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The Region of α-Lactalbumin Recognized by GroEL

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The binding constants between disulfide-intact or various disulfide-reduced bovine α-lactalbumins and an *Escherichia coli* chaperonin, GroEL, were determined by using the equilibrium dialysis method. The disulfide-intact and one-disulfide (Cys6-Cys120)-reduced α-lactalbumins were shown not to bind with GroEL both in the presence and absence of Ca^{2+}. The two-disulfide (Cys6-Cys120 and Cys28-Cys111)-reduced α-lactalbumin, which has the native-like tertiary structure in its β-domain region and an unfolded α-domain in the presence of Ca^{2+}, showed considerable binding with GroEL. The binding free energy of the two-disulfide-reduced α-lactalbumin in the presence of Ca^{2+} is close to that of the molten globule state of disulfide-intact α-lactalbumin. This result suggests that GroEL binds to the unfolded α-domain of α-lactalbumin regardless of the conformation of the β-domain. The fully disulfide-reduced and two-disulfide-reduced α-lactalbumins were found to bind more strongly with GroEL in the absence of Ca^{2+} than the two-disulfide-reduced α-lactalbumin in the presence of Ca^{2+}, thus indicating that the unfolding of the β-domain of α-lactalbumin leads to stronger interaction with GroEL.

Key words: equilibrium dialysis, GroEL, α-lactalbumin, binding constant, binding site.

The *Escherichia coli* chaperonin GroEL is one of the most extensively studied molecular chaperones that is able to assist the refolding of proteins (*1-6*). GroEL is a large homotetradecamer composed of 60 kDa subunits (*7-10*) and seems to prevent off-pathway reactions of the protein folding that lead to protein aggregation (*1*). The initial step in the process of GroEL action is capture of the target protein. It has previously been reported that GroEL recognizes the α-helical conformation (*11, 12*), although its interaction with an all β-protein (*13*) has also been reported. Recently, the apical domain of GroEL has been suggested to be responsible for its interaction with substrate proteins (*14*), and this domain contains many hydrophobic residues (*7*). Many substrate structures recognized by GroEL have been shown to have considerable hydrophobic regions exposed to the solvent (like that in the molten globule or in more disordered states), from which it has been suggested that hydrophobicity is important for the GroEL recognition (*15, 16*). Also, because the binding strength between GroEL and substrate protein is influenced by salt concentration (*17, 18*), electrostatic interaction has also been suggested to affect the binding strength between GroEL and substrate proteins. Although considerable progress has been made in understanding the interaction between substrate proteins and GroEL, important questions remain concerning the conformation of the target protein recognized by GroEL. What kinds of the structural elements and features of substrate protein are recognized by GroEL? One way to elucidate this problem is to study the exact binding affinity of the protein substrate, which can assume various conformations, to GroEL.

The equilibrium dialysis method is most appropriate for studying the protein-ligand interaction. Compared to gel filtration chromatography, by which many binding affinities of GroEL to substrate proteins have previously been studied, the equilibrium dialysis method allows exact determination of the binding constant and detection of weak interactions. Also, bovine α-lactalbumin (BLA) may be a suitable substrate for the study of the recognition mechanism of the protein by GroEL (*4, 6, 17-22*). This protein has four disulfide bonds (Cys6-Cys120, Cys28-Cys111, Cys61-Cys77, and Cys73-Cys91) and binds a single Ca^{2+} (*23*) in the native state (Fig. 1). Its structure changes easily between the native and unfolded states by treatments other than changes in pH, temperature, and concentrations of denaturants and salts: (a) selective reduction of disulfide bonds, (b) the chemical modification of the reduced disulfide bonds (*24-26*), and (c) control of Ca^{2+} binding (*25, 27*). The separate contributions of hydrophobic and electrostatic interactions to the binding of GroEL with the target protein can be studied by skillful use of these processes of conformational change.

In this paper, to clarify the structural elements and features of BLA recognized by GroEL, the binding strengths of intact, and one-, two-, and four-disulfide-reduced (the reduced disulfide bonds were *S*-carboxyamidomethylated by iodoacetamide or *S*-carboxymethylated by iodoacetate) BLAs with GroEL were studied by the equilibrium dialysis method in the presence and absence of Ca^{2+} at a low temperature, 4°C. By comparing the conformations of the derivatives of BLA determined mainly from the CD data, the region of BLA that is important for recognition of GroEL was investigated.

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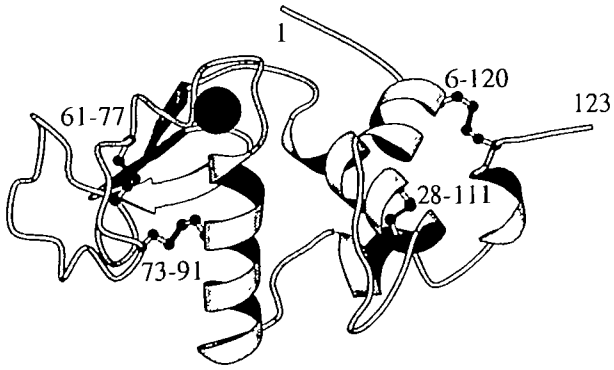

Fig. 1. **The crystal structure of α-lactalbumin (*23*) produced with the program Molscript (*37*).** Calcium is presented as a sphere and disulfide bonds are highlighted.

MATERIALS AND METHODS

Materials—BLA was prepared from fresh milk. The one-disulfide (Cys6-Cys120)-reduced and *S*-alkylated BLA ($3SS_{cm}$-BLA and $3SS_{cam}$-BLA), the two-disulfide (Cys6-Cys120 and Cys28-Cys111)-reduced and *S*-alkylated BLA ($2SS_{cm}$-BLA and $2SS_{cam}$-BLA), and the fully reduced and *S*-alkylated BLA (R_{cm}-BLA and R_{cam}-BLA) were prepared as reported previously (*24, 25, 28*). The subscripts cm and cam denote *S*-carboxylmethylated and *S*-carboxyamidomethylated, respectively. The *S*-carboxyamidomethylated and *S*-carboxylmethylated proteins were purified by reversed-phase HPLC (a column of μBONDASPHERE, 5μ, C4 300 Å) and DEAE-Sepharose chromatography, respectively. Purities of these proteins were examined by polyacrylamide gel electrophoresis. Two peptides (B14 and C17) corresponding to the B- and C-helices in BLA, respectively, were synthesized as shown in the previous studies (*29*). GroEL was obtained in a freeze-dried form from Takara Biomedicals, Ohtsu, and used without further purification. Purity of GroEL was examined by SDS polyacrylamide gel electrophoresis and gel filtration under denaturing conditions (a column of TSK-gel G3000SW).

Equilibrium Dialysis—Equilibrium dialysis was performed at 4°C with a dialysis tube (Spectra/por), with a molecular weight cutoff of about 100,000 (for BLA and its derivatives) or 25,000 (for the peptides). To obtain the binding data, 0.4 ml of about 5 μM GroEL solution containing 20 mM Tris-HCl, 200 mM KCl, and 1 mM $CaCl_2$ (or 1 mM EDTA) at pH 7.2 was dialyzed against 25 ml of 0.05-20 μM substrate in the same buffer for 120 h.

Determination of Binding Constants—After equilibration of dialysis, an equivalent volume of 8 M guanidine hydrochloride (GdnHCl) solution was added to the solution inside and outside the tube, and GroEL and the substrate in the 4 M GdnHCl solution were separated by HPLC (a column of TSK-gel G3000SW, and an elution buffer of 20 mM potassium phosphate, 10 mM mercaptoethanol, and 4 M GdnHCl at pH 6.0). Concentrations of BLA, its derivatives, and GroEL were determined from the corresponding peak areas on the elution profile of HPLC detected at 225 nm. The peak area was calibrated by applying a solution of a known concentration of GroEL or BLA. The concentrations of BLA and GroEL were spectrophotometrically determined using the molar extinction coefficients ε_{280} = 27,700 $M^{-1}\cdot cm^{-1}$ (*30*) and ε_{280} = 125,440 $M^{-1}\cdot cm^{-1}$ in 6 M GdnHCl solution, respectively. The ε_{280} value of GroEL were determined from the amino acid composition by the method of Gill and von Hippel (*31*). The molar extinction coefficients of the disulfide-reduced BLAs were assumed to be identical with that of BLA, because the reported influence of the disulfide reduction on ε_{280} was within 3% (*24, 28*).

CD Measurements—CD measurements were made at 5°C using a Jasco J-720 spectropolarimeter that was calibrated with *d*-10-camphorsulfonic acid. Quartz cells with path lengths of 1 and 0.1 cm were used for near (250-350 nm)- and far (190-250 nm)-UV regions, respectively. The mean residue ellipticity, $[\theta]$, was calculated from the equation $[\theta]=\theta/(10lcN)$, where θ is the observed ellipticity in mdeg, l the path length in cm, c molar concentration of the protein, and N the number of amino acid residues in the sequence.

RESULTS

Determination of Equilibration Time—To determine the time required to attain dialysis equilibrium, about 50 μM BLA was dialyzed against a buffer, and the time dependence of the BLA concentration outside the tube was obtained (Fig. 2). The BLA concentration of the exterior solution was found to reach an almost constant value after about 100 h, at which time the BLA concentration inside the tube was almost equal to that outside. The dialysis for about 120 h, therefore, was found to be sufficient to attain the equilibrium.

The Binding Constant of BLA and Its Derivatives with GroEL—Although the equilibrium dialysis method can detect a weak protein-protein interaction in principle (even with a binding constant of less than 10^3 M^{-1}), a very high GroEL concentration is required to investigate such a weak interaction. Under our experimental conditions, we can accurately estimate a binding constant larger than 10^4 M^{-1}. First, we estimated roughly the binding constant K_b by dialyzing about 5 μM GroEL against 2-5 μM disulfide-intact or -reduced BLA solutions and found that these concentrations are sufficient to reliably estimate K_b in our study. When the roughly estimated K_b was larger than 10^4 M^{-1}, the exact K_b was calculated from the binding data for at least at four different concentrations of the substrate as shown in Fig. 3. The binding constant K_b of the substrate protein with GroEL was calculated by fitting the data to the following equation, assuming that GroEL and the substrate proteins interact with a stoichiometry of 1:1 (*17*)

$$\upsilon=\frac{[L]K_b}{1+[L]K_b}, \quad (1)$$

where υ and [L] are the average number of bound substrate molecules per GroEL molecule and free substrate concentration, respectively: $\upsilon=([BLA]_i-[BLA]_o)/[GroEL]_{total}$ and $[L]=[BLA]_o$. [ ] means the concentration, and i and o express inside and outside the dialysis tube, respectively. The K_b values calculated from Eq. 1 are shown in Table I. The binding affinity to GroEL of the disulfide-intact BLA or its apo form (in the presence of 1 mM EDTA) is concluded to be very weak: the K_b is less than 10^4 M^{-1} (data not shown in Table I). $3SS_{cm}$-BLA and $3SS_{cam}$-BLA are known to form the native-like structure at low temperatures, and the CD

spectra of our 3SS-derivatives shown later are consistent with the previous result (*32*). Our experiments showed that the K_b values of the $3SS_{cm}$-BLA and $3SS_{cam}$-BLA were also less than 10^4 M^{-1} (not shown in Table I), and their binding affinities were considered to be almost the same as that of intact BLA. Thus, we did not determine exactly the K_bs of the three disulfide derivatives in this study. On the other hand, the K_bs of the two-disulfide- and fully reduced derivatives are considerably large. In particular, the structures of the two-disulfide derivatives (2SS-BLA) have previously been shown to depend on $[Ca^{2+}]$ (*25, 33*), and the Ca^{2+}-dependent change in the binding affinity of GroEL for 2SS-BLA seems to be important in relation to the dependence of the binding on the target protein conformation. In this study, the binding of the fully disulfide-reduced derivatives (R-BLA) with GroEL was investigated only in the absence of Ca^{2+}, because R-BLA is known to be completely disordered (*34*). The binding behavior of R-BLA depends largely on the chemically modified group of the reduced disulfide bonds. Although the K_b of the R_{cam}-BLA is very large (10^7 M^{-1}), suggesting the strong binding with GroEL, the binding of the R_{cm}-BLA is not so strong: it is in the same order as that of 2SS-BLA in the presence of Ca^{2+}. Generally, the binding of *S*-carboxyamidomethylated derivatives of BLA is stronger than that of the corresponding *S*-carboxymethylated derivatives under the same experimental conditions. Also, to address whether or not the α-helical regions formed in the molten globule state are recognized by GroEL, we examined the binding of two peptides (B14 and C17) with GroEL. The B14 and C17 peptides correspond to the B- and C-helix regions of BLA, and they have been shown to remain even in the molten globule form of BLA. Although, as shown in the previous paper (*29*), the B14 and C17 peptides are in a random coil state under the present experimental conditions, the helical structure may be

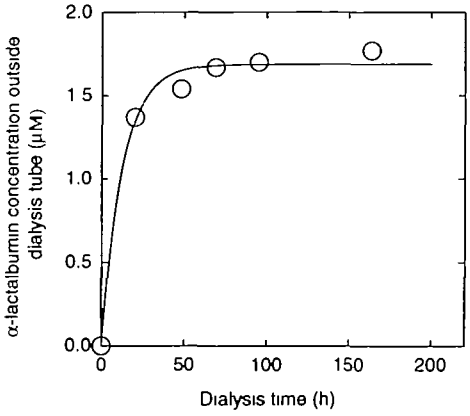

Fig. 2. **Time dependence of the BLA concentration outside the dialysis tube in 20 mM Tris-HCl, 200 mM KCl buffer (pH 7.2) at 4°C.**

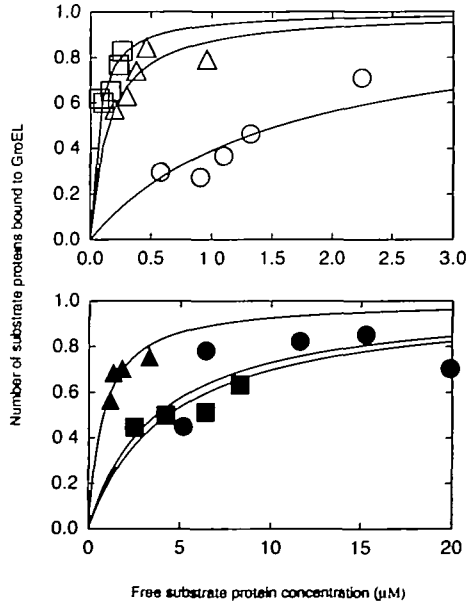

Fig. 3. **Binding isotherms of disulfide-reduced BLAs and GroEL.** The conditions of the equilibrium dialysis experiments were in 20 mM Tris-HCl, 200 mM KCl buffer (pH 7.2) containing 1 mM $CaCl_2$ or 1 mM EDTA at 4°C. ○, $2SS_{cam}$-BLA containing 1 mM $CaCl_2$; △, $2SS_{cam}$-BLA containing 1 mM EDTA; □, R_{cam}-BLA containing 1 mM EDTA; ●, $2SS_{cm}$-BLA containing 1 mM $CaCl_2$; ▲, $2SS_{cm}$-BLA containing 1 mM EDTA; ■, R_{cm}-BLA containing 1 mM EDTA. The lines are fitting curves based on Eq. 1.

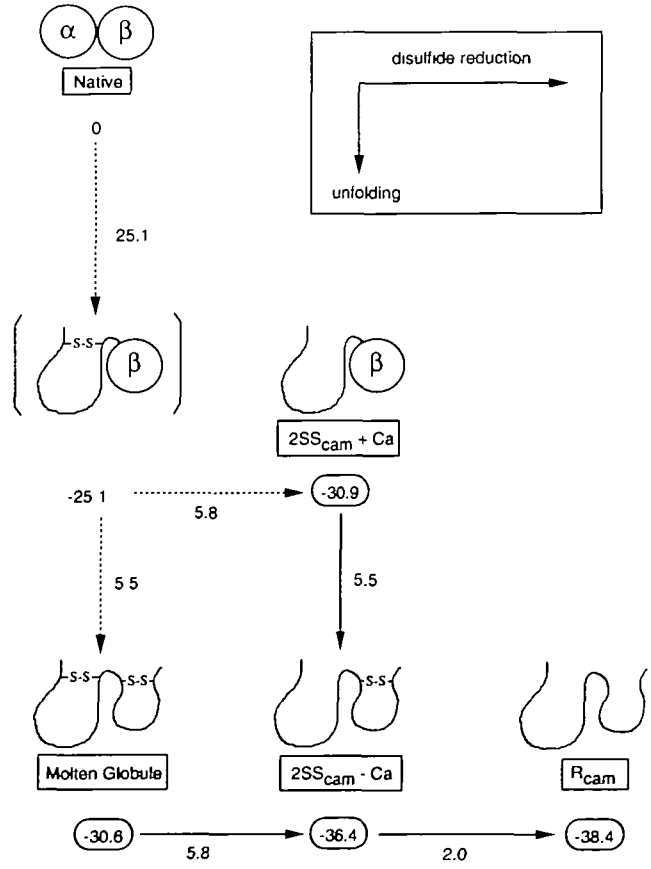

Fig. 4. **The binding free energy, ΔG (kJ·mol^{-1}), between BLAs and GroEL.** The numbers show the ΔG, and the observed values are enclosed within an ellipse. The α and β enclosed with a circle show the folded α and β domains, respectively. The string indicates the unfolded domain. This diagram is drawn by assuming that the structural change of substrate protein that accompanies the binding to GroEL is negligible, as in the case of other proteins (*23, 38*).

induced by binding to GroEL. The binding constants of both peptides with GroEL, however, were found to be less than $10^4\ M^{-1}$.

We calculated the binding free energy ΔG as a quantitative measure of the binding interaction between BLA and GroEL.

$$\Delta G = -RT\ln(K_b) \quad (2)$$

Table I and Fig. 4 show ΔG calculated from K_b. As described above, weak binding with a larger ΔG than -21 kJ·mol^{-1} (corresponding to a K_b of less than $10^4\ M^{-1}$) cannot be detected under our experimental conditions.

The Structures of Disulfide-Reduced α-Lactalbumins—To clarify the profile of BLA recognized by GroEL, the structures of the derivatives of BLA used here were studied at 5°C. The near-UV CD spectrum is related to the tertiary conformation, and the far-UV CD spectrum mainly to the secondary structure. Figure 5, A and B, shows the CD spectra of various derivatives of BLA with spectra of the native and fully unfolded (in 8 M urea) BLA. The far-UV spectrum of native BLA shows the typical spectrum (double minima at 208 and 222 nm) of an α-helical protein, and BLA in 8 M urea shows the typical unfolded spectrum. The 3SS-BLA has been reported to assume almost the native form in the presence of Ca^{2+} (*25*), and moreover, the structure of apo 3SS-BLA is shown to be largely dependent on conditions. At 5°C, both $3SS_{cm}$-BLA and $3SS_{cam}$-BLA used here assume native-like secondary and tertiary structures both in the absence and presence of Ca^{2+} (data not shown). On the other hand, 2SS- and R-BLA assume non-native structures, and the structure of 2SS-BLA depends largely on [Ca^{2+}]. The tertiary structures of 2SS-BLA and R-BLA in the absence of Ca^{2+} appeared to be virtually unfolded as judged by their near-UV CD spectra, which are similar to that of the unfolded BLA. In contrast, the far-UV spectra of these derivatives of BLA are largely different from that of the unfolded BLA, indicating that 2SS- and R-BLA in the absence of Ca^{2+} possess some residual secondary structures, although they are in more unfolded states than the typical molten globule of intact BLA (*35,*

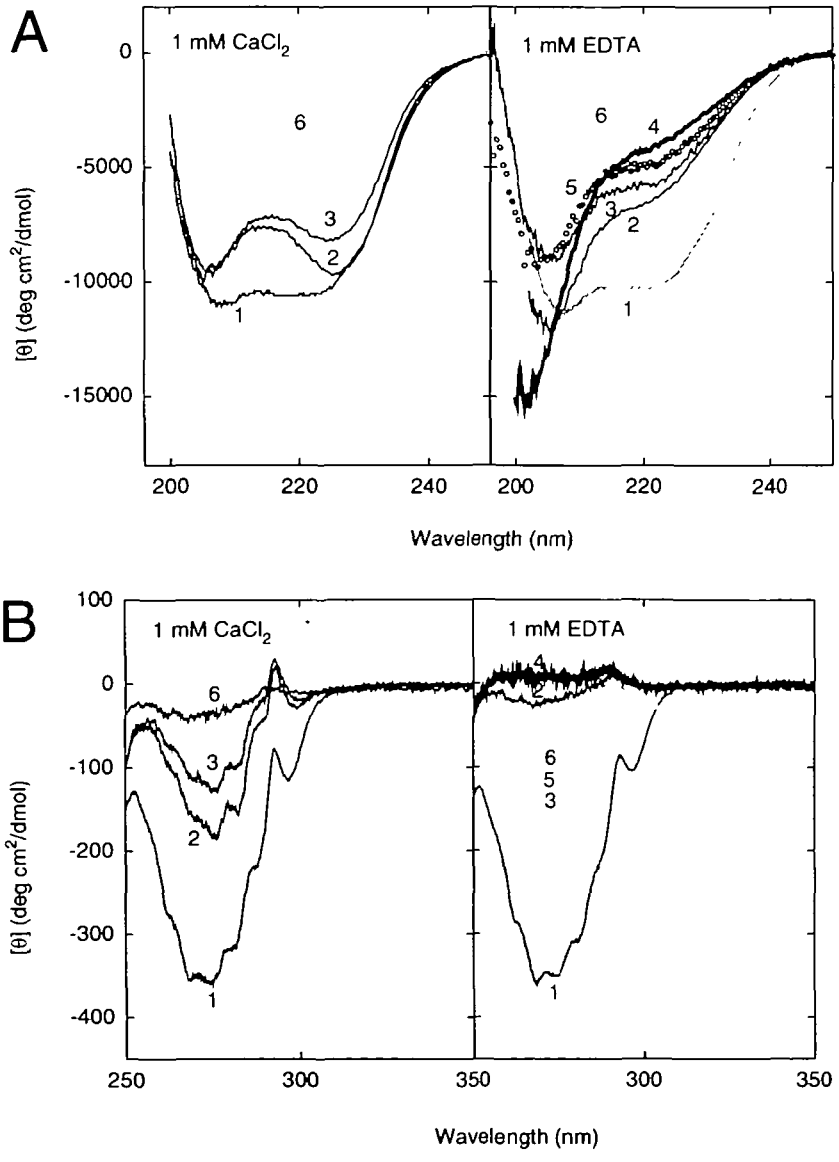

Fig. 5. Far-UV (A) and near-UV (B) CD spectra of disulfide-reduced BLAs in 20 mM Tris-HCl, 200 mM KCl buffer (pH 7.2) containing 1 mM $CaCl_2$ or 1 mM EDTA at 5°C. 1, BLA; 2, $2SS_{cm}$-BLA; 3, $2SS_{cam}$-BLA; 4, R_{cm}-BLA; 5, R_{cam}-BLA; 6, BLA in 8 M urea.

36). Although the residual secondary structures of $2SS_{cam}$-, $2SS_{cm}$-, R_{cam}-, and R_{cm}-BLA differ slightly in the absence of Ca^{2+}, the structures of these derivatives are essentially similar. On the other hand, in the presence of 1 mM Ca^{2+}, $2SS_{cam}$- and $2SS_{cm}$-BLA assume some tertiary structure as judged by their near-UV CD spectra.

DISCUSSION

In this study, the binding constants of BLA and its derivatives to GroEL have been investigated by means of equilibrium dialysis. The binding of BLA and its three-disulfide derivatives has not been detected in the absence and presence of Ca^{2+}, indicating that the binding constant of GroEL with these proteins is less than $10^4\ M^{-1}$. Because these proteins assume a folded conformation under the conditions used in this study (20 mM Tris-HCl, 200 mM KCl, pH 7.2, 4°C), it is concluded that GroEL does not bind with the folded proteins. This is consistent with the previous results (*6, 19*). The strong binding of fully disulfide-reduced α-lactalbumin has been reported by many groups (*6, 19-22*). Of these studies, however, the binding constant was reported only for reduced and non-alkylated BLA (R_{SH}-BLA). Because the binding constant obtained in this study is that for reduced and alkylated BLA (R_{cm}-BLA and R_{cam}-BLA), our results can't be directly compared with the reported values for R_{SH}-BLA. Nevertheless, it is interesting to compare the binding constant for R_{cam}-BLA obtained in this study with the reported values for R_{SH}-BLA, because Hayer-Hartl *et al.* (*19*) have shown that GroEL has similar affinities to R_{SH}-BLA and R_{cam}-BLA. Murai *et al.* (*6*) have determined the binding constant of GroEL to R_{SH}-BLA to be $10^9\ M^{-1}$ using a BIAcore™ system. On the other hand, Aoki *et al.* (*22*) have obtained a significantly different value ($10^5\ M^{-1}$) by means of isothermal titration calorimetry. The binding constant for R_{cam}-BLA obtained here ($10^7\ M^{-1}$) is intermediate between those obtained from BIAcore™ and calorimetry. On the other hand, Hayer-Hartl *et al.* (*19*) have shown that GroEL does not bind with R_{cm}-BLA. In this study, however, we have observed the binding of R_{cm}-BLA to GroEL, although it is relatively weak ($K_b = 2.3 \times 10^5\ M^{-1}$). The binding constant of R_{cm}-BLA is slightly lower than that of $2SS_{cam}$-BLA, only 15% of which was detected by chromatography to bind to GroEL (*19*). The binding of R_{cm}-BLA to GroEL, therefore, may not be detected by the chromatographic method used by Hayer-Hartl *et al.* (*19*). The weaker binding of R_{cm}-BLA contrasts with the strong binding of R_{cam}-BLA. Hayer-Hartl *et al.* (*19*) have argued that R_{cm}-BLA has lost most of the characteristic properties of the molten globule due to the repulsive forces resulting from the introduction of eight additional negative charges, so that it had little affinity for GroEL. It has been shown, however, that the affinity of the molten globule form of disulfide-intact BLA for GroEL depends on the salt concentration, and that this salt effect is ascribable to the screening of the repulsive forces between negatively charged GroEL (−18 per monomer at pH 7) and BLA (−7 at pH 7) (*17*). Because R_{cm}-BLA has eight additional negative charges, the electrostatic repulsion between R_{cm}-BLA and GroEL is expected to be much stronger than that between R_{cam}-BLA and GroEL. Therefore, the lower affinity of GroEL for R_{cm}-BLA is not necessarily due to the expanded structure of R_{cm}-BLA. In fact, $2SS_{cm}$-BLA, which has four more negative charges than $2SS_{cam}$-BLA, shows 2-5 times lower affinity for GroEL than $2SS_{cam}$-BLA, although $2SS_{cm}$-BLA and $2SS_{cam}$-BLA show similar CD spectra.

The binding constants of 2SS-BLA obtained here are very useful for understanding the region of the BLA molecule recognized by GroEL. The α-lactalbumin molecule is composed of two sub-domains (*23*). The α-domain comprises residues 1-34 and 86-123 and contains four α-helices. The β-domain comprises residues 35-85, and this region assumes little secondary structure except for a small β-sheet. It has been shown that $2SS_{cm}$-BLA maintains a native-like tertiary structure in its β-domain and Ca^{2+}-binding site including the C-helix (86-98) in the presence of Ca^{2+} (*28, 33*, Ikeguchi *et al.*, unpublished results). In the following discussion, these structured regions in the Ca^{2+}-bound $2SS_{cm}$-BLA are defined as the β-domain (see Ref. *28*). Because the CD spectrum of $2SS_{cam}$-BLA is similar to that of $2SS_{cm}$-BLA (Fig. 5), the structure of $2SS_{cam}$-BLA is thought to be similar to that of $2SS_{cm}$-BLA. If GroEL binds both $2SS_{cam}$-BLA and $2SS_{cm}$-BLA in the presence of Ca^{2+} without changing their structures, then the binding probably involves recognition by GroEL of the unfolded α-domain of these molecules. As the CD spectrum of 2SS-BLA shows the presence of some residual secondary structures and is considerably different from that of unfolded BLA in 8 M urea, the unfolded α-domain does not represent the fully unfolded state but a more unfolded state than the typical molten globule of disulfide-intact BLA. The binding strengths of both $2SS_{cam}$-BLA and $2SS_{cm}$-BLA increase when the bound Ca^{2+} is depleted so that the β-domain is unfolded. This result indicates that the interaction between the unfolded β-domain and GroEL also contributes to the binding of 2SS-BLA and GroEL. The reduction of the number of disulfide bonds in the β-domain further increases the affinity of BLA for GroEL as indicated by the large binding constant of R_{cam}-BLA. It is interesting to consider the energetic contribution of the interaction of individual parts of the BLA molecule with GroEL as shown in Fig. 4. Katumata *et al.* (*17, 18*) have investigated in detail the effect of GroEL on the refolding rate of disulfide-intact BLA and have determined the binding constant of the molten globule form of disulfide-intact BLA as a function of temperature and ionic strength. Based on their data, the binding constant of the molten globule form of disulfide-intact BLA is estimated to be $6 \times 10^5\ M^{-1}$ under our experimental conditions. Therefore, the free energy change accompanying the binding of the molten globule form of BLA to GroEL is $30\ kJ \cdot mol^{-1}$. Because the contribution of the β-domain unfolding is estimated to be $5.5\ kJ \cdot mol^{-1}$ based on the difference in the binding free energy between Ca^{2+}-bound $2SS_{cam}$-BLA and apo $2SS_{cam}$-BLA (Fig. 4), the contribution of the unfolded and disulfide-intact α-domain is expected to be $25\ kJ \cdot mol^{-1}$, if the contribution of the β-domain unfolding is independent of the disulfide bond formation in the unfolded α-domain and if it is assumed that the interaction energy between the folded β-domain and GroEL is zero. Of the entire interaction between the molten globule form of disulfide-intact BLA and GroEL, therefore, 80% is attributed to the interaction between the α-domain and GroEL. If the interaction energy between the folded β-domain and GroEL is zero, the contribution of the β-do-

TABLE I. **Binding constant and binding free energy of α-lactalbumin and disulfide-reduced α-lactalbumin for GroEL.**

	Binding constant[a] ($\times 10^5$ M^{-1})		Binding free energy (kJ·mol^{-1})	
	1 mM $CaCl_2$	1 mM EDTA	1 mM $CaCl_2$	1 mM EDTA
$2SS_{cam}$-BLA	6.4±0.9	69±12	−30.9±0.3	−36.4±0.3
$2SS_{cm}$-BLA	2.7±0.8	12.5±1.4	−28.9±0.7	−32.4±0.3
R_{cam}-BLA		161±22		−38.4±0.3
R_{cm}-BLA		2.3±0.3		−28.5±0.3

[a]The binding constants are calculated by assuming that BLA and disulfide-reduced BLAs bind with GroEL in a 1:1 ratio, as described in detail in the text.

main portion to the binding free energy of R_{cam}-BLA is estimated to be 7.5 kJ·mol^{-1} based on the difference in the binding free energy between R_{cam}-BLA and $2SS_{cam}$-BLA in the presence of Ca^{2+} (Table I and Fig. 4). Therefore, of the entire interaction between R_{cam}-BLA and GroEL, 80% is also attributed to the interaction between the α-domain portion and GroEL. Although these contributions of the α-domain may be overestimated, we conclude that the α-domain make a larger contribution to the interaction between unfolded BLA and GroEL. This conclusion is consistent with the observation that a chymotryptic fragment of BLA (54-104), which contains only the β-domain, is unable to bind to GroEL, whereas a larger fragment (17-108) associates with GroEL (*19*).

A short 13-residue peptide corresponding to an α-helix region in rhodanese is known to bind with GroEL (*11*). Also, Okazaki *et al.* (*21*) have proposed that the C-helix region of α-lactalbumin that does not form the secondary structure is the site of R_{SH}-BLA that is strongly recognized by GroEL, because it is positively charged and shows high hydrophobic indices. Therefore, the unstructured helical parts of α-lactalbumin are expected to bind to GroEL. However, the binding of B14 and C17 peptides was not detected, indicating that the binding constant of these peptides with GroEL is less than 10^4 M^{-1}. Because these peptides assume the unfolded conformation in aqueous solution (*29*), it is concluded that the sequences of the B- and C-helix regions of α-lactalbumin are not specifically recognized by GroEL. Nevertheless, this result does not necessarily indicate that the secondary structure elements of α-lactalbumin are not recognized by GroEL. Because the proteins recognized by GroEL are not completely unfolded, interaction with other parts of the protein may be required for efficient binding of the secondary structure elements to GroEL.

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