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The Effect of Polyhydric Alcohols on the Thermal Denaturation of Lysozyme as Measured by Differential Scanning Calorimetry

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The thermal denaturation of lysozyme in aqueous solutions of polyhydric alcohols at pH 2 was investigated by differential scanning calorimetry (DSC). The polyhydric alcohols employed were inositol, sorbitol, xylitol, erythritol, glycerol, and ethylene glycol. These alcohols tended to enhance the thermal stability of lysozyme; both the temperature and the enthalpy of denaturation increased almost linearly with increasing alcohol concentration. The increase in denaturation temperature at a given alcohol concentration was a linearly increasing function of the number of hydroxyl groups per alcohol molecule. The denaturation enthalpy at identical temperature increased only slightly by the addition of polyhydric alcohol. The hypothesis may be supported that the dominant mechanism by which polyhydric alcohols stabilize proteins to thermal denaturation is through their effect on the water structure, which determines the strength of the hydrophobic interactions between the nonpolar groups of the protein.

The folding, structural stability, and dynamics of globular proteins are thought to be extensively controlled by the interactions of the protein with water. Various substances affect the protein-water interactions when added to aqueous protein solutions and consequently alter the structural stability of proteins.[1-3] There have been many investigations which show the stabilizing effect of polyhydric alcohols and sugars on the native conformation of proteins. Simpson and Kauzmann[4] observed that the extent of denaturation of ovalbumin in urea solutions was reduced in the presence of glycerol or sucrose. The influence of polyhydric alcohols and sugars on the rate of subunit dissociation of tetrameric L-asparaginase in the presence of urea was investigated by Shifrin and Parrott.[5] Gerlsma and Stuur[6] showed by spectrophotometric measurements that polyhydric alcohols raised the thermal transition temperature of lysozyme and ribonuclease. A similar effect of polyhydric alcohols has also been observed for chymotrypsinogen.[7,8] However, the manner in which polyhydric alcohols and sugars induce the increased thermal stability of proteins or reduce the extent of denaturation by other reagents is poorly understood.

The application of differential scanning calorimetry (DSC) to the investigation of thermally induced conformational transitions of protein has provided valuable information on the mechanism of protein denaturation; this has been recently reviewed by Biltonen and Freire[9] and Privalov.[10] The extensive calorimetric investigation of the thermal denaturation of lysozyme has been reported by Privalov and Khechinashvili.[11] The reversible nature of the denaturation has been established. Recently, the DSC study of the thermal denaturation of lysozyme in sugar solutions has been reported by Uedaira and Uedaira.[12]

We have been investigating the effect of various substances on the thermal stability of globular proteins by means of DSC. In the previous paper,[13] the effect of monohydric alcohols on the thermal denaturation of lysozyme was reported. The denaturation temperature of lysozyme in monohydric alcohol solutions decreased gradually with increasing alcohol concentration, which became more pronounced with increasing hydrocarbon content of alcohol. On the other hand, the denaturation enthalpy passed through a maximum with increasing alcohol concentration and the maximal value decreased with increasing hydrocarbon content of alcohol. A similar behavior has been observed with sulfoxides.[14] These tendencies may be interpreted in terms of the mixed hydrophobic-hydrophilic character of monohydric alcohols and sulfoxides.

In the present paper, the effect of polyhydric alcohols on the thermal denaturation of lysozyme has been investigated by means of DSC and the results are compared with those of monohydric alcohols.

Experimental

Materials. The hen egg-white lysozyme used was a salt-free, six-times-recrystallized preparation obtained from Seikagaku Kogyo Co. The molecular weight of lysozyme was taken to be 14307. Analytical grade inositol, sorbitol, and xylitol were purchased from Nishio Kogyo Co. and reagent grade *meso*-erythritol from Merck. Spectral grade glycerol and ethylene glycol were obtained from Wako Pure Chemicals. These polyhydric alcohols were used without further purification.

Methods. The stock solution of lysozyme was prepared with 0.1 mol dm^{-3} glycine buffer at pH 2.0 containing 0.1 mol dm^{-3} NaCl and if necessary the pH was adjusted by adding HCl. The protein concentration was determined spectrophotometrically using an extinction of $E^{1\%}_{1cm}=26.9$ at 280 nm in 0.1 mol dm^{-3} glycine buffer at pH 3.0.[15] A Hitachi 323 automatic recording spectrophotometer was used. Polyhydric alcohol was added by weight.

Thermal measurements were performed with a Daini Seikosha SSC-560U differential scanning calorimeter (conduction type) at protein concentrations of 2.39—2.47%. The sample (0.06 cm^3) was hermetically sealed in a silver vessel. A heating rate of 1 K min^{-1} was typically used, although some measurements were performed at 0.2, 0.5, and 2.5 K min^{-1} to check the effect of the heating rate. The calorimeter was calibrated with indium, benzophenone, and benzoic acid.

Results and Discussion

Figure 1 shows the DSC curves for the thermal denaturation of lysozyme in aqueous solutions (pH 2) containing different amounts of sorbitol at a heating rate of 1 K min^{-1}. In every measurement, a reproducible

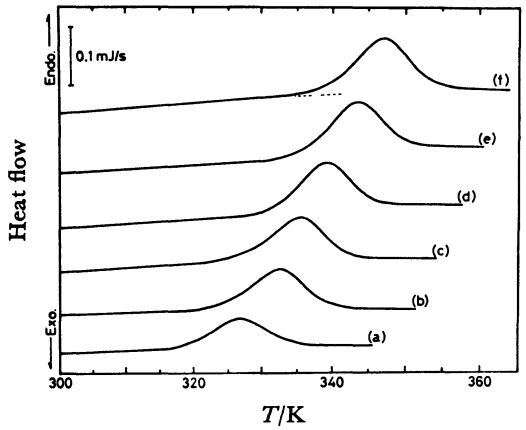

Fig. 1. DSC curves at heating rate of 1 K min^{-1} for the thermal denaturation of lysozyme in aqueous sorbitol solutions at pH 2.
The sorbitol concentrations are (a): 0, (b): 0.95, (c): 1.45, (d): 1.98, (e): 2.56, and (f): 3.04 mol dm^{-3}.

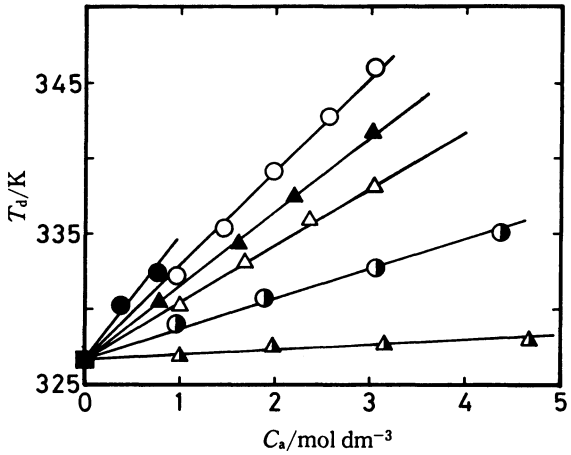

Fig. 2. The denaturation temperature of lysozyme as a function of concentration of polyhydric alcohol.
■: Aqueous, ●: inositol, ○: sorbitol, ▲: xylitol, △: erythritol, ◑: glycerol, ◭: ethylene glycol.

endothermic peak was observed occurring over a temperature width of 23—25 K. The endothermic peak shifted to higher temperature with increasing sorbitol concentration, while the peak area became larger and also the peak width slightly larger. The denaturation temperature, T_d, was estimated by assuming that the area under the thermogram was proportional to the amount denatured, and T_d defined as the temperature at which the protein is half-denatured. The difference between the estimated T_d and the peak temperature of the transition peak did not exceed 0.6 K in every measurement. The denaturation enthalpy, ΔH_d, was estimated by measuring the area between the transition peak and a base line fitted by inspection.

In order to check the effect of the heating rate, the thermal denaturation of lysozyme in aqueous solution and in aqueous sorbitol solutions was measured at heating rates of 0.2, 0.5, and 2.5 K min^{-1} other than 1 K min^{-1}. There was no significant difference in the denaturation enthalpies obtained at different heating rates. The denaturation temperature, however, showed a slight dependence on the heating rate; the denaturation temperature increased slightly with increasing heating rate. The values extrapolated to zero heating rate were lower than those obtained at a heating rate of 1 K min^{-1} by 0.4—0.6 K, which were in fair agreement with the denaturation temperature obtained by Gerlsma and Stuur[6] from spectrophotometric measurements.

The effect of polyhydric alcohols on the denaturation temperature of lysozyme is shown in Fig. 2. The T_d increased almost linearly with increasing concentration of alcohol and the increase became more pronounced with an increase in the number of hydroxymethyl groups in the alcohol. It is evident that these alcohols stabilize the protein against thermal denaturation. This tendency of polyhydric alcohols differ clearly from that of monohydric alcohols. As described in the previous paper,[12] monohydric alcohols lower the denaturation temperature of lysozyme, which becomes more pronounced with increasing alcohol concentration and increasing hydrocarbon content of the alcohol. That is, monohydric alcohols unstabilize the native structure of the protein. The unstabilizing effect of monohydric alcohols has been generally interpreted in terms of the hydrophobic interactions between the nonpolar groups of the protein and the alkyl chain of alcohol.[16,17]

The dependence of the denaturation enthalpy of lysozyme on the concentration of polyhydric alcohols is shown in Fig. 3. In every case studied, the ΔH_d was

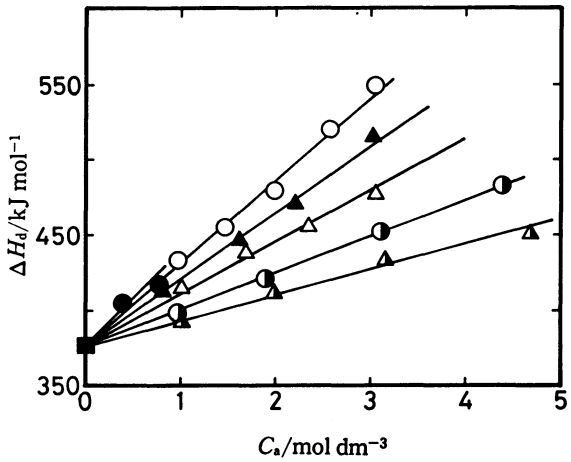

Fig. 3. The denaturation enthalpy of lysozyme as a function of concentration of polyhydric alcohol.
The symbols used are identical with those in Fig. 2.

enhanced by the addition of polyhydric alcohol. The ΔH_d increased almost linearly with increasing alcohol concentration. The increase in ΔH_d for a given concentration of alcohol becomes more pronounced as the number of hydroxymethyl groups of the added alcohol increase. The effect of the polyhydric alcohols on the denaturation enthalpy also differs from that of monohydric alcohols. As has been reported by Velicelebi and Sturtevant[17] and us,[13] the ΔH_d of lysozyme in the presence of monohydric alcohol shows a complex

dependence on the alcohol content; the ΔH_d passes through maximum with increasing alcohol concentration and the maximal value decreases with an increase in the hydrocarbon content of alcohol. A comparison between the results with polyhydric and monohydric alcohols suggests that the second and additional hydroxyl groups in the polyhydric alcohols play an important role in their stabilizing influence on the thermal denaturation of protein.

At a constant concentration of alcohol, the increase of T_d and ΔH_d with inositol were larger than those with sorbitol. This indicates that the stabilizing ability of inositol with a cyclic structure on the thermal denaturation of lysozyme is higher than that of sorbitol with a linear structure. This configuration effect may be analogous to the effect induced by branching the alkyl chain of monohydric alcohols. It has been established that the lowering of the denaturation temperature of globular proteins by monohydric alcohols with branched alkyl chain is smaller than that by the corresponding straight-chain alcohols.[13,18] It is possible that cyclization causes the increase in the content of hydroxymethyl groups per molecular chain and consequently brings about the high stabilizing ability of inositol on the thermal stability of lysozyme.

Figure 4 shows the plot of the increase in the denaturation temperature of lysozyme at an identical alcohol concentration of 1 mol dm^{-3}, ΔT_d, against the number of hydroxyl groups of the polyhydric alcohols used. As can been seen, the ΔT_d shows the linear dependence

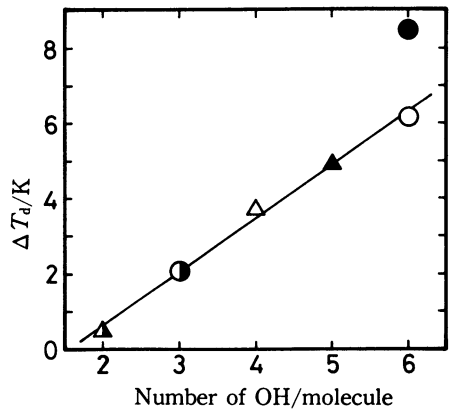

Fig. 4. Plot of the increase in the denaturation temperature of lysozyme at an identical alcohol concentration of 1 mol dm^{-3} against the number of hydroxyl groups of polyhydric alcohols.

on the number of hydroxyl groups of the polyhydric alcohols used with the exception of inositol. From the slope in Fig. 4, the T_d of lysozyme is raised by 1.4 K per hydroxyl group in the linear polyhydric alcohols, which is considerable larger than that measured by Gerlsma and Stuur.[6] A similar relation has been found with sugars as well as with polyhydric alcohols. Uedaira and Uedaira[12] have recently reported that the T_d of lysozyme in aqueous sugar solutions increases almost linearly with an increase in the number of equatorial hydroxyl groups in the sugar. From the direct dependence of ΔT_d on the number of hydroxyl groups of alcohols, it is presumed that there is no direct molecular interaction between the protein and the polyhydric alcohol, since any direct interaction would depend on the molar concentration.

The dependence of the denaturation enthalpy of lysozyme on the denaturation temperature in the presence of polyhydric alcohol is shown in Fig. 5. For

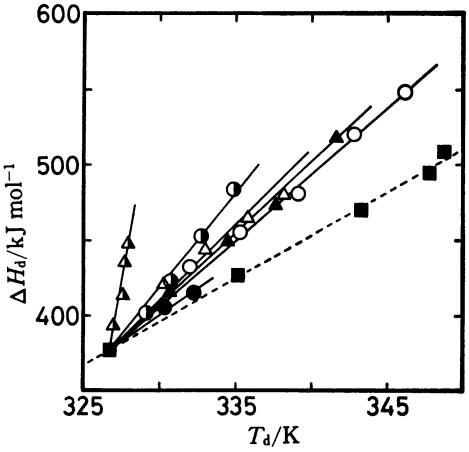

Fig. 5. The dependence of the denaturation enthalpy of lysozyme on the denaturation temperature.
The symbols used are identical with those in Fig. 2.

all the alcohols, the ΔH_d showed the linear function of T_d. This is similar to the behavior reported by Pfeil and Privalov[19] with variations of T_d produced by variation of the pH and addition of guanidinium chloride. However, there is a difference in the dependence of ΔH_d on T_d for each polyhydric alcohol. The slope of ΔH_d *vs.* T_d in the presence of alcohol decreased with an increase in the number of hydroxyl groups in the alcohol. This also suggests that the stabilizing effect of polyhydric alcohols on the thermal denaturation of protein is closely related to the number of hydroxyl groups in the alcohol.

Since ΔH_d is dependent on temperature, as mentioned above, the effect of polyhydric alcohols on ΔH_d must be estimated by comparison of the variation of ΔH_d with alcohols at identical temperature. In order to obtain the temperature dependence of ΔH_d in the absence of polyhydric alcohol, we also measured the thermal denaturation of lysozyme in glycine buffer solutions at different pH values and in acetate buffer solution at pH 5.0. These results are summarized in Table 1 together with those obtained by other investigators from calorimetric studies. Taking into account the difference in the measurement conditions such as heating rate, protein concentration, and ionic strength, our results are in good agreement with those obtained by other investigators. The dependence of ΔH_d on T_d in the absence of alcohol is shown in Fig. 5 together with that in the presence of alcohol. From the slope of this function, the temperature dependence of the denaturation enthalpy of lysozyme in the absence of alcohol, $(d\Delta H_d/dT_d)$, was obtained to be 5.6 kJ K^{-1} mol^{-1}, which was in rough agreement with that obtained from

Table 1. The denaturation temperatures and enthalpies of lysozyme at different pH values obtained by calorimetric studies

pH[a)]	T_d / K	ΔH_d / kJ mol^{-1}
1.0	319.2[b)]	234[b)]
2.0	326.7	377
	329.2[c)]	444[c)]
	325.2[d)]	382[d)]
2.5	335.2	427
3.0	343.2	470
	347.7[c)]	556[c)]
3.8	347.8	496
4.5	351.7[c)]	590[c)]
5.0	348.7	509
5.4	349.7[e)]	577[e)]

a) The following buffer solutions were used: 0.1 mol dm^{-3} glycine buffer for pH 2.0, 2.5, 3.0, and 3.8; 0.1 mol dm^{-3} acetate buffer for pH 5.0. b) O'Reilly and Karasz.[20] c) Privalov and Khechinashvili.[11] d) Velicelebi and Sturtevant.[17] e) Delben and Crescenzi.[21]

precision measurement by Pfeil and Privalov.[19] Assuming that $(d\Delta H_d/dT_d)$ remains identical with the value in the absence of alcohol at all alcohol solutions used, the denaturation enthalpy of lysozyme in the presence of alcohol at a given temperature, $\Delta H_d°(a)$, is expressed as follows:

$$\Delta H_d°(a) = \Delta H_d + (d\Delta H_d/dT_d)(T_d° - T_d), \quad (1)$$

where ΔH_d and T_d are the observed enthalpy and temperature of denaturation in the presence of alcohol and $T_d°$ is a given temperature. Then, the variation of denaturation enthalpy by the addition of polyhydric alcohol at identical temperature, $\Delta(\Delta H_d°)$, can be evaluated by the following equation,

$$\Delta(\Delta H_d°) = \Delta H_d°(a) - \Delta H_d°(w), \quad (2)$$

where $\Delta H_d°(w)$ is the denaturation enthalpy in the absence of alcohol at same temperature.

Figure 6 shows the dependence of $\Delta(\Delta H_d°)$ on polyhydric alcohol concentration at 326.7 K which is the denaturation temperature of lysozyme in aqueous solution at pH 2.0. Although these plots show appreciable scatter, it is obvious that the $\Delta(\Delta H_d°)$ is a slight

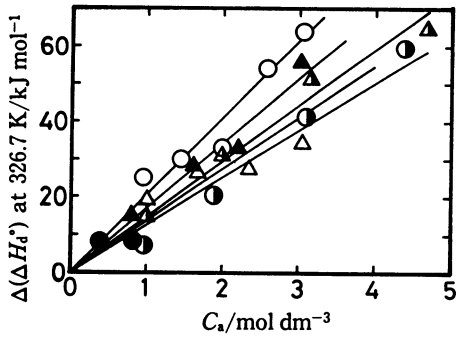

Fig. 6. The variation of the denaturation enthalpy of lysozyme with polyhydric alcohols at an identical temperature, 326.7 K, as a function of concentration of polyhydric alcohol.
The symbols used are identical with those in Fig. 2.

increasing function of alcohol concentration. However, the $\Delta(\Delta H_d°)$ value should be considerably smaller as compared with that presumed from the large increase in T_d. Substantially, the $\Delta(\Delta H_d°)$ values in the range of lower concentration of alcohol are within the experimental error in ΔH_d. Therefore, it is difficult to consider that the increase in the free energy change of denaturation by the addition of polyhydric alcohol results from only the slight increase of the denaturation enthalpy. These polyhydric alcohols for which data are available have lower dielectric constants than pure water.[22] Thus, electrostatic interactions are stronger in these solutions than in water, which causes the denaturation enthalpy to increase. However, this contribution to the stabilizing effect appears to be relatively small.

It is interesting that the effect of polyhydric alcohols is different from that of monohydric alcohols. There have been many investigations which show the direct interaction of monohydric alcohols with protein.[23] The unstabilizing effect of monohydric alcohols on the native structure of protein has been generally interpreted in terms of the interaction between protein and alcohol. Bull and Breese[24] have reported from their study on the binding of alcohols to protein using equilibrium dialysis that monohydric alcohols are strongly bound to protein, but there is no direct binding of polyhydric alcohols to protein. Our presumption based on the direct dependence of ΔT_d on the number of hydroxyl groups of the added polyhydric alcohols is strongly supported by their observations. Therefore, it is conceivable that there exist other interactions which stabilize the native structure of protein in the system protein–alcohol–water. It is likely that polyhydric alcohols interact principally with the other component in the system, that is, water.

From thermodynamic studies of transfer of amino acids from water to aqueous solutions of polyhydric alcohols, Gekko and Noguchi[25] have recently found that the enthalpy change of the transfer is positive for nonpolar side chains of amino acids and that polyhydric alcohols decrease the solubility of nonpolar groups by an unfavorable enthalpy effect. According to their findings, it is presumed that the added polyhydric alcohols dominantly affect the hydrophobic interactions between the nonpolar groups of protein. Back *et al.*[26] have also reported that the hydrophobic interactions between pairs of hydrophobic groups are stronger in aqueous glycerol solutes than in pure water. Since hydrophobic interaction is a result of a rearrangement of the water molecules surrounding hydrophobic groups, it is likely that there is a relation between the effect of the added solutes on hydrophobic interaction and their effect on the structure of water. The effect of polyhydric alcohols on hydrophobic interaction and consequently on the thermal stability of protein may depend upon how they affect the structure of water.

There is no completely unambiguous numerical measure of "structure making" or "structure breaking" by a solute, but a change in the extent to which water is structured is accompanied by a volume change.[27] According to Oakenfull and Fenwick,[28] the effect of solute on the water structure is closely related with its

partial molar volume. The structure-making effect of polyhydric alcohols determined from their partial molar volume enhances in the order sorbitol>xylitol> erythritol>glycerol>ethylene glycol, that is, increasing number of hydroxyl groups per alcohol molecule. The structure-making effect of inositol, a cyclic alcohol, is larger than that of sorbitol. The stabilizing ability of polyhydric alcohols on the thermal denaturation of lysozyme also increases in the same order.

These results suggest the hypothesis that the dominant mechanism by which polyhydric alcohols stabilize protein against thermal denaturation is through their effect on the structure of the water, which, in turn, determines the strength of the hydrophobic interactions.

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