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Calorimetric Study on Thermal Denaturation of Lysozyme in Polyol-Water Mixtures[1]

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In order to clarify the mechanism of polyol-induced stabilization of protein, the thermal denaturation of lysozyme was studied at pH 4 in aqueous mixtures of some polyols (ethylene glycol, glycerol, erythritol, xylitol, and sorbitol) by a differential scanning calorimetry (DSC). The denaturation temperature, T_d, increased with increasing the polyol concentration and the number of hydroxymethyl groups per polyol molecule. The calorimetric enthalpy of denaturation, ΔH_{cal}, increased with the increase in polyol concentration, but it was not significantly affected by the chain length of the polyol: ΔH_{cal} was about 30 kcal/mol larger in 30% (w/w) aqueous polyols than in water. The standard thermodynamic parameters for denaturation, ΔG°, ΔS°, and ΔH°, which were calculated for glycerol and sorbitol systems using T_d and ΔH_{cal} and assuming a constant heat capacity change, were an increasing function of polyol concentration. According to the thermodynamics of three component systems, it appeared that one or two polyol molecules are preferentially excluded from the domain of this protein on thermal denaturation. These thermodynamic data support the hypothesis that the thermal stabilization of lysozyme by polyols is due to a preferential solvent interaction effect which strengthens the hydrophobic interaction of the protein.

It has been proposed that the stabilization of proteins by sugars and polyols may be dominantly mediated through the changes in solvent properties or alteration of the water structure (*1–7*). Recently, we have shown that globular proteins are preferentially hydrated in polyol-water mixtures through the delicate balance between the repulsion from nonpolar regions and the attraction from polar regions on the protein surface (*8*, *9*). The resulting increase in chemical potential of the protein means that the system is thermodynamically unfavorable; thus reactions accompanied by an increase in protein-solvent interface, such as denaturation and dissociation, would be prevented in such unfavorable solvent systems. Thermodynamic studies on transfer of amino acids from water to aqueous polyols have clearly confirmed that polyol molecules are antagonistic to nonpolar side chains of amino acids exposed on denaturation of proteins (*10*, *11*). Therefore, it can be expected from the free energy level that the polyol-induced stabilization of protein is dominantly due

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to the strengthening of intramolecular hydrophobic interactions.

Despite many investigations on the effects of polyols and sugars on the stability of proteins, there are only few limited data on the thermodynamic parameters of protein denaturation in aqueous solutions of these stabilizing reagents (*12–15*). A further advanced understanding of the stabilization mechanism must be attained by elucidating the enthalpy and entropy contributions to the free energy function of denaturation, since they should more sensitively reflect the solvent effects. In this paper, these thermodynamic parameters of the thermal denaturation of lysozyme have been measured in aqueous solutions of some polyols by differential scanning calorimetry (DSC). The results will be discussed from the viewpoint of protein-solvent interaction in relation to the stabilization mechanism of the protein.

EXPERIMENTAL PROCEDURE

Materials—Hen eggwhite lysozyme was purchased from Seikagaku Kogyo Co., Ltd. (lot No. E0201, six-times recrystallized). This product was further subjected to exhausitive dialysis against distilled water at 4°C and lyophilized before use. All alcohols used were the same as those in the previous papers (*9–11, 14*). Other chemicals were analytical reagent grade products from Wako Pure Chemical Industries. All the calorimetric experiments were carried out at a protein concentration of 2% in 0.01 M glycine buffer adjusted to pH 4.0 as measured by a Horiba model f-7ss pH meter, with a sensitivity of 0.002 pH units, in conjunction with a Horiba 6026 combination pH electrode. Protein concentration was determined spectrophotometrically on a Jasco 501 double beam spectrophotometer. The extinction coefficient of lysozyme was assumed to be 26.9 dl/(g cm) at 280 nm in water (*16*) and the coefficient was corrected for each mixed solvent system using the method described in a previous paper (*17*).

DSC Measurements—DSC measurements were performed with a Dainiseikosha SSC-560 differential scanning calorimeter with high sensitivity (0.025 mcal/s/full scale) at a heating rate of 1.4 k/min. The instrument was calibrated by using four materials with known enthalpy of fusion: benzophenone (48.2°C), palmitic acid (62.8°C), stearic acid (69.6°C), and naphthalene (80.2°C). The calibration constants obtained did not show the temperature dependence within 2% error, then the averaged value, 0.531 mcal/mV·s, was used. Freshly prepared sample solutions (60 μl) were packed in a calorimetric silver cell and immediately subjected to DSC measurements. For each solvent system, three samples were run against a solvent blank. The curve for each solvent blank, which was run after the experiments with the samples, was assumed to be the base line in the transition region (see Fig. 1). The area under the thermograms was determined by means of a planimeter and converted to the enthalpy of denaturation using the calibration constant and the molecular weight of lysozyme, 14,300. The calorimetric enthalpy of denaturation thus obtained, ΔH_{cal}, was not significantly different from that calculated using the base line based on the heat capacity difference between the native and denatured states. Because of the symmetrical nature of the peak, the temperature of maximal excess heat flow, T_m, was assumed as a denaturation temperature, T_d, at which the transition is half complete, although T_d and T_m do not always coincide, even for a two-state process with no permanent change in heat capacity (*18*).

Density Measurements—In order to examine the solvent component interaction with native lysozyme, two types of partial specific volumes of the protein in polyol solutions, ϕ_2° and $\phi_2'^\circ$, were measured by using an Anton Paar DMA-02C density meter: ϕ_2° and $\phi_2'^\circ$ refer to the partial specific volumes on keeping the molality and the chemical potential of polyol identical between the solvent and the solutions, respectively. The detailed experimental procedures were described in previous papers (*8, 9, 17*).

RESULTS

Repetitive scans with DSC have shown a good reversibility in thermal denaturation of lysozyme when the sample was rapidly cooled after the preceding scan. However, when the sample was held for a long time at a temperature above that corresponding to the end of the denaturation, the denaturation was not completely reversible, especially in polyol solutions, probably due to partial aggregation of the protein. The peak

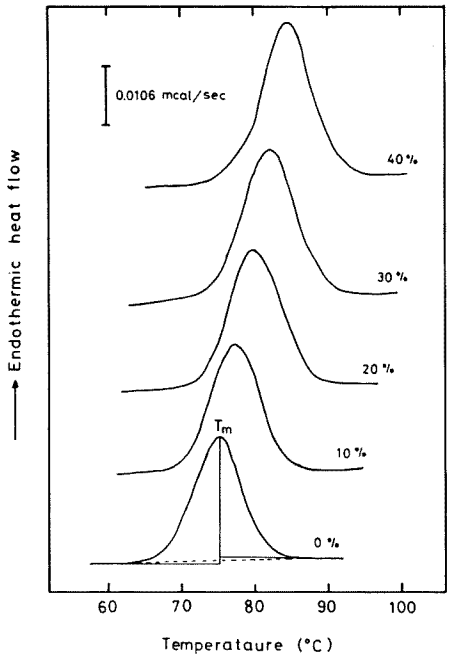

Fig. 1. Typical DSC thermograms of the thermal denaturation of lysozyme in sorbitol-water mixtures at pH 4.0 (0.01 M glycine buffer). The number attached to the curves shows the concentration of sorbitol (% w/w). A solvent blank curve, shown by the dotted line in the bottom figure, was assumed for a base line in the transition region.

temperature and the form of the DSC curves were hardly influenced by heating rates slower than 1.4 k/min, allowing equilibrium analysis of the data.

Figure 1 represents typical DSC curves for denaturation of lysozyme in sorbitol-water mixtures. The temperature of maximal heat flow and the peak area appeared to increase with increasing sorbitol concentration. Similar tendencies were observed for other polyol systems, while addition of methanol and ethylene glycol depressed the peak temperature. The values of T_d and ΔH_{cal} obtained are listed in Table I, in which each value is the averaged from three experiments. The values of both T_d and ΔH_{cal} in buffer solution are in very good accord with the data of Uedaira and Uedaira (*12*), while ΔH_{cal} is slightly less than that obtained by Donovan and Ross (*19*) and Pfeil and Privalov (*20*).

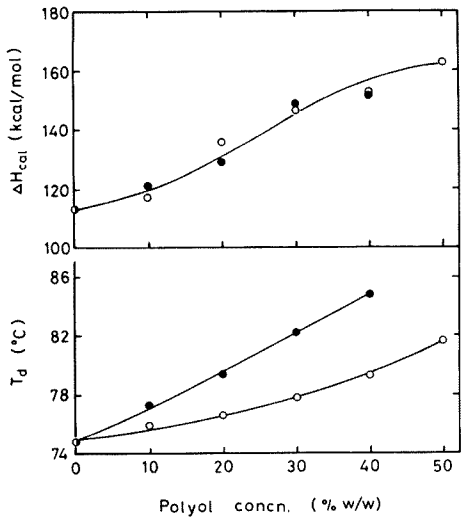

Fig. 2. Variation of T_d and ΔH_{cal} with the concentration of glycerol (○) and sorbitol (●). The symbol ◑ refers to the buffer solution without polyol.

Figure 2 shows the plots of T_d and ΔH_{cal} as a function of the concentration of glycerol and sorbitol. It is evident that both polyols can more effectively stabilize lysozyme against thermal denaturation as their concentration increases. The ΔH_{cal} also increased monotonously with the addition of the polyols. This is the case with the corresponding entropy of denaturation, ΔS_{cal} ($= \Delta H_{cal}/T_d$), (see Table I). Therefore, the observed increase in T_d occurs because ΔS_{cal} does not increase as much as ΔH_{cal} does upon the addition of polyols. Thus these polyol molecules seem to inhibit the disordering of the system induced by heating, suggesting the possibility of some kind of solvent-ordering effect around the protein molecules.

The relation between the structure and the stabilizing ability of polyols can be deduced from the plots of T_d and ΔH_{cal} as a function of the number of hydroxymethyl groups per polyol molecule at identical concentrations of polyols, 30% (w/w), (Fig. 3). T_d increased with an increase in the number of OH groups per polyol molecule, in agreement with the results in other reports (*2*, *3*, *6*, *14*). Thus, it may be generally accepted that polyols can more effectively stabilize the proteins as their hydroxymethyl chains increase in

TABLE I. Thermodynamic parameters of thermal denaturation of lysozyme in polyol-water mixtures.

Alcohol conc. (% w/w)		T_d[a] (°C)	ΔH_{cal}[b] (kcal/mol)	ΔS_{cal} (cal/K·mol)
Water (pH 4.0)		74.8	113	323
Methanol	30	57.0	121	368
Ethylene glycol	30	73.7	143	413
Glycerol	10	75.9	117	336
	20	76.6	136	388
	30	77.8	147	419
	40	79.3	153	435
	50	81.7	163	460
Erythritol	30	79.8	136	386
Xylitol	30	81.3	144	406
Sorbitol	10	77.3	121	344
	20	79.4	129	366
	30	82.2	149	419
	40	84.8	152	425

[a] Temperatures have experimental errors of ±0.2°C. [b] These values have maximum expected errors of ±4% including errors in sample preparation, calibration constant and reproducibility.

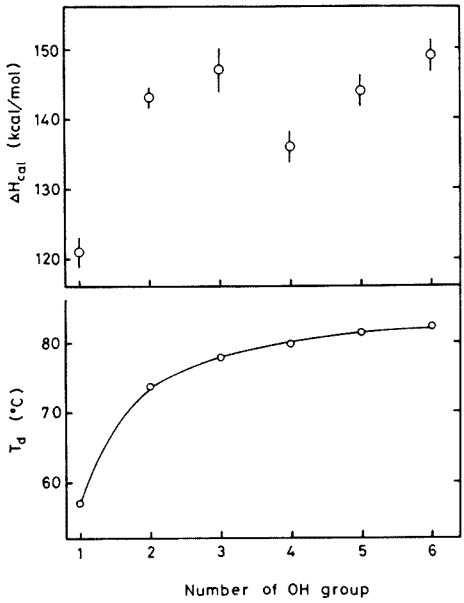

Fig. 3. Variation of T_d and ΔH_{cal} with the number of hydroxymethyl groups of polyols at polyol concentration of 30% (w/w). The bar attached to the symbol for ΔH_{cal} shows the experimental error in three experiments.

length. However, ΔH_{cal} did not always show a corresponding dependence on the number of OH groups: the values of ΔH_{cal} in polyol systems were larger by about 30 kcal/mol than in buffer solution. In this regard, it is of interest that sugar-induced thermal stabilization of lysozyme is enhanced as the number of equatorial OH groups per sugar molecule increases, accompanied by an increase of about 10% in ΔH_{cal} (*12*).

The ΔH_{cal} values for all the systems investigated were plotted against ΔS_{cal} in Fig. 4. A linear correlation between these thermodynamic parameters suggests that the polyol-induced stabilization of lysozyme is closely related to the water structure around the protein molecule (*21*). The compensation temperature, which is defined as the slope of the graph, is about 370 K. Although this value is considerably higher than the temperature range of 250 to 320 K observed for many processes, it is in very good accord with the compensation temperature deduced from the results for denaturants such as propanol (*18*), urea (*22*) and guanidine hydrochloride (*20*).

Since ΔH_{cal} is known to be dependent on temperature (*18*, *20*) (this means that the heat capacity change of denaturation, ΔC_p, is not zero), the enthalpy of denaturation must be compared

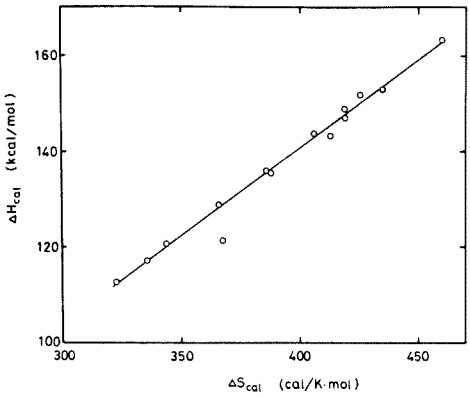

Fig. 4. Enthalpy-entropy compensation plots for thermal denaturation of lysozyme in polyol-water mixtures.

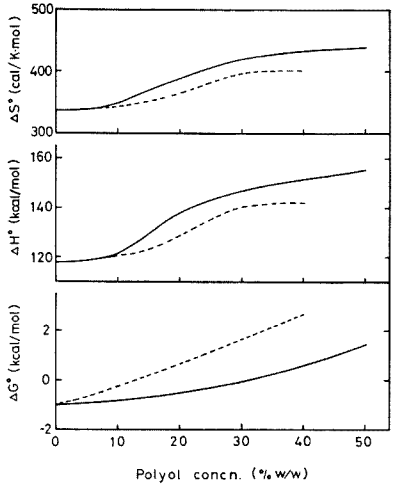

Fig. 5. Variation of ΔG°, ΔH°, and ΔS° with the concentration of glycerol (——) and sorbitol (----) at 78°C.

at identical temperatures to evaluate more accurately the effects of these polyols. Standard thermodynamic functions, ΔG°, ΔH°, and ΔS°, at any temperature, T, can be calculated with ΔC_p and T_d, assuming that $\Delta G^\circ = 0$ at T_d in each solvent system and that ΔC_p is independent of temperature (*18*).

$$\Delta H^\circ = \Delta H_{cal} - \Delta C_p(T_d - T) \quad (1)$$

$$\Delta G^\circ = \Delta H_{cal}(1 - T/T_d) - \Delta C_p(T_d - T) + T\Delta C_p \ln (T_d/T) \quad (2)$$

$$T\Delta S^\circ = \Delta H^\circ - \Delta G^\circ \quad (3)$$

Although ΔC_p in each solvent system could not be accurately determined in the present study, it seems not to be largely affected by addition of polyol (*13*). Then ΔC_p in mixed solvents was assumed to be identical with that in buffer solution, 1.6 kcal/K·mol, which was estimated by Pfeil and Privalov (*20*). This assumption will cause no significant error in ΔH° when T is close to T_d. Thus, ΔG°, ΔH°, and ΔS° for the glycerol and sorbitol systems were calculated at 78°C, which was near the midpoint of the denaturation temperature range of both polyol systems. It is evident from Fig. 5 that ΔG°, ΔH°, and ΔS° are increasing functions of concentration of both polyols. This finding demonstrates that the driving force for polyol-induced stabilization of lysozyme is not an entropy effect but an enthalpy effect.

The results of density measurements are listed in Table II. The partial specific volume of lysozyme at constant molality of polyol, ϕ_2°, is less than that at constant chemical potential of polyol, $\phi_2'^\circ$. The preferential interaction parameter of polyol (component 3) with protein (component 2), $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, was calculated using ϕ_2° and $\phi_2'^\circ$ from the equation

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = -g_3(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = \rho_0(\phi_2^\circ - \phi_2'^\circ)/(1 - \rho_0 v_3) \quad (4)$$

where g_i is the concentration of component i in grams per gram of principal solvent, *i.e.*, water (component 1); μ_i is the chemical potential of component i; ρ_0 is the density of the solvent; and v_3 is the partial specific volume of component 3. The preferential interaction parameter on a molal basis, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, can be expressed by

$$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (M_2/M_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (5)$$

where m_i and M_i are the molal concentration and molecular weight of component i, respectively. As shown in the fourth and sixth columns of Table II, the preferential interaction parameters thus calculated were confirmed to be negative in all solvent systems examined, indicating a deficiency of glycerol and sorbitol molecules in the immediate domain of the protein, *i.e.*, preferential hydration. The chemical potential change of pro-

TABLE II. Partial specific volumes and preferential interaction parameters of lysozyme with solvent components in polyol-water mixtures.

Solvent	ϕ_2° (ml/g)	$\phi_2'^\circ$ (ml/g)	$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$ [a]
Glycerol[b]						
30%(v/v) (pH 2.0)	0.709	0.715	−0.040	0.074	− 6.3	600
30%(v/v) (pH 5.8)	0.709	0.720	−0.073	0.137	−11	1,100
Sorbitol[c]						
10%(w/w) (pH 4.0)	0.713	0.722	−0.029	0.264	− 2.3	2,300
20%(w/w) (pH 4.0)	0.713	0.729	−0.057	0.216	− 4.5	1,900

[a] Calories per mol of polyol per mol of protein in 1,000 g H_2O. [b] At 20°C. From Ref. *8*. [c] At 25°C.

tein due to preferential solvation, which is listed in the last column of this table, was evaluated by the following relation

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = -(RTM_2/1{,}000g_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (6)$$

The positive values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ mean that the system is made thermodynamically less favorable by the addition of polyols.

DISCUSSION

The present calorimetric study has shown that the free energy of denaturation of lysozyme in aqueous polyols, $\Delta G^\circ(c)$, is larger than that in water, $\Delta G^\circ(o)$. This means that the free energy of transfer of the denatured protein from water to aqueous polyols, ΔG_{tr}^D, must be larger than that of the native protein, ΔG_{tr}^N, since $\Delta G_{tr}^N + \Delta G^\circ(c) = \Delta G^\circ(o) + \Delta G_{tr}^D$. Thus, a dominant factor for the stabilization of protein by polyols may be the effect of solvent medium or solvation on the native and denatured protein, as predicted in the previous papers (*8*, *9*). A more detailed discussion will be given below based on the thermodynamics of multicomponent systems.

For the present three-component systems, the dependence of the equilibrium constant of denaturation on the solvent variables, $d \ln K/d \ln a_1$, is ascribed to the difference in the preferential binding of solvent components, $\Delta\xi_3$, between the native and denatured states (*23*, *24*)

$$\left(\frac{d \ln K}{d \ln a_3}\right) = \Delta\xi_3 = \left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3}^D - \left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3}^N \quad (7)$$

where K is the equilibrium constant of denaturation; a_3 is the chemical potential of component 3 (polyol); and superscripts N and D refer to the native and denatured states, respectively. The equilibrium constant, K, at temperature T, may be taken as $Q_T/(Q-Q_T)$ where Q is the total area of the thermogram and Q_T is the partial area swept out by the pen-recorder up to temperature T. The logarithms of the equilibrium constants thus obtained are plotted against the logarithms of the concentration of glycerol and sorbitol in Fig. 6, where the activity of glycerol was calculated from osmotic coefficient data (*25*). For the sorbitol system, the molality unit was used instead of activity on the assumption that the activity coefficient is unity, since there is no activity datum for sorbitol in water. The slope of the lines calculated by a least-squares method, $\Delta\xi_3$, which is listed in the parenthesis attached to each line, is negative and becomes more negative with increasing temperature. The absolute value of $\Delta\xi_3$ for lysozyme is considerably smaller in the glycerol system than in the sorbitol system as well as for chymotrypsinogen (*14*).

Preferential interaction is strictly related to the chemical potential effect, since

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} = -\left(\frac{\partial \mu_3}{\partial m_2}\right)_{T,P,m_3} \Big/ \left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2} \quad (8)$$

where $\mu_i = \mu_i^\circ(T,P) + RT \ln m_i + RT \ln \gamma_i$, and γ_i is the activity coefficient of component i. Then, from Eqs. 7 and 8,

$$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}^D - \left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}^N = -RT\Delta\xi_3\left(\frac{1}{m_3} + \frac{\partial \ln \gamma_3}{\partial m_3}\right) \quad (9)$$

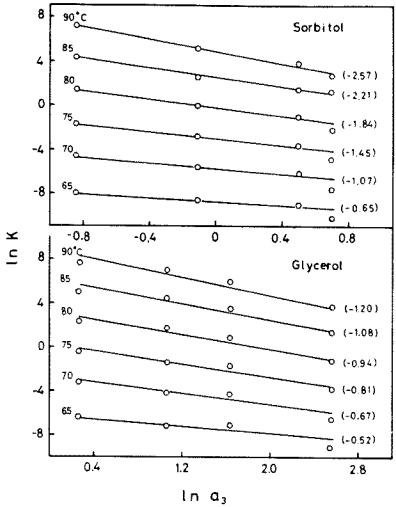

Fig. 6. Wyman plots of the effect of glycerol (lower figure) and sorbitol (upper figure) on the thermal denaturation of lysozyme at several temperatures. The number at the left end of each line shows the temperature and the number in parenthesis at the right end indicates the slope of the line, *i.e.*, $\Delta\xi_3$, calculated by the least-squares method.

In general, the last term on the right-hand side of this equation is negligibly small compared to the first term. Then, the observed negative $\Delta\xi_3$ indicates that $(\partial m_3/\partial m_2)^{D}_{T,P,\mu_3}$ should be more negative than $(\partial m_3/\partial m_2)^{N}_{T,P,\mu_3}$, and that $(\partial\mu_2/\partial m_3)^{D}_{T,P,m_2}$ is more positive than $(\partial\mu_2/\partial m_3)^{N}_{T,P,m_2}$, since $(\partial m_3/\partial m_2)^{N}_{T,P,\mu_3}$ and $(\partial\mu_2/\partial m_3)^{N}_{T,P,m_2}$ for these polyol systems are known to be negative and positive, respectively, through preferential interaction studies on native protein (see Table II). This means that both polyols are preferentially excluded more strongly from the domain of denatured protein than from the native one. The resulting thermodynamic instability of the denatured state should be reduced by a displacement of the equilibrium toward the native state, leading to the stabilization of native lysozyme.

Such variations of thermodynamic parameters with solvent composition would be dominantly generated in both of the following processes: exposing the nonpolar residues buried in the interior of the native protein, and breaking a peptide-peptide hydrogen bond in the native state and replacing it by a peptide-water hydrogen bond. A resultant increase in surface area of the denatured protein should contribute to increase the extent of hydration around the protein. The exposed nonpolar residues would be antagonistic to polyol molecules, but the peptide-water interaction seems not to be significantly affected by polyols, although polyols with short chain length may be excluded from the peptide group (*10*). Since preferential interaction is related to the total interaction of solvent components with protein, $\bar{\nu}$, as follows (*26, 27*)

$$\Delta\xi_3 = (\bar{\nu}_3^{D} - \bar{\nu}_3^{N}) - (m_3/m_1)(\bar{\nu}_1^{D} - \bar{\nu}_1^{N}) \qquad (10)$$

the observed enhancement of preferential hydration upon denaturation ($\Delta\xi_3 < 0$) could result from the change in solvent ordering, $\bar{\nu}_1^{D} > \bar{\nu}_1^{N}$ and/or $\bar{\nu}_3^{D} < \bar{\nu}_3^{N}$.

A similar analysis may be applied to the corresponding enthalpy and entropy changes. The observed increase in ΔH° and ΔS° upon addition of polyols means that the enthalpy and entropy of transfer of denatured protein from water to aqueous polyols are larger than those of native protein. This suggests that the variation in enthalpy of denaturation with polyols would be more dominantly produced by exposing the nonpolar residues rather than the peptide groups, since the enthalpy of transfer of nonpolar side chains of amino acids and peptide groups from water to aqueous polyols are positive and negative, respectively (*11*). Thus, with regard to both free energy and enthalpy, the polyol-induced stabilization of lysozyme can be explained as the result of strengthening by polyols of the intramolecular hydrophobic interaction of the protein. However, the polyol-induced stabilization of chymotrypsinogen was, conversely, due to a greater decrease in ΔS° compensating for the decrease in ΔH°, suggesting the dominance of the polyol effect on peptide-water interaction in an enthalpy sense (*14*). Such an opposite change in ΔH° for the two proteins means that the polyol effects on exposed nonpolar groups do not necessarily dominate those on peptide groups with regard to enthalpy, whereas the free energy change can be interpreted as the dominant effect of polyols on nonpolar groups. Therefore, the protein stabilization by polyols must result from mutually different fashion of enthalpy-entropy compensation in a solvent-ordering process

around the nonpolar and peptide groups exposed. Irregular dependence of ΔH_{cal} on the number of OH groups of polyols despite the regular increase in T_d (see Fig. 3) also may be a result of such complicated enthalpy-entropy compensation. In this regard, it seems pertinent to note that the heat capacity of unfolded β-lactoglobulin decreases with polyol chain length in similar irregular fashion (*28*).

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