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The Effect of Organic Solvents 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 *N,N*-dimethylformamide(DMF), *N,N*-dimethylacetamide(DMA), acetone, and dioxane at pH 3 was investigated by differential scanning calorimetry (DSC). The denaturation temperature, T_d, decreased linearly with an increase in the concentration of organic solvent. The lowering in T_d at identical concentrations of organic solvent increased in the order: acetone< DMF<DMA<dioxane. The calorimetric enthalpy of denaturation, ΔH_d, showed a complex dependence on the solvent composition. For three organic solvents (not dioxane), the ΔH_d first increased slightly with increasing the concentration of organic solvent and then started decreasing at different concentrations of each organic solvent. This behavior is analogous to those observed earlier with monohydric alcohols and with sulfoxides. For dioxane, on the other hand, the ΔH_d decreased gradually with increasing the concentration of dioxane. The effect of these organic solvents on the thermal stability of lysozyme may correlate with their effect on the structure of water, which determines the strength of the hydrophobic interaction of the protein.

Various substances affect the protein–water interactions when added to aqueous protein solution and consequently alter the structural stability of protein. Solvent perturbations have been used extensively as probes of the solution conformation of protein.[1,2] These studies have provided valuable information about the role of the solvent in maintaining the native structure of protein. The conformational changes of lysozyme induced by the addition of organic solvents have been investigated by Hamaguchi and co-workers.[3–5] They have suggested that organic solvents change the conformation of the protein by perturbing the characteristic water structure and by interacting directly with the protein molecule. Izumi and Inoue[6,7] have reported recently the effect of dioxane on the conformation of lysozyme measured by CD, difference spectroscopy, and fluorometry.

Differential scanning calorimetry(DSC) is a useful tool for investigating the thermally induced conformational transition of protein. The thermogram can be used to calculate the van't Hoff enthalpy[8] or an effective enthalpy[9] for comparison to the calorimetric enthalpy to determine the cooperativity of the transition. Application of DSC to the study of protein unfolding has been recently reviewed by Privalov[10] and Biltonen and Freire.[11] A comprehensive thermodynamic investigation of the unfolding of lysozyme has been reported by Pfeil and Privalov.[12]

We have been investigating the effect of various substances on the thermal stability of globular proteins by means of DSC. The thermal denaturation of lysozyme in aqueous solutions of monohydric alcohols and sulfoxides was reported previously.[13,14] These organic solvents lowered the denaturation temperature; this lowering became more pronounced with increasing the concentration and the hydrocarbon content of organic solvent. The calorimetric enthalpy of denaturation passed through a maximum with increasing the concentration of organic solvent. There are many studies of the thermal denaturation of lysozyme in aqueous alcohol solutions by different techniques.[8,15,16] Although various substances may be expected to affect the thermal stability of protein in different ways, the effect of organic solvents other than alcohols on the thermal denaturation of protein has rarely been investigated. So far as we know, Hamaguchi and Sakai[17] have reported the thermal denaturation of lysozyme in aqueous solution of amides and dioxane in a limited concentration of the organic solvent, as determined by spectrophotometry.

In the present paper, the effect of *N,N*-dimethylformamide (DMF), *N,N*-dimethylacetamide (DMA), acetone, and 1,4-dioxane on the thermal denaturation of lysozyme as measured by DSC will be reported.

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. Spectral grade *N,N*-dimethylformamide (DMF), acetone, and 1,4-dioxane were purchased from Wako Pure Chemicals and reagent grade *N,N*-dimethylacetamide (DMA) from Tokyo Kasei Kogyo Co. Solutions were prepared with deionized and distilled water.

Methods. The protein concentration was determined spectrophotometrically on a Hitachi 323 spectrophotometer, using an extinction coefficient of 2.69 dm^3 $(g\ cm)^{-1}$ at 280 nm in glycine buffer at pH 3.0.[17] The organic solvent was added by weight. All the calorimetric experiments were carried out at a protein concentration of 2% in 0.05 mol dm^{-3} glycine buffer adjusted to pH 3.0 as measured by a Horiba model f-7_{ss} pH meter standardized at pH 4.0 in aqueous solution. DSC measurements were performed with a Daini Seikosha SSC-560U differential scanning calorimeter (conduction type). The sample solutions (0.06 cm^3) were hermetically sealed in a silver vessel. An equal amount of solvent was used as reference material. A heating rate of 1 K min^{-1} was typically used, although some measurements were performed at 0.5 to 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 presents typical DSC curves for the thermal denaturation of lysozyme in aqueous solutions containing different amounts of DMA at pH 3. In every

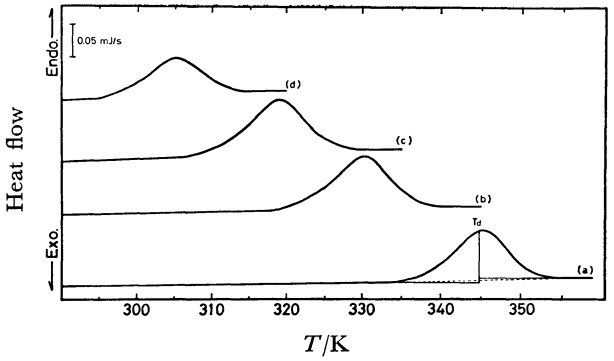

Fig. 1. Typical DSC curves for the thermal denaturation of lysozyme in aqueous DMA solutions at heating rate of 1 K min^{-1}. Protein concentration is 2.0% (w/v).
Curve a is in 0.05 mol dm^{-3} glycine buffer at pH 3.0 with no organic solvent. Curves b, c, and d are with 2, 4, and 6 mol dm^{-3} DMA, respectively.

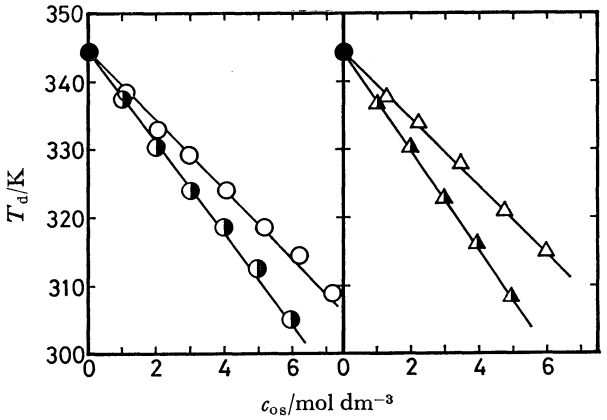

Fig. 2. The denaturation temperature, T_d, of lysozyme as a function of organic solvent concentration.
●: Aqueous, ○: DMF, ◑: DMA, △: acetone, ▲: dioxane.

measurement, a reproducible endothermic peak was observed, occurring over a temperature range of 22—25 K. The endothermic peak shifts to lower temperature with increasing DMA content, while the peak area varies with the solvent composition. The denaturation temperature, T_d, was estimated by assuming that the area under the thermogram was proportional to the amount denatured, and T_d is defined as the temperature at which the protein is half-denatured. According to Velicelebi and Sturtevant,[8] the temperature at which the transition is half complete and the temperature of maximal excess heat flow, T_p, do not coincide, even for a two-state process with no permanent change in heat capacity. They have reported that T_d differs from T_p by 0.75—1.0 K. In the present work, the T_d obtained was lower than the T_p by only 0.3—0.5 K.

There was no significant difference in the T_d obtained at heating rates of 0.5 and 1 K min^{-1}, but the T_d obtained at 2.5 K min^{-1} was higher than those at 0.5 and 1 K min^{-1} by 0.4—0.8 K. This indicates that the denaturation temperature is not much influenced by heating rates lower than 1 K min^{-1}, allowing equilibrium analysis of the data. The denaturation temperature of lysozyme in 0.05 mol dm^{-3} glycine buffer at pH 3.0 was 344.4±0.1 K, which was comparable with previous values obtained from NMR measurements by McDonald *et al.*[18] and from spectrophotometric measurement by Gerlsma and Stuur[15] at similar pH. Parodi *et al.*[16] have reported a slightly lower T_d, 341 K, from ORD measurements, whereas a high T_d value of 347.7 K from calorimetric measurements has been reported by Privalov and Khechinashvili.[19] A number of factors such as protein concentration, ionic strength, and specific buffer effect as well as heating rate could contribute to the difference in T_d values reported from the different laboratories. Variation in the measurement techniques could also contribute to the difference in T_d value.

The calorimetric enthalpy of denaturation, ΔH_d, was estimated by measuring the area between the transition peak and a base line. The ΔH_d obtained was not significantly different from that estimated using the base line based on the heat capacity difference between the native and denatured states. The ΔH_d values have maximum expected errors of ±5%, including errors in calibration constant, sample preparation, and reproducibility. The denaturation enthalpy was almost independent of the heating rate in the range of 0.5 to 2.5 K min^{-1}. The ΔH_d of lysozyme in 0.05 mol dm^{-3} glycine buffer at pH 3.0 was 489 kJ mol^{-1}, the average of nine experiments. Taking into account the difference in measurement conditions, the value of ΔH_d appears to be line with previous values obtained from calorimetric measurements by other investigators, indicating values of 577 kJ mol^{-1} at pH 5[20] and 427[12] and 382 kJ mol^{-1} at pH 2.[8]

There was no significant change in the T_d and ΔH_d on repetitive scans when the sample was rapidly cooled after the preceding scan, indicating that the thermal denaturation of lysozyme in these organic solvent solutions is almost reversible. According to Privalov *et al.*,[9] the effective enthalpy of denaturation, ΔH_{eff}, can be calculated by the half width of the heat absorption peak on the DSC thermogram, if it is assumed that the denaturation is a simple transition between two states without thermodynamically stable intermediate forms. The ratio of the calorimetric to the calculated effective enthalpies of denaturation, $\Delta H_d/\Delta H_{eff}$, showed near-unity value, suggesting that the thermal denaturation of lysozyme in these organic solvent solutions is very nearly a two-state process.

Figure 2 shows the effect of organic solvents on the denaturation temperature. For all the systems investigated, the T_d decreases almost linearly with increasing the concentration of organic solvent. The lowering of T_d at identical concentrations of organic solvent increases in the order: acetone<DMF<DMA<dioxane. The effect of DMF, DMA, and dioxane on the denaturation temperature of lysozyme has been reported earlier from spectrophotometric measurements by Hamaguchi and Sakai.[17] They have observed trends similar to the ones we have observed. The lowering in the T_d of lysozyme by monohydric alcohols and sulfoxides was reported previously.[13,14]

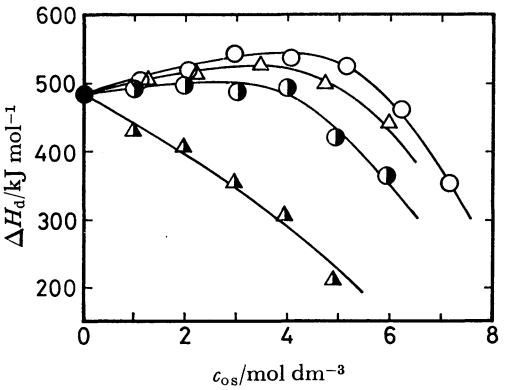

Fig. 3. The denaturation enthalpy, ΔH_d, of lysozyme as a function of organic solvent concentration.
The symbols used are identical with those in Fig. 2.

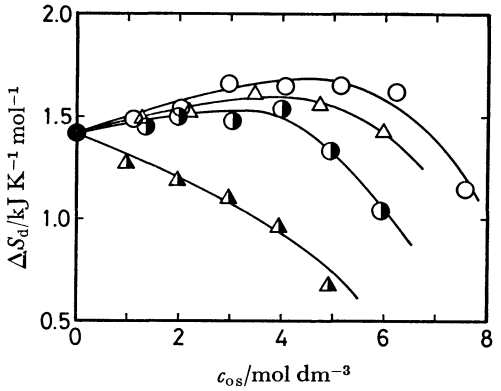

Fig. 4. The denaturation entropy, ΔS_d, calculated at the transition temperature as a function of organic solvent concentration.
The symbols used are identical with those in Fig. 2.

The thermal unstabilization of lysozyme by alcohols has been observed by many investigators through different techniques.[8,15,16] The unstabilizing effect of organic solvents such as alcohols on the thermal stability of protein has been generally interpreted in terms of hydrophobic interactions between the nonpolar groups of protein and the alkyl chain of organic solvent, and thus relatively favoring the denatured state in the mixed solvent. DMA produces a greater decrease in T_d than DMF does, suggesting that the unstabilizing ability of amides is enhanced by an increase in the hydrocarbon content, similarly to those of monohydric alcohols and sulfoxides. This observation also provides the above view with more support.

The dependence of the denaturation enthalpy on the concentration of organic solvent is shown in Fig. 3. For three organic solvents (not dioxane), the ΔH_d shows a maximum, that is, the ΔH_d first increases slightly with increasing the concentration of organic solvent and then starts decreasing at a different concentration of organic solvent. The maximum value of ΔH_d increases in the order: DMA<acetone<DMF. The maximum ΔH_d occurs at approximately 3, 3.5, and 4 mol dm^{-3} for DMA, acetone, and DMF, respectively. The maximum in the ΔH_d of lysozyme has been observed with monohydric alcohols and sulfoxides as well as with the organic solvents used in this experiments.[13,14] A similar trend in ΔH_d with dimethyl sulfoxide has been recently found for ribonuclease A.[21] On the other hand, the ΔH_d with dioxane decreases gradually with an increase in dioxane concentration; this becomes more pronounced at high dioxane contents. It is obvious that the effect of dioxane on the denaturation enthalpy of lysozyme differs from those of other organic solvents.

The entropy of the thermal denaturation, ΔS_d, was calculated for the reversible denaturation by assuming that the free energy change at the midpoint of the transition is zero: $\Delta S_d = \Delta H_d / T_d$. The dependence of ΔS_d on the concentration of organic solvent is shown in Fig. 4. The ΔS_d also shows a behavior similar to ΔH_d. Therefore, the observed decrease in T_d by the addition of organic solvents should result from the complex dependence of ΔH_d and ΔS_d on the solvent composition.

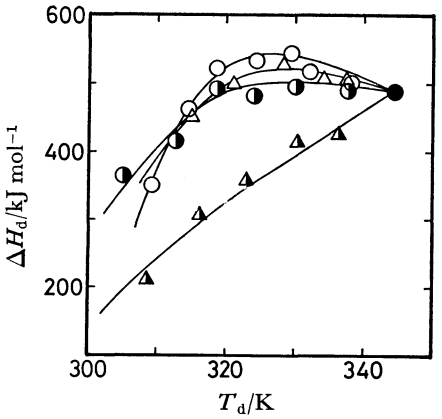

Fig. 5. The variation in the denaturation enthalpy with denaturation temperature.
The symbols used are identical with those in Fig. 2.

Figure 5 represents the dependence of ΔH_d and T_d in the presence of organic solvents. For three organic solvents (not dioxane), the ΔH_d value passes through a maximum with increasing temperature. Such a behavior was also observed with monohydric alcohols and sulfoxides.

For dioxane, on the other hand, the ΔH_d is an increasing function of temperature with slightly downward curvature. There are clear differences in the temperature dependence of ΔH_d for each organic solvent. The dependence of ΔH_d on T_d in the presence of organic solvents thus differs drastically from that reported by Pfeil and Privalov[12] with variations of T_d produced by variation of the pH and addition of guanidine hydrochloride. They found ΔH_d to be a linear function of T_d under all conditions. It is suggested that the nature of the unfolding of lysozyme resulting from the addition of organic solvents is different from that produced by pH changes or by the addition of guanidine hydrochloride.

The conformational changes of lysozyme by the addition of dioxane have been investigated by ORD,[3] CD,[6] and difference spectroscopy.[3,7] These studies show that the tertiary structure of lysozyme is initially disrupted at 20—30% (v/v) dioxane, corresponding to

approximately 3 mol dm^{-3} dioxane, with possible slight disorganization of the ordered secondary structure, and then a pronounced increase in helical structure is induced at very high dioxane concentrations. A similar situation has been observed with DMA and DMF.[3]

These organic solvents have lower dielectric constants than pure water. Thus, electrostatic interaction is stronger in water–organic solvent mixtures than in water, which causes the denaturation enthalpy to increase. However, this contribution appears to be small, as can be seen from decreasing the denaturation enthalpy for the water–dioxane system, because the dielectric constants of aqueous dioxane solutions are relatively lower than those of aqueous solutions of other organic solvents.

The maximum in ΔH_d and ΔS_d for the thermal denaturation of lysozyme has been observed with monohydric alcohols and sulfoxides as well as for the organic solvents examined in the present work. This similarity implies that the effect of organic solvents on the thermal stability of the protein is controlled to a large extent by the common component in the binary mixtures, that is, water. A number of properties of aqueous mixtures of these organic solvents show maxima or minima as a function of composition. This behavior reflects the change in the structure of water by the addition of organic solvents. The complex dependence of ΔH_d and ΔS_d on the solvent composition appears to correlate with the behavior of the water–organic solvent system.

In mixing with water, dioxane[22] shows an exothermic enthalpy of mixing similarly to acetone[23] and both amides,[24] suggesting the strong interactions of dioxane, as well as other organic solvents, with water molecule. The ΔH_d and ΔS_d of lysozyme in water–dioxane mixtures decreased monotonously with increasing concentrations of dioxane, although the maximum in ΔH_d and ΔS_d was predicted. Based on the density data, Oakenfull and Fenwick[25] have suggested that the effect of dioxane on the structure of water differs from those of DMF, ethanol, and dimethyl sulfoxide. The structural contribution to the temperature of maximum density for water–dioxane system shows a negative change, in contrast to a positive change for alcohols and acetone mixing with water.[26,27] Our observation also supports the view that the effect of dioxane on the water structure differs slightly from those of other organic solvents. However, the effects of these organic solvents, including dioxane, on the water structure differ from the so-called structure-making effect of polyhydric alcohols which stabilize the protein strongly against thermal denaturation.[28]

The organic solvents used in the present work, like monohydric alcohols and sulfoxides, unstabilize lysozyme against thermal denaturation. The thermal unstabilization of the protein by organic solvents is enhanced on increasing the concentration and hydrocarbon content of the organic solvent. This observation suggests that organic solvents interact principally with the nonpolar groups of the protein, which causes weakening the hydrophobic interaction of the protein. Bull and Breese[29] have shown from equilibrium dialysis studies that monohydric alcohols are strongly bound to protein. From NMR studies on the solvation of lysozyme in water–dioxane mixtures in the frozen state, Izumi *et al.*[30] have suggested that some water molecules are replaced or expelled by dioxane molecules in the interacting sites due to the more favorable interaction of dioxane with hydrophobic groups lying on the protein surface.

The perturbation of the water structure by the addition of organic solvents also causes the thermal unstabilization of lysozyme. This may contribute more to the thermal unstabilization of the protein than that from the direct interaction of organic solvent with the protein. Since hydrophobic interaction is a result of a rearrangement of the water molecules surrounding hydrophobic groups, the perturbation of the characteristic water structure necessarily causes weakening of the hydrophobic interaction of the protein. Solubility measurements on amino acids in aqueous solutions of ethanol or dioxane have shown favorable free energy of transfer of nonpolar side chains from water to these organic solvent solutions.[31] Thus, the denatured state of protein is stabilized more in the presence of organic solvents than is the native state, since more groups are exposed to solvent in the denatured state than in the native state. It is probable that organic solvents lower the temperature of denaturation as a result of decreased hydrophobic stabilization of the native state.

These results support the hypothesis that the dominant mechanism by which organic solvents unstabilize protein against thermal denaturation is through the perturbation of the characteristic structure of water by organic solvents, which weakens the hydrophobic interaction of the protein.

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