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Pressure-induced unfolding of lysozyme in aqueous guanidinium chloride solution

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Abstract

The pressure-induced unfolding of lysozyme was investigated in an aqueous guanidinium chloride solution by means of ultraviolet spectroscopy. Assuming a two-state transition model, volume changes were calculated from the slope of free energy vs. pressure plots over a temperature range of 10 to 60 °C. Between 25 and 60 °C, almost constant volume changes were observed in the transition region, which was reflected in almost identical slopes of the free energy change vs. pressure plots. On the other hand, the different slopes were observed in the pressure dependence of free energy change at temperatures lower than 25 °C. These data were interpreted as suggesting that a two-state model is not appropriate at low temperature, but instead one or more intermediates are present under these conditions. The volume changes for unfolding became less negative at temperatures higher than 25 °C.

Keywords: guanidinium chloride; lysozyme; pressure; unfolding; volume

Thermodynamic aspects of protein unfolding are important for a detailed understanding of unfolding states and unfolding process (Creighton, 1990; Kim & Baldwin, 1990; Dill & Shortle, 1991). Pressure is becoming increasingly popular as a tool for investigating protein unfolding; negative volume changes have been commonly observed in conjunction with the pressure unfolding of proteins (Brandts et al., 1970; Hawley, 1971; Zipp & Kauzmann, 1973; Taniguchi & Suzuki, 1983). Partial volume change (ΔV) is one of several thermodynamic parameters that is expected to reflect protein unfolding. Therefore, to characterize a protein in an unfolded state, it is of use to study the temperature dependence of ΔV . However, only limited data have been reported as regards the temperature dependence of ΔV that accompanies protein unfolding. In their early work on pressure-induced unfolding of ribonuclease A, Brandts et al. (1970) found that the volume change for unfolding depends on the pH and the temperature at which the experiment is carried out and falls within the range of -45 to -5 mL mol⁻¹. In a study of pressure-induced unfolding of ribonuclease A, Yamaguchi et al. (1995) showed that the ΔV value for unfolding becomes more negative with an increase in temperature. They suggested that a major factor affecting the pressure-induced unfolding of this protein is the thermodynamic state of solvent water surrounding the exposed nonpolar groups. Recently, Dewa et al. (1998) investigated the effect of pressure on the thermal unfolding of cytochrome *c*. They reported that the ΔV value for

unfolding is strongly dependent on temperature and changes its sign from negative to positive at around 40 °C. Further studies on the temperature dependence of ΔV for unfolding would provide additional information about the unfolding process and unfolded states. Moreover, it is expected that some novel aspects of protein structure and dynamics will be revealed by further study of the application of moderate pressure, which not only affects the structure of solvent water but in turn also perturbs chemical and thermal unfolding.

To characterize unfolded states of lysozyme at various temperatures, we studied the temperature dependence of ΔV associated with pressure-induced unfolding of lysozyme in aqueous GdnCl solutions.

Results

Figure 1 shows the $d\epsilon/d\lambda$ values at 290 nm of lysozyme as a function of GdnCl concentration at various temperatures. The $d\epsilon/d\lambda$ values were calculated from the intensity ($dA/d\lambda$) at 290 nm of first-order differential absorption spectra of lysozyme where A is absorbance, ϵ (mol⁻¹ cm⁻¹) is the extinction coefficient of the protein, and λ (nm) is the wavelength. The wavelength, 290 nm, was selected to calculate the equilibrium constant between the native and unfolded states by detecting the first-order differential absorption spectra from 293 to 286 nm in several GdnCl solutions (K. Sasahara, M. Sakurai, & K. Nitta, unpubl. data). Figure 2 shows the plots of unfolded fractions of lysozyme estimated from $d\epsilon/d\lambda$ values at 290 nm by ultraviolet spectroscopy and from ellipticities at 289 nm and at 222 nm by CD measurement as a function of GdnCl concentration at several temperatures. The transitions observed with UV and CD spectra coincided well with each

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Abbreviations: CD, circular dichroism; GdnCl, guanidinium chloride.

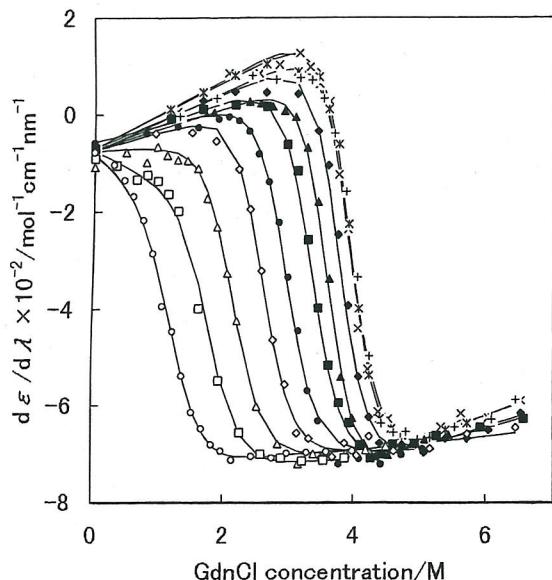


Fig. 1. GdnCl concentration and temperature dependence of the differential absorption at 290 nm of lysozyme. Open circle, 60 °C; open square, 55 °C; open triangle, 50 °C; open diamond, 45 °C; filled circle, 40 °C; filled square, 35 °C; filled triangle, 30 °C; filled diamond, 25 °C; cross, 20 °C; saltire, 15 °C; asterisk, 10 °C.

other; hence, the GdnCl-induced unfolding of this protein is considered to be a two-state process over a temperature range of 10 to 60 °C. The effects of pressure on the $d\epsilon/d\lambda$ values at 25 °C are shown in Figure 3, which represents changes in the fractions of native or unfolded states in the transition region.

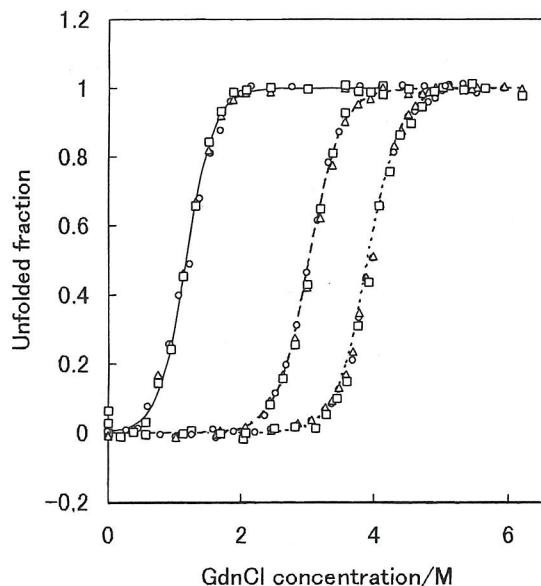


Fig. 2. Unfolded fraction of lysozyme estimated from $d\epsilon/d\lambda$ at 290 nm by UV measurement and from the ellipticities at 289 and 222 nm by CD measurement as a function of GdnCl concentration at several temperatures. Open circle, UV; open square, CD 289 nm; open triangle, CD 222 nm; solid line, 60 °C; long dashed line, 40 °C; dashed line, 10 °C.

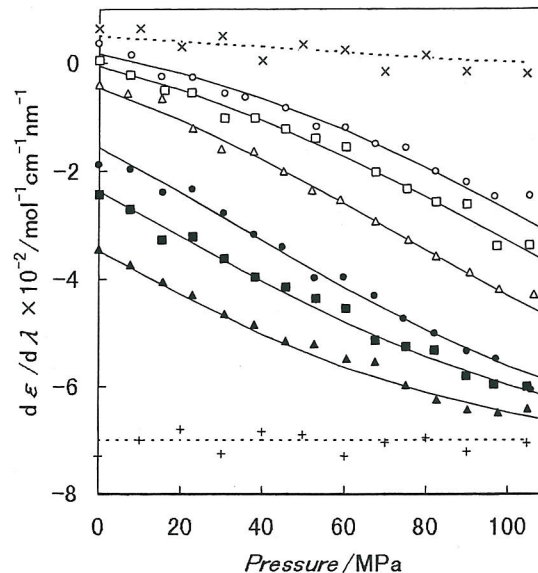


Fig. 3. Pressure dependence of the differential absorption at 290 nm of lysozyme in GdnCl solution at 25 °C. Open circle, 3.11 M GdnCl; open square, 3.27 M; open triangle, 3.45 M; filled circle, 3.69 M; filled square, 3.75 M; filled triangle, 3.87 M; saltire, 1.50 M; cross, 6.20 M.

Assuming a two-state transition by compression from the native to the unfolded state, the equilibrium constant (K_u), and hence the Gibbs free energy change (ΔG_u), were calculated using the following equation:

$$\begin{aligned}\Delta G_u &= -RT \ln K_u \\ &= -RT \ln(f_u/f_n) \\ &= -RT \ln\{(X - X_n)/(X_u - X)\},\end{aligned}\quad (1)$$

where R is the gas constant, T the absolute temperature, f_n the native fraction, and f_u the unfolded fraction. X_n , X_u , and X are the respective $d\epsilon/d\lambda$ values calculated from the intensity ($dA/d\lambda$) at 290 nm of first-order differential spectra for the native (pre-transition region) and unfolded states (post-transition region), and for the transition region. The ΔG_u values at 25 °C were plotted against pressure in Figure 4A. The slopes of the lines are almost linear and relate as follows to the volume changes of transition (ΔV_u):

$$(\partial\Delta G_u/\partial p) = \Delta V_u.\quad (2)$$

We based our calculation of ΔV_u values on the assumption that the ΔG_u value varies linearly with pressure in the transition region. We observed almost constant ΔV_u values (i.e., -40 to -50 mL mol⁻¹) at 25 °C. The constant ΔV_u values in the transition region were also observed at temperatures of 40 and 60 °C, as shown in Figures 4B and 4C. Figure 5 provides the pressure dependence of ΔG_u values in various GdnCl solutions at temperatures of 20, 15, and 10 °C. Different slopes were observed in the pressure dependence of ΔG_u ; namely, the ΔV_u values were not constant in the transition region. Figure 6 shows the relations between the unfolded fraction at 0.1 MPa and the ΔV_u values at several temperatures. In the lower temperature range, the ΔV_u values change during the early stages of unfolding. This volumetric behavior appears to become notable

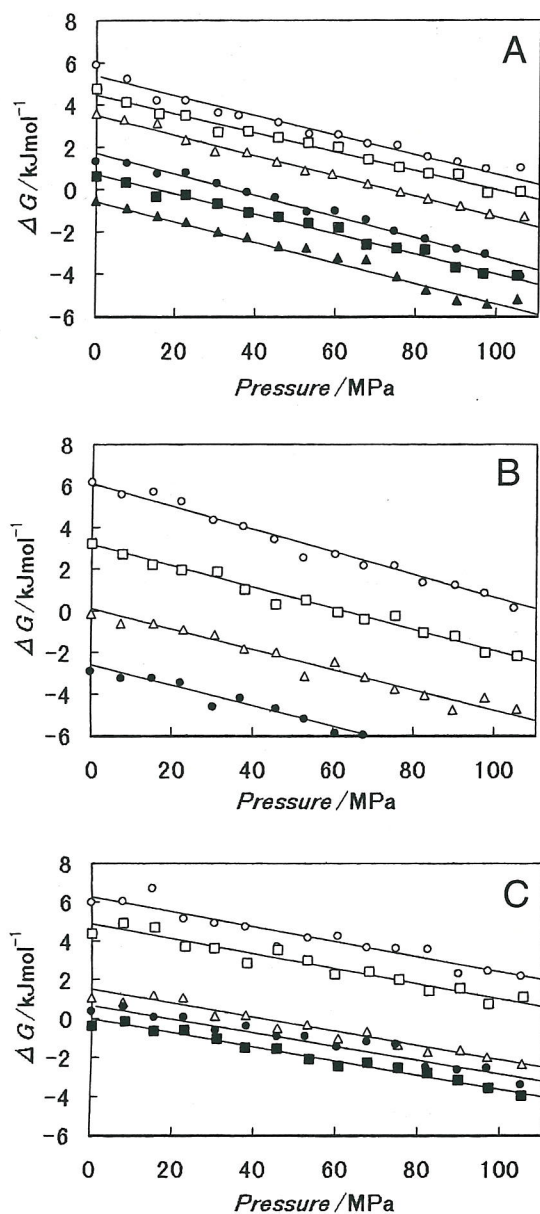


Fig. 4. Pressure dependence of the free energy change of lysozyme in GdnCl solution at (A) 25°C, (B) 40°C, and (C) 60°C. A: Open circle, 3.11 M GdnCl; open square, 3.27 M; open triangle, 3.45 M; filled circle, 3.69 M; filled square, 3.75 M; filled triangle, 3.87 M. B: Open circle, 2.59 M; open square, 2.88 M; open triangle, 3.09 M; filled circle, 3.27 M. C: Open circle, 0.80 M; open square, 0.91 M; open triangle, 1.21 M; filled circle, 1.26 M; filled square, 1.32 M.

as temperature decreases. On the other hand, the ΔV_u values are almost constant in the transition region at temperatures of 25, 40, and 60°C. Figure 7 shows the plots of all of the ΔV_u values as a function of temperature. The ΔV_u values become less negative at temperatures higher than 25°C.

Discussion

Negative volume changes have been observed upon pressure unfolding of hen lysozyme. For example, Li et al. (1976) obtained

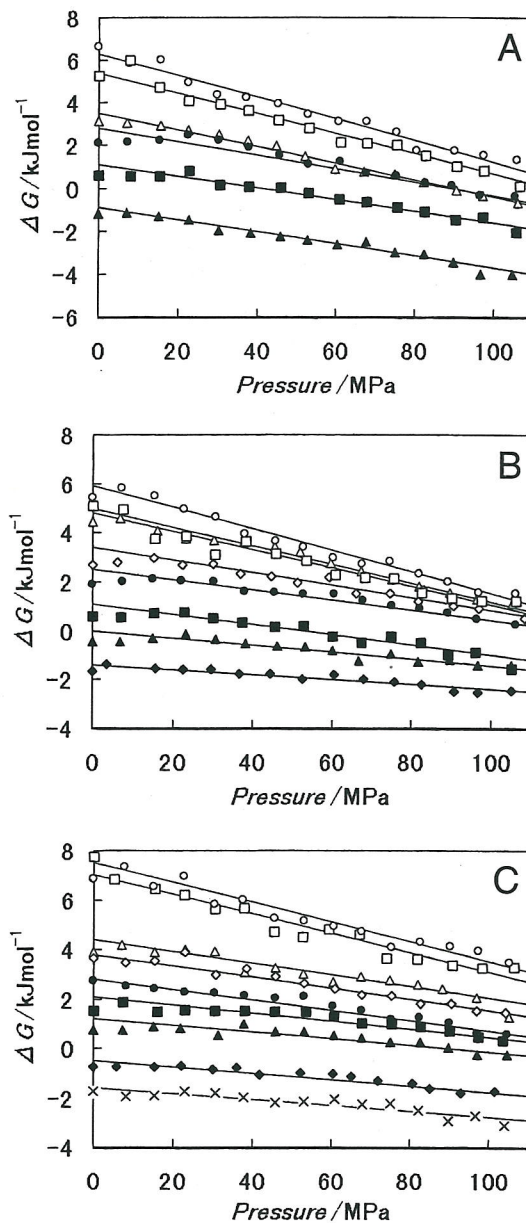


Fig. 5. Pressure dependence of the free energy change of lysozyme in GdnCl solution at (A) 20°C, (B) 15°C, and (C) 10°C. A: Open circle, 3.27 M GdnCl; open square, 3.45 M; open triangle, 3.69 M; filled circle, 3.79 M; filled square, 3.94 M; filled triangle, 4.10 M. B: Open circle, 3.40 M; open square, 3.45 M; open triangle, 3.50 M; open diamond, 3.71 M; filled circle, 3.79 M; filled square, 3.90 M; filled triangle, 4.00 M; filled diamond, 4.09 M. C: Open circle, 3.16 M; open square, 3.22 M; open triangle, 3.27 M; open diamond, 3.35 M; filled circle, 3.50 M; filled square, 3.71 M; filled triangle, 3.79 M; filled diamond, 3.90 M; saltire, 4.00 M.

$\Delta V = -19.7 \text{ mL mol}^{-1}$ at pH 7.6 and 23°C by means of a fluorescence technique. Using high resolution proton magnetic resonance spectroscopy, Samarasinghe et al. (1992) reported $\Delta V = -10.5 \text{ mL mol}^{-1}$ at pH 3.9 and 68.5°C. These values are less negative than the values we obtained, namely, -40 to -50 mL mol^{-1} at pH 4.0 and 25°C. It is known that an appreciably residual protein structure remains after pressure unfolding, as compared with GdnCl-induced unfolding (Zhang et al., 1995).

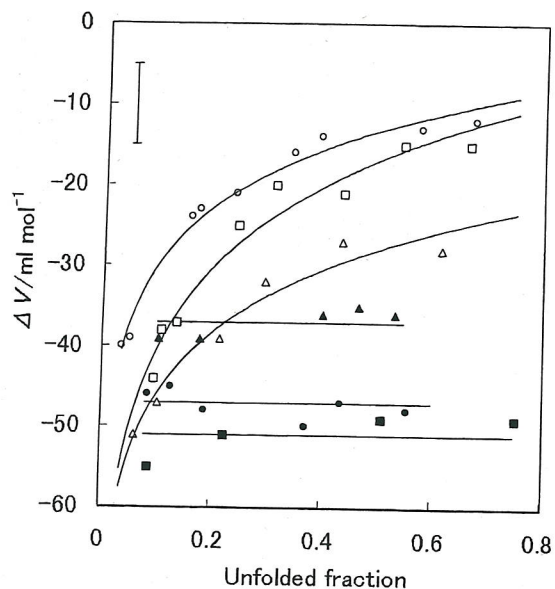


Fig. 6. Relation between unfolded fraction at 0.1 MPa and volume change. Open circle, 10 °C; open square, 15 °C; open triangle, 20 °C; filled circle, 25 °C; filled square, 40 °C; filled triangle, 60 °C.

The difference between our results and the values reported as regards pressure unfolding may be attributed to differences in experimental conditions; namely, the pressure-induced unfolding observed in the present study was carried out in an aqueous GdnCl solution. Based on results from dilatometric measurement, Skerjanc and Lapanje (1972) estimated the volume change of lysozyme upon unfolding by GdnCl to be -54 mL mol^{-1} at pH 5.2 and 25 °C. These values seem to be in good agreement with our results, if one takes into account experimental uncertainty and different experimental conditions under which both studies were performed.

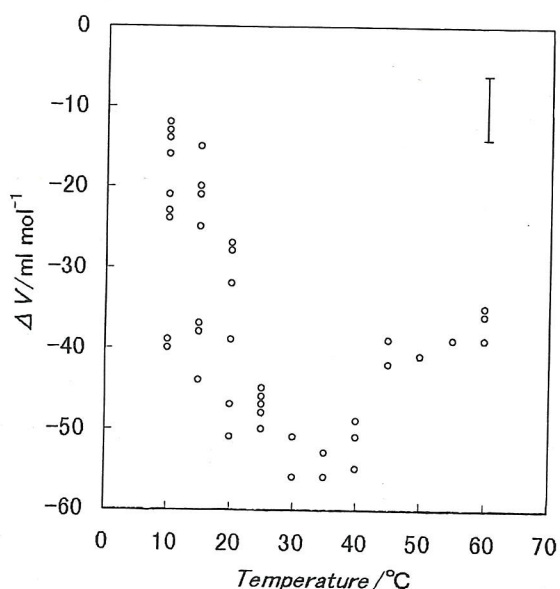


Fig. 7. Relation between temperature and volume change.

The volume change for protein unfolding can arise from a variety of structural origins such as void volume due to imperfect packing in the protein interior and hydration structure of charged, polar, and nonpolar groups (Chalikian & Breslauer, 1996; Frye & Royer, 1998). In addition, a high GdnCl concentration makes the interpretation of volume changes more complex due to the physical properties of solvent water at these high salt concentrations since such salt concentrations may affect the overall hydration of the protein states. We see from Figure 7 that the ΔV_u values become less negative at temperatures higher than 25 °C. Brandts et al. (1970) showed an apparent trend of ΔV values to become less negative with increases in temperature in the pressure-induced unfolding of ribonuclease A. Yamaguchi et al. (1995) used high pressure proton NMR to study the thermodynamic stability of ribonuclease A; they showed that the ΔV value becomes more negative with an increase in temperature. The reason for discrepancies between the two results as regards ribonuclease A remains unclear. Dewa et al. (1998) have studied the perturbation of thermal unfolding of cytochrome *c* with compression to obtain ΔV for unfolding. They reported that the ΔV value is strongly dependent on temperature and its sign changes from negative to positive at around 40 °C. Chalikian and Breslauer (1996) have presented an approach to volume changes for protein unfolding that involves changes in the solvent accessible surface area. They suggested that volume decreases due to changes in protein hydration and loss of internal void space in the native structure are compensated for by an increase in thermal volume that results from the thermally induced mutual motion of exposed residues and solvent molecules. Based on their analysis, the volume change for unfolding becomes less negative with increases in temperature. Furthermore, the volume change even becomes positive in the case of larger proteins. Thus, the volumetric behavior depicted in Figure 7, which becomes less negative at temperatures higher than 25 °C, seems predictable.

The GdnCl-induced unfolding of lysozyme in the transition region has been regarded as a highly cooperative two-state reaction lacking stable intermediates (Tanford, 1968; Aune & Tanford, 1969). Lysozyme in the native state consists of two domains: the α -domain, which contains four α -helices, and the β -domain, which contains a three-strand antiparallel β -sheet (Miranker et al., 1991). It follows, then, that lysozyme represents a single cooperative macroscopic system in GdnCl-induced equilibrium unfolding, where the constituent domains do not independently change their states. In this study, almost constant ΔV_u values were observed in the transition region at temperatures of 25, 40, and 60 °C. We interpret these results as supportive of the notion that the pressure-induced unfolding of lysozyme shows a two-state transition, which is reflected in almost identical slopes of the ΔG_u vs. pressure plots. On the other hand, we observed different slopes in the pressure dependence of ΔG_u at temperatures of 20, 15, and 10 °C. These data indicate that a two-state model is not appropriate for the pressure-induced unfolding at lower temperatures. Thus, we arrive at the conclusion that pressure-induced unfolding at lower temperatures in the presence of GdnCl cannot be represented by a two-state model, but instead corresponds to a variation in an ensemble of the conformational species that is partially unfolded.

Although the equilibrium unfolding of lysozyme in the transition region is well represented as a two-state reaction without any stable intermediates, the existence of a transient intermediate that is similar to the intermediate (i.e., molten globule state) of homologous α -lactalbumin has been demonstrated in the kinetic folding

process of lysozyme (Kuwajima et al., 1985; Ikeguchi et al., 1986; Miranker et al., 1991; Radford et al., 1992). Haezebrouck et al. (1995) have suggested the existence of a partially folded state, which has some characteristics of the intermediate state of homologous α -lactalbumin, in the thermal unfolding process of lysozyme at lower pH. The small-angle X-ray scattering studies reported by Chen et al. (1996) have suggested the existence of an equilibrium intermediate in the urea-induced unfolding of hen lysozyme at pH 2.9. Hence, one can assume that hen lysozyme, which has been considered as a protein that exhibits cooperative two-state unfolding behavior, reveals a latent tendency toward noncooperative unfolding under extreme conditions.

The application of hydrostatic pressure to a protein solution is a method of perturbing the structure of protein and its interactions with solvent water. Since most of the nonpolar groups in the native structure are not exposed to the solvent water (Janin, 1979; Wolfenden et al., 1981; Rose et al., 1985), one assumes that the state of hydration of exposed nonpolar groups plays an important role in determining the thermodynamic stability of a protein. Yamaguchi et al. (1995) have suggested that a major factor affecting the pressure-induced unfolding of ribonuclease A is the pressure-induced change in the thermodynamic state of solvent water surrounding the exposed nonpolar groups. Hummer et al. (1998) have reported that pressure destabilizes the contact configuration of nonpolar groups relative to a solvent-separated configuration. They describe the process of pressure unfolding as the penetration of water molecules into the protein interior. Under other circumstances, it has been shown that the native structure of a globular protein in an aqueous environment is destroyed not only by increases in temperature but also by decreases in temperature (Privalov & Gill, 1988; Franks, 1995; Nash & Jonas, 1997). The main cause of cold unfolding is assumed to be the weakness of the collective repulsion between water and nonpolar group (Franks, 1995). Thus, it is important to understand the thermodynamics of protein stability under pressure at lower temperatures.

The conformation of unfolded proteins at lower temperatures appears to be quite different from those of thermally and chemically unfolded states. For instance, Nash and Jonas (1997) investigated the structure of the pressure-assisted cold-unfolding state of lysozyme by means of amide hydrogen exchange. They showed that the extent of protection of many of the 52 backbone amide protons is markedly different from that of lysozyme unfolded by high temperatures and high urea concentration; the pattern of protection factors is similar to that observed in lysozyme during the early stages of refolding from highly unfolded states. NMR measurements by Tamura et al. (1991) have identified a cooperative transition between cold- and heat-unfolded states of subtilisin. Unfortunately, pressure-induced unfolding, used in the present study, makes it more difficult to interpret the temperature dependence of ΔV_u in the presence of the denaturant. We are not certain whether the action of denaturant on proteins is direct and can be regarded as ligand binding, or whether it is indirect and involves change in the physical properties of solvent water (Lee & Timasheff, 1974; Makhatadze & Privalov, 1992). It would therefore be difficult to give a satisfactory explanation for the temperature dependence of ΔV_u at low temperatures. A plausible explanation for the volumetric deviation from a two-state model at lower temperatures is that during the early stages of unfolding, lysozyme has one or more intermediates that are similar to the intermediate of homologous α -lactalbumin. Calorimetric studies on α -lactalbumin suggest that the stable intermediate has nearly the same heat capacity as the

unfolded state (Griko et al., 1994). Such studies lead to the conclusion that nonpolar side chains in the intermediate state are hydrated (Privalov et al., 1989). Moreover, this suggests that the hydration of amino acid residues is expected to be accompanied by further decreases in volume (Kauzmann, 1959; Gross & Jaenicke, 1994). The response of a system to hydrostatic pressure is to minimize volume; therefore, partially unfolded intermediate states may be more easily observed under the equilibrium condition established by compression at lower temperatures.

Materials and methods

Materials

Hen egg white lysozyme (crystallized six times) was purchased from Seikagaku Corporation, Ltd. (Tokyo, Japan) and was used without further purification. The concentration of lysozyme in the sample solution was about 0.04 wt% and was determined by using an extinction coefficient (26.5) at 280 nm for a 1% solution in a 1 cm cell. Biochemical-grade GdnCl was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The concentration of GdnCl was determined by the difference between the refractive index of a GdnCl solution and that of water (Nozaki, 1972). The pH of the protein solutions was adjusted to 4.0 by acetic acid and sodium acetate. Distilled and deionized water was used for preparing the solutions.

Pressure-induced unfolding

A high-pressure vessel made of high-tensile stainless steel with two sapphire windows was specially designed to fit into the spectrophotometer (Hikari High-Pressure Instruments, Ltd., Hiroshima, Japan). The sample cell for the measurement was made of quartz. It had a 10 mm path length and a capillary tube 2 mm in diameter. The quartz cell, filled with an aqueous protein solution, was fixed into the high-pressure vessel by a cell holder made of brass. The capillary of the quartz cell was sealed with mercury to separate the sample solution from the pressure mediator (2,2,4-trimethylpentane). Temperature was controlled to an accuracy of 0.1 °C by circulating an ethylene glycol + water mixture in the pressure vessel. Temperature was monitored via a thermometer installed inside the pressure vessel.

Pressure was regulated with a hand pump and was transmitted through a stainless steel tube containing a mediator to the sample solution. The pressure of the sample was increased step by step from 0.1 to 110 MPa and was read with a Heise pressure gauge. After 10 min, the intensity ($dA/d\lambda$) at 290 nm of the first-order differential spectra from 292 to 286 nm was measured with a Shimadzu UV-3000 spectrophotometer as an equilibrium value. The $dA/d\lambda$ value was transformed into the $d\epsilon/d\lambda$ value. The measurements were repeated seven times and the average of the $d\epsilon/d\lambda$ values was recorded.

CD measurements

The GdnCl-induced unfolding of lysozyme was detected by CD measurement with a Jasco J-725A spectropolarimeter. Ellipticities at 289 and 222 nm were measured in quartz cells with 10 and 1 mm path lengths, respectively. The temperature surrounding the cell was maintained by circulating an ethylene glycol + water

mixture from a constant temperature bath. Thermal stability was kept within 0.1 °C.

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