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# Effect of hydrostatic pressure on unfolding of $\alpha$ -lactalbumin: Volumetric equivalence of the molten globule and unfolded state

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## Abstract

The effect of pressure on the unfolding of bovine  $\alpha$ -lactalbumin was investigated by ultraviolet absorption methods. The change of molar volume associated with unfolding,  $\Delta V$ , was measured in the presence or absence of guanidine hydrochloride at pH 7. The  $\Delta V$  was estimated to be  $-63 \text{ cm}^3/\text{mol}$  in the absence of a chemical denaturant. While in the presence of guanidine hydrochloride (GuHCl), it was found that  $\Delta V$  was  $-66 \text{ cm}^3/\text{mol}$  at  $25^\circ\text{C}$  and was independent of the concentration of GuHCl, despite the fact that the molten globule fraction in the total unfolding product decreased with the increase of GuHCl concentration. The results indicate that the volume of  $\alpha$ -lactalbumin only changes at the transition from a native to a molten globule state, and almost no volume change has been found during the transition from a molten globule to the unfolded state.

**Keywords:**  $\alpha$ -lactalbumin; folding; hydration; hydrostatic pressure; molten globule; volume change

Our understanding of the thermodynamic stability of protein conformation has advanced considerably due to recent calorimetric studies (Privalov & Gill, 1989). Most protein unfolding studies have been carried out at atmospheric pressure with perturbations of temperature, concentration of chemical reagents such as guanidine hydrochloride (GuHCl), and of pH. It has been shown that pressure can also be used as a perturbant to provide volume change information during unfolding (Brandts et al., 1970; Hawley, 1971; Zipp & Kauzmann, 1973). The exposure of buried hydrophobic residues to water and their resulting hydration, which occurs during unfolding, causes a decrease in the partial molar volume of protein. Hydration of exposed hydrophobic groups plays a decisive role in the thermodynamic stability of protein and can therefore be regarded as an important factor for understanding protein folding, dynamics, stability, and functions.

The final process in protein biosynthesis involves the folding of the polypeptide chain into a specific three-dimensional structure, which is considered to be determined predominantly by the amino acid sequence (Anfinsen, 1973). In the course of polypeptide chain folding, there exists a partially folded intermediate state (Hiraoka & Sugai, 1985). To further understand this state, the so-called

molten globule state should be used as a model. Molten globule states, which have been observed in more than 40 natural proteins, protein fragments, apoproteins, and polypeptides, are compact, partially folded states, showing native-like secondary structure but reduced specific tertiary structure (Kuwanjima et al., 1976; Kuwanjima, 1977; Baldwin, 1994; Ptitsyn et al., 1990; Goto & Aimoto, 1991; Kuroda et al., 1992; Barrick & Baldwin, 1993; Chyan et al., 1993; Kharakoz & Bychkova, 1997). It is known that the molten globule is highly hydrated (Kharakoz & Bychkova, 1997). According to sound velocity measurements, when protein is denatured under mild conditions, i.e., at moderate ionic strength, in the absence of strong chemical denaturants or in acidic pH, the partial molar compressibility of protein decreases but its magnitude is not as large as expected (Kharakoz & Bychkova, 1997). This result can be explained by the increased hydration of the molten globule state, which reduces partial molar volume and compressibility.

It is known that  $\alpha$ -lactalbumin undergoes a three-state denaturation; also, that it is a protein in which molten globule exists in the stable state under acidic pH, in the presence of moderate concentrations of chemical denaturant such as GuHCl or urea, or when exposed to higher temperatures (e.g., Ikeguchi et al., 1986; Kuwanjima et al., 1986). The molten globule of  $\alpha$ -lactalbumin has been characterized by a number of physical methods; for instance, it has been shown from the binding of ANS to  $\alpha$ -lactalbumin that the exposure of hydrophobic residues to solvent occurs at the transition from a native to molten globule state, but there exists to some extent a hydrophobic cluster in the molten globule state (Semisotnov et al., 1987, 1991). The volume change accompanying the

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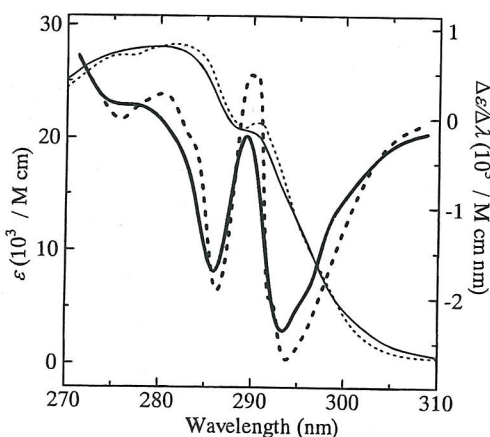
**Abbreviations:** A, molten globule; ANS, 1-anilinonaphthalene-8-sulfonate; bis-tris, bis(2-hydroxyethyl)-methane; CD, circular dichroism; GuHCl, guanidine hydrochloride; N, native; U, unfolded.

native to molten globule transition has been studied by densitometry or dilatometry for many proteins, for example,  $\alpha$ -lactalbumin (Kharakoz & Bychkova, 1997), cytochrome *c* (Chalikian et al., 1995; Foygel et al., 1995), and  $\alpha$ -chymotrypsinogen A (Chalikian et al., 1997). According to the densitometry results, there is only a slight volume change ( $-0.002 \pm 0.002 \text{ cm}^3/\text{g}$ ) during the native to molten globule transition of human  $\alpha$ -lactalbumin (Kharakoz & Bychkova, 1997). However, it seems to us that this value is so small that it can hardly be detectable by direct volumetric methods. In this study, to better understand hydration to the molten globule state and the volumetric behavior of unfolding, we studied the effects of pressure on the unfolding equilibrium of  $\alpha$ -lactalbumin, which may be more sensitive to volume change than direct volumetric measurements.

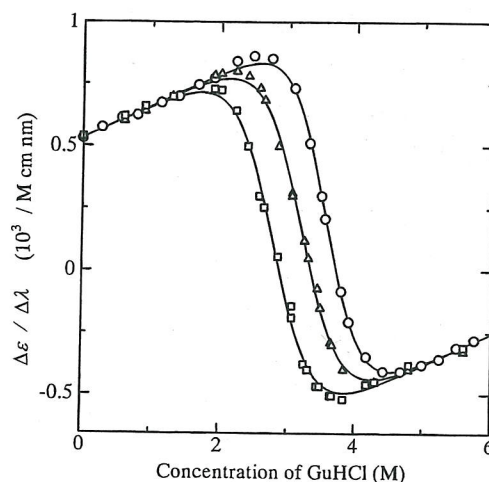
## Results

### Effect of pressure on GuHCl-induced unfolding

The ultraviolet absorption spectrum of native  $\alpha$ -lactalbumin shows a minimum around 290 nm, but this minimum disappears as the unfolding proceeds (Fig. 1). In Figure 1, the first derivative of the spectra is also shown. In native state, the derivative shows a positive value at 290 nm, and after unfolding, the derivative shows a negative value. The derivatives at 290 nm are plotted against the concentration of GuHCl under three different pressures as shown in Figure 2. Under atmospheric pressure the value changes drastically from 3 to 4 M GuHCl, which indicates the unfolding of  $\alpha$ -lactalbumin like ellipticity, difference of absorption, and fluorescence does. Figures 3A and 3B show the GuHCl-induced unfolding of recombinant human holo- $\alpha$ -lactalbumin (a) and of bovine holo- $\alpha$ -lactalbumin (b) determined by CD spectra. In these figures the degree of unfolding, derived from the first derivative of ultraviolet (UV) absorption spectrum at 290 nm, is also plotted. By comparison with the near UV CD results, the derivative was found to reflect the tertiary structure of  $\alpha$ -lactalbumins, similarly to ellipticity at 270 nm. Accordingly, we can obtain the same information as that from near CD measurements with UV derivative spectroscopy. The value of the first derivative is scarcely dependent on pressure in the pre- and post-transition region, and the degree of unfolding can easily be obtained (Fig. 2).



**Fig. 1.** UV absorption spectra  $\epsilon$  of native (thin broken line) and unfolded state (thin solid line) state, and the first derivative of molar absorption coefficient with respect to wavelength  $d\epsilon/d\lambda$  of native (thick broken line) and unfolded state (thick solid line) of bovine holo- $\alpha$ -lactalbumin at 25 °C and pH 7.0. All solutions were buffered with 50 mM sodium cacodylate containing 50 mM NaCl. Spectra of native state were measured at 0 M GuHCl and unfolded state at 6 M GuHCl.



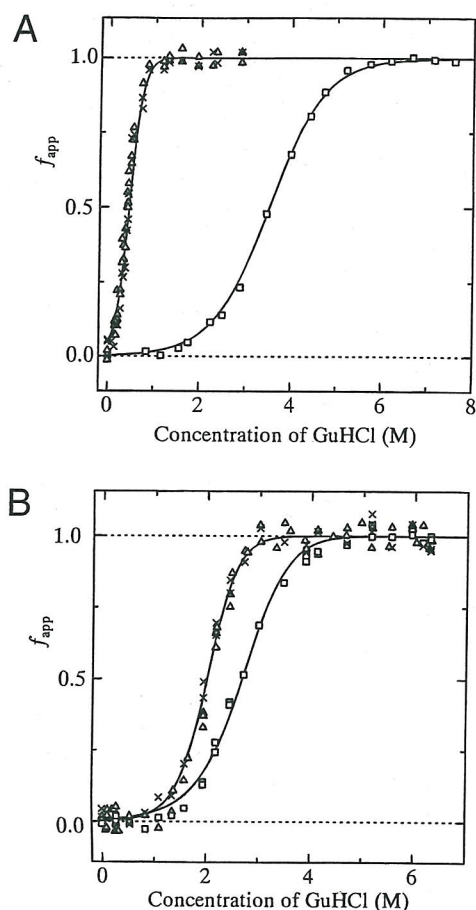
**Fig. 2.** Dependence of the first derivative of molar absorption coefficient at 290 nm on the concentration of guanidine hydrochloride at atmospheric pressure (circles), 50 MPa (triangles), and 100 MPa (squares).

ticity at 270 nm. Accordingly, we can obtain the same information as that from near CD measurements with UV derivative spectroscopy. The value of the first derivative is scarcely dependent on pressure in the pre- and post-transition region, and the degree of unfolding can easily be obtained (Fig. 2).

Free energy changes were obtained from the degree of unfolding shown in Figures 3A and 3B, and were fitted to the following equation, describing the thermodynamic stability of  $\alpha$ -lactalbumin as a function of the concentration of chemical denaturant:

$$\begin{aligned} \Delta G &= \Delta G_w - m [\text{GuHCl}] \\ &= m(C_{1/2} - [\text{GuHCl}]), \end{aligned} \quad (1)$$

where  $\Delta G_w$  is the free energy change in the absence of GuHCl,  $m$  is the so-called cooperativity coefficient, and  $C_{1/2}$  is the concentration of GuHCl at the midpoint of transition. The obtained parameters are listed in Table 1. The values of  $\Delta G_w$  and  $C_{1/2}$  for the native to molten globule transition (N-A transition) of human  $\alpha$ -lactalbumin are substantially lower, and  $m$  extremely higher, than those for bovine  $\alpha$ -lactalbumin. For the molten globule to unfolded state transition (A-U transition), on the other hand,  $\Delta G_w$  and  $C_{1/2}$  for recombinant human  $\alpha$ -lactalbumin are larger than those for bovine  $\alpha$ -lactalbumin. These facts indicate that the molten globule of recombinant human  $\alpha$ -lactalbumin can be more easily formed and is more stable than that of bovine  $\alpha$ -lactalbumin. Furthermore, the formation of the molten globule state of goat  $\alpha$ -lactalbumin is facilitated by the introduction of Met to the N terminus (Chaudhuri et al., 1999). Recombinant proteins produced by the *Escherichia coli* expression system often possess an additional Met residue at their N-terminus, because methionine aminopeptidase cannot remove the N-terminal methionine residue when the proteins are overexpressed or the radius of gyration of the side chains of the penultimate residues are larger than 1.43 (Moerschell et al., 1990). Because the human  $\alpha$ -lactalbumin was expressed in this study as an inclusion body in *E. coli*, and the original N-terminal residue of human  $\alpha$ -lactalbumin is Lys, the resulting recombinant human  $\alpha$ -lactalbumin possesses the N-terminal Met residue exclusively.



**Fig. 3.** Guanidine unfolding of (A) human recombinant holo- $\alpha$ -lactalbumin, and (B) bovine holo- $\alpha$ -lactalbumin, measured by ellipticity at 270 nm (crosses), 222 nm (squares), and the first derivative spectrum at 290 nm (triangles).

Figures 4A and 4B show the pressure dependence of  $\Delta G$  at four representative GuHCl concentrations from which we can calculate the volume change associated with unfolding. Using the  $m$  value and  $\Delta G_w$ , the molten globule fraction in the total unfolding product ( $f_A/(f_A + f_U)$ ) under atmospheric pressure was estimated, and is shown in Figures 5A and 5B. For recombinant human  $\alpha$ -lactalbumin at 0.5 M GuHCl, most of the tertiary structure would be destroyed, whereas the secondary structure would be retained to a great extent (Fig. 3A). In this region, therefore, most of the resulting unfolding product can be regarded as molten globule, and the  $\Delta V$  values are

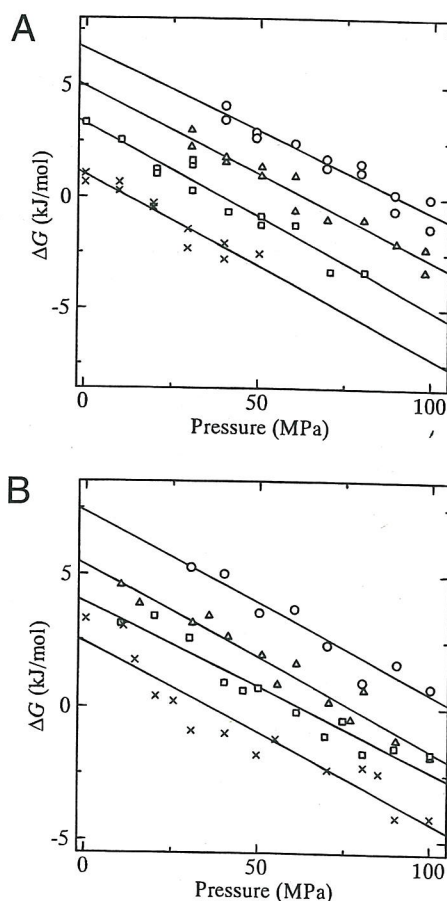
**Table 1.** Equilibrium unfolding parameters of bovine and human  $\alpha$ -lactalbumin induced by GuHCl

	Bovine $\alpha$ -lactalbumin			Human $\alpha$ -lactalbumin		
	$\Delta G_w$ (kJ/mol)	$m$ (kJ/mol M)	$C_{1/2}$ (M)	$\Delta G_w$ (kJ/mol)	$m$ (kJ/mol M)	$C_{1/2}$ (M)
N-A	16.5	7.8	2.10	7.52	17.0	0.44
A-D	5.8	2.1	2.72	14.3	4.08	3.51

virtually independent of the concentration of GuHCl. On the other hand, with bovine  $\alpha$ -lactalbumin in 3 M GuHCl, the tertiary structure is also completely destroyed, but about 20% of the secondary structure remains under atmospheric pressure (Fig. 3B); that is, in the GuHCl concentration range from 0 to 3.2 M, the unfolding product is a mixture of both the molten globule and completely unfolded states. In the absence of GuHCl, about 90% of the unfolding product of bovine  $\alpha$ -lactalbumin remains in the molten globule state (Fig. 5B). The molten globule fraction in the total unfolding product decreases gradually with the increase of GuHCl concentration. When the GuHCl concentration was increased up to 3.5 M, more than 90% of the unfolding product became completely unfolded (Fig. 5B). It is noteworthy that  $\Delta V$  is independent of the GuHCl concentration within experimental error, despite the fact that a molten globule fraction in the total unfolding product varies significantly. The mean value of  $\Delta V$  was estimated to be  $-66 \text{ cm}^3/\text{mol}$  for bovine  $\alpha$ -lactalbumin and  $-81 \text{ cm}^3/\text{mol}$  for the transition from native to molten globule state of human  $\alpha$ -lactalbumin (Table 2).

*Pressure dependence of thermal unfolding*

The melting temperature of bovine apo- $\alpha$ -lactalbumin is known to be raised by the addition of  $\text{Na}^+$  or  $\text{K}^+$  (Hiraoka & Sugai, 1985;



**Fig. 4.** Pressure dependence of  $\Delta G$  at various GuHCl concentrations. A: Recombinant human holo- $\alpha$ -lactalbumin in, from top to bottom, 0, 0.09, 0.21, and 0.30 M GuHCl. B: Bovine holo- $\alpha$ -lactalbumin in, from top to bottom, 1.49, 1.60, 1.73, and 1.98 M GuHCl.

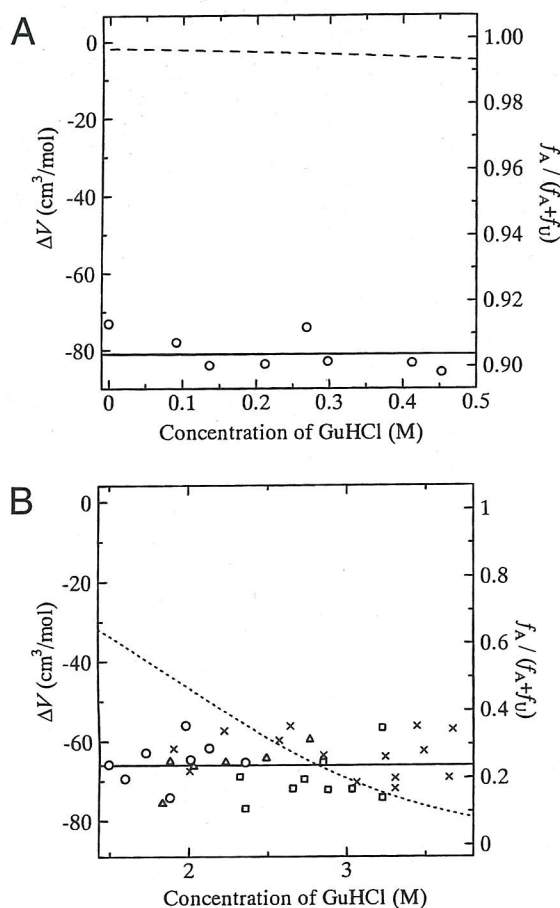


Fig. 5. GuHCl concentration dependence of  $\Delta V$  (solid line) and  $f_A/(f_A + f_U)$  (broken line) under atmospheric pressure of (A) human recombinant holo- $\alpha$ -lactalbumin and (B) bovine holo- $\alpha$ -lactalbumin in 0 (circles), 0.1 (triangles), 1 (squares), and 10 mM (crosses)  $\text{Ca}^{2+}$ .

Mitani et al., 1986), and to be drastically raised by the addition of  $\text{Ca}^{2+}$  (Kuwajima et al., 1986). The  $\Delta V$  values were obtained as a function of temperature as described in Materials and methods. The obtained  $\Delta V$  values decrease in magnitude linearly with an increase in temperature (Fig. 6). This behavior coincides with the results reported for ribonuclease A (Brandts et al., 1970) and cytochrome *c* (Dewa et al., 1998). The  $\Delta V$  value at 25 °C was estimated to be  $-36.5 \text{ cm}^3/\text{mol}$  for bovine apo- $\alpha$ -lactalbumin and  $-62 \text{ cm}^3/\text{mol}$  for bovine holo- $\alpha$ -lactalbumin. The former value is about four times that of lysozyme, which is homologous to  $\alpha$ -lactalbumin, obtained at pH 3.5 and 68.5 °C (Samarasinghe et al., 1992).

#### Separation of apparent volume change into those of N-A and A-U transitions

From the data shown in Figure 5B, the volume changes of  $\Delta V_{\text{NA}}$  and  $\Delta V_{\text{AU}}$ , for the N-A and A-U transitions, respectively, can be estimated:

$$N = A = U.$$

Assuming the above conformational equilibrium and defining  $f_N$ ,  $f_A$ , and  $f_U$  as respective fractions, the equilibrium constants and

Table 2. Volume change of unfolding of bovine and human  $\alpha$ -lactalbumin

	Bovine $\alpha$ -lactalbumin ( $\text{cm}^3/\text{mol}$ )	Human $\alpha$ -lactalbumin ( $\text{cm}^3/\text{mol}$ )
N-A		
holo <sup>a</sup>	-66(6)	-81(4)
holo <sup>b</sup>	-63	—
apo <sup>b</sup>	-37	—
holo <sup>c</sup>	-65.6(1.5)	—
A-D	0.9(6.7) <sup>c</sup>	—

<sup>a</sup>Measured in the presence of various concentrations of GuHCl at 25 °C.

<sup>b</sup>Measured in the absence of GuHCl and at various temperatures. The value were extra- or interpolated values of the experimental value to 25 °C, assuming a linear dependence of volume change on temperature (see text).

<sup>c</sup>Calculated by nonlinear least-squares methods (see text). Numbers in parentheses indicate standard deviations.

free energy changes of the N-A and A-U transitions are defined as follows:

$$K_{\text{NA}} = f_A/f_N, \quad K_{\text{AU}} = f_U/f_A.$$

$$\Delta G_{\text{NA}} = -RT \ln K_{\text{NA}} = \Delta G_{\text{NA}}^0 - m_{\text{NA}}[\text{GuHCl}] + p\Delta V_{\text{NA}}, \quad (2)$$

$$\Delta G_{\text{AU}} = -RT \ln K_{\text{AU}} = \Delta G_{\text{AU}}^0 - m_{\text{AU}}[\text{GuHCl}] + p\Delta V_{\text{AU}}. \quad (3)$$

Apparent equilibrium constant of the unfolding estimated with the present method can be shown as follows because the A-state and U-state are not distinguished:

$$K_{\text{app}} = (f_A + f_U)/f_N = K_{\text{NA}}(1 + K_{\text{AU}}). \quad (4)$$

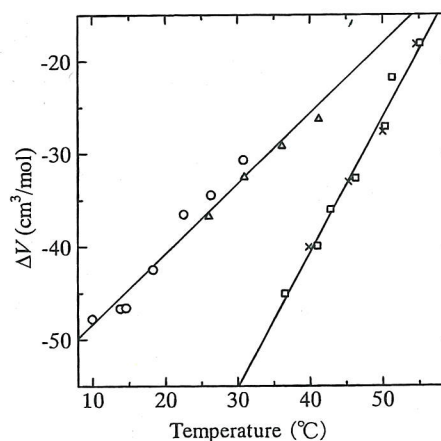


Fig. 6. Temperature dependence of  $\Delta V$  of bovine  $\alpha$ -lactalbumin at pH 7.0. Sample solutions of apo- $\alpha$ -lactalbumin were buffered with 20 mM bis-Tris containing 10 mM NaCl (circles) or 50 mM sodium cacodylate containing 50 mM NaCl (triangles), and those of holo- $\alpha$ -lactalbumin were buffered with 50 mM sodium cacodylate containing 50 mM NaCl (squares) or 20 mM bis-Tris containing 2 mM diethylenetriamine pentaacetic acid and 1 mM  $\text{CaCl}_2$  (crosses).

Apparent volume change  $\Delta V_{app}$ , which is estimated from the above equilibrium constant  $K_{app}$ , is expressed as follows:

$$\begin{aligned} \Delta V_{app} &= (\Delta G_{app}/\partial p)_{[GuHCl]} = -(1/RT)(\ln K_{app}/\partial p)_{[GuHCl]} \\ &= \Delta V_{NA} + \Delta V_{AU}K_{AU}/(1 + K_{AU}) \\ &= \Delta V_{NA} \\ &\quad + \frac{\Delta V_{AU} \exp\{-(\Delta G_{AU}^0 - m_{AU}[GuHCl] + p\Delta V_{AU})/RT\}}{1 + \exp\{-(\Delta G_{AU}^0 - m_{AU}[GuHCl] + p\Delta V_{AU})/RT\}}, \end{aligned} \quad (5)$$

$$(6)$$

where  $\Delta G_{ij}^0$  is the free energy change in the absence of GuHCl of  $i$ - $j$  transition, and  $m_{ij}$  is the so-called cooperativity coefficient of  $i$ - $j$  transition.

The values of  $\Delta V_{app}$ 's were obtained again from plots of  $\Delta G_{app}$  against pressure using Equation 6 under various concentrations of GuHCl. In this case, the values of  $\Delta G_{app}$  within the range of pressure less than 40 MPa were used, and the mean value of the pressures was taken as the pressure exhibiting the obtained  $\Delta V_{app}$ . With the fixed parameters of  $\Delta G_{AU}^0$  and  $m_{AU}$ , which were determined under atmospheric pressure and shown in Table 1,  $\Delta V_{NA}$  and  $\Delta V_{AU}$  were estimated with two two-dimensional nonlinear least-squares method (listed in Table 2). The values of  $\Delta V_{app}$  were replotted against the fraction of unfolded state in the product estimated as follows using the obtained  $\Delta V$ s, and shown in Figure 7:

$$\begin{aligned} f_U/(f_A + f_U) \\ = \frac{\exp\{-(\Delta G_{AU}^0 - m_{AU}[GuHCl] + p\Delta V_{AU})/RT\}}{1 + \exp\{-(\Delta G_{AU}^0 - m_{AU}[GuHCl] + p\Delta V_{AU})/RT\}}. \end{aligned} \quad (7)$$

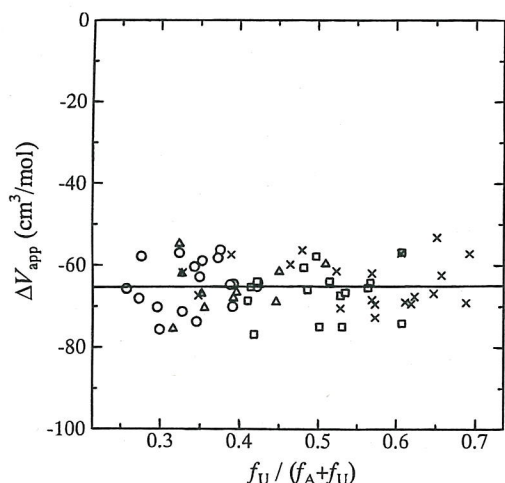


Fig. 7. The values of  $\Delta V_{app}$  are replotted against the fraction of unfolded state in the product ( $f_U/(f_A + f_U)$ ) estimated from Equation 7 (see text). Symbols are the same as Figure 5B.

## Discussion

In the absence and presence of GuHCl, the observed  $\Delta V$  values for bovine holo- $\alpha$ -lactalbumin coincided, but those for apo- $\alpha$ -lactalbumin differed considerably from those of holo- $\alpha$ -lactalbumin, yet.  $\alpha$ -Lactalbumin is known as  $Ca^{2+}$ -binding protein, and that the binding contribution of  $Ca^{2+}$  is equal for  $\alpha$ -lactalbumin and  $Ca^{2+}$ -binding lysozyme (Desmet et al., 1989, 1991a, 1991b). Kuroki et al. (1989) prepared a mutant human lysozyme with an engineered  $Ca^{2+}$ -binding site by replacing Glu86 and Ala92 with Asp residues (D86/92 human lysozyme). It has been shown by X-ray crystallography and calorimetry that the binding of  $Ca^{2+}$  to D86/92 human lysozyme, of which the  $Ca^{2+}$  binding site is identical with that of  $\alpha$ -lactalbumin, causes the release of at least seven water molecules from both  $Ca^{2+}$  (six water molecules) and the protein (one water molecule replaced with  $Ca^{2+}$ ) (Inaka et al., 1991; Kuroki et al., 1992). It has also been shown that the overall structure of apo and holo D86/92 human lysozyme is essentially identical. Accordingly, the overall structural change must not be caused by  $Ca^{2+}$  binding itself to  $\alpha$ -lactalbumin. Therefore, the overall structure of apo and holo  $\alpha$ -lactalbumin must also be identical. Therefore, a difference in  $\Delta V$  values between apo and holo- $\alpha$ -lactalbumin must be caused by the release of bound  $Ca^{2+}$  from  $\alpha$ -lactalbumin and accompanying solvation, which substantially decreases the partial molar volume (Kauzmann, 1959). Accordingly, for apo- $\alpha$ -lactalbumin, the obtained volume change must be caused only by conformational change. In the following discussions we compare volume changes between holos.

It has been shown by CD measurements that the thermal unfolding of  $\alpha$ -lactalbumin results in the molten globule state rather than the random coil state (Dolgikh et al., 1985). The molten globule state has also been realized in pressure-induced unfolding of bovine  $\alpha$ -lactalbumin because the binding amount of 1,1'-bis(4-anilino)naphthalene-5,5'-disulfonate (bis-ANS) is increased by increasing pressure (Tanaka & Kunugi, 1996). Accordingly, in the absence of GuHCl, the unfolding product at pressures up to 120 MPa is also the molten globule. Therefore, we can conclude that the volume changes, obtained at various temperatures in the absence of GuHCl, are those of the N-A transition.

In the presence of GuHCl, despite the unfolding product, a mixture of unfolded state and molten globule state is found. In addition, the composition changes in relation to the concentration of GuHCl, and the obtained  $\Delta V$  remains almost constant (Fig. 5B). Moreover, obtained  $\Delta V$  values for bovine holo- $\alpha$ -lactalbumin were comparable to the value for the N-A transition of holo-human- $\alpha$ -lactalbumin, and that of bovine  $\alpha$ -lactalbumin obtained in the absence of GuHCl, as shown in Table 2. In the case of bovine holo- $\alpha$ -lactalbumin,  $\Delta V_{AU}$  was estimated to be nearly 0  $cm^3/mol$  within experimental error. Accordingly, partial molar volume does not change at the A-U transition, at least in the case of bovine  $\alpha$ -lactalbumin. For cytochrome *c* and apomyoglobin, the changes of partial molar volume accompanying the N-A transition and A-U transition have been separately estimated by dilatometry, densitometry, or pressure unfolding. For cytochrome *c*, the volume change accompanying the A-U transition has been estimated to be -124  $cm^3/mol$  from dilatometry (Foygel et al., 1995) and -99.6  $cm^3/mol$  from densitometry (Chalikian et al., 1995). The addition of salt to unfolded cytochrome *c* results in a transformation to the molten globule state (Kuroda et al., 1992). Accordingly, the change of partial molar volume accompanying the A-U transition of cytochrome *c* may be due to the binding of anions. On the

other hand, the A-U transition of  $\alpha$ -lactalbumin merely occurs by increasing the concentration of chemical denaturant. For apomyoglobin, the volume change accompanying the A-U transition has been estimated to be  $-69 \text{ cm}^3/\text{mol}$  from pressure unfolding (Vidugiris & Royer, 1998). Comparison of the heat capacities of native, molten globule, and unfolded forms of apomyoglobin (Griko et al., 1994; Griko & Privalov, 1994) reveals that only 10% of the total increase in heat capacity occurs in the N-A transition, whereas the remaining 90% is observed in the A-U transition. On the other hand, the heat capacity change is dominant in the  $\alpha$ -lactalbumin N-A transition (Privalov, 1996). Thus, the nature of the molten globule of apomyoglobin and  $\alpha$ -lactalbumin differs considerably.

The present results indicate that  $\alpha$ -lactalbumin changes its conformation from the molten globule state to the unfolded state without volume changes. It is shown that tight packing is lost in the molten globule of  $\alpha$ -lactalbumin, but there exists a hydrophobic core, which disappears at the transition from the molten globule to the unfolded state (Semisotnov et al., 1987; Alexandrescu et al., 1992a, 1992b; Baum et al., 1989). Further, it has also been demonstrated by sound velocity measurements that the interior of the molten globule state of  $\alpha$ -lactalbumin at a molten globule is highly hydrated and fluctuated (Kharakoz & Bychkova, 1997). Calorimetric studies for apomyoglobin indicate that the bulk of the change in hydration occurs upon the unfolding of the molten globule, and that the interior of the molten globule is relatively dry (Privalov & Gill, 1989; Spolar et al., 1992; Privalov & Makhatadze, 1990; Livingstone et al., 1991; Makhatadze & Privalov, 1993, 1995). However, pressure-unfolding studies indicate that the molten globule of apomyoglobin is moderately hydrated (Vidugiris & Royer, 1998). Our pressure unfolding results for  $\alpha$ -lactalbumin apparently agree with the results of the calorimetric studies for  $\alpha$ -lactalbumin. In our experiments, the  $m$ -value (which correlates with the degree of surface area exposure) of the A-U transition was observed to be 30% of the N-A transition. This result also indicates that the change of the degree of surface area exposure of the N-A transition is greater than that of the A-U transition, as the calorimetric and the present pressure results indicate.

Dilatometric studies of helix-coil transitions of poly-L-amino acid showed the volume change of the helix-coil transition to be about  $-1 \text{ cm}^3/\text{mol}$  residue (Noguchi & Yang, 1963; Noguchi, 1966) and that of the  $\beta$ -coil transition to be  $-1.90$  to  $-2.35 \text{ cm}^3/\text{mol}$  residue (Makino & Noguchi, 1971) in aqueous solution. Hydrogen exchange studies by NMR of a human  $\alpha$ -lactalbumin molten globule showed that 24 amide protons from the  $\alpha$ -helical domain are protected by an H/D exchange (Schulman et al., 1995). From CD measurements, helical residues remain at 36% in the bovine  $\alpha$ -lactalbumin molten globule (Kuwajima, 1977). From these facts, it would be reasonable if an A-U transition volume change were observed. However, it was not observed in our experiment. Consequently, dilatometric studies of poly-L-amino acid and the present results do not agree. There exists a long-standing "protein volume paradox," in which inexplicably small net volume changes are observed to accompany a large conformational change based on small molecule data (Vidugiris & Royer, 1998). More studies may be required on the behavior of secondary structure volumes of proteins.

There appears to be two potential conclusions. One is that most internal cavities are liberated by hydration, and that almost all the buried residues are exposed in the process from native to the molten globule state for  $\alpha$ -lactalbumin. If this conclusion is true, then the differences in our data and dilatometric studies of poly-

L-amino acid cannot be explained, and this may be a case where model compound data and protein differ. On the other hand, a significant positive contribution to the partial volume change accompanying unfolding reaction has recently been reported (Chalikian & Breslauer, 1998). So the other conclusion may be that the positive and negative contributions of hydration of the exposed polypeptide surface area offset each other, and therefore the overall measured volume change likely arises primarily from the elimination of packing defects, which was suggested in a previous work to be apomyoglobin (Vidugiris & Royer, 1998) and staphylococcal nuclease (Frye et al., 1996). This apparently explains the reason for the discrepancy between our results and those of dilatometric studies of poly-L-amino acid. In either conclusion, our results indicate that the interior of molten globule of  $\alpha$ -lactalbumin is as highly hydrated, as previously reported in other papers (Kharakoz & Bychkova, 1997). Further investigations of other proteins showing the molten globule state will contribute to the understanding of the folding mechanism of proteins.

### Materials and methods

Bovine  $\alpha$ -lactalbumin was prepared from fresh bovine milk as described previously (Lin, 1970), and contained 1-2 mol  $\text{Ca}^{2+}$  per mole of the protein. The human  $\alpha$ -lactalbumin gene was cloned into an expression vector pET22-b(+) (Novagene, Madison, Wisconsin), expressed as an inclusion body in *E. coli* BL-21 (DE3), and then refolded using a method described previously (Peng & Kim, 1994). It was confirmed with atomic absorption spectroscopy (Hitachi 170-10) that human  $\alpha$ -lactalbumin also contained 1-2 mol  $\text{Ca}^{2+}$  per mole of the protein when 1 mM  $\text{CaCl}_2$  was added to refolding buffer. Decalcified bovine  $\alpha$ -lactalbumin was obtained by chromatography on a column PD-10 (Pharmacia Biotech, Brussels, Belgium) equilibrated with 0.1 M formic acid, followed by lyophilization. For experiments of apo- $\alpha$ -lactalbumin, contamination by  $\text{Ca}^{2+}$  was checked before and after the measurements by using atomic absorption spectroscopy. All sample solutions used for the study of GuHCl-induced unfolding were buffered with 50 mM sodium cacodylate containing 50 mM NaCl and 0, 0.1, 1, or 10 mM  $\text{CaCl}_2$  at pH 7.0 and measured at 25 °C. Concentration of bovine  $\alpha$ -lactalbumin, human  $\alpha$ -lactalbumin, and GuHCl were determined by the methods described previously (Nozaki, 1972; Sugai et al., 1973; Nozaka et al., 1978). Sample solutions used for thermal unfolding of apo- $\alpha$ -lactalbumin were buffered with 20 mM bis-Tris containing 10 mM NaCl at pH 7.0 or with 50 mM sodium cacodylate containing 50 mM NaCl at pH 7.0, and holo- $\alpha$ -lactalbumin with 50 mM sodium cacodylate containing 50 mM NaCl at pH 7.0 or with 20 mM bis-Tris containing 2 mM diethylenetriamine pentaacetic acid and 1 mM  $\text{CaCl}_2$  at pH 7.0, as a buffer for the concentration of calcium ions, which enabled us to assume a first-order reaction for the unfolding of calcium-binding  $\alpha$ -lactalbumin.

The UV derivative spectra were measured with a UV-3000 spectrophotometer (Shimadzu, Kyoto, Japan) using an optical cuvette with a 10-mm pathlength, with a spectral bandwidth of 0.5 nm and a wavelength difference ( $\Delta\lambda$ ) of 0.8 nm. The concentration increase due to compression was not corrected. The pressure vessel with two windows made of a synthetic sapphire was made of high-tensile stainless steel (Hikari High Pressure Instruments, Ltd Hiroshima, Japan). The vessel was filled with spectrum-grade 2,2,4-trimethylpentane (Junsei Chemical Co., Ltd., Tokyo, Japan) and compressed with a manual pressure pump to a pressure of less than

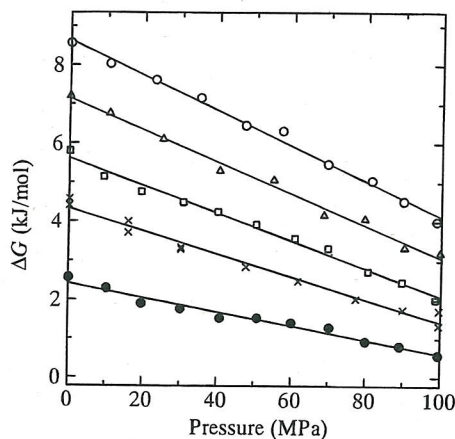


Fig. 8. Pressure dependence of  $\Delta G$  at various temperatures, from top to bottom, 36.5, 41.0, 46.3, 50.3 and 56.2°C, of holo-bovine  $\alpha$ -lactalbumin at pH 7.0.

120 MPa. Pressure was determined with a pressure gauge (Heise). The optical cell was filled with an aqueous solution of human or bovine  $\alpha$ -lactalbumin, sealed with mercury, and placed in the vessel.

Because the estimated  $\Delta G$ 's are linear to pressure up to 100 MPa within experimental error, as shown in Figures 4 and 8, the volume change,  $\Delta V$ , was calculated assuming a linear dependence of  $\Delta G$  on pressure  $p$  as follows:

$$\Delta G = (\Delta G)_0 + p\Delta V. \quad (8)$$

The experimental  $\Delta G$  values were fitted to Equation 7 at each temperature or at each concentration of GuHCl, and the value of  $\Delta V$ , the volume change accompanying denaturation, was obtained by the least-squares method.

Circular dichroism (CD) were measured with a Jasco J-500A spectropolarimeter (Jasco, Tokyo, Japan). Optical cuvettes with 1- and 10-mm pathlengths were used for the measurements in the far- and near-UV regions, respectively. For all measurements thermostated water was circulated to maintain the cell temperature, which was monitored with a thermistor probe.

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