Effect of Aqueous Alcohol Solutions on the Thermal Transition of Lysozyme: A Calorimetric Study

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Thermal denaturation of lysozyme has been studied at pH = 3 in water/ethanol and water/*tert*-butyl alcohol mixtures in the water rich region of composition (mole fraction of cosolvent $x_2 < 0.12$) by high-sensitivity differential scanning calorimetry. The results show that on increasing alcohol concentration, the enthalpy and entropy of denaturation of lysozyme first reach a maximum at an intermediate composition $x_2 = x_2^*$ typical for each alcohol ($x_2^* \cong 0.06$ for ethanol and $x_2^* \cong 0.02$ for *tert*-butyl alcohol) and then decrease with increasing x_2. In addition, two enthalpy−entropy compensation patterns each having its own compensation temperature (T_c) clearly appear from the data: a compensation data line obtained with rising x_2 in the $0-x_2^*$ range (with $T_c = 281 \pm 6$ K) followed by a compensation line with $T_c = 403 \pm 14$ K after x_2 passes the x_2^* value. The value of x_2^* is close to that at which a change in the nature of solvent component interaction occurs as inferred from compressibility and IR absorption measurements. The data have been interpreted on the basis of the assumption that the addition of short chain alcohols affects the thermal transition of proteins, modifying the extent of enthalpy and entropy contribution associated with structural reorganization of water in the unfolding process.

I. Introduction

It is generally accepted that the biological solvent water plays an important role in the folding and reactivity of native biopolymers. A common method of investigating the role that the solvent plays in the maintenance of the macromolecular native state is to study the conformation and thermal stability of these macromolecules in altered water structure by adding small quantities of monohydric alcohols of varying chain length.[1] Actually, the mechanism of the action of alcohols on biomolecules is still obscure, and there are different views as to whether this action can be regarded as ligand binding or indirect, involving a change in the properties of solvent water. Previous work at this laboratory[2] showed that the effects due to alcohols on two very different systems and processes, the thermal denaturation of transfer ribonucleic acid molecules and the micellization of several surfactant molecules, are strikingly similar and are closely paralleled in simpler properties of alcohol/water mixtures themselves. Accordingly, these results support the hypothesis that the observed effects are not due to a binding of alcohol molecules to groups in macromolecules but to an indirect mechanism due to changes in the solvent properties by addition of alcohol.

In ref 3 it was suggested that the effect of an alcohol on the micellization process is essentially that of a decrease in the strength of the hydrophobic interactions. The primary contribution to the strength of these interactions derives from changes in the structure of water when nonpolar groups interact with one another. Hydrophobic interactions are believed to be closely related to the micellization process and to play an important role in stabilizing the native structure of proteins. Such interactions arise from the unique three-dimensional structure of water and should be changed considerably by variations in the solvent structure due to addition of alcohol. According to Benzinger[4] and as developed by Lumry and Frank,[5,6] the structuring of water around nonpolar groups is largely a compensated process, so that the entropy and enthalpy associated with such a structuring nearly compensate and thus make only a small contribution to the free energy. As a consequence, one would expect the enthalpy and entropy rather than the free energy to be affected by alcohol addition.

Differential scanning calorimetry (DSC) provides information on the enthalpy, temperature, and heat capacity changes that accompany conformational transitions of biopolymers in solution. There has been a great interest in calorimetry of biological systems in recent years, due, in part, to considerable advances in instrumentation.[7−10] In particular the unfolding transition of lysozyme in aqueous solutions has been extensively investigated by DSC.[10] Heat denaturation of this protein can be described to a first approximation by the two-state transition model; the deviation from this model does not exceed 5%.

In this paper, the effect of ethanol and *tert*-butyl alcohol on the thermal denaturation of lysozyme as measured by differential scanning calorimetry will be reported. The approach followed is to alter the composition of the solvent by adding little quantities of alcohol to the solution and to study the induced changes in the thermal transition of the protein. The main aim is to establish the existence of correlations between certain effects of alcohols on the protein conformation stability and changes in the properties of solvent. To this purpose, the quantitative thermodynamic parameters accompanying the thermal denaturation have been evaluated as a function of alcohol concentration and discussed also in connection with adiabatic compressibility and infrared absorption data of water/alcohol mixtures in the same concentration range.

II. Experimental Section

Hen egg-white lysozyme was obtained from Fluka Chemie AG and used without further purification. Alcohols employed were ethanol and *tert*-butyl alcohol, reagent grade. The sample solutions at 2% weight were prepared by dissolving lysozyme in 0.1 M HCl/glycine buffer solution at pH = 3. The glycine buffer was prepared by dissolving glycine in water/alcohol

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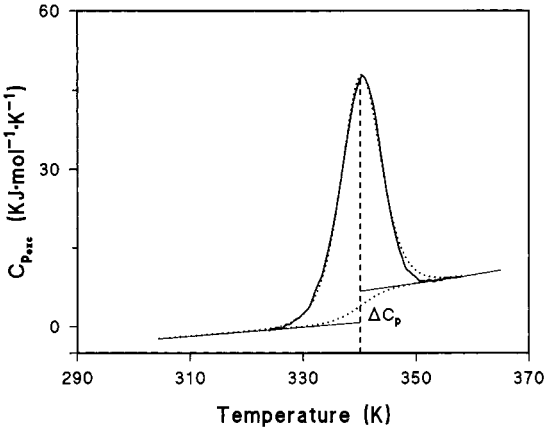

Figure 1. DSC thermal scan of lysozyme in water (—). Two-state model curve (···). The sigmoidal baseline was generated by the two-state model.

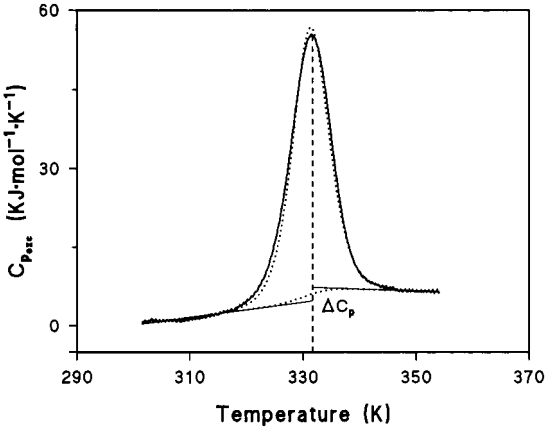

Figure 2. DSC thermal scan of lysozyme in water/*tert*-butyl alcohol mixture at a 0.02 *tert*-butyl alcohol mole fraction (—). Two-state model curve (···). The sigmoidal baseline was generated by the two-state model.

mixtures and adding concentrated HCl dropwise with stirring until the pH reached 3.0, as monitored by a pH-meter. The concentration of protein in the sample solution was determined by weighing the components and in some cases spectrophotometrically by using an extinction of $E^{1\%}_{1cm} = 26.9$ at 280 nm. Both methods give identical results within experimental error.

The DSC experiments were performed on a micro-DSC II (Setaram, France) at a scan rate of 18 K·h^{-1} with sample masses of 0.85 g. The weights of the sample and the reference cells were always matched. An excess power vs temperature scan for the lysozyme transitions was obtained by subtracting the power input of a thermal scan of buffer vs buffer from the power input scan of the solution vs buffer. To determine the transitional baseline, transition temperature (T_m), and van't Hoff transition enthalpy (ΔH_{vf}), the excess heat capacity vs temperature profile were fitted to the two-state transition model described by Schwarz and Kirchoff.[9] In the fitting procedure the number of moles of protein was fixed at a value N determined by weight. The molecular weight of lysozyme was taken to be 14 300. Calorimetric enthalpies (ΔH_{cal}) were determined from the area under the transition profile with a sigmoidal baseline; use of a straight baseline gives identical results within experimental error. The cooperativity η of the transition was taken as the ratio $\Delta H_{cal}/\Delta H_{vf}$. Reversibility of denaturation was checked by repeated heating of the solution after its cooling. It was observed that all denaturations studied here were >90% reversible. This allows the use of equilibrium thermodynamics in the analysis of the present data. The results were independent of DSC scan rate.

III. Results and Discussion

(a) Calorimetric Measurement. A typical thermal scan of lysozyme in aqueous solution is shown in Figure 1 together with the computer simulated excess heat capacity curve from the two-state model. The unfolding transition consists of a single symmetrical peak followed by an increase in the transitional baseline. Values of $\Delta H_{vf} = 410 \pm 1$ KJ·mol^{-1}·K^{-1}, $T_m = 339.9 \pm 0.2$ K, and the cooperativity $\eta = 0.99 \pm 0.01$ observed in the aqueous suspension agree well with the literature values.[10] Extrapolation of the baseline to the transition temperature shows a positive increase from the pretransitional to posttransitional baseline, indicating an increase in the heat capacity ΔC_p of the solution on denaturation. This is also observed for other globular proteins and is currently attributed to the hydrophobic hydration of nonpolar groups of inner protein exposed to the bulk water after unfolding.[11]

The addition of alcohol to water induced significant changes in the unfolding process. As an example in Figure 2 is reported a thermal scan of lysozyme in a water/*tert*-butyl alcohol mixture at $x_2 = 0.02$. Both T_m and ΔC_p decrease, and a modification of the area under the transition profile is observed. The $C_{p_{exc}}$ curve can be well approximated by a single transition, as in aqueous solutions assuming a two-state behavior. The thermodynamic information on the unfolding of the protein in water/ethanol and water/*tert*-butyl alcohol determined from a DSC scan of 18 samples at selected x_2 values is summarized in Table 1. ΔH_{cal} and T_m values are also reported in Figure 3. It is found, in agreement with earlier work,[12] that alcohols have a destabilizing effect upon the native conformation of protein, the melting temperature T_m being progressively lowered on increasing x_2 (Figure 3a). In the concentration range investigated the plots are nearly linear with $dT_m/dx_2 = -165$ K for ethanol and $dT_m/dx_2 = -470$ K for *tert*-butyl alcohol mixtures. The transition enthalpy (Figure 3b) depends in a complex manner on the nature and amount of alcohol present. According to other authors,[12] as the concentration of alcohol is increased, ΔH_{cal} increases until a maximum value is reached at a mole fraction x_2^* of about 0.02 for *tert*-butyl alcohol and 0.06 for ethanol. A corresponding maximum also arises in the entropy change accompanying the denaturation. An inspection of Table 1 shows that the parameter η describing the cooperativity of the transition is sensitive to small mole fractions of alcohol added and goes through a maximum at the same values of x_2 where a maximum in ΔH was observed.

It must be noted that $(dT_m/dx_2)_{tert\text{-but}}/(dT_m/dx_2)_{ethanol} \cong 3$ and $(x_2^*)_{ethanol}/(x_2^*)_{tert\text{-but}} \cong 3$; these ratios compare well with the ratio of the $-CH_3$ groups in the two molecules. In Figure 4 T_m and ΔH_{cal} quantities are reported as a function of $-CH_3$ groups mole fraction (x_{CH_3}). The data for the two alcohols superimpose within experimental error. Thus, the thermodynamic properties of protein unfolding in water/alcohol mixtures appear determined primarily by the number of $-CH_3$ groups in solution. From this result it was presumed that there is no direct molecular interaction between the protein and alcohol molecules, since any direct interaction would depend on the alcohol concentration.

The observed behavior of ΔH_{cal} as a function of x_2 is very similar to that found for the enthalpy of demicellization in the case of surfactants.[3] The close similarity between the behavior of these two very different systems and processes is striking. It is relevant that the only common feature is the same solvent system. In ref 3 it was suggested that the effect of alcohol on

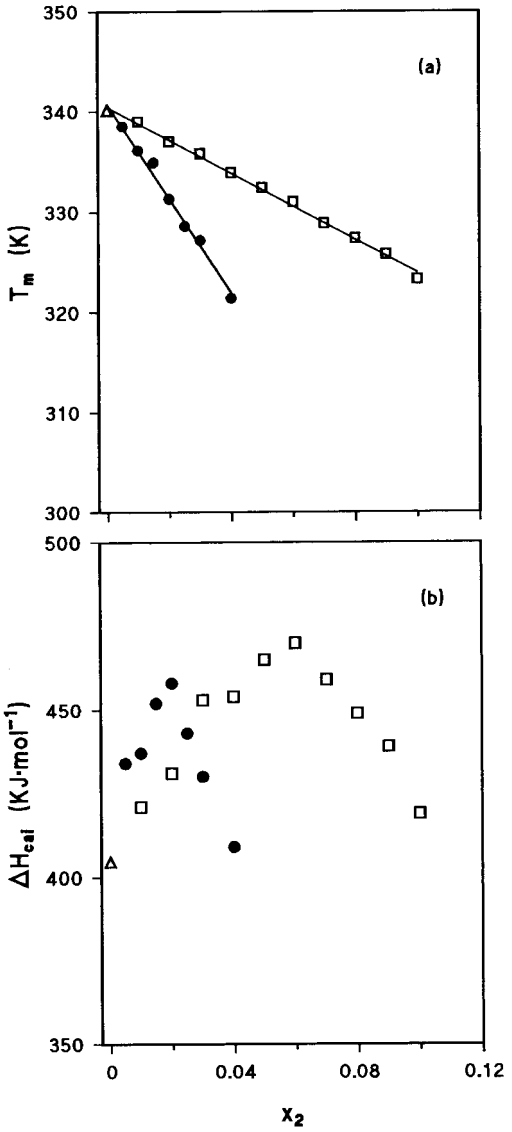

Figure 3. (a) Melting temperature T_m of lysozyme in water/alcohol mixtures as a function of alcohol mole fraction x_2. Continuous lines are linear fits. (b) Calorimetric enthalpy ΔH_{cal} of lysozyme in water/alcohol mixtures as a function of alcohol mole fraction x_2: (△) pure water, (□) ethanol, (●) *tert*-butyl alcohol.

the micellization process is essentially to modulate hydrophobic interactions. Consistently with this suggestion ΔC_p (see Table 1) progressively decreases as x_2 increases, indicating a progressive attenuation of the hydrophobic effect with alcohol concentration.

The participation of water in the process can also be inferred from an observed enthalpy−entropy compensation phenomenon. The changes $\Delta\Delta H$ in the enthalpy of denaturation in water/alcohol mixtures relative to those in pure water solution are reported in Figure 5 vs the corresponding $\Delta\Delta S$ changes. Two compensation patterns seem to be clearly delineated, each having its own compensation temperature. The fit to two linear segments is very good. A segment is obtained with rising x_2 in the $0 - x_2^*$ range (with $T_c = 281 \pm 6$ K) followed by a segment (with $T_c = 403 \pm 14$ K) after the x_2 passes the x_2^* value.

Values of T_c lying in the range 270−300 K are normally found in a variety of protein reactions in highly aqueous solutions and are said to be a strong indication of the involvement of water in the compensation process.[13] Water is perturbed by the cosolvent at lower mole fraction but still retains its integrity as water. As x_2 increases, a progressive loss of the characteristic tetrahedric water structure is expected, and this could be connected to the observed change in the compensation pattern for $x_2 > x_2^*$ (Figure 5).

TABLE 1: Thermodynamic Parameters of the Thermal Transition of Lysozyme in Water/Alcohol Mixtures

x_2	T_m (K)	ΔC_p (KJ· mol^{-1}·K)	ΔH_{cal} (KJ· mol^{-1})	ΔH_{vf} (KJ· mol^{-1})	η
		Water			
0	339.9	6.0	404	410	0.99
		Water/Ethanol			
0.01	339.0	3.8	421	418	1.01
0.02	337.0	5.5	431	430	1.00
0.03	335.8	3.3	453	443	1.02
0.04	333.9	2.2	454	445	1.02
0.05	332.4	3.1	465	447	1.04
0.06	331.0	1.9	470	454	1.04
0.07	328.9	3.9	459	452	1.02
0.08	327.4	2.9	449	444	1.01
0.09	325.8	2.1	439	438	1.00
0.10	323.3	0.5	419	428	0.98
		Water/*tert*-Butyl Alcohol			
0.005	338.5	2.1	434	417	1.04
0.010	336.1	3.5	437	422	1.04
0.015	334.9	7.1	452	423	1.07
0.020	331.3	2.6	458	431	1.06
0.025	328.6	2.9	443	424	1.05
0.030	327.2	3.1	430	415	1.04
0.040	321.4	1.4	410	401	1.02

(b) Water/Alcohol Mixtures: Compressibility and Infrared Absorption Measurements. Present results collectively indicate that the unfolding process of the protein is dominated by the properties of the aqueous solvent, and as such, a complete description of the phenomenon requires considering the properties of the plain alcohol/water mixtures and their evolution as alcohol concentration increases.

It has to be noted that in the same concentration range at which a maximum in ΔH, ΔS, and η and a change in compensation pattern are observed an anomalous behavior in several properties of water/ethanol and/or water/*tert*-butyl alcohol mixtures themselves, such as sound velocity, sound absorption, compressibility, and molar volume, is also observed. Our recent studies[14,15] on properties of water/alcohol mixtures show that this anomalous behavior can be associated with some kind of "hydrophobic clustering" of alcohol molecules in the water rich region of composition, beyond a threshold value of alcohol concentration. A plot of ethanol and *tert*-butyl alcohol apparent molar compressibilities, Φ_k, as a function of x_2 at 25 °C is reported in Figure 6. This quantity is nearly constant at low alcohol concentration, but it increases steeply as more alcohol is added to the solution. In the same Figure 6 the frequency $\bar{\nu}$ of the C−H stretching vibrations is also reported. As it can be seen, a shift of the frequency is observed as the alcohol mole fraction is increased. The dependence on alcohol concentration of $\bar{\nu}$ matches that of Φ_k. The two sets of data, adequately normalized, superimpose within experimental error.[14] The compressibility and IR data have been discussed in ref 14 by considering three mole fraction ranges defined by "signpost" mole fraction x_2^a and x_2^b. For water/ethanol mixtures $x_2^a \cong 0.06$ and $x_2^b \cong 0.29$, while for water/*tert*-butyl alcohol mixtures $x_2^a \cong 0.025$ and $x_2^b \cong 0.13$ at 25 °C. The data suggest that in the $0-x_2^a$ range, where Φ_k and $\bar{\nu}$ do not change with x_2, the alcohol molecules are essentially dispersed and surrounded by "water cages" of fairly regular and longer-lived H bonds. In the $x_2^a-x_2^b$ region a transition attributed to a progressive clustering of alcohol molecules with strong modification in the solvation of hydrophobic groups is observed.

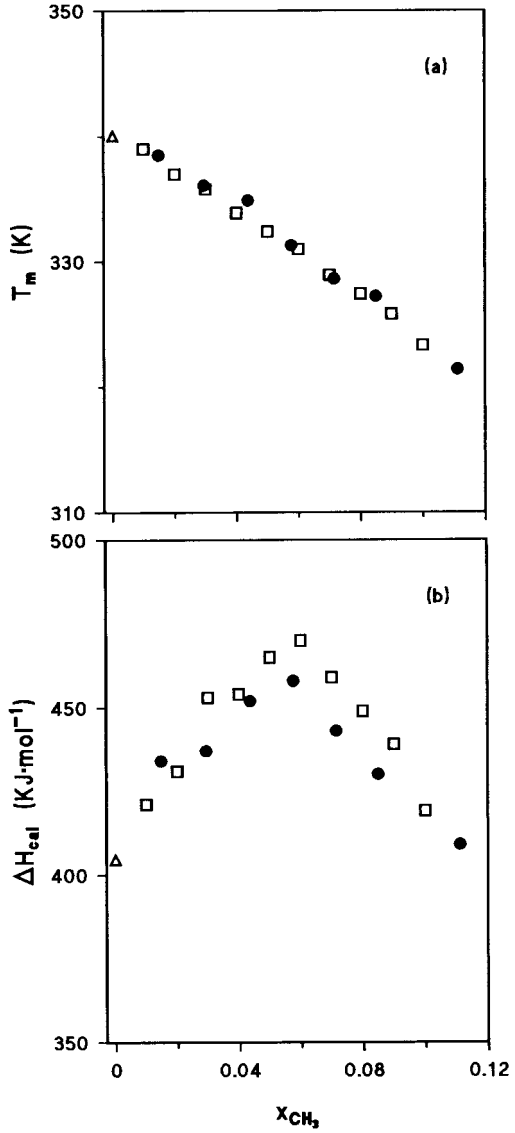

Figure 4. Melting temperature T_m (a) and calorimetric enthalpy ΔH_{cal} (b) of lysozyme in water/alcohol mixtures as a function of CH_3 group mole fractions in water/alcohol mixtures: (△) pure water, (□) ethanol, (●) *tert*-butyl alcohol.

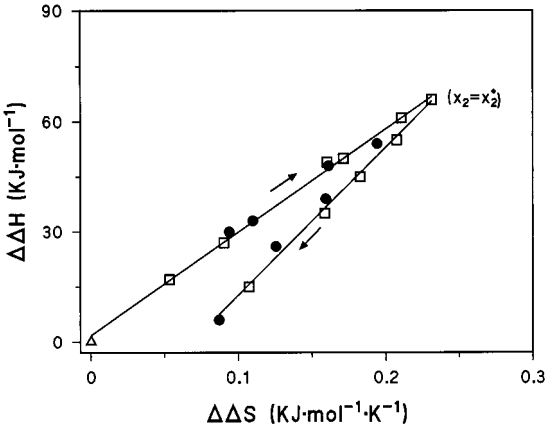

Figure 5. Changes in the enthalpy of denaturation ($\Delta\Delta H$) of lysozyme in water/alcohol mixtures relative to those in pure water solutions vs the corresponding entropy changes ($\Delta\Delta S$). The continuous lines are linear fits. Arrows point in the direction of x_2 increasing. (△) pure water, (□) ethanol, (●) *tert*-butyl alcohol.

Finally, in the x_2^b-1 range, effects due to hydrophobic hydration become negligible; water presumably loses its hydrogen

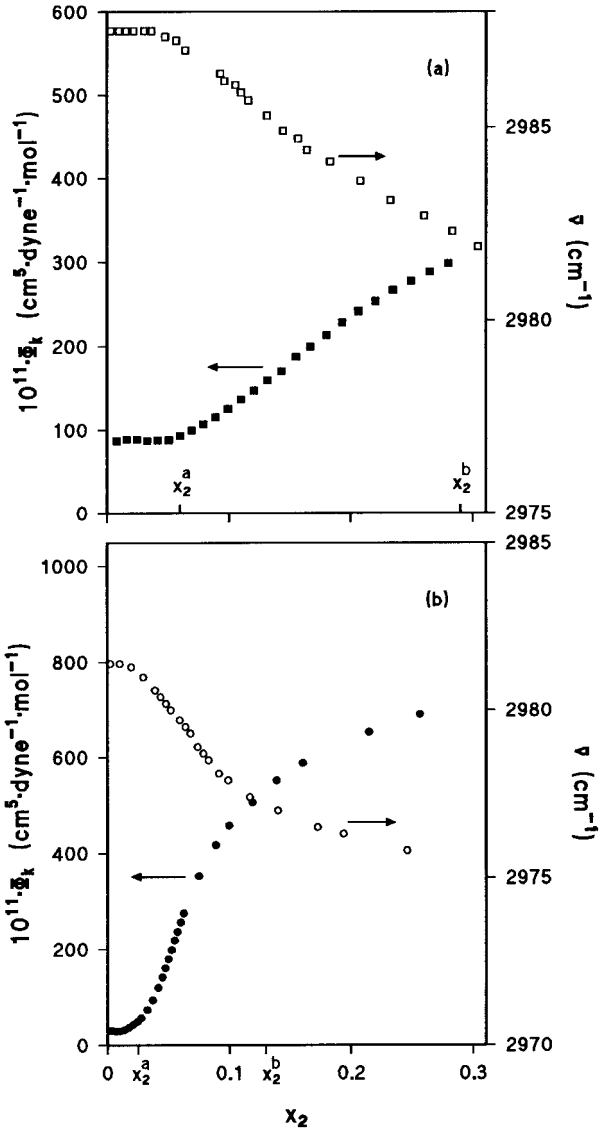

Figure 6. Apparent alcohol molar compressibility Φ_k (left side) and frequency $\bar{\nu}$ of the C−H stretching vibrations (right side) in water/alcohol mixtures as a function of alcohol mole fraction x_2: (a) ethanol, (b) *tert*-butyl alcohol.

bond network completely, and it mixes into the solution as a single molecules.

An approximate evaluation of the number S of water molecules located in the first hydration layer of an alcohol molecule shows that $x_2^a \cong 1/(S+1)$; that is, the self-association of alcohol molecules starts where all the water is involved in hydration structures. It has been suggested that an alcohol may affect the organization of neighboring water molecules, producing a configuration like clathrate, which has its maximum stabilization at the x_2^a composition. A correlation between the effects of ethanol and *tert*-butyl alcohol on the thermal transition of lysozyme and changes in the properties of solvent is evident from these data. The observed pattern suggests that at an alcohol concentration that promotes the maximum orders in the solvent the ΔH goes through a maximum. Therefore these results clearly indicate that the conformation stability of protein in water/alcohol mixtures is strictly dependent on the properties and anomalous behavior of the solvent system. In fact, these results on the nature of alcohol/water mixtures can be employed to propose a rather more quantitative explanation of the observed effects of ethanol and *tert*-butyl alcohol on the thermal transition of lysozyme.

(c) The Hydrophobic Effect. It is usually accepted that the hydrophobic processes are driven by positive entropy changes resulting from the release of structured water when nonpolar groups interact with one another. This traditionally held view of hydrophobic processes seems incorrect. As first observed by Shinoda,[16] the more ordered hydration structure formation around the solute molecules is, indeed, accompanied by a large decrease in entropy; this is, however, more than compensated by an ever greater enthalpic effect. Thus, the net consequence of the effect of hydrophobic hydration is to enhance the solubility of nonpolar species and to disfavor their aggregation. This point of view well agrees with our description of self-association behavior of monohydric alcohols in water. In this case it has been suggested that the formation of hydration structures or "cages" around hydrophobic groups hinders alcohol−alcohol association at low cosolvent concentration.[15]

In order to give a more quantitative description, let us decompose the volume of a solution containing n_1 moles of water and n_2 moles of alcohol into two phases: the free water region and hydration region encompassing all water molecules taking part statistically in alcohol-promoted cages. Under such an assumption one obtains

$$n_1 = n_1^{\text{free}} + n_1^{\text{hydr}} = n_1^{\text{free}} + n_2 S \tag{1}$$

where

$$S = n_1^{\text{hydr}}/n_2 \tag{2}$$

is the number of water molecules in the hydration region per mole of alcohol (hydration number). On adding dn_2 moles of alcohol to the solution,

$$dn_1^{\text{hydr}} = S\, dn_2 + n_2\, dS \tag{?}$$

One would consider the possibility that a water molecule may belong to different cages at the same time. The dependence of this overlap mechanism and, therefore, of S on alcohol concentration is expected to be very complicated, but in any case $dS < 0$. There appears to be a critical concentration n_2^* such as

$$\left(\frac{dS}{dn_2}\right)_{n_2^*} = -\frac{S}{n_2^*} \tag{4}$$

at which $dn_1^{\text{hydr}} = 0$.

As $n_2 > n_2^*$, the hydrophobic hydration no longer favors the solubility of nonpolar species, and a progressive clustering of alcohol molecules is observed.

It is likely that in the water−protein-alcohol system the existence of a critical concentration of hydrophobic groups will be reflected in the conformational behavior of the protein. The unfolding process in general involves the removal of nonpolar side-chain peptide groups from the interior of the native conformation and their exposure to the aqueous environment in the unfolded conformation. This can be described, to a first approximation, as the introduction in the water/alcohol solvent of an equivalent number dn_2 of $-CH_3$ groups with a concomitant transfer of a number dn_1^{hydr} of water molecules from the free region to the hydration region. Exposure of hydrophobic groups during the unfolding process can be, therefore, associated with the enthalpic change,

$$\Delta H^{\text{hydr}} = \Delta H_1^{\text{hydr}}\, dn_1^{\text{hydr}} \tag{5}$$

and the corresponding entropic change

$$\Delta S^{\text{hydr}} = \Delta S_1^{\text{hydr}}\, dn_1^{\text{hydr}} \tag{6}$$

where ΔH_1^{hydr} and ΔS_1^{hydr} refer to enthalpic and entropic changes due to the transfer of 1 mol of water from the free phase region to the hydration region. ΔH^{hydr} and ΔS^{hydr} are negative quantities, and their absolute values decrease as alcohol concentration increases and go through minima at the critical cosolvent concentration n_2^* where the change in n_1^{hydr} induced in the water by the addition of new hydrophobic groups is zero. Consistently with this description one would expect that for $n_2 < n_2^*$ only water molecules will be in the solvation shell of hydrophobic groups of protein, while for $n_2 > n_2^*$ both alcohol and water molecules will participate in the solvation process.

The overall enthalpy and entropy of protein unfolding change with x_2 in a way consistent with the change in ΔH^{hydr} and ΔS^{hydr} as expected from previous description. This seems to indicate that addition of alcohol essentially affects the hydrophobic hydration. Unfolding of lysozyme leads however to a change in both the exposed nonpolar and polar surface and a complete description could take into account changes in both types of hydration. However, the description can be always reduced to a competitive water reorganization by polar and hydrophobic groups, and the basic conclusions of the previous analysis remain valid. In any case a change in the nature of solvent component interactions beyond a critical concentration of hydrophobic groups is expected.

Referring to eqs 5 and 6, note that ΔH_1^{hydr} and ΔS_1^{hydr} need not depend on x_2, and so the ratio $\Delta S^{\text{hydr}}/\Delta H^{\text{hydr}} = \Delta S_1^{\text{hydr}}/\Delta H_1^{\text{hydr}} =$ constant. Thus, the rearrangement of water molecules between "free" and "hydration" region during the unfolding process appears responsible for compensation behavior. Accordingly with this description, Lumry and Rajender[13] suggested that the states predominantly involved in a two-state protein denaturation are in fact two states of water rather than two states of the protein.

Note that the above description suggests, consistently with Shinoda's point of view,[16] that hydrophobic interactions at low concentration of hydrophobic groups ($n_2 < n_2^*$) and low temperatures ($T < T_c$) are repulsive and give a destabilizing contribution to the native structure of the protein. Therefore, the attenuation of these interactions, due to alcohol addition or increase of temperature, should favor clustering of hydrophobic groups and more compact structures. In fact, it is well-known that, whereas the general effect of alcohols is one of destabilizing the native state, low concentration at low temperatures promotes a more ordered or more tightly folded conformation.[1,12] Inspection of the literature shows that the maximum stabilizing capability arises over the critical x_2^* concentration. At the same value of x_2^* a minimum is observed in the critical micelle concentration of several surfactant molecules.[2] A close similarity between the effects of alcohols on protein and micellar structure appears from these data. Effects of changes in temperature on these two systems are also similar. A temperature of maximum stability for protein native structure[17] and a temperature of minimum for the critical micelle concentration of surfactants[18] are widely reported in literature. These effects could arise from a destabilizing contribution of hydrophobic effects at low temperatures and its attenuation with alcohol addition or increase in temperature.

It is to be noted that, in apparent contrast with the stabilizing effect of low alcohol concentration on the helical conformation of protein, the melting temperature of the protein shows a linear depression as a function of alcohol content (Figure 3a). It must be noted, in this respect, that the behavior of $T_m = \Delta H/\Delta S$ critically depends on the $\Delta H - \Delta S$ balance. The observed

decrease in T_m indicates that relatively large entropy changes with respect to enthalpy changes occur on melting transition.

Conclusions

Our results show that protein conformational stability is closely linked with the properties of the solvent system and its anomalous behavior. The data suggest that addition of short chain alcohols affects the thermal transition of protein, modifying the extent of enthalpy and entropy contribution associated with structural reorganization of water in the unfolding process.

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