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

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The thermal denaturation of lysozymc in aqueous alcohol solution has been investigated by differential scanning calorimetry. The alcohols employed were methanol, ethanol, isomeric propyl alcohols and butyl alcohols as monohydric alcohols, and ethylene glycol and glycerol as polyhydric alcohols. In monohydric alcohols, the temperature of denaturation, T_d, of lysozyme decreased linearly with increasing alcohol concentration, which became pronounced with an increasing in the hydrophobic character. The enthalpy of denaturation, ΔH_d, of lysozyme showed a complex dependence on the solvent composition; the ΔH_d first increased with increasing alcohol concentration and then started decreasing at different concentrations for each alcohol. The branching of the alkyl chain decreased the destabilizing effect of the alcohol on the native conformation of the protein. In polyhydric alcohols, both T_d and ΔH_d increased with increasing alcohol concentration. The polyhydric alcohols stabilized the native conformation of the protein in contrast with the monohydric alcohols. The results are discussed in terms of the hydrophobic and hydrophilic characters of the alcohols.

In an earlier work, the hydration, the ordering of the water molecules around the protein molecule, were reported to play an important role in the stabilizing the native structure of the globular protein.[1,2] The addition of alcohol to a protein solution is expected to affect the stability of the native structure of the protein due to alteration in the characteristic structure of water. The effect of alcohols on the stability of globular proteins have been extensively investigated by spectroscopic measurements.[3-5] The studies have revealed that the effectiveness of the alcohols as protein denaturants increased with increasing chain length or hydrocarbon content. Hamaguchi *et al.*[6,7] have, however, found that the CD and difference spectra of the lysozyme in aqueous alcohol solutions depend nonlinearly on the concentration of alcohol at a concentration below that required to unfold the protein. Moreover, Parodi *et al.*[8] have reported from different spectrophotometric and ORD studies of the thermal denaturation of lysozyme in aqueous alcohol solutions that the van't Hoff enthalpy of denaturation showed a complex dependence on alcohol concentration.

In this paper, the effect of mono- and polyhydric alcohols on the thermal denaturation of lysozyme as measured by differential scanning calorimetry (DSC) will be reported.

Experimental

Materials and Methods. The hen egg-white lysozyme used in the present study was a six-times recrystallized preparation obtained from Seikagaku Kogyo Co. The alcohols employed were methanol, ethanol, the isomeric propyl alcohols and butyl alcohols, ethylene glycol and glycerol. The alcohols employed were spectroscopic grade or analytical grade reagents and were used without further purification. The concentration of the stock solution of the lysozyme, which dissolved with the 0.1 M glycine–HCl buffer (pH 3), was determined spectrophotomerically using an extinction of $E^{1\%}_{1cm}=26.9$ at 280 nm. The sample solution were prepared by mixing suitable aliquots of stock solution with the alcohol.

Calorimetric measurements were conducted with a Rigaku Denki standard-type differential scanning calorimeter at a heating rate of 10 K/min and a concentration of 15—25 mg/cm^3. The molecular weight of lysozyme was taken as 14300.[9]

Results and Discussion

The thermal denaturation of lysozyme in aqueous alcohol solutions was measured with a differential scanning calorimeter. For each solution, a reproducible endothermic peak was observed occurring over a temperature range of 15—20 K. The temperature, T_d, and the enthalpy, ΔH_d, of denaturation were estimated from the peak temperature and the peak-area in the thermogram obtained.

The T_d and ΔH_d in solutions containing methanol, ethanol, 1-propanol, and 1-butanol are plotted as a function of the alcohol concentration in Figs. 1 and 2, respectively. The T_d decreased gradually with increasing alcohol concentration, which became more pronounced with an increase in length of the alkyl chain. A similar dependence of the T_d on the concentration and/or length of the alkyl chain of alcohols has been reported earlier for ribonuclease[3,10] and lysozyme[8,10] from spectroscopic studies of thermal denaturation. The ΔH_d showed a complex dependence on the solvent composition; the ΔH_d first increased with increasing

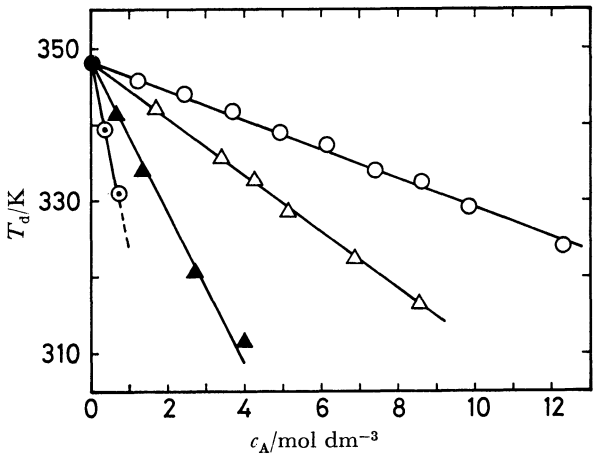

Fig. 1. The temperature of denaturation, T_d, of lysozyme as a function of the alcohol concentration, c_A. ●, Water; ○, methanol; △, ethanol; ▲, 1-propanol; ⊙, 1-butanol.

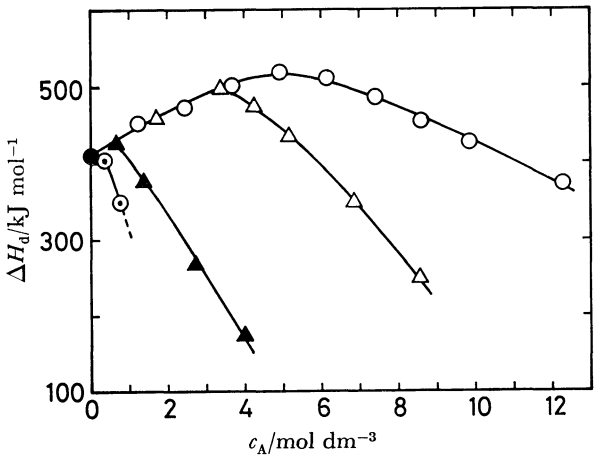

Fig. 2. The enthalpy of denaturation, ΔH_d, of lysozyme as a function of the alcohol concentration, c_A. ●, Water; ○, methanol; △, ethanol; ▲, 1-propanol; ⊙, 1-butanol.

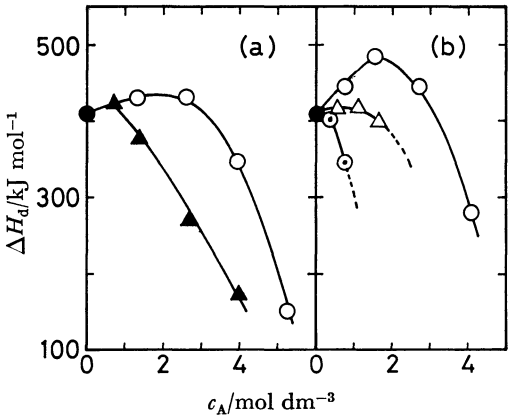

Fig. 4. The enthalpy of denaturation, ΔH_d, of lysozyme as a function of the alcohol concentration, c_A. (a) Isomeric propyl alcohols, ●, water; ○, *i*-PrOH; ▲, *n*-PrOH. (b) Isomeric butyl alcohols, ●, water; ○, *t*-BuOH; △, *s*-BuOH; ⊙, *n*-BuOH.

alcohol concentration and then started decreasing at a different concentration for each alcohol. The maximum in the values of ΔH_d occurred in the order of methanol, ethanol, 1-propanol, and 1-butanol, that is, increasing hydrophobic character of the alcohol added. Moreover, the maximum value of ΔH_d decreased with increasing length of the alkyl chain and all values of ΔH_d observed in aqueous 1-butanol solutions were smaller than that in the aqueous solution.

This observation was similar to that for van't Hoff enthalpies of denaturation obtained by Parodi *et al.*[8] from spectroscopic studies of lysozyme in aqueous alcohol solutions. Recently, Velicelebi and Sturtevant[11] have reported similar plots for the enthalpies of denaturation determined from high sensitivity calorimetric measurements of the thermal denaturation of lysozyme in similar alcohol–water mixtures at pH 2. The ΔH_d values obtained in our present work appear to be in fairly good agreement with those reported by Velicelebi and Sturtevant. The T_d values, however, in our work were considerably higher. The differences probably result from the large difference in the heating rate.

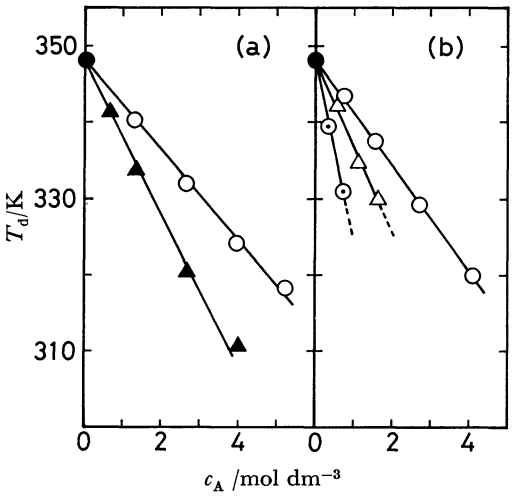

Fig. 3. The temperature of denaturation, T_d, of lysozyme as a function of the concentration, c_A. (a) Isomeric propyl alcohols, ●, water; ○, *i*-PrOH; ▲, *n*-PrOH. (b) Isomeric butyl alcohols, ●, water; ○, *t*-BuOH; △, *s*-BuOH; ⊙, *n*-BuOH.

The effect of branched-chain alcohols was also investigated and the results are showed in Figs. 3 and 4 together with the results of the corresponding straight-chain alcohols. The lowering of the T_d by the branched-chain alcohols was smaller than that by the corresponding straight-chain alcohols. The ΔH_d in the branched-chain alcohol solutions showed a complex dependence on the solvent composition, as found in the case of straight-chain alcohols. The maximum in the values of ΔH_d occurred at a higher alcohol concentration than that in the corresponding straight-chain alcohol. The results indicate that branching of the alkyl chain reduces the effectiveness of the alcohol as protein denaturants.

Aliphatic alcohols can be regarded either as hydrocarbons containing hydrophilic hydroxyl groups or as water in which one hydrogen atom has been replaced by a hydrophobic alkyl group. The effect of alcohols on the stability of the native structure of protein in terms of the hydrophobic, destabilizing, and hydrophilic, stabilizing, effects. In the monohydric alcohols the results indicate that the hydrophobic effect, which enhances with chain length of the alkyl groups, dominates the hydrophilic effect. The selective binding of alcohols to the nonpolar groups of protein in water–alcohol mixtures has been experimentally verified.[12] It is well documented that alcohols perturb the characteristic water structure around the protein molecule.[13] The binding of alcohols to the nonpolar side chains of proteins which become exposed during denaturation may reduce the ordering effect of the nonpolar side chains on the water molecules, simultaneously leading to the weakening hydrophobic interaction between the nonpolar side chains, which favors the denatured state. Therefore, the depression of T_d by the addition of alcohols which increases with increasing alcohol concentration and/or length of the alkyl chain appears to be

associated to a predominance of an increasing entropy of the denaturation. This result is in agreement with that of Schrier *et al.*[3] suggesting that the lowering of the denaturation temperature of ribonuclease by alcohols is largely entropy dependent.

The complex dependence of the ΔH_d on the alcohol concentration and/or length of the alkyl chain may also be discussed in terms of the hydrophobic effect of alcohols. At lower alcohol concentration, the alcohol molecules interact selectively with the nonpolar groups of protein, and thus the hydrophobic interaction between the nonpolar groups of protein is weakened. As suggested by Privalov,[14] rupture of the hydrophobic bond is accompanied by liberation rather than absorption of heat. It appears resonable to consider weakening of the hydrophobic bond by the addition of alcohol reduces the exothermic contribution of hydrophobic-bond rupture to the total ΔH_d. In addition, it has been experimentally shown that the secondary structure of polypeptide was stabilized more in water–alcohol mixtures than in water.[15] This suggestes that the disordering of the water molecules around the protein molecule due to the binding of alcohol to the nonpolar groups of protein enhances the stability of the secondary structure of protein which is created essentially by the interaction between the polar groups of protein. The addition of alcohol will lead to an increase in ΔH_d because the rupture of the polar interaction is an endothermic reaction. At higher alcohol concentration, however, alcohol may increase the ordering of water molecules, leading to not only stronger hydrophobic interaction but a weakening of the polar interaction. The rise in the temperature of maximum density of water by the addition of alcohol has been accounted for by the ordering of water molecules by alkyl groups.[16]

The T_d and ΔH_d in solutions containing ethylene glycol and glycerol are plotted as a function of the alcohol concentration in Fig. 5. In contrast with the case of monohydric alcohols, the T_d in the solutions of polyhydric alcohols increased with increasing alcohol concentration. The ΔH_d increased also with increasing alcohol concentration. The increase in the T_d and ΔH_d became more pronounced in the order: ethylene glycol, glycerol, that is, increasing the number of hydroxyl groups of the polyhydric alcohol added. It is apparent that polyhydric alcohols stabilize the native structure of protein; the hydrophilic effect of polyhydric alcohols dominate the hydrophobic effect. When the methylene group was present in equimolar quantities the polyhydric alcohol stabilized the native structure of protein in contrast to the monohydric alcohol. This is to be expected if the hydrophobic interaction between the nonpolar group of protein and the nonpolar part of the alcohol molecule is an important factor in destabilization. A higher degree of interaction exists between polyhydric alcohol and water than monohydric alcohol and water. Introduction of a second and third hydroxyl group into a monohydric alcohol increases the interaction with water, whereas the hydrophobic properties are less pronounced.

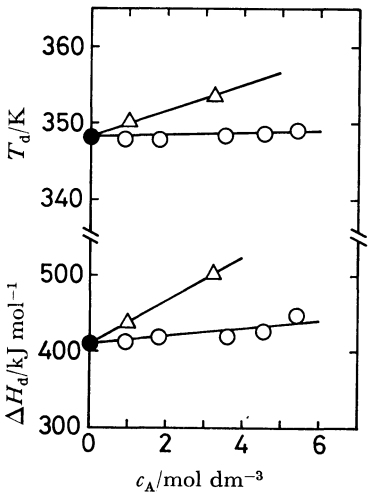

Fig. 5. The temperature, T_d, and the enthalpy, ΔH_d, of denaturation of lysozyme as a function of the alcohol concentration, c_A.
●, Water; ○, ethylene glycol; △, glycerol.

Gerlsma and Stuur[10] have suggested that there was no direct molecular interaction between protein and polyhydric alcohol from the studies on the thermal denaturation of ribonuclease and lysozyme in the present of polyhydric alcohols. This suggests that the hydrophilic effect of alcohols on the stability of protein appears mainly to be of an indirect kind, *viz.* either a strengthening of the polar interaction by a lowering of the dielectric constant, an increasing of the enthalpic change of denaturation, and/or an intensifying of the hydrophobic interaction of protein by altering the water structure, which principally is entropically controlled. The addition of polyhydric alcohol may lead to the strengthening of the polar interaction by reducing the interactions with water molecules and by lowering the dielectric constant of the medium. It is well known that hydrophobic interactions are intensified by an increase in temperature.[14] Therefore, it is also probable that the addition of polyhydric alcohol may enhance the hydrophobic interactions. The hydrophilic effect increases the hydrophobic contribution to the stabilizing of the native structure of protein. This may be induced not only by the hydrophilic groups of the added alcohols but the polar groups of the protein molecule itself will exert a similar effect on the surrounding water molecules.

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