

Salt-dependent monomer–dimer equilibrium of bovine β -lactoglobulin at pH 3

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Abstract

Although bovine β -lactoglobulin assumes a monomeric native structure at pH 3 in the absence of salt, the addition of salts stabilizes the dimer. Thermodynamics of the monomer–dimer equilibrium dependent on the salt concentration were studied by sedimentation equilibrium. The addition of NaCl, KCl, or guanidine hydrochloride below 1 M stabilized the dimer in a similar manner. On the other hand, NaClO₄ was more effective than other salts by about 20-fold, suggesting that anion binding is responsible for the salt-induced dimer formation, as observed for acid-unfolded proteins. The addition of guanidine hydrochloride at 5 M dissociated the dimer into monomers because of the denaturation of protein structure. In the presence of either NaCl or NaClO₄, the dimerization constant decreased with an increase in temperature, indicating that the enthalpy change (ΔH_D) of dimer formation is negative. The heat effect of the dimer formation was directly measured with an isothermal titration calorimeter by titrating the monomeric β -lactoglobulin at pH 3.0 with NaClO₄. The net heat effects after subtraction of the heat of salt dilution, corresponding to ΔH_D , were negative, and were consistent with those obtained by the sedimentation equilibrium. From the dependence of dimerization constant on temperature measured by sedimentation equilibrium, we estimated the ΔH_D value at 20°C and the heat capacity change (ΔC_p) of dimer formation. In both NaCl and NaClO₄, the obtained ΔC_p value was negative, indicating the dominant role of burial of the hydrophobic surfaces upon dimer formation. The observed ΔC_p values were consistent with the calculated value from the X-ray dimeric structure using a method of accessible surface area. These results indicated that monomer–dimer equilibrium of β -lactoglobulin at pH 3 is determined by a subtle balance of hydrophobic and electrostatic effects, which are modulated by the addition of salts or by changes in temperature.

Keywords: β -Lactoglobulin; monomer–dimer equilibrium; ultracentrifuge; heat capacity change

Bovine β -lactoglobulin (β -LG) is the major whey protein abundant in cow's milk, but not present in human mother's

milk (Godovac-Zimmermann 1988; Hambling et al. 1992; Sawyer and Kontopidis 2000). β -LG consists of 162 amino acid residues (18 kD), and contains two disulfide bonds (Cys66–Cys160 and Cys106–Cys119) and a free thiol (Cys121). It is a predominantly β -sheet protein consisting of nine β -strands (A–I) and one major α -helix at the C-terminal end of the molecule (Fig. 1) (Brownlow et al. 1997; Qin et al. 1998a, 1998b; Kuwata et al. 1998, 1999; Uhrínová et al. 2000). A–H β strands form an up-and-down β -barrel with a hydrophobic cavity inside, where hydrophobic compounds such as retinol bind. Bovine β -LG forms a dimer at neutral pH, while the monomeric native state is stable at pH values below 3. The dimer is stabilized by hydrogen bonds

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Abbreviations: ASA, accessible surface area; β -LG, β -lactoglobulin; CD, circular dichroism; ΔC_p , heat capacity change of dimer formation; $\Delta C_{p,h}$, hydration heat capacity change of dimer formation; ΔG_D , Gibbs free energy change of dimer formation; ΔH_D , enthalpy change of dimer formation; ΔS_D , entropy change of dimer formation; Gdn-HCl, guanidine hydrochloride; HSQC, heteronuclear single quantum coherence; ITC, isothermal titration calorimeter; K_D , dimerization constant; NMR, nuclear magnetic resonance.

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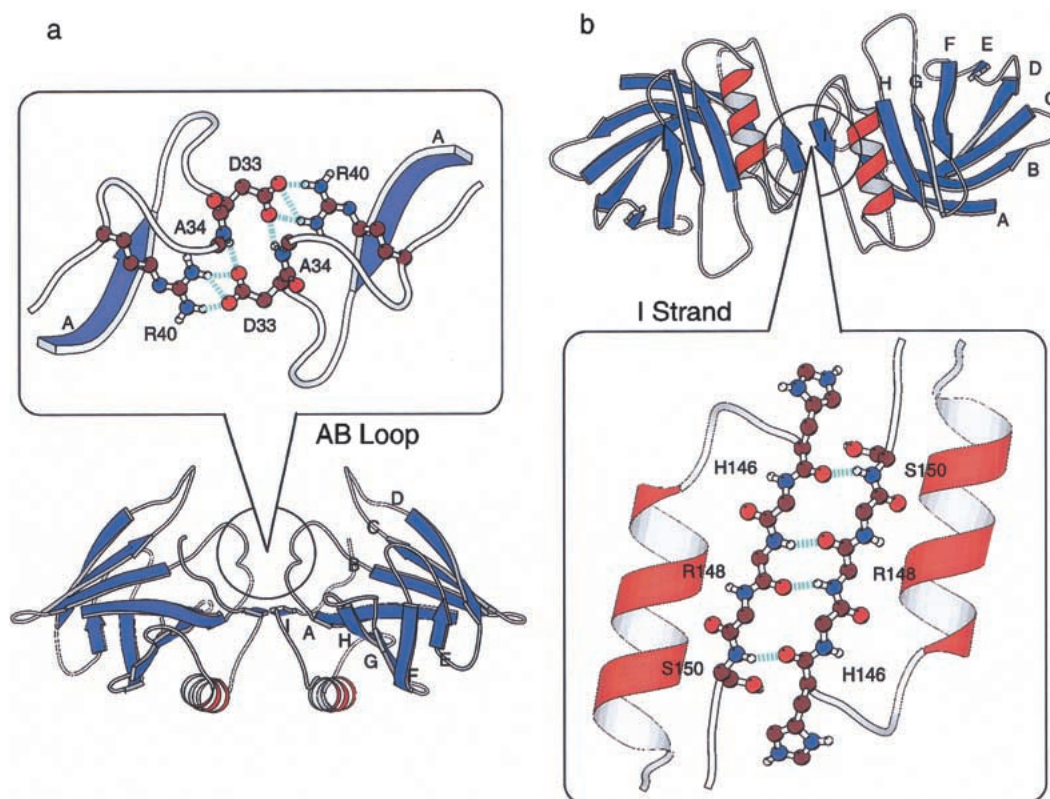


Fig. 1. Dimer interface of β -LG from the X-ray coordinates, 1BEB. Side view (a) and top view (b) with respect to β I strands. Close-up views of AB loops (a) and β I strands (b) are also shown, where intersubunit hydrogen bonds between side chains and main chains can be seen.

distributed between the surface AB loop and the antiparallel β -sheet between β I strands (Fig. 1), and by tight packing of the residues in the interface.

We have been studying the folding mechanism of bovine β -LG (Hamada et al. 1995, 1996; Shiraki et al. 1995; Kuroda et al. 1996; Hamada and Goto 1997; Kuwata et al. 1998, 1999, 2001; Forge et al. 2000). Although it is a predominantly β -sheet protein, it has a markedly high helical propensity (Nishikawa and Noguchi 1991; Shiraki et al. 1995; Kuroda et al. 1996). During its refolding reaction, an intermediate with non-native α -helical structure accumulates because of the inconsistency of local and nonlocal interactions (Hamada et al. 1996). Thus, β -LG is an intriguing model for studying the interplay between local and non-local interactions during protein folding. It may provide clues for clarifying the α -to- β -transition (Dalal et al. 1997; Mihara and Takahashi 1997) suggested for several biologically important processes, such as the conformational transitions of prion protein (Prusiner 1997; Cohen 1999; Jackson et al. 1999) or Alzheimer A β peptide (Benzinger et al. 2000). A recent study (Kuwata et al. 2001) clarified that, in the intermediate accumulated over the time range of milliseconds, non-native α -helix is formed at the β A region and that the non-native α -helix is transformed slowly to the

native β -strand through a process by which the core β -sheet extends to the native β -barrel.

In addition, one of the intriguing topics of β -LG is the monomer–dimer equilibrium. The dimer is the predominant species of β -LG at neutral pH regions and at protein concentration above 1 mg mL⁻¹. However, because the dimerization constant (K_D) is not large ($\sim 5 \times 10^4$ M⁻¹), the population of monomers increases at low protein concentrations. It has been known that the chemical modification of a free thiol at Cys121 on the β H strand, which is completely buried under the C-terminal α -helix, results in a decrease in K_D (Zimmerman et al. 1970; Cupo and Pace 1983; Burova et al. 1998; Yamasaki et al. 1999). In our previous study (Sakai et al. 2000), we characterized the monomer–dimer equilibrium dependent on the modification of Cys121 by 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and indicated that the dissociation into monomer is coupled with the substantial disorganization of the β -LG structure by modification of the buried thiol group.

On the other hand, while the native monomer is the major species under low salt conditions at pH 3, it has been shown that an increase in the salt concentration stabilizes the dimer (Townend et al. 1960; Joss and Ralston 1996). However, the thermodynamic mechanism by which salt stabilizes the

dimer is unknown. In the present study, we characterized the salt-induced dimer formation of β -LG at pH 3 by sedimentation equilibrium and isothermal titration calorimetry (ITC). Our results indicated that the monomer–dimer equilibrium of β -LG at pH 3 is determined by a subtle balance between the hydrophobic interactions between the dimer interface, which favor dimer formation, and electrostatic repulsion between the positively charged monomers, which favors the monomer. The addition of salt decreases the electrostatic repulsion by anion binding, resulting in stabilization of the dimer. Intriguingly, Gdn-HCl, a neutral salt known as a strong denaturant, stabilized the dimer before it worked as a denaturant. From the temperature dependence of the monomer–dimer equilibrium, we determined the enthalpy change (ΔH_D) and the change in heat capacity (ΔC_p) on dimer formation. The observed ΔC_p values were consistent with the value calculated by the method of accessible surface area (Oobakate and Ooi 1993) based on the X-ray structure of the dimer.

Results

Salt effects

We measured the sedimentation equilibrium of β -LG in the presence of various concentrations of salts at pH 3.0 and 20°C. Salts used were NaCl, KCl, sodium perchlorate (NaClO_4), and Gdn-HCl. The plots of concentration versus radius data clearly indicated that the profile was dependent on the salt concentration (Fig. 2). The results were explained satisfactorily on the basis of a monomer (M)–dimer (D) equilibrium.



Although it is difficult to interpret the concentration versus radius profile intuitively, data with a larger inclination in the outer region have a higher association constant. We ob-

tained the K_D values by fitting the theoretical curve (Eq. 2) to the plots (Figs. 2, 3; Table 1). Under the present experimental conditions, a reliable protein concentration range to be determined after sedimentation equilibrium was from 0.1 to 2.0 mg mL^{-1} . This corresponded to a range of K_D from 10^2 to 10^8 M^{-1} , or a range of dimer fraction (w/w) from 1% to 99%. The optimal K_D range to be determined was about 10^3 – 10^6 M^{-1} , corresponding to a range of dimer fraction from 10 to 90%.

Under the minimal salt conditions (i.e., 20 mM glycine-HCl at pH 3.0 and 20 mM NaCl), the K_D value was 10^3 M^{-1} . For all the salts examined, the addition of salt increased K_D , and thus the fraction of dimer. However, the dependence on salt concentration varied, depending on the salt species (Fig. 3). The effects of NaCl and KCl were essentially the same and, at a β -LG concentration of 1.0 mg mL^{-1} , more than 80% (w/w) of β -LG formed a dimer in the presence of 1 M NaCl. The salt-induced transition was cooperative, and the midpoint, where the dimer fraction was 50%, was at 0.1 M NaCl or KCl. In contrast, NaClO_4 was much more effective than NaCl or KCl. All β -LG molecules formed dimers in the presence of 25 mM NaClO_4 and the midpoint salt concentration was as low as 6 mM. Thus, NaClO_4 was 20-fold more effective than NaCl or KCl.

Gdn-HCl is the most commonly used denaturant of proteins. However, at low concentrations Gdn-HCl behaved similarly to NaCl or KCl, stabilizing the dimer. The dimer fraction in 1 M Gdn-HCl at a β -LG concentration of 1 mg mL^{-1} was 80% (w/w). Then, 5 M Gdn-HCl decreased the fraction of dimer to 20%. Although the decrease in the dimer fraction at 5 M Gdn-HCl was due to the Gdn-HCl-induced denaturation of β -LG, the increase in the dimer fraction below 1 M Gdn-HCl was due to the salt effects of Gdn-HCl.

Goto and coworkers (Goto and Fink 1990, 1994; Goto et al. 1990a, 1990b; Hagihara et al. 1993, 1994) studied the salt effects on several acid-denatured proteins (e.g., cyto-

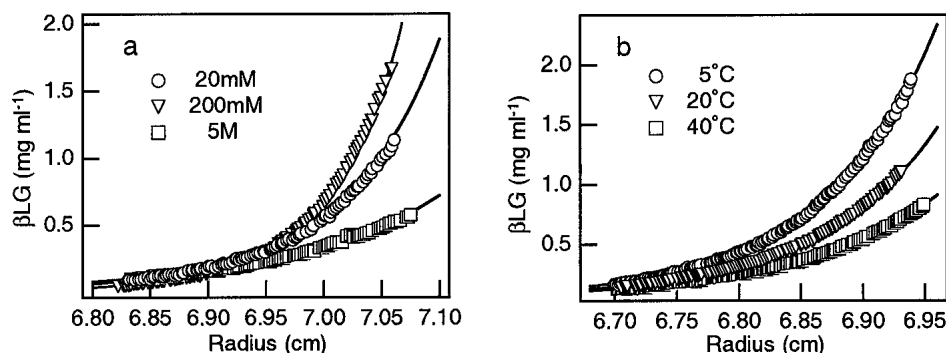


Fig. 2. Sedimentation equilibrium analysis of β -LG in the presence of various concentrations of Gdn-HCl (a) and at various temperatures (b). The panels show the concentration versus radius plot. The solid lines are theoretical curves based on Equation 2. The data with a larger inclination in the outer region have a higher association constant.

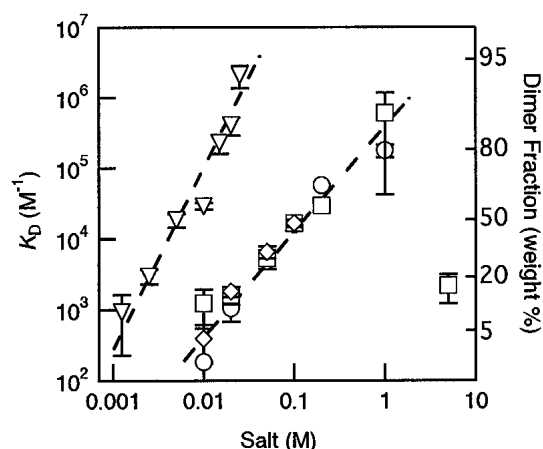


Fig. 3. Dependence of K_D on salt concentration at 20°C. The salts examined were NaClO_4 (down open triangles), NaCl (open circles), KCl (open diamonds), and Gdn-HCl (open squares). The right axis shows dimer fraction (w/w) at a β -LG concentration of 1.0 mg mL^{-1} . The slope of the broken lines ($\text{dlog}K_D/\text{dlog}[\text{salt}]$) is Δn , and the values for the perchlorate- and chloride-induced transitions were 2.6 and 1.5, respectively.

chrome c, apomyoglobin, β -lactamase) at pH 2, where, in the absence of salt, the proteins are unfolded maximally because of the charge repulsion between the positive charges. The effects of various salts on the acid-unfolded proteins were explained by the binding of anions to the positively charged groups on proteins. The binding of counter ions shields the charge repulsion, and consequently the hydrophobic interactions come to play a significant effect, stabilizing the compact molten globule state. Alternatively, the anion-induced folding transition can be explained by the preferential binding of anions to the compactly folded state with the higher positive charge density. One of the most important characteristics of the anion bind-

ing-dependent mechanism is that the effectiveness of various anions follows the electroselectivity series: among the monovalent anions, chaotropic anions such as perchlorate, ClO_4^- , are more effective than chloride, Cl^- . Another interesting consequence of the salt effects is the differential effects of Gdn-HCl on the conformational stability of proteins (Hagihara et al. 1993, 1994; Mayr and Schmid 1993). Although Gdn-HCl below 1 M works as a neutral salt because of the chloride effects, it works as a denaturant above 1 M. This results in the consecutive refolding and unfolding transitions of acid-unfolded proteins by the addition of Gdn-HCl .

The effects of four different salts on the monomer–dimer equilibrium of β -LG were very similar to the effects of these salts on the acid-unfolded proteins (Goto and Fink 1990, 1994; Goto et al. 1990a, 1990b; Hagihara et al. 1993, 1994; Mayr and Schmid 1993), although the conformations stabilized by salts were different in the two cases. This similarity suggested that the salts stabilize the dimer of β -LG by the same mechanism as they stabilize the molten globule states (Goto et al. 1990b). Anion binding decreased the charge repulsion between the positively charged monomers, and consequently, intrinsic hydrophobic effects stabilized the β -LG dimer.

The preferential anion binding can be characterized by Δn , the number of anions preferentially and thermodynamically bound to the dimer, which can be obtained by plotting $\log K_D$ against $\log[\text{salt}]$ (Fig. 3; Record et al. 1976, 1991; Goto et al. 1990b). Δn for the perchlorate-induced dimer formation (2.6) was slightly larger than that (1.5) for the chloride-induced transition, representing a slightly higher cooperativity of the perchlorate-induced transition.

Temperature dependence

Sedimentation equilibrium was measured at various temperatures between 5 and 40°C in the presence of different concentrations of NaCl and NaClO_4 . For all salt conditions, the fraction of dimer decreased with increasing temperature (Fig. 2), i.e., the decrease in K_D with increase in temperature (Fig. 4). In the presence of 5 mM NaClO_4 at a β -LG concentration of 1 mg mL^{-1} , the dimer fraction was 70% at 5°C, and it decreased to 10% at 40°C (Table 2, Fig. 4b). In the presence of 50 mM NaCl , the dimer fraction was 50% at 5°C and 7% at 40°C (Table 2, Fig. 4a). van't Hoff plots (i.e., $\ln K_D$ versus $1/T$) give the enthalpy change (ΔH_D) of dimer formation (Fig. 4). It was evident that for both NaCl or NaClO_4 the plots were not linear, indicating that ΔC_p values were not zero. For each salt, we estimated the ΔH_D and ΔC_p values by global curve fitting on the basis of Equation 6 (Table 3). Then, with Equations 3–6, temperature dependencies of ΔG_D , ΔH_D , and $T\Delta S_D$ were plotted (Fig. 5).

Under all salt conditions examined, ΔG_D was negative and increased slightly (i.e., decrease in magnitude) with

Table 1. Sedimentation equilibrium analysis of β -lactoglobulin A at pH 3.0 in various salts at 20°C

NaCl (mM)	K_D (M^{-1})	NaClO_4 (mM)	K_D (M^{-1})
10	1.84×10^2	1.25	$9.34 (\pm 7.02) \times 10^2$
20	$3.26 (\pm 1.24) \times 10^2$	2.5	$3.01 (\pm 0.72) \times 10^3$
50	$4.93 (\pm 1.18) \times 10^3$	5	$1.84 (\pm 0.40) \times 10^4$
100	$1.60 (\pm 0.17) \times 10^4$	10	$2.91 (\pm 0.30) \times 10^4$
200	$5.71 (\pm 0.10) \times 10^4$	15	$2.27 (\pm 0.67) \times 10^5$
1000	$1.79 (\pm 0.36) \times 10^5$	20	$4.04 (\pm 1.18) \times 10^5$
		25	$2.10 (\pm 0.73) \times 10^6$
KCl (mM)	K_D (M^{-1})	Gdn-HCl (mM)	K_D (M^{-1})
10	$3.91 (\pm 2.18) \times 10^3$	10	$1.23 (\pm 0.67) \times 10^3$
20	$1.81 (\pm 0.29) \times 10^3$	20	$1.64 (\pm 0.31) \times 10^3$
50	$6.58 (\pm 1.30) \times 10^3$	50	$5.44 (\pm 1.12) \times 10^3$
100	$1.64 (\pm 0.19) \times 10^4$	100	$1.66 (\pm 0.13) \times 10^4$
		200	$2.94 (\pm 0.26) \times 10^4$
		1000	$6.01 (\pm 5.59) \times 10^5$
		5000	$2.20 (\pm 0.97) \times 10^3$

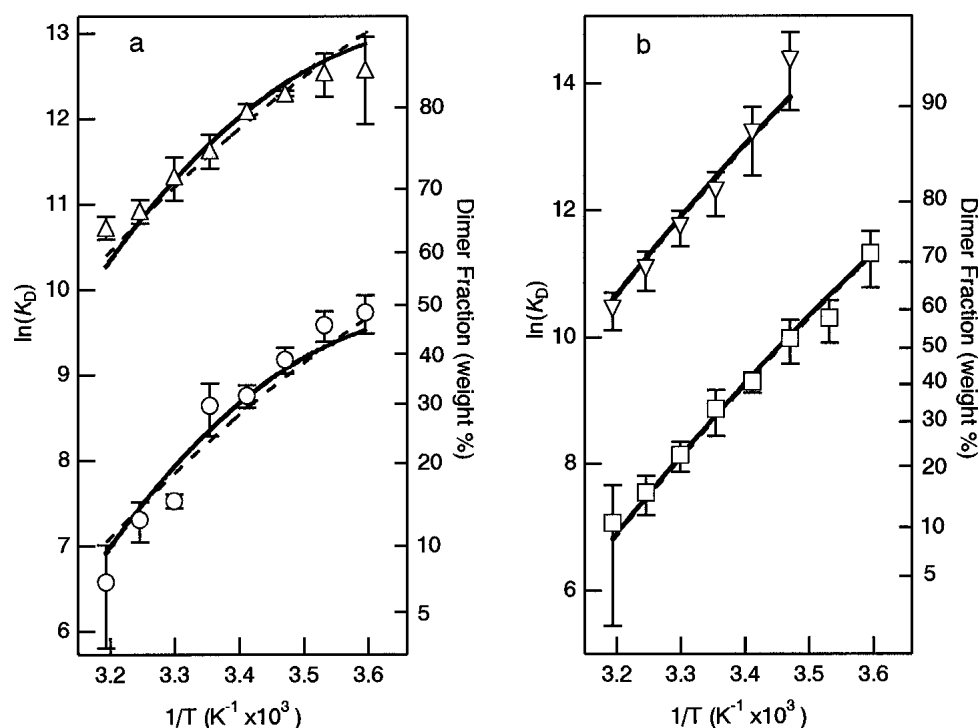


Fig. 4. Dependence of K_D on temperature represented as a form of van't Hoff plot ($\ln K_D$ versus K^{-1}). The K_D values were obtained in (a) 50 mM NaCl (open circles), 500 mM NaCl (up open triangles), (b) 5 mM NaClO_4 (open squares), and 20 mM NaClO_4 (down open triangles). The right axis shows dimer fraction (w/w) at a β -LG concentration of 1.0 mg mL^{-1} . The solid lines show the theoretical curves with Equation 6 with ΔC_p as the adjustable parameter and the broken lines show the curves with a fixed ΔC_p of $-0.72 \text{ kJ mol}^{-1} \text{ K}^{-1}$, calculated by the method of Oobatake and Ooi (1993). In case of NaClO_4 (b), the line solid completely overlaps the broken line.

increasing temperature. These slight changes in ΔG_D were due to the large changes in ΔH_D and $T\Delta S_D$, as observed for the corresponding parameters of protein unfolding (Oobatake and Ooi 1993; Makhatadze and Privalov 1995). Figure 5a also shows the data reported by Joss and Ralston (1996), in which the values for β -LG isomer B at pH 2.6 and $I = 0.16$, controlled by NaCl, were determined by sedimentation equilibrium. β -LG isomer B is different from isomer A used in the present study at two positions: whereas

isomer A has Asp and Val at positions 64 and 118, respectively, isomer B has Gly and Ala at the corresponding positions. In addition, the buffer conditions used by Joss and Ralston (1996) were not the same as those used in the present study. Nevertheless, the agreement of two results in the presence of NaCl was remarkable, indicating the reliability of the present measurements. This agreement is explained by the fact that the thermodynamic values of the dimer formation are not affected by Asp and Val at positions 64

Table 2. Dimerization constants of β -lactoglobulin A at pH 3.0 obtained by sedimentation equilibrium analysis

Temperature (°C)	$K_D \text{ (M}^{-1}\text{)}$			
	50 mM NaCl	500 mM NaCl	5 mM NaClO_4	20 mM NaClO_4
5	$1.68 (\pm 0.37) \times 10^4$	$2.89 (\pm 1.36) \times 10^5$	$8.20 (\pm 3.40) \times 10^4$	ND ^a
10	$1.45 (\pm 0.26) \times 10^4$	$2.79 (\pm 0.69) \times 10^5$	$2.99 (\pm 0.97) \times 10^4$	ND ^a
15	$9.65 (\pm 1.44) \times 10^3$	$2.19 (\pm 0.07) \times 10^5$	$2.16 (\pm 0.73) \times 10^4$	$1.75 (\pm 0.62) \times 10^6$
20	$6.37 (\pm 0.83) \times 10^3$	$1.78 (\pm 0.16) \times 10^5$	$1.08 (\pm 0.18) \times 10^4$	$5.52 (\pm 2.72) \times 10^5$
25	$5.67 (\pm 1.69) \times 10^3$	$1.12 (\pm 0.22) \times 10^5$	$7.07 (\pm 2.44) \times 10^3$	$2.23 (\pm 0.75) \times 10^5$
30	$1.87 (\pm 0.15) \times 10^3$	$8.30 (\pm 0.20) \times 10^4$	$3.42 (\pm 0.81) \times 10^3$	$1.27 (\pm 0.35) \times 10^5$
35	$1.50 (\pm 0.35) \times 10^3$	$5.53 (\pm 0.76) \times 10^4$	$1.90 (\pm 0.57) \times 10^3$	$6.53 (\pm 1.94) \times 10^4$
40	$7.22 (\pm 3.89) \times 10^2$	$4.56 (\pm 0.61) \times 10^4$	$1.18 (\pm 0.94) \times 10^3$	$3.46 (\pm 0.99) \times 10^4$

^a It was not possible to determine the value because of the limits of sedimentation equilibrium analysis.

Table 3. Thermodynamic parameters obtained from sedimentation equilibrium at pH 3.0

Conditions	ΔH_{20} (kJ mol ⁻¹)	ΔC_p (kJ K ⁻¹ mol ⁻¹)	ΔS_{20} (J K ⁻¹ mol ⁻¹)	K_{20} (M ⁻¹)	ΔG_{20} (kJ mol ⁻¹)
50 mM NaCl	-50.4	-1.93	-99.2	6.92×10^3	-21.3
500 mM NaCl			-71.3	1.62×10^5	-29.5
5 mM NaClO ₄	-90.9	-0.75	-232	1.17×10^4	-22.9
20 mM NaClO ₄			-201	5.91×10^5	-32.1

and 118, respectively, due to no change of ASA values of those residues (see Table 4). Thus, although the number of data points was not sufficient to determine ΔC_p precisely, the negative ΔC_p values will be valid (Table 3).

Isothermal titration calorimetry

Heat of the dimer formation upon addition of salts at pH 3.0 was directly measured by ITC at 30 and 40°C (Fig. 6). This measurement was possible only with NaClO₄, because its concentration required for dimer formation was low enough

to perform the ITC experiment (Hamada et al. 1994). The dilution of NaClO₄ was endothermic, absorbing heat. The titration of β -LG with NaClO₄ was first exothermic, and then became endothermic, indicating that the interaction between β -LG and NaClO₄ upon dimer formation is exothermic (Fig. 6a). After subtracting the dilution heat of NaClO₄ and integrating the net heat effect of each step, we obtained the plot of total heat effect against NaClO₄ concentration (Fig. 6b). The plot showed a saturating curve with a slightly sloped baseline. Assuming that the dimer fraction is 100% on the baseline, dimer fraction at 30°C was 48% at 5 mM NaClO₄ and 87% at 20 mM NaClO₄. Considering the β -LG concentration of 5.0 mg mL⁻¹ in ITC measurements, these percentages were in good agreement with those calculated from K_D values obtained from the sedimentation equilibrium analysis (i.e., 49% for 5 mM NaClO₄ and 89% for 20 mM NaClO₄, Fig. 4). We consider that the extrapolated total heat effect from the saturated regions above 40 mM corresponds to the total heat of dimer formation, i.e., ΔH_D . In fact, the ΔH_D values obtained from ITC measurements at 30 and 40°C were close to those obtained by sedimentation equilibrium analysis (Fig. 5b). Although the ΔC_p value suggested from these ΔH_D values is positive, inconsistent with the negative value obtained from sedimentation equilibrium measurements, we do not consider that the ΔH_D values obtained from ITC measurements have a high enough quality to determine ΔC_p (see below).

As control experiments, we carried out titration experiments in the presence of 8 M urea at pH 3.0, where β -LG was unfolded and monomeric measured by sedimentation equilibrium (data not shown). No significant heat effect was observed (Fig. 6b), confirming that the heat effects in the absence of denaturant were due to the monomer–dimer transition. We performed the ITC measurements at various temperatures. However, because the results below 30°C were likely to include the contribution of aggregation of proteins at high salt concentrations, we did not report these data in the present paper.

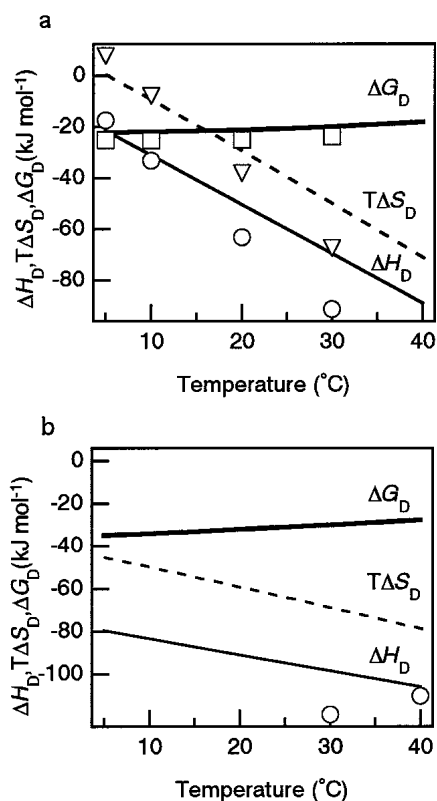


Fig. 5. Dependence of ΔG_D , ΔH_D , and $T\Delta S_D$ on temperature in NaCl (a) and NaClO₄ (b). In (a), the values of β -LG isomer B at pH 2.6 and $I = 0.16$ taken from Joss and Ralston (1996) are shown: (open squares) ΔG_D , (open circles) ΔH_D , (down open triangles) $T\Delta S_D$. In (b), ΔH_D obtained by ITC measurements (circles) are shown.

Calculation of thermodynamic parameters

With the PDB coordinates 1BEB, a dimeric β -LG obtained at pH 6.5, we calculated the change in ASA (ΔASA) on

Table 4. Estimation of thermodynamic parameters using X-ray coordinates, 1 BEB

	ΔASA_n ($\text{\AA}^2$)	ΔASA_p ($\text{\AA}^2$)	ΔASA_t ($\text{\AA}^2$)	$\Delta C_{p,h}$ (nonpolar) ($\text{J K}^{-1} \text{mol}^{-1}$)	$\Delta C_{p,h}$ (polar) ($\text{J K}^{-1} \text{mol}^{-1}$)	$\Delta C_{p,h}$ (total) ($\text{J K}^{-1} \text{mol}^{-1}$)
Asp28A	0.0	-8.3	-8.3	0	8	8
Ile29A	-89.0	0.0	-89.0	-138	0	-138
Asp33A	-1.6	-70.2	-71.7	-3	34	31
Ala34A	-46.2	-2.2	-48.3	-71	0	-71
Gln35A	-18.8	-1.1	-19.9	-29	0	-29
Arg40A	0.0	-47.1	-47.1	0	2	2
Met145A	0.0	-13.6	-13.6	0	13	13
His146A	-73.8	-33.1	-106.8	-99	25	-74
Ile147A	-24.8	0.0	-24.8	-38	0	-38
Arg148A	-73.6	-52.6	-126.2	-109	28	-81
Leu149A	-60.4	0.0	-60.4	-94	0	-94
Ser150A	-2.4	-54.8	-57.2	-4	18	14
Phe151A	-9.3	-13.5	-22.8	-13	13	0
Asn152A	-10.6	-0.4	-11.0	-16	0	-16
Gln155A	0.0	-17.8	-17.8	0	14	14
Asp28B	0.0	-1.8	-1.8	0	2	2
Ile29B	-72.5	0.0	-72.5	-112	0	-112
Asp33B	-1.6	-50.1	-51.7	-3	24	21
Ala34B	-31.4	-2.4	-33.8	-49	0	-49
Gln35B	-3.0	-0.7	-3.7	-5	0	-5
Arg40B	0.0	-20.4	-20.4	0	1	1
Met145B	0.0	-5.1	-5.1	0	5	5
His146B	-36.8	-22.2	-59.0	-48	15	-33
Ile147B	-24.6	0.0	-24.6	-38	0	-38
Arg148B	-19.6	-36.5	-56.1	-30	18	-12
Leu149B	-41.9	0.0	-41.9	-65	0	-65
Ser150B	-2.8	-43.6	-46.4	-4	17	12
Phe151B	-7.8	-2.9	-10.7	-10	3	-7
Asn152B	0.0	-26.5	-26.5	0	8	8
Gln155B	0.0	-20.6	-20.6	0	16	16
Total	-652.4	-547.3	-1199.7	-977	262	-715

A and B after residue number mean the chain names of dimer, respectively. ΔASA_n , ΔASA_p , and ΔASA_t represent ASA changes of nonpolar, polar, and total heavy atoms, respectively, upon dimer formation. $\Delta\text{ASA} = \text{ASA}(\text{dimer}) - \text{ASA}(\text{monomer})$ and $\Delta C_{p,h} = C_{p,h}(\text{dimer}) - C_{p,h}(\text{monomer})$.

dimer formation, assuming that the monomeric structures were the same as those in the dimer (Table 4). In the total decrease in ASA ($1200 \text{ \AA}^2 \text{ dimer}^{-1}$) upon dimer formation, nonpolar and polar surfaces were 652 and $547 \text{ \AA}^2 \text{ dimer}^{-1}$, respectively. The two monomers in the dimer were not exactly the same, although they were very similar. For both monomer units, the residues with ΔASA larger than $50 \text{ \AA}^2 \text{ dimer}^{-1}$ were Ile29, Asp33, His146, Arg148, Leu149, and Ser150. For many of these residues, the contribution of the nonpolar surface to the total ΔASA was slightly larger than that of the polar surface. We estimated the $\Delta C_{p,h}$ value upon dimer formation on the basis of the method of Oobatake and Ooi (1993). The estimated $\Delta C_{p,h}$ value was $-0.72 \text{ kJ K}^{-1} (\text{mole of dimer})^{-1}$ (Table 4). Because ΔASA_n and ΔASA_p contribute to $\Delta C_{p,h}$ negatively and positively, respectively, the negative value of calculated $\Delta C_{p,h}$ means the major contribution of nonpolar surfaces to $\Delta C_{p,h}$. Considering errors in the experimental determination of the small ΔC_p

value, the estimated value was consistent with the values obtained by sedimentation equilibrium analysis (-1.93 and $-0.75 \text{ kJ K}^{-1} (\text{mole of dimer})^{-1}$ for NaCl and for NaClO_4 , respectively; Table 3), arguing that the observed ΔC_p of dimer formation is mostly $\Delta C_{p,h}$.

Discussion

Although β -LG assumes a predominantly monomeric native structure at around pH 3 in the absence of salt, the addition of salt stabilizes the dimeric structure. In fact, the salt-dependent monomer–dimer equilibrium has been proposed to be a good standard to calibrate monomer–dimer analysis with sedimentation equilibrium (Joss and Ralston 1996). X-ray crystallographic studies (Brownlow et al. 1997; Qin et al. 1998a, 1998b) have characterized dimeric structures of bovine β -LG, in which β I strands join two monomers forming an antiparallel β -sheet (Fig. 1). The monomers are as-

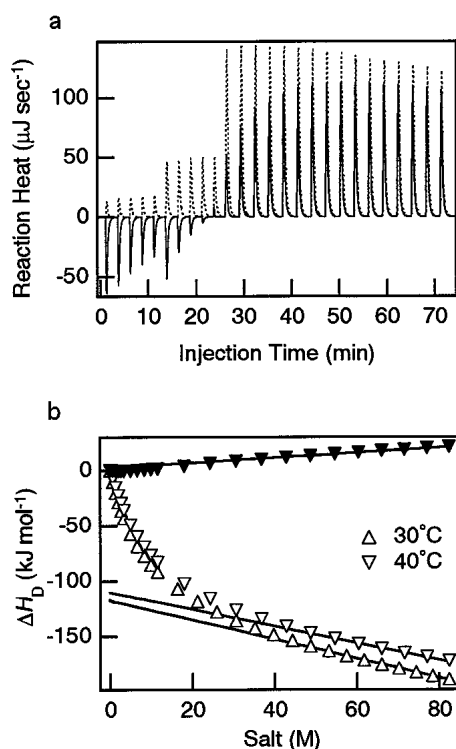


Fig. 6. Heat effects of dimer formation measured by ITC. (a) Solid line shows typical calorimetric titration of the monomeric β -LG with NaClO_4 at 30 °C at pH 3.0. 1 μL of 0.45 M NaClO_4 was added five times, and then 2 μL of the same stock salt solution was added for each injection. The broken line indicates the control titration without protein. (b) Net heat effect obtained after subtracting the control heat effect data and integrating each peak by time. We assumed that the net heat effect obtained by extrapolation to zero salt concentration (solid line) corresponded to ΔH_D . Temperatures were 30 (up open triangles) and 40 (down open triangles) °C. Solid data show the control experiments at 40 (down filled triangles) °C under the same conditions except including 8 M urea, in which β -LG is unfolded and monomeric.

sociated by 12 hydrogen bonds distributed between AB loops (eight side chain bonds) and β I strands (four main chain bonds). The dimer interface buries about 1200 Å² of the accessible surface area per dimer, corresponding to about 6% of the total surface area of the isolated native structures. This value lies at the lower end of the range for strongly associated dimers (Brownlow et al. 1997), suggesting that this dimeric interaction is not very tight. Consistent with this, the dimerization constant was only $5 \times 10^4 \text{ M}^{-1}$ at pH 6.5, and a significant fraction of the molecules existed as monomers at low protein concentrations (Sakai et al. 2000). Therefore, it is not surprising that a decrease of pH easily destabilizes the dimer, forming a monomer at pH values below 3 under conditions of low salt. On the other hand, because of this weak interaction, the detailed mechanism by which the salts stabilize the dimer at around pH 3 has not been clarified. To obtain further insight into the mechanism, we studied the salt-dependent monomer–dimer equilibrium

of β -LG with analytical ultracentrifugation and ITC. The sedimentation equilibrium was used to characterize the change in the monomer–dimer equilibrium dependent on salt conditions and temperature. ITC directly measured ΔH_D of dimer formation. The results clarified several characteristics of the thermodynamic mechanism of monomer–dimer equilibrium of β -LG.

Salt effects

We first showed that the salt-induced stabilization of the β -LG dimer is similar in mechanism to the salt-induced stabilization of the molten globule state of acid-unfolded proteins as studied by Goto and coworkers (Goto and Fink 1990, 1994; Goto et al. 1990a, 1990b). In the case of acid-denaturation, pH conditions as low as pH 2 caused an increase in the net charge of protein molecules. Under low salt conditions, this produces the unfavorable charge repulsion, resulting in global unfolding of the protein molecule. The anions produced by salts or even strong acids shield this charge repulsion by the direct interaction with positive charges. The importance of the direct interaction was demonstrated by the observation that the order of the effectiveness of various anions follows the electroselectivity series of anions toward anion-exchange resins. In the electroselectivity series, multivalent anions are more effective than monovalent anions. Among monovalent anions, chaotropic anions are more effective than kosmotropic anions: $\text{SCN}^- > \text{ClO}_4^- > \text{I}^- > \text{Br}^- > \text{Cl}^- > \text{F}^-$. Anions are preferentially bound to the compact molten globule state because the electrostatic interaction becomes stronger when the protein assumes a compact state with a high charge density.

In the salt-induced stabilization of the dimer of β -LG, NaClO_4 is about 20-fold more effective than NaCl or KCl , consistent with the anion binding-induced dimerization mechanism. Otherwise, such a large difference in the effects of NaClO_4 and NaCl or KCl would not be explained. The Debye–Hückel screening effect of salts is independent of anion species. The difference in the number of thermodynamically bound anions, Δn , was obtained from the plot of $\log K$ versus $\log[\text{salt}]$ (Fig. 3) and the estimated Δn value of NaClO_4 is slightly larger than that of NaCl or KCl , indicating that the NaClO_4 -induced transition is a little more cooperative than the NaCl or KCl transitions. In addition, Δn value per β -LG dimer of both salts was about 2, consistent with the idea that the monomer–dimer equilibrium depends critically on the subtle balance of the stabilizing and destabilizing forces. Candidates of the anion-binding residues responsible for the dimer formation are R40, H146, and R148, as shown in Figure 1. The most reasonable one is R148 because the distance between ϵ -nitrogens of R148 on each monomer is shorter than any other pairs of the positive charges. It is likely that anion binding suppresses the repulsion caused by these positive charges.

β -LG is resistant against acid-denaturation, and it assumes a native conformation even at pH 2.0 as has been characterized by NMR analysis (Kuwata et al. 1998, 1999). In fact, its stability against denaturant at pH 2 is higher than at pH 7.0 (Sakai et al. 2000). The pI value of β -LG is 4.6 and the net charges at pH 7.5 and pH 2.0 were calculated to be -9 and $+20$, respectively, on the basis of the amino acid composition. Therefore, the net charge repulsion, which is stronger at pH 2.0 than at pH 7.5, cannot explain the increased stability at pH 2, and the precise reason of these phenomena is still unclear. We considered that the binding of counter ions shields the charge repulsion at pH 2.0 and, consequently, the dimer is stabilized by the interactions similar to those that stabilize the native dimer at neutral pH. As the salt bridges involved in the dimer interface would be difficult to form at the acidic pH where carboxyl groups are protonated (Fig. 1), the hydrophobic effects are the most important factors for stabilizing the dimer at pH 3.

In this context, it is intriguing that many nonruminant β -LG are monomeric at neutral pH (Hambling et al. 1992). Consideration of sequence variation suggested that Ala34 or Arg40 in the AB loop is important for stabilizing the dimer of bovine β -LG (Hambling et al. 1992). Recently, the conformation and folding of horse β -LG have been characterized (Ikeguchi et al. 1997; Fujiwara et al. 1999). Intriguingly, horse β -LG is unstable against acid, and forms an intermediate conformation with an increased α -helical content at pH 2.

Hydrophobic effects

The major contribution of hydrophobic effects in protein folding or protein–protein interactions produces a positive value of the heat capacity change upon disappearance of the interactions, for example, exposure of hydrophobic surfaces upon protein unfolding (Makhatadze and Privalov 1990a, 1990b, 1995; Murphy and Gill 1991; Oobakate and Ooi 1993). To examine the role of hydrophobic effects, we measured the temperature dependence of the monomer–dimer equilibrium (Fig. 4). For both NaCl and NaClO₄, K_D decreased with increases in temperature, resulting in dissociation into monomers. The thermodynamic parameters showed that both ΔH_D and $T\Delta S_D$ are negative and change largely with temperature and the entropy–enthalpy compensation results in a slight increase in ΔG_D with increasing temperature, as observed for the corresponding parameters of protein unfolding (Makhatadze and Privalov 1990a; Oobakate and Ooi 1993). Temperature dependencies of the changes of ΔH_D in NaCl and NaClO₄ were similar, and could be explained by a common negative value of ΔC_p (Table 3).

The only thermodynamic parameter that can be estimated on the basis of X-ray structures at the moment is ΔC_p (Makhatadze and Privalov 1990a, 1990b, 1995; Oobakate and

Ooi 1993). ΔH_D and ΔS_D have been difficult to predict correctly because several formidable factors, especially electrostatic energy and freedom, are involved. The ΔC_p of protein unfolding is largely determined by the hydration of hydrophobic groups, which contribute positively, and the hydration of hydrophilic groups including polar side chains and peptide groups, which contribute negatively. The same argument will be valid for the process of dimer formation, although the signs of these factors are opposite (Oobakate and Ooi 1993). As expected, the ΔC_p value ($-0.72 \text{ kJ K}^{-1} (\text{mole of dimer})^{-1}$) of dimer formation calculated from the X-ray coordinates of the dimeric structure is negative (Table 4), consistent with the ΔC_p value obtained in the presence of NaClO₄, and, considering the possible errors, with the ΔC_p value in the presence of NaCl (Table 3).

Conclusions

Monomer–dimer equilibrium of β -lactoglobulin at pH 3 is determined by a subtle balance between stabilizing and destabilizing forces because only a small ASA is buried on dimer formation. Although the net hydrophobic effects stabilize the dimer, the electrostatic charge repulsion between positive charges dissociates the dimer. This equilibrium is modulated by the addition of salts or by changes in temperature. The addition of salt at pH 3 shifts the equilibrium to the dimer because counterion binding to the positive charges of the monomer suppresses the charge repulsion; consequently, the intrinsic forces stabilizing the native dimer manifesting themselves. The anion-dependent dimer formation is similar in mechanism to the anion-dependent stabilization of the molten globule state as observed for several acid-unfolded proteins. On the other hand, increases in temperature destabilize the dimer because of the negative ΔH_D of dimer formation. The analysis of the temperature dependence of the monomer–dimer equilibrium indicated the negative ΔC_p of dimer formation, confirming the dominant role of the hydrophobic effects. Finally, one of the most important points in this study is that we succeeded in directly measuring ΔH_D of the dimer formation by ITC with NaClO₄ as a titrant.

Materials and methods

Materials

Bovine β -LG isomer A was purchased from Sigma and used without further purification. Other reagents were from Nacalai Tesque. The buffer used was 20 mM glycine-HCl (pH 3.0). The stock buffer solution of 1 M was prepared and the salts were added when the stock buffer solution was diluted 50-fold.

Analytical ultracentrifugation

Prior to the ultracentrifugation experiments, proteins were dialyzed against the desired buffer or passed through a PD10 column equili-

brated with the buffer. Sedimentation equilibrium experiments were performed with a Beckman Optima XL-A analytical ultracentrifuge at 20,000 rpm. The measurements in the presence of various concentrations of different salts were performed at 20°C. The data were obtained after 20 h of centrifugation by monitoring the absorbance at 278 nm. β -LG with a molecular weight of 18,400 was assumed to have a partial specific volume (v) of 0.751 mL g⁻¹ at 25°C (Joss and Ralston 1996), independent of the buffer conditions. A value of 0.961 mL mg⁻¹ cm⁻¹ at 280 nm (Townend et al. 1960) was used as the absorbance coefficient of β -LG. The density (ρ) of glycine-HCl buffer (pH 3.0) at 20°C was assumed to be 0.9990 g mL⁻¹.

Concentration versus radius data acquisition was as an average of 20 absorbance measurements over 0.001-cm intervals. All experiments used double-sector 12 mm-thick charcoal–epon centerpieces and matched quartz windows. Protein concentration (C_T) was plotted against radius (r) from the arbitrary radius point (r_0). The C_T versus r plots were analyzed to determine K_D according to the theoretical equation for monomer–dimer equilibrium:

$$C_T = C_M(r_0) \exp[M\phi(r^2 - r_0^2)] + (2K_D/M)[C_M(r_0)]^2 \exp[2M\phi(r^2 - r_0^2)] \quad (2)$$

where $C_M(r_0)$ is the monomer concentration at r_0 , M is the molecular weight of monomer, $\phi = \omega^2(1 - v\rho)/(2RT)$, and R , T , and ω are the gas constant, absolute temperature, and rotor speed, respectively (Teller et al. 1969). 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 individually with Equation 2, and the average of the three values was presented. Least-squares curve fitting was performed using the Igor software program (WaveMetrics).

Sedimentation equilibrium measurements were also performed at various temperatures between 5 and 40°C at an interval of 5°C. The results were analyzed as described above. The densities of glycine-HCl buffer (pH 3.0) at various temperatures were estimated assuming that the density ratio of the buffer and water was the same as that at 20°C, where the density of water at various temperatures is available from the literature (Svedberg and Pederesen 1940).

The temperature dependence of the dimerization constant, K_D , was analyzed assuming the following standard equations:

$$\Delta G_D = -RT \ln K_D = \Delta H_D - T\Delta S_D \quad (3)$$

$$\Delta S_D = \Delta S_{20} + \Delta C_p \ln \left(\frac{T}{293.15} \right) \quad (4)$$

$$\Delta H_D = \Delta H_{20} + \Delta C_p(T - 293.15) \quad (5)$$

and

$$\ln K_D = -\frac{1}{R} \left[\Delta C_p \left(\frac{293.15}{T} - \ln \left(\frac{293.15}{T} \right) - 1 \right) + \Delta S_{20} - \frac{\Delta H_{20}}{T} \right] \quad (6)$$

where ΔG_D , ΔH_D , and ΔS_D are Gibbs free energy, enthalpy, and entropy changes, respectively, of dimer formation, and ΔS_{20} and ΔH_{20} are entropy and enthalpy changes of dimer formation at 20°C, respectively.

ITC measurements

The β -LG solution in the cell was titrated with a solution of NaClO₄ in a similar way as used for titration of the acid-unfolded cytochrome *c* with NaClO₄ (Hamada et al. 1994). The lyophilized β -LG was dissolved into 20 mM glycine-HCl (pH 3.0) buffer and dialyzed against the same buffer. Then, the concentration of the β -LG solution was adjusted to 5.0 mg mL⁻¹. The stock NaClO₄ solution at 0.45 M was prepared with the buffer solution after dialysis. An MCS ITC unit calorimeter (MicroCal Inc.) was used. Measurements were carried out at 30 and 40°C. After setting the solutions, temperature was equilibrated for at least 3 h before measurement. The salt concentration was increased from 0 to 80 mM by stepwise addition of the stock NaClO₄ solution. As a control titration measurement, the buffer solution after dialysis was titrated with the same NaClO₄ solution.

Estimation of thermodynamic parameters

The hydration heat capacity change $\Delta C_{p,h}$ value and other thermodynamic parameters of dimer formation were calculated using the X-ray dimeric structure (1BEB) by the method of Oobatake and Ooi (1993). The $\Delta C_{p,h}$ value on protein unfolding contributes to most of the total ΔC_p value (Makhatadze and Privalov 1990a, 1990b, 1995; Oobatake and Ooi 1993). The values of ΔASA and $\Delta C_{p,h}$ are divided into the contributions of nonpolar atoms (C) and polar atoms (N, O, and S).

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