \times 10^5 M^{-1}$), showing that the dimerization ability, once lost upon the introduction of the electrostatic repulsion on

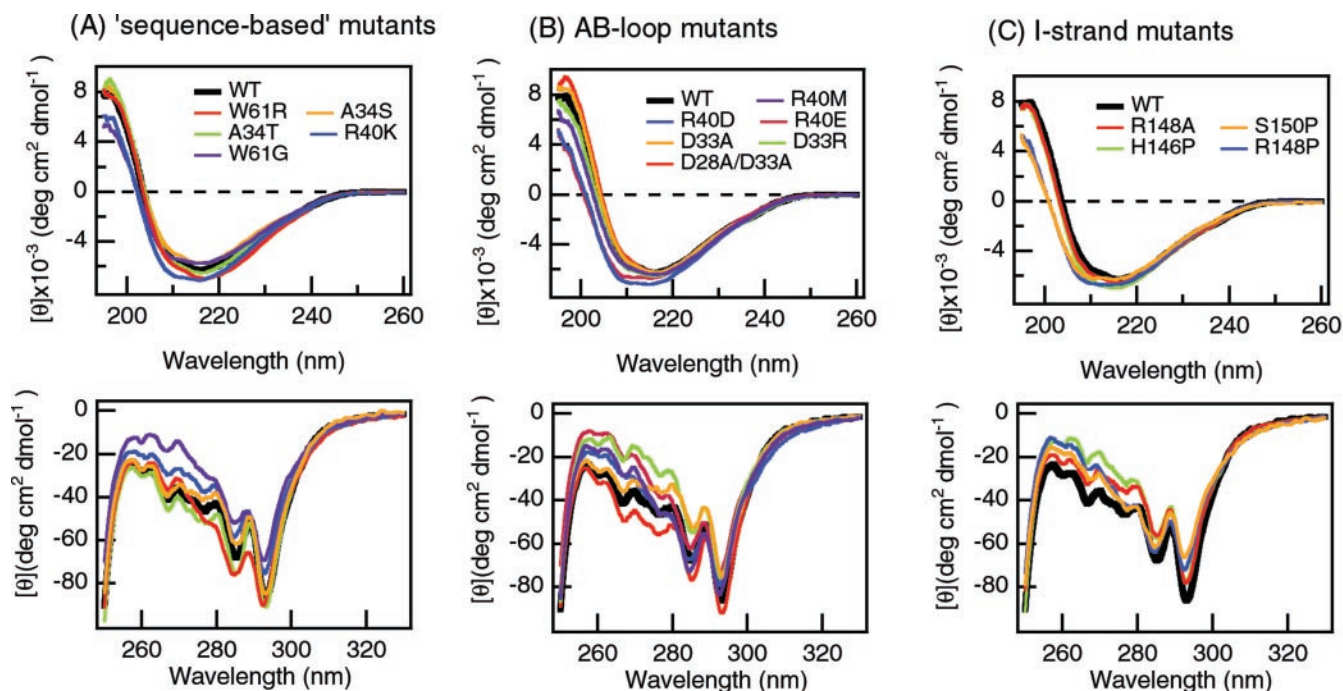


FIG. 2. Far- and near-UV CD spectra of wild-type and mutant β -LG. A–C, measurements were done in 20 mM sodium phosphate buffer (pH 6.5) containing 20 mM NaCl at 20 °C.

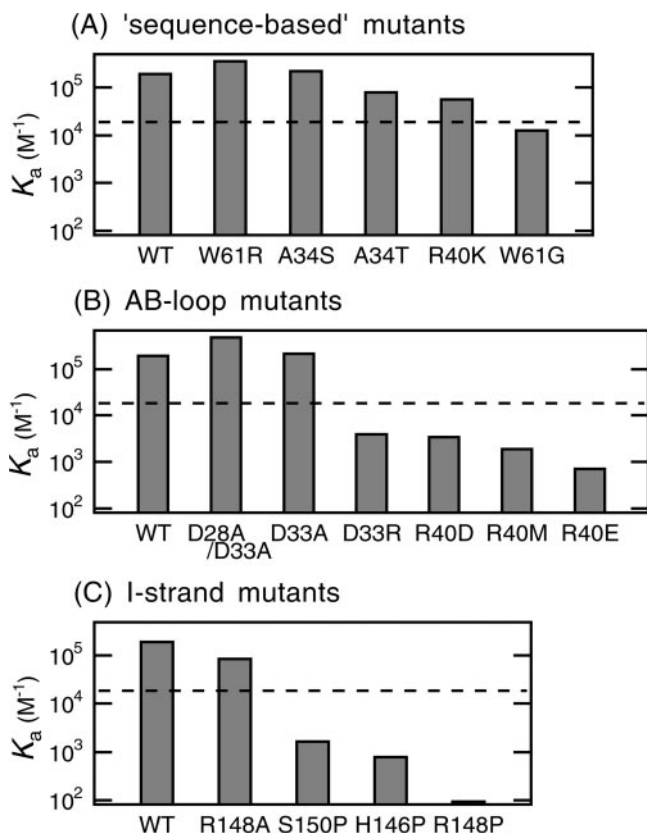


FIG. 3. K_a values of wild-type and mutant β -LG measured by analytical ultracentrifugation. A–C, the broken lines in the graphs indicate the value of K_a where dimer contents reach to 50% (w/w) at a protein concentration of 1 mg/ml.

AB-loops, was recovered by forming a heterodimer with complementary charges (Fig. 5).

Second, the heterodimer formation was examined by gel-filtration HPLC. We reported previously that β -LG monomer and dimer can be separated by gel-filtration HPLC; the β -LG

monomer eluted more slowly than the dimer (21). Heterodimer formations between D33R and R40D or between D33R and R40E were examined (Fig. 4). To prevent unfavorable electrostatic interactions between proteins and the silica matrix of column, the salt concentration of buffer (20 mM sodium phosphate, 100 mM NaCl) was slightly higher than that used for ultracentrifugation (20 mM sodium phosphate, 20 mM NaCl). We confirmed that this difference in salt concentration did not notably affect the K_a value of wild-type and mutant β -LG measured by ultracentrifugation.

A mixture of D33R and R40D at a molar ratio of 1:1 eluted faster than either mutant applied separately, and moreover, the retention time for the mixture was close to that of wild-type β -LG (Fig. 4A). This finding indicated that D33R and R40D formed a heterodimer when mixed. To examine the stoichiometry of complex formation, we further increased the amount of D33R with a fixed amount of R40D (Fig. 4B). When the molar ratio of D33R exceeded that of R40D, a peak of monomer appeared as indicated by the arrow in Fig. 4B, and its intensity seemed to represent the excess amount of D33R. The opposite results were obtained when the amount of R40D was varied at a fixed amount of D33R. The elution peaks were deconvoluted to obtain the fractions of monomer and dimer (Fig. 4C). These results indicated that D33R and R40D form a 1:1 complex, and the excess amount of either species, D33R or R40D, eluted separately as a monomer. Similar results were obtained for a combination of D33R and R40E (Fig. 4D), indicating that D33R can form a heterodimer with both R40D and R40E.

DISCUSSION

Conversion of β -LG Dimer to Monomer—Whereas bovine β -LG exists as a dimer at neutral pH, equine and porcine β -LG exist as monomers (14, 15). X-ray structures suggest that the dimer is stabilized by two I-strands and two AB-loops where hydrogen bonds, electrostatic attraction, and hydrophobic interactions play roles (Fig. 1). However, the dimer interface buries only ~ 1200 Å² of the accessible surface area per dimer, corresponding to $\sim 6\%$ of the total surface area of the isolated native structure. This value lies at the lower end of the range

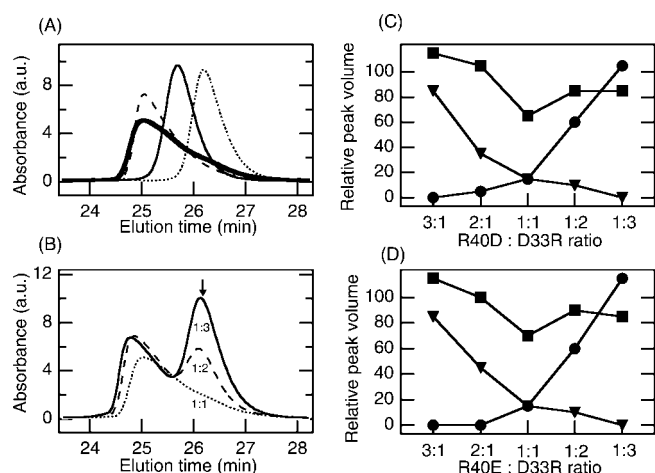


FIG. 4. Heterodimerization of β -LG mutants monitored by gel-filtration HPLC. Elution buffer was 20 mM sodium phosphate buffer (pH 6.5) containing 100 mM NaCl, and the flow rate was 0.4 ml/min. Elution was monitored by absorbance at 220 nm. A, elution profiles of D33R (100 μ M, 20 μ l, dotted line), R40D (100 μ M, 20 μ l, thin solid line), a 1:1 mixture of R40D (100 μ M, 10 μ l) and D33R (100 μ M, 10 μ l) (thick solid line), and wild type (100 μ M, 20 μ l, broken line). B, elution profiles of a mixture of R40D (100 μ M, 10 μ l) and D33R at various molar ratios. The ratios of R40D and D33R are indicated in the graph. C, fractions of the dimer (■) and monomer at various ratios of R40D (▼) and D33R (●). The fractions were estimated by deconvolution of the elution peaks. D, the results of an experiment similar to that in C at various ratios of R40E and D33R.

for strongly associated dimers (6), consistent with the relatively low value of K_a of wild-type β -LG. Thus, the mutation of the residues at the interface was expected to critically affect the monomer-dimer equilibrium.

To convert dimeric bovine β -LG to the monomeric form, we chose two approaches. By sequence-based mutation, we substituted the residues on or near the dimer interface on the basis of sequence comparison with equine and porcine β -LG (3, 6). However, little changes in K_a were seen. Recently, to convert monomeric horse β -LG to dimeric β -LG (an idea opposite to ours), Kobayashi, Ikeguchi, and Sugai (Soka University, Hachioji, Japan) prepared mutants in which the AB-loop and I-strand residues of horse β -LG were mutated to those of bovine β -LG.² Contrary to their expectations, these mutants did not form a dimer. Taken together, the difference in monomer-dimer behavior between bovine β -LG and equine/porcine β -LG cannot be explained simply by the sequence differences at the dimer interface.

On the other hand, our structure-based mutation approach successfully yielded several monomeric bovine β -LG mutants. The distortion or breaking of either the intermolecular β -sheet at I-strands or electrostatic interactions at AB-loops markedly destabilized the dimer without affecting the overall folding of β -LG as measured by CD. The importance of electrostatic interactions at AB-loops was further demonstrated by the formation of heterodimers between the two mutants, *i.e.* D33R and R40D or D33R and R40E, in which electrostatic repulsion was converted to electrostatic attraction (Fig. 5).

In our previous study (2), we characterized the thermodynamics of the salt- and temperature-dependent monomer-dimer equilibria of bovine β -LG at pH 3.0. Based on a comparison of the observed negative heat capacity change upon dimer formation that calculated from the x-ray dimer structure, we proposed that both the non-polar and polar interactions are responsible for dimer formation, although the contribution of the non-polar interaction to the heat capacity change is greater

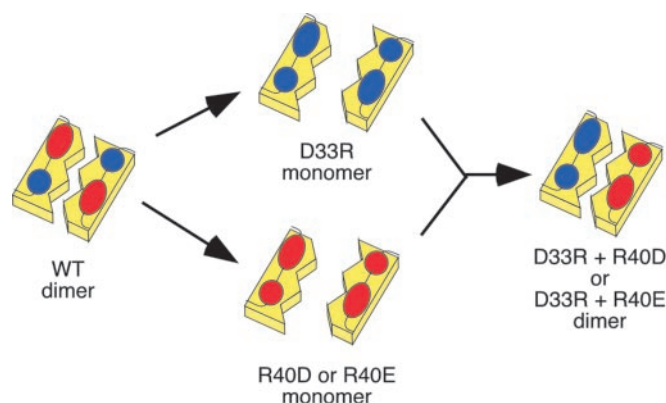


FIG. 5. Schematic model of heterodimeric β -LG formation. Substitution of Asp³³ or Arg⁴⁰ in the AB-loop with a residue with opposite charge causes a dissociation of the dimer because of electrostatic repulsion. However, when these two mutants with the opposite charges were mixed, a heterodimer was formed by the recovery of the attractive electrostatic interactions. Intermolecular interactions between β I-strands, indicated by complementary pleats, were assumed not to be altered during the dissociation and association processes.

than that of the polar interaction. The present results confirmed the participation of electrostatic interactions in dimer formation. Thus, the dimer of bovine β -LG is stabilized by the sum of various forces including overall hydrophobic interactions, hydrogen bonds at I-strands, and electrostatic attractions (*i.e.* salt bridges) as well as the consequent hydrogen bonds at AB-loops. Nevertheless, total energy stabilizing the dimer is still marginal because of the fairly small area of interaction. This results in the marked change in monomer-dimer equilibrium by point mutations as observed here.

It should be noted, however, that the K_a values of some mutants, in particular those of AB-loop mutants, were inconsistent with the simple argument on the basis of the hydrogen bonds and electrostatic effects. For example, whereas R40M showed a decrease in K_a as expected from the positive role of salt bridges between Arg⁴⁰ and Asp³³, D33A did not. These inconsistencies are probably related to marked structural changes of the mutants. One possible reason why D33A showed no decrease in K_a is that compensating interaction was created upon the loss of the electrostatic interaction between Arg⁴⁰ and Asp³³ by changing the interfacial coordinates. Such phenomena were reported in other protein-protein complexes such as barnase-barstar and lysozyme-antibody complexes (22, 23) where cavities created upon mutation were filled by water molecules to make new hydrogen bonds or to restore the interfacial packing. Thus, clarifying the x-ray structures of mutant proteins will be necessary to understand the effects of Asp³³ mutations in detail. Moreover, the x-ray structures will be important to understand the roles of various forces, *i.e.* hydrogen bonds, salt bridges, and hydrophobic interactions, in determining the monomer-dimer equilibrium as has been addressed for the stability of monomeric proteins (24). X-ray structures of heterodimers are of particular importance to demonstrate the roles of electrostatic interactions.

Heterodimer Formation—We successfully reformed the β -LG dimer by mixing mutants with complementary charges, none of which could form homodimers alone because of charge repulsion. Although there have been many studies of the complex dissociation or attenuation of interactions by mutagenesis, there have been few reports regarding the generation of new specific interactions by inducing mutations into both components of complexes. One such report was concerned with human growth hormone (hGH) and its receptor (hGHbp) (25). In this previous study (25), hGH with a single mutation that

² T. Kobayashi, M. Ikeguchi, and S. Sugai, personal communication.

prevented binding to wild-type hGHbp showed the restoration of binding ability by five point mutations in hGHbp, which resulted in the repacking of the interface. In the present study, the recovery of dimer formation was induced by a single point mutation in each monomer. This was probably related to the fact that the interface of β -LG dimer is relatively small (2, 6) and only a small number of residues are responsible for the interactions at the interface. Therefore, we consider the β -LG dimer to be an ideal model for studying protein-protein interaction.

Conclusions—The results of this study demonstrated how bovine β -LG dimers are stabilized by various interactions at the dimer interface. The intermolecular β -sheet formed between I-strands plays an important role in positioning each monomer for proper dimer assembly. Two charged residues in the AB-loop (Asp³³ and Arg⁴⁰) supply additional geometrical restrictions for dimer formation. Together with the energetic gain caused by the electrostatic interactions as well as the gain by the hydrophobic interactions, the dimer interface with specific geometry is stabilized. The dimer interaction is so marginal that it is easily destroyed by modifying either of these interactions, for example, by changing the electrostatic attraction at the AB-loop to electrostatic repulsion. Intriguingly, the two mutants with opposite charge repulsion at the AB-loop can form a heterodimer. These observations may provide new ideas to facilitate protein engineering and *de novo* design and will help in understanding protein complex formation and aggregation phenomena such as gelation and amyloidosis.

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