Thermodynamics of the Denaturation of Lysozyme in Alcohol–Water Mixtures†

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ABSTRACT: The thermal denaturation of lysozyme was studied at pH 2 in aqueous mixtures of methanol, ethanol, and 1-propanol by high sensitivity differential scanning calorimetry (DSC). The most obvious effect of alcohols was the lowering of T_d, the temperature of denaturation, increasingly with higher alcohol concentration and longer alkyl chain. Both the calorimetric and van't Hoff enthalpies of denaturation initially increased and then decreased with increasing alcohol concentration, the ratio of the two enthalpies being nearly unity, 1.007 ± 0.011, indicating the validity of the two-state approximation for the unfolding of lysozyme in these solvent systems. The reversibility of the denaturation was demonstrated by the reversibility of the DSC curves and the complete recovery of enzymic activity on cooling. The changes in heat capacity on unfolding decreased with increasing alcohol concentration for each alcohol. Experimentally determined values of denaturation temperature and of entropy and heat capacity changes were used to derive the additional thermodynamic parameters $\Delta G°$ and $\Delta S°$ for denaturation as a function of temperature for each alcohol–water mixture. Comparison of the thermodynamic parameters with those reported [Pfeil, W., & Privalov, P. L. (1976) *Biophys. Chem.* *4*, 23–50] in aqueous solution at various values of pH and guanidine hydrochloride concentration showed that these latter changes have no effect on the heat capacity change, whereas the addition of alcohols causes a sharp decrease.

The elucidation of the nature of the forces that stabilize the native conformations of proteins has been a major focus of protein research in the last few decades. Reversible thermal denaturations of globular proteins have been extensively studied to this end through different methods of observation and analysis, the results of which have been reviewed in detail (Kauzmann, 1959; Tanford, 1968, 1970; Brandts, 1969; Wetlaufer & Ristow, 1973; Baldwin, 1975; Pace, 1975, Anfinsen & Scheraga, 1975; Edelhoch & Osborne, 1976).

Differential scanning calorimetry (DSC)[1] of thermal denaturation has greatly improved the reliability of the available thermodynamic information concerning proteins. Application of DSC to the study of protein unfolding has been reviewed by Privalov (1974) and Privalov & Khechinashvili (1974). Most recently, a comprehensive thermodynamic investigation of the unfolding of lysozyme has been reported by Pfeil & Privalov (1976a–c).

It is well known that various substances cause changes in the conformation of proteins when added to aqueous protein solutions (Tanford, 1968; Franks & Eagland, 1975). Investigations of these changes have provided valuable information about the role of the solvent in maintaining the native conformation of proteins. Various solvent additives, such as guanidine hydrochloride (Gdn·HCl), urea, and alcohols, affect proteins in different ways, acting as effective probes of the solution conformations of proteins.

We have chosen alcohol–water mixtures as the solvent system in our studies because of the mixed hydrophobic–hydrophilic character of alcohols. It has been suggested (Franks & Eagland, 1975; Brandts & Hunt, 1967) that alcohols destabilize proteins by weakening hydrophobic interactions between nonpolar residues as well as by perturbing the characteristic water structure around the protein molecule.

The thermodynamic parameters of heat capacity and entropy are known to be sensitive to changes in the extent of structuring of water around nonpolar groups. The need for experimentally determined thermodynamic properties of alcohol–water–protein systems has been strongly expressed in the past (Franks & Eagland, 1975). We have employed high sensitivity differential scanning calorimetry (DSC) and isothermal calorimetry in a detailed study of the effect of alcohols on the thermodynamics of the thermal unfolding of lysozyme. Lysozyme was chosen as the protein for study because it is a typical globular protein of modest size (129 amino acid residues, molecular weight 14 445), and its thermal unfolding has been shown to be a two-state process in aqueous solution (Privalov & Khechinashvili, 1974).

Materials and Methods

Hen egg white lysozyme (*N*-acetylmuramide glycanohydrolase; EC 3.2.1.17) was obtained from Sigma Chemical Co. as grade 1, three times crystallized, dialyzed, and lyophilized powder. This product was further treated by dissolution in and dialysis against 0.05 M ammonium formate (obtained from Fisher Scientific Co.) buffer at pH 4.0, and subsequent lyophilization using a Virtis refrigerated freeze-dryer. All buffer solutions were prepared from analytical grade reagents. Protein concentrations were determined spectrophotometrically on a thermostated Cary 14 spectrophotometer, using $E_{280}^{1\%} = 26.5$ in aqueous solution (Bjurulf & Wadsö, 1972). The absorbance measurements were carried out at 10 °C where the protein was native in all the alcohol solutions employed as judged by denaturation profiles. All the calorimetric experiments were carried out in 0.04 M glycine buffer adjusted to pH 2.0 as measured by a Radiometer pH meter standardized at pH 4.0 in aqueous solution.

Lysozyme assays were run at 10 °C following the standard procedure supplied by Sigma Chemical Co. Dried (*Micrococcus lysodeikticus*) cells (from Sigma Chemical Co.) were suspended in 0.066 M potassium phosphate buffer at pH 6.24. At zero time, 50 μL of a lysozyme solution of known concentration was added to 2 mL of substrate suspension in the sample cell of a Cary 14 spectrophotometer, and the subse-

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[1] Abbreviations used: DSC, differential scanning calorimetry; Gdn·HCl, guanidine hydrochloride.

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Table I: Thermodynamic Parameters of Lysozyme Denaturation in Various PrOH–Buffer Mixtures

1-propanol (M)	T_d (°C)	ΔH_{cal} (kcal mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$	ΔC_p^d (kcal K^{-1} mol^{-1})
0	52.03 ± 0.52	91.2 ± 1.9	89.5 ± 1.0	0.983 ± 0.014	1.55 ± 0.12
0.67	47.44 ± 0.50	92.6 ± 1.6	88.9 ± 1.3	0.971 ± 0.013	1.24 ± 0.14
1.34	41.64 ± 0.54	95.4 ± 2.7	89.9 ± 1.3	0.945 ± 0.021	1.30 ± 0.15
2.00	33.36 ± 0.61	94.9 ± 0.7	89.9 ± 1.4	0.937 ± 0.015	1.10 ± 0.31
2.67	25.44 ± 0.37	77.1 ± 1.1	81.5 ± 0.8	1.060 ± 0.010	1.02 ± 0.48
3.34	15.69 ± 0.51	59.0 ± 1.7	64.4 ± 2.1	1.091 ± 0.006	1.13 ± 0.15

quent decrease in apparent absorbance at 450 nm was monitored. Enzyme activity was calculated in terms of change of absorbance units per unit time per g of protein from the initial linear portions of the assay curves.

DSC measurements were performed with a Privalov calorimeter (Privalov et al., 1975) at a heating rate of 1 °C min^{-1} and at protein concentrations of 1.5–5.5 mg mL^{-1}. The thermodynamic parameters of interest listed in Table I were obtained from the DSC traces of excess heat capacity vs. temperature following the procedure outlined by Privalov & Khechinashvili (1974), as demonstrated in Figure 1f.

The first step in the treatment of the raw DSC data is correction of the sample curve for changes of slope observed in a run with buffer in each cell by substracting the buffer curve from the protein curve point by point. The linear portions of the corrected curve corresponding to pre- and posttransition base lines are extrapolated to T_d, the temperature at which the transition is half complete. T_d is determined by successive area measurements until the vertical line drawn at T_d divides the peak area into two halves. T_d and T_m, the temperature of maximal excess heat capacity, do not coincide, even for a two-state process with no permanent change in heat capacity. In the present work, T_d differs from T_m by 0.75–1.0 °C.

The area under the heat capacity curve (shaded in Figure 1a) is determined by means of a planimeter and converted to cm^2 units. The subsequent conversion into calories is done by the calculation shown in eq 1 and 2, where V_A and V_B are

$$\Delta q_{cal}(\text{cal}) = (\text{area, cm}^2) \times (V_A/V_B)^2(\text{calibration constant, cal min}^{-1})(\text{temp scale, K cm}^{-1})/[(\text{calibration mark, cm})(\text{scan rate, K min}^{-1})] \quad (1)$$

$$\Delta H_{cal}{}^d(\text{cal mol}^{-1}) = \frac{(\Delta q_{cal})(\text{mol wt, g mol}^{-1})}{(\text{cell volume, mL})(\text{protein concn, g mL}^{-1})} \quad (2)$$

average voltage readings for sample and reference cell, respectively. Since the calibration mark is obtained by current fed into the reference cell B, it is necessary to multiply the calibration constant by $(V_A/V_B)^2$ in order to correct for the small difference in the heat capacities of the two cells (cell heaters of equal resistances).

The evaluation of ΔC_p^d, the heat capacity change upon denaturation, and ΔC_d, the peak height, both at T_d, involves a similar calculation, again based on the height of the calibration mark (see eq 3).

$$(\text{signal, cm})(V_A/V_B)^2(\text{calibration constant, cal min}^{-1}) \times (\text{mol wt, g mol}^{-1})/[(\text{calibration mark, cm})(\text{scan rate, K min}^{-1})(\text{cell volume, mL})(\text{concn, g mL}^{-1})] \quad (3)$$

The van't Hoff enthalpy of denaturation can also be obtained from the same calorimetric curve since the extent of transition, α, may be assumed to be proportional to the amount of heat absorbed. It follows from the van't Hoff equation that

$$\Delta H_{vH} = 4RT_d^2\left(\frac{d\alpha}{dT}\right)_{T_d} \quad (4)$$

The derivative $d\alpha/dT$ can be determined from

$$\frac{d\alpha}{dT} = \frac{1}{\Delta q_{cal}}\frac{d\Delta q_{cal}}{dT} = \frac{C_{excess}}{\Delta q_{cal}} \quad (5)$$

where C_{excess} is the observed excess specific heat and Δq_{cal} is the observed total heat. If desired, molar units may be employed; for $\alpha = 1/2$

$$\Delta H_{vH} = 4RT_d^2\frac{\Delta C_d}{\Delta H_{cal}} \quad (6)$$

where ΔC_d is in units of cal K^{-1} mol^{-1} and ΔH_{cal} in units of cal mol^{-1}. Since for a two-state process $\Delta H_{vH} = \Delta H_{cal}$, this expression becomes

$$\Delta H_{vH} = 2T_d(R\Delta C_d)^{1/2} \quad (7)$$

It should be noted that, in cases where ΔC_p^d is large, the approach to the transition will be very gradual because of the small values of the enthalpy of transition at temperatures well below T_d, and that this gradual approach may contribute to an apparent increase in the heat capacity of the native state as the temperature is raised. This does not appear to be a serious source of error in the interpretation of the transition curves reported in this paper.

Density measurements were carried out with a Model 01D Sodev vibrating tube densimeter. Measurements of the resonance period (τ) of the tube enable the determination of the density (d) of the sample according to the relation

$$d = A + B\tau^2 \quad (8)$$

where A and B are constants of the system and are determined by calibration of the densimeter using fluids of known density, typically nitrogen gas and water. The densimeter was immersed in a water bath the temperature of which was controlled to ±0.002 °C by a Tronac thermostatic control.

The apparent specific volume, $\bar{v}$, of lysozyme was determined using the expression

$$\bar{v} = \frac{1}{C}\left(\frac{1}{d_{soln}} - \frac{1}{d_{solv}}\right) + \frac{1}{d_{solv}} \quad (9)$$

where C is the protein concentration in g per g of solution.

Results

Enzyme Activity. At all the alcohol concentrations investigated, there appears to be no change within experimental error in the level of enzymic activity of lysozyme before and after it has been heated through the denaturation range and cooled down. As noted above, the alcoholic protein solution was diluted 1:40 with aqueous substrate for the activity determinations, in effect being transferred back to H_2O. It was found that for some unknown reason the activity of the enzyme decreased by about 30% after exposure to even the lowest alcohol concentrations employed, whether or not it was heated prior to the activity measurement. This drop in activity, which

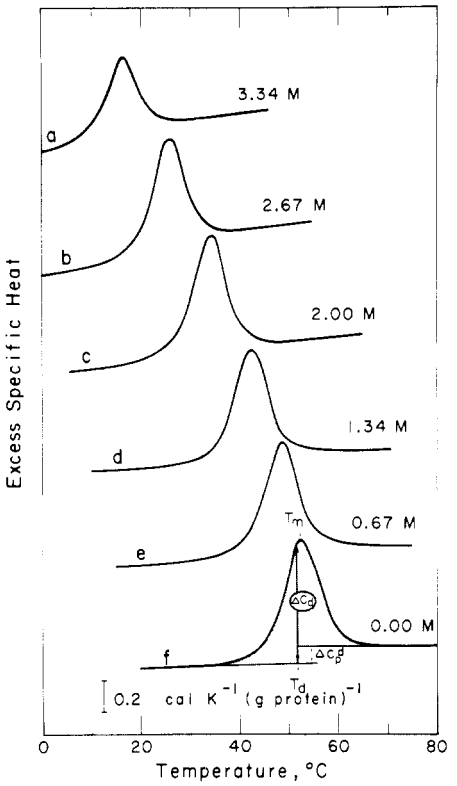


FIGURE 1: Average DSC scans of lysozyme denaturation in 0–3.34 M aqueous PrOH solutions, all normalized to 1 mg mL^{-1} protein concentration. Buffer: 0.04 M glycine, pH 2.0. Figure 1f demonstrates how the thermodynamic parameters of denaturation are determined from a typical DSC output.

was the same for all three alcohols, and may be due to the retention of some tightly bound alcohol even on dilution with buffer, did not interfere with the demonstration of the reversibility of the thermal unfolding process.

DSC Results. Figures 1a–f are representative of the DSC scans for lysozyme denaturation in PrOH–H_2O mixtures. The heat absorption curves shown correspond to denaturation of a protein solution of 1 mg mL^{-1} concentration in increasing PrOH concentrations in the range 0–3.34 M. The temperature of maximum heat capacity decreases with increasing alcohol content, while the peak area becomes smaller and the peak width slightly larger. In addition, the temperature dependence of the apparent heat capacity of the protein in the pre- and post-denaturational regions increases with increasing PrOH concentration. The thermodynamic parameters obtained through analysis of numerous DSC denaturation curves of lysozyme in various PrOH–H_2O mixtures are compiled in Table I. Each value in the table represents the average of four to nine experiments, and the standard error of the mean is listed. The results obtained for denaturation in aqueous buffer are in good agreement with those reported by Pfeil & Privalov (1976b).

Figure 2 shows the dependence of the temperature of denaturation, T_d, on alcohol concentration. There is clearly a decreasing trend with increasing alcohol content, which becomes more pronounced with increasing alkyl chain length.

Figure 3 displays the effect of alcohol concentration on the calorimetric enthalpy of denaturation, ΔH_{cal}. It should be noted that ΔH_{cal} is assigned to T_d, although $\Delta C_p \neq 0$. This treatment can be justified by constructing a theoretical two-state curve using the values derived as described from the experimental curve and noting that integration regenerates, within experimental uncertainty, the original value of ΔH_{cal}.

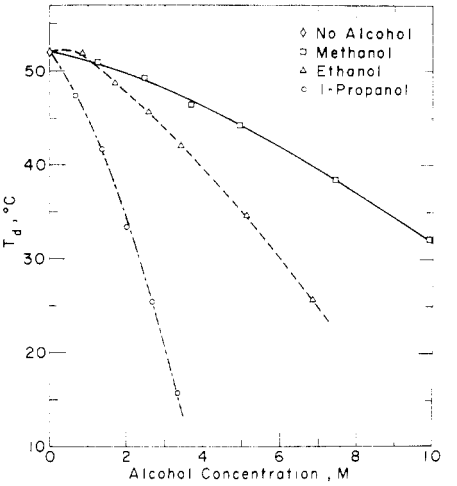


FIGURE 2: Variation of T_d, the temperature of denaturation, with alcohol concentration.

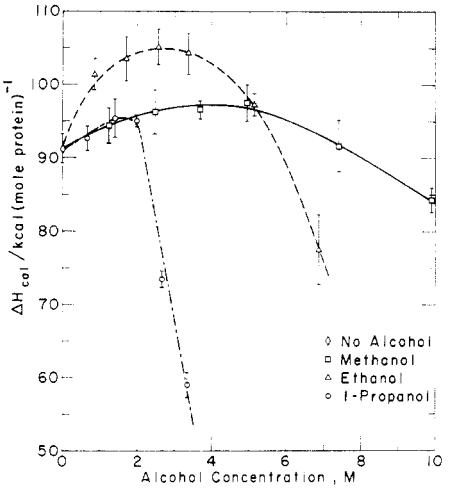


FIGURE 3: Variation of ΔH_{cal}, the calorimetric enthalpy of denaturation, with alcohol concentration.

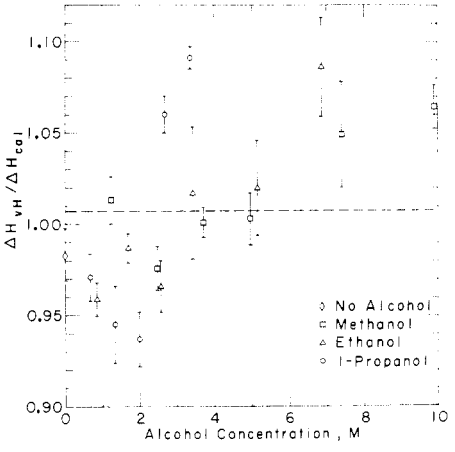


FIGURE 4: Variation of $\Delta H_{vH}/\Delta H_{cal}$, the ratio of van't Hoff to calorimetric enthalpies of denaturation, with alcohol concentration.

ΔH_{cal} shows a complex dependence on solvent composition, an initial increase up to a certain alcohol concentration followed by a decrease which becomes sharper in the order MeOH, EtOH, PrOH. The mamimum in the values of ΔH_{cal} occurs at a lower alcohol concentration in the order MeOH, EtOH, PrOH, that is, increasing hydrophobic character of the alcohol added. Parodi et al. (1973) have reported similar plots for van't Hoff enthalpies determined from spectroscopic

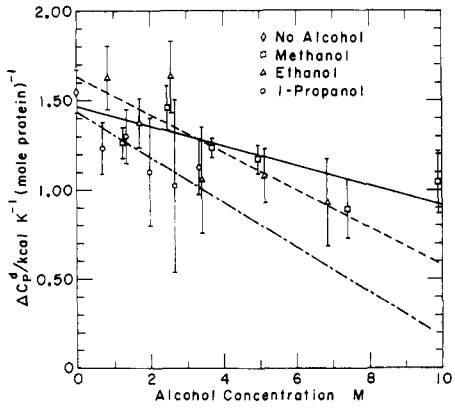


FIGURE 5: Variation of ΔC_p^d, the denaturational heat capacity change, with alcohol concentration.

measurements of lysozyme denaturation in similar alcohol–water mixtures at pH 3.

In Figure 4, the ratio of van't Hoff to calorimetric enthalpies of denaturation, $\Delta H_{vH}/\Delta H_{cal}$, is plotted against the alcohol concentration. Unity or near-unity values for this ratio provide a necessary and sufficient criterion for a two-state process (Sturtevant, 1977). The trend toward higher values of the ratio with increasing alcohol concentration is of doubtful statistical significance, and in any case is very small. The average value, 1.007 ± 0.011, indicates that the unfolding of lysozyme in aqueous alcohols is very nearly two-state in character.

Figure 5 represents the variation of ΔC_p^d, the heat capacity change on denaturation, with alcohol concentration. The dependability of the values for ΔC_p^d is primarily limited by the uncertainty in establishing the pre- and posttransition base lines. For this reason, the data for the highest alcohol concentrations are less dependable, since the protein starts to undergo denaturation at very low temperatures (see Figure 2). Especially in the case of PrOH above 2 M, as can be judged from the average curves shown in Figure 1, there is a strong temperature dependence in the pretransition base lines even when the experiment is started at −2 °C in 3.34 M (25% v/v) PrOH.

The lines in Figure 5 are drawn according to least-squares fitting of the data. There is a decreasing trend in the values of ΔC_p^d with increasing alcohol concentration. The average value for the intercept of the three lines is 1.51 ± 0.06 kcal K^{-1} mol^{-1}, in very good agreement with the experimentally determined value of 1.55 ± 0.12 kcal K^{-1} mol^{-1} in aqueous buffer.

Figure 6 shows the temperature dependence of ΔH_{cal}. For all three alcohols, ΔH_{cal} first increases with increasing temperature and then starts decreasing at a different temperature for each alcohol. The temperature of maximum ΔH_{cal} increases in the order PrOH, EtOH, MeOH. While there are clear differences in the temperature dependence of ΔH_{cal} for each alcohol on the high temperature side, the slope of ΔH_{cal} vs. T_d is nearly the same for all three alcohols in the lower temperature region. The dependence of ΔH_{cal} on T_d in the presence of alcohols thus differs drastically from that reported by Pfeil & Privalov (1976a,b) with variations of T_d produced by variation of the pH and addition of Gdn·HCl. They found ΔH_{cal} to be a linear function of T_d under all conditions, with slope equal to the constant quantity ΔC_p^d.

Densimetry Results. Density measurements were carried out primarily in order to determine any necessary corrections to the DSC data due to expansion of the solutions during the heatings. The temperature dependence of the solvent and solution densities was examined, and the correction that would

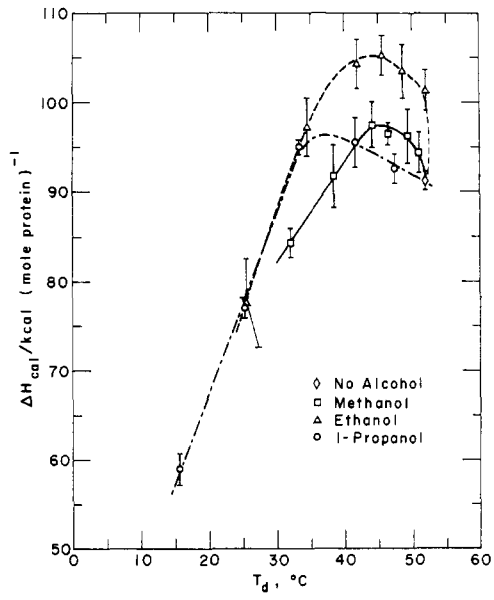


FIGURE 6: Variation of ΔH_{cal} with T_d.

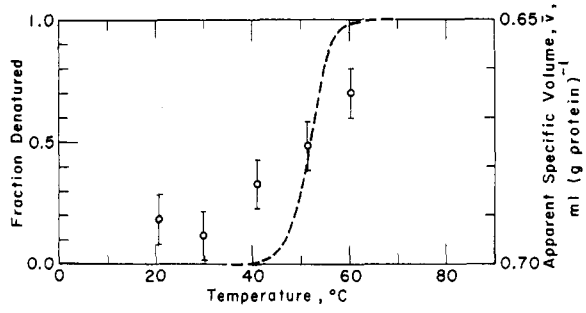


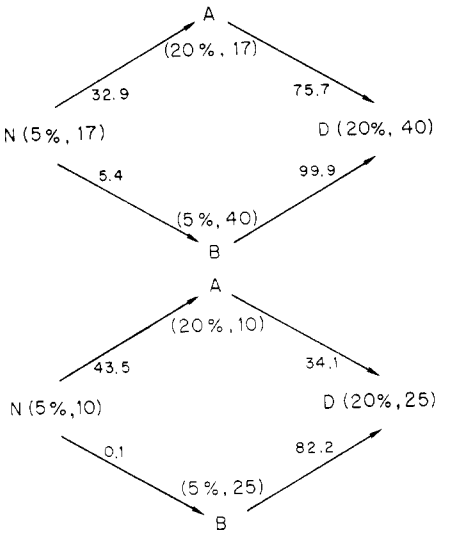
FIGURE 7: Variation of $\bar{v}$, apparent specific volume, of lysozyme with temperature and the corresponding transition curve determined from DSC results.

be applicable to either cell was calculated to be 1% or less in all solvent compositions. The differential nature of the measurements ensures nearly complete cancellation of these small effects and they may therefore be safely neglected.

The density data were used to determine the apparent specific volume of the protein in the various alcohol–water mixtures according to eq 9. The value obtained in aqueous buffer agrees quite well with those reported in the literature. At 25 °C, we have determined $\bar{v}$ = 0.711 ± 0.001 mL (g of protein)$^{-1}$ as compared with 0.714 and 0.721 (Charlwood, 1957), 0.668 (Colvin, 1960) and 0.702 (Lee & Timasheff, 1974) at the same temperature and 0.703 (Sophianopoulos et al., 1962) at 20 °C.

The temperature dependence of the apparent specific volume in aqueous buffer was investigated in the range 20–60 °C to look for any denaturational changes. The results are plotted in Figure 7 along with the fraction denatured determined by integration of the DSC results. The density curve is obviously much broader than the calorimetric curve. There is a volume decrease of approximately 0.027 mL g^{-1} or 390 mL mol^{-1} accompanying 98% denaturation of the protein. This value agrees with the expected volume changes, −100 to −400 mL mol^{-1}, due to exposure of nonpolar groups upon unfolding (Zipp & Kauzmann, 1973), but is significantly larger than those reported for ribonuclease (Brandts et al., 1970), −45 mL mol^{-1} at 25 °C and pH 2, and metmyoglobin (Zipp & Kauzmann, 1973), −110 mL mol^{-1} at 20 °C and pH ~4, on the basis of the effect of pressure on denaturation. The apparent specific volume of the protein was unaffected within

Scheme I



experimental error by changes in the solvent composition.

Discussion

The reversibility and the approximately two-state character of the thermal unfolding of lysozyme in aqueous alcohols have been demonstrated by the data cited above. It is thus permissible to apply equilibrium thermodynamics for the evaluation of thermodynamic parameters as functions of temperature and alcohol concentration. Unity activity coefficients have been assumed throughout. Errors resulting from this assumption are less serious with respect to enthalpy and heat capacity values than to free energy and entropy values.

Using the data obtained by isothermal calorimetry of the interaction of lysozyme with PrOH (Velicelebi, 1978), we demonstrated that the denaturational transition observed in our experiments corresponds to thermodynamically defined states. Two examples are given in Scheme I where N corresponds to the native and D to the denatured state of the protein. The numbers in each set of parentheses represent the percent by volume of PrOH and the temperature, while the number by each arrow is the experimental value for the enthalpy of that particular step. In the first example, the enthalpy changes for path A total 108.6 ± 7.9 and those for path B 105.3 ± 3.7. In the second case, the corresponding sums for steps A and B are 82.3 ± 3.7 and 77.6 ± 5.3, respectively. The equivalence of the total enthalpy from state I to II for two different pathways indicates that the enthalpy is a function of state.

The DSC data pertain to the change of the protein from the native to the denatured state in a solvent of fixed composition, and therefore give no information concerning the absolute stability of either state. The additional data necessary to describe the thermodynamic properties of both N and D states relative to a specified reference state, such as the state N at 25 °C in aqueous solution, could in principle be gathered through measurements of the solubility of the native protein in the various solvents. However, this would be a very difficult task in view of the very high solubility of the enzyme, and an impossible one for solvent compositions and temperatures at which the protein is partially denatured. In such measurements it sould certainly not be appropriate to assume unity activity coefficients.

We have neglected any possible effects on the charge state of the protein due to lowering of the dielectric constant by the addition of alcohols to the aqueous solvent. Lowering the dielectric constant will increase the pKs of –COOH groups, the only type of groups ionizing at low pH, and will thus tend to increase the positive charge on the protein. According to the pH titrations reported by Tanford & Wagner (1954), lysozyme is saturated with protons at pH 2, and thus will not be affected by a lowering of the dielectric constant.

The most obvious effect of alcohols is the lowering of T_d, the temperature at which the equilibrium constant, K, for the N $\rightleftarrows$ D transition is equal to unity and $\Delta G° = 0$. The effectiveness of an alcohol in lowering T_d increases with alkyl chain length; for example, in 3.34 M PrOH, T_d is decreased from 52 to 16 °C, whereas EtOH at the same concentration lowers T_d to 42 °C and MeOH to 47 °C. It is evident that, in general, alcohols destabilize N relative to D. It seems reasonable to assume that the effects of alcohols are larger on D than on N since more groups are exposed to solvent in D than in N. It thus appears that D is stabilized more in the presence of alcohols than is N.

Depression of the denaturation temperatures of proteins by alcohols has been reported earlier for chymotrypsinogen A (Gerlsma, 1976), ribonuclease (Gerlsma & Stuur, 1972, 1974, 1976; Schrier et al., 1965), and lysozyme (Gerlsma & Stuur, 1974; Parodi et al., 1973) from spectroscopic studies of thermal denaturation. These workers have observed trends similar to the ones we have described, that is, enhanced lowering of T_d with increasing alcohol concentration and alkyl chain length. Such results have generally been interpreted in terms of hydrophobic interactions between the solvent and the nonpolar groups of the unfolded protein, and thus a relatively favored denatured state in the case of the alcoholic solvents.

The extensive calorimetric studies of Pfeil & Privalov (1976a,b) showed that, in aqueous solution when T_d is changed by changing the pH or adding Gdn·HCl, ΔH_{cal} increases linearly with T_d, giving a constant value for ΔC_p equal to ΔC_p^d. In our calorimetric investigations where T_d was changed by the addition of alcohols, this situation was not found, as can be judged from the decrease of ΔC_p^d with alcohol concentration shown in Figure 5. It appears that the present experiments have added complications, suggesting that the nature of the unfolding resulting from the addition of alcohols is different from that produced by pH changes or additions of Gdn·HCl. We will assume that ΔC_p is independent of temperature and equals ΔC_p^d at a fixed alcohol concentration, although we have no direct evidence in support of this assumption. We cannot avoid this assumption if we wish to derive the thermodynamic functions for denaturation over a range of temperatures.

Thermodynamic functions, $\Delta G°$, $\Delta S°$, and ΔH, were calculated at different temperatures for denaturation of the protein in each solvent system, using $\Delta G° = 0$ at T_d and interpolated values of ΔC_p from the line fitting in Figure 5. The relationships used in these calculations are given in eq 10–12. Because of the large values of ΔC_p, strong temperature

$$\Delta H = \Delta H_{cal} - \Delta C_p(T_d - T) \quad (10)$$

$$\Delta G° = \Delta H_{cal}\frac{T_d - T}{T_d} - \Delta C_p(T_d - T) + T\Delta C_p \ln\frac{T_d}{T} \quad (11)$$

$$T\Delta S° = \Delta H - \Delta G° \quad (12)$$

dependence is seen for $-T\Delta S°$ and ΔH while the temperature variation of $\Delta G°$ is small because of the inevitable enthalpy–entropy compensation.

By interpolation in temperature, thermodynamic functions are obtained as a function of alcohol concentration at constant temperature (10, 25, and 40 °C). The results for $\Delta G°$ are given in Figure 8. The values follow smooth curves with broad maxima in most cases. The position of the maximum occurs at lower concentrations in the order propanol < ethanol <

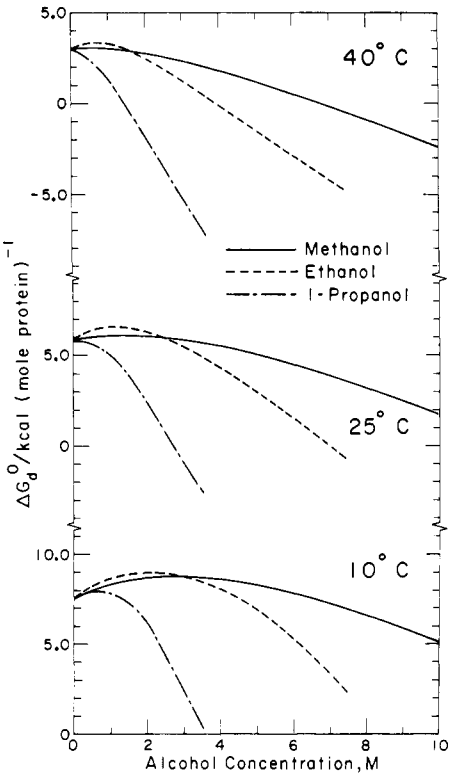


FIGURE 8: Variation of $\Delta G°$ with alcohol concentration at 10, 25, and 40 °C.

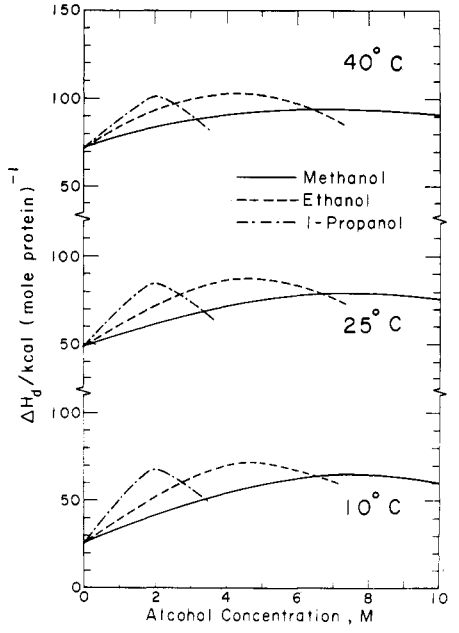


FIGURE 9: Variation of ΔH with alcohol concentration at 10, 25, and 40 °C.

methanol, and the sharpness with which the values drop increases in the reverse order at all temperatures. These trends indicate increased stabilization of the denatured state relative to the native state with increasing alcohol concentration for a given alcohol and increasing alkyl chain length for a given concentration. As expected, the values of $\Delta G°$ decrease with increasing temperature.

Figures 9 and 10 display the alcohol concentration dependence of ΔH and $\Delta S°$, respectively, at 10, 25, and 40 °C. Both functions exhibit similar behavior, an initial increase with alcohol concentration up to a maximum followed by a decrease. Maxima in the values of both ΔH and $\Delta S°$ occur at ~7.5 M

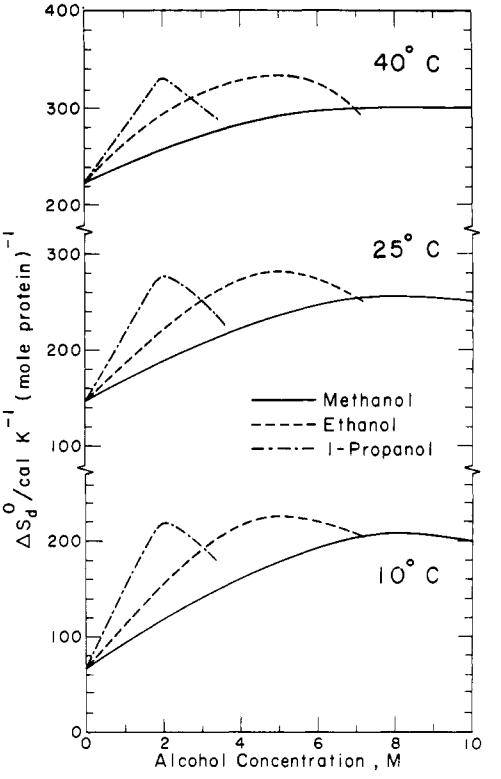


FIGURE 10: Variation of $\Delta S°$ with alcohol concentration at 10, 25, and 40 °C.

methanol, 4.4 M ethanol, and 2 M propanol and appear to be independent of temperature.

As has been observed with other protein denaturations, the unfolding of lysozyme in aqueous solution is accompanied by large increases in heat capacity. However, the value in water, 1.5 kcal K^{-1} mol^{-1}, is decreased linearly by the addition of alcohols, as shown in Figure 5. Large denaturational ΔC_p increases have been attributed to the structuring of H_2O molecules around the nonpolar groups of the protein that become exposed to solvent as a result of unfolding of the globular native structure. The decrease observed in the values of ΔC_p for denaturation in alcohol–water mixtures can be interpreted in terms of an altered water structure in the presence of alcohols. An increased organization of water due to addition of alcohols has been indicated in the literature (Frank & Eagland, 1975; Franks & Ives, 1966). Therefore, upon unfolding of the protein globule in aqueous solutions, particularly in higher concentration, one would not expect as much reordering of H_2O structure around the apolar residues, and hence the decrease in ΔC_p as compared with the value in H_2O.

The procedure outlined by Sturtevant (1977) can be applied to the data in aqueous solution to determine the values for hydrophobic and vibrational contributions to ΔC_p and $\Delta S°$. We have assumed a value of +200 cal K^{-1} mol^{-1} for the conformational contribution to $\Delta S°$; however, the general shapes of the curves obtained are unaffected if other values are assumed for $\Delta S°_{conform}$. At 25 °C, for the denaturation of lysozyme in 0.04 M glycine buffer at pH 2.0, ΔC_p = 1.55 kcal K^{-1} mol^{-1} and $\Delta S°$ = 146 cal K^{-1} mol^{-1}. The calculated hydrophobic and vibrational contributions are

$$\Delta C_p(\text{hydro}) = 1283 \text{ cal K}^{-1} \text{ mol}^{-1}$$

$$\Delta C_p(\text{vib}) = 266 \text{ cal K}^{-1} \text{ mol}^{-1}$$

$$\Delta S°(\text{hydro}) = -334 \text{ cal K}^{-1} \text{ mol}^{-1}$$

$$\Delta S°(\text{vib}) = 280 \text{ cal K}^{-1} \text{ mol}^{-1}$$

As has been found in other cases (Sturtevant, 1977), the vibrational and hydrophobic contributions to the entropy are both large and of opposite sign, and the hydrophobic contribution to the heat capacity is much larger than the vibrational contribution. If it is assumed that the vibrational contributions to ΔC_p and $\Delta S°$ are unaffected by the addition of alcohols, the hydrophobic contributions to ΔC_p and $\Delta S°$ can be calculated for denaturation in the various alcohol–water mixtures studied.

The values for ΔC_p(hydro) at 25 °C were calculated using the experimentally determined $\Delta C_p^d = \Delta C_p$ values (assumed to be independent of temperature) and the $\Delta S°$ values determined according to eq 10–12. The values of ΔC_p(hydro) decrease linearly with alcohol concentration as expected since the values of ΔC_p show a similar variation. In other words, the assumption that ΔC_p(vib) is independent of alcohol concentration is tantamount to assigning all the variation of ΔC_p at constant temperature to a diminishing hydrophobic effect.

The variation of $-\Delta S°$(hydro) with alcohol concentration displays a minimum for each alcohol, which is broader in the order MeOH, EtOH, PrOH. The magnitude of the apparent hydrophobic contribution to $\Delta S°$ is the largest in the presence of MeOH over the concentration range studied.

In conclusion, our experimental results have shown that the thermal denaturation of lysozyme in alcohol–water mixtures is a reversible, two-state transition between thermodynamically defined states. The presence of alcohols lowers the temperature of denaturation presumably largely as a result of decreased hydrophobic stabilization of the native state. This effect is enhanced by increasing alcohol concentration and alkyl chain length.

Comparison of the denaturational thermodynamic parameters we have determined in alcohol–water mixtures with those reported by Pfeil & Privalov (1976a–c) in aqueous solution at various pH and Gdn·HCl concentrations shows several significant differences, the most striking of which is perhaps the variation of the heat capacity change when the unfolding process is altered by the addition of alcohols as compared with its constancy when the pH is changed or Gdn·HCl is added. It appears that these latter changes have little or no effect on the hydrophobic contribution to the heat capacity change, whereas the addition of alcohols causes a sharp decrease.

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