Specific Solvent Effects on the Thermal Denaturation of Ribonuclease. Effect of Dimethyl Sulfoxide and *p*-Dioxane on Thermodynamics of Denaturation†

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ABSTRACT: Differential scanning calorimetry has been used to study the thermal denaturation of ribonuclease in aqueous dimethyl sulfoxide and aqueous *p*-dioxane. A two-state reversible denaturation occurs in aqueous dimethyl sulfoxide. The denaturation is irreversible in aqueous *p*-dioxane. The transition temperature decreases in both organic solvents, with *p*-dioxane producing a considerably greater effect. The enthalpy of the denaturation initially increases with increasing dimethyl sulfoxide concentration and then decreases at high concentrations. Similar behavior is observed in the entropy in the dimethyl sulfoxide solutions. The enthalpy of the denaturation decreases markedly with low *p*-dioxane concentrations. Changes in pH due to the presence of organic solvent cannot account for the changes in enthalpy of denaturation. Addition of organic solvent tends to increase the pH of the ribonuclease solution. Higher pH in aqueous solutions gives a higher transition temperature, whereas addition of organic solvent results in the opposite behavior. The relative changes in the two solvent systems suggest that specific solvent effects occur and that destabilization of the native state relative to the denatured state is greater with *p*-dioxane than with dimethyl sulfoxide.

Differential scanning calorimetry has proven a useful tool for providing information on the mechanism of protein denaturation. Lumry et al. (1966) and Tanford (1968) determined the cooperativity of the transition in thermal denaturation from a comparison of the calorimetric heat and the van't Hoff transition enthalpy. The thermogram can be used to calculate the van't Hoff enthalpy (Delben et al., 1969; Reilly & Karasz, 1970) or an effective enthalpy (Privalov et al., 1971, 1973) for comparison to the calorimetric enthalpy to determine the cooperativity of the denaturation. The thermodynamic functions at any temperature can be estimated from the thermograms by use of the Gibbs equation. Privalov & Khechinashvili (1974) and Biltonin & Freire (1978) have reviewed the application of differential scanning calorimetry to protein unfolding.

The thermal denaturation of ribonuclease has been extensively studied. The reversible nature of the denaturation has been well established (Brandts & Hunt, 1967; Delben et al., 1969; Tsong et al., 1970; Privalov et al., 1971, 1973; Privalov & Khechinashvili, 1974). Such extensive study provides a good basis for extension into determination of specific solvent effects. Studies of specific solvent effects may contribute to our understanding of the interactions and forces which stabilize the tertiary structure of biological macromolecules. Solvent perturbations have been used extensively as probes of the solution conformation of proteins (Tanford, 1968).

Dimethyl sulfoxide (Me_2SO) and *p*-dioxane have been chosen as the solvent systems in this differential scanning colorimetry study since perturbations of the initial state of ribonuclease have been studied in these solvents (Herskovits & Lashowski, 1968; Steinschneider & Druck, 1972; Jacobson & Krueger, 1975; Bigelow, 1964). Several calorimetric studies of solvent perturbation (by urea, guanidine hydrochloride, hexamethylenetetramine, and ethanol) are also available for comparison of solvent effects (Brandts & Hunt, 1967; Delben et al., 1969).

Materials and Methods

Protease-free bovine pancreas ribonuclease A (Type XII-A) was purchased from Sigma Chemical Co. The protein molecular weight was taken to be 13 700. Ammonia-free crystalline glycine was also purchased from Sigma. Spectral grade dimethyl sulfoxide (Me_2SO) and *p*-dioxane were supplied by Baker. Double deionized water was used throughout, and ultrapure HCl was used for pH adjustment.

Stock solutions of ribonuclease were 100 mg/mL in 0.04 M glycine buffer, pH 4.1, for the Me_2SO experiments and 50 mg/mL in 0.04 M glycine buffer, pH 4.1, for the *p*-dioxane experiments. Organic solvent was added to these stock solutions, with stirring and on ice, to minimize the effects of the heat of mixing of the water and the organic solvent.

Thermal measurements were made on freshly prepared samples with a Perkin-Elmer differential scanning calorimeter (Model DSC-2). The calorimeter was calibrated with indium and benzoic acid. Samples (70 μL) were pipetted into 75-μL stainless steel pans with a Hamilton syringe. For each protein/solvent system, at least three samples were run against a solvent blank and the reversibility was checked by reheating one of these samples. A solvent blank was run before and after each set of experiments. A heating rate of 2.5 °C/min was typically used, although some runs were performed at 5 °C/min to check the effect of the heating rate.

Results

Figure 1 shows a typical scan for ribonuclease at the highest concentration used in these experiments (100 mg/mL) and indicates how the thermodynamic parameters were calculated. There was no significant difference in the enthalpy of denaturation (ΔH_{cal}) if a linear base line was used or if the heat capacity difference between native and denatured forms (ΔC_p^d) was considered. This agreement is probably due to the small values of ΔC_p^d and the symmetrical nature of the peak. The temperature at the midpoint of the transition (T_d) was first estimated from the peak symmetry. Final values were obtained by assuming that the area under the thermogram was proportional to the amount denatured, and T_d occurs when the protein is half-denatured. The calculated T_d was lower than

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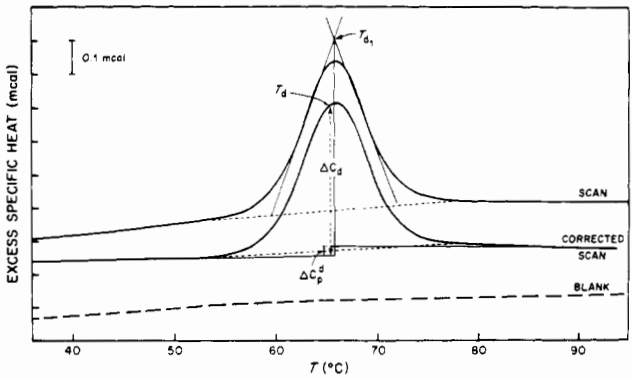

FIGURE 1: Typical thermogram at 5 °C/min heating rate for ribonuclease at pH 4.1 in 0.04 M glycine buffer. The sample contained 74.8 mg of a 100 mg/mL protein solution. The methods of calculation for ΔH_{cal}, T_d, and ΔC_p^d are illustrated.

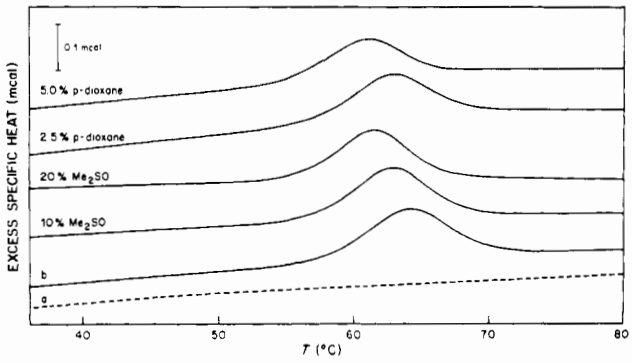

FIGURE 2: Set of thermograms at 2.5 °C/min heating rate. Only the blank for the 0.04 M glycine buffer (pH 4.1) is shown (scan a). Scan b is of a 74.6-mg sample of a 50 mg/mL protein sample. The concentrations of the organic solvent added are as indicated.

the estimated T_d by less than 1 °C. Privalov & Khechinashvili (1974) reported that this calculation lowered T_d by approximately 0.7 °C.

A set of scans at a lower protein concentration (50 mg/mL) is shown in Figure 2. Only the blank for the glycine buffer is shown, though blanks were run for all solvent compositions. The glycine buffer blank is typical of all the blanks. Reproducibility is high since high instrumental sensitivity is not required at this protein concentration. The figure is given to illustrate the typical scans with *p*-dioxane. The majority of the runs with Me_2SO were at higher protein concentrations.

Repetitive scans with the differential scanning calorimeter have shown that the denaturation of ribonuclease in aqueous solutions and with Me_2SO is reversible. The denaturation with *p*-dioxane is only partially reversible at low concentrations of *p*-dioxane (80% reversible at 0.005 mol fraction of *p*-dioxane) and totally irreversible at higher *p*-dioxane concentrations (0.023 mol fraction of *p*-dioxane). Concentrations of *p*-dioxane greater than 0.123 mol fraction caused precipitation of the protein.

The enthalpy of denaturation of ribonuclease (ΔH_{cal}) in glycine buffer at pH 4.1 is 91.4 ± 0.2 kcal mol^{-1}. There was no significant change in this value when the heating rate was increased from 2.5 to 5.0 °C/min. This value for ΔH_{cal} is similar to previous values obtained by Delben & Crescenzi (1969) (99 ± 8 kcal mol^{-1}) and by Privalov et al. (1973) (108 kcal mol^{-1}) at similar pH. Tsong et al. (1970) reported a considerably higher ΔH_{cal} (126 kcal mol^{-1} at pH 4.0). However, these authors also reported that this value decreased to 96 kcal mol^{-1} when a temperature-dependent heat capacity was used for the native state. A number of factors such as

Table I: Comparison of Effect of Change in pH to Effect of Organic Solvent

mol fraction	organic solvent	pH[a] measd	ΔH_{cal}[b] measd (kcal mol^{-1})	ΔH_{cal}[c] calcd at pH 4.1 (kcal mol^{-1})	T_d[b] measd (°C)	T_d[c] calcd at pH 4.1 (°C)
		4.1	91.4		63.9	
		5.0	94.7	91.4	65.1	63.6
0.060	Me_2SO	4.7	100.2	98.0	59.1	58.1
0.202	Me_2SO	5.1	104.3	100.6	60.1	59.4
0.050	*p*-dioxane	4.3	57.8	57.1	51.7	51.4
0.123	*p*-dioxane	4.9	59.7	56.7	43.8	42.4

[a] The pH meter was calibrated with standard HCl in the particular aqueous-organic solvent mixtures. [b] All measurements were at a 2.5 °C/min heating rate. [c] Values obtained in aqueous solution were used to obtain a correction to pH 4.1. A linear change with pH was assumed.

protein concentration (aggregation), ionic strength, specific buffer effects, and purity of the protein sample could contribute to the differences in ΔH_{cal} values reported from the different laboratories.

Aggregation is expected to occur at high protein concentration. However, variation in ΔH_{cal} due to differences in aggregation is expected to be of minor importance. Tsong et al. (1970) found no difference in ΔH_{cal} in the protein concentration range 1–27 mg/mL. We report no change between 50–100 mg/mL. In addition, at the higher protein concentrations used in this study, higher not lower ΔH_{cal} values would be expected if aggregation significantly contributed to ΔH_{cal}. The most likely reason for differences in measurement of ΔH_{cal} among various laboratories is ionic strength, buffer, and protein sample variation. Ginsberg & Carroll (1965) have shown considerable stabilization of ribonuclease due to phosphate (24%) or sulfate addition (50%). At pH 2.1 Tsong et al. (1970) reported ΔH_{cal} values 45% (21 kcal mol^{-1}) greater than those of Ginsberg & Carroll (1965). Tsong et al. (1970) attributed this difference to possible contamination of their sample by phosphate. Small differences in contamination could easily account for the variations in ΔH_{cal} reported in the various laboratories.

The temperature at the midpoint of the transition (T_d) is a function of heating rate. T_d values of 63.9 ± 0.2 and 65.5 ± 0.2 °C were obtained at heating rates of 2.5 and 5.0 °C/min, respectively. A linear extrapolation gives a T_d value of 62.2 °C at a zero heating rate. In aqueous solution the enthalpy increased by 3.3 kcal/mol when the pH was increased from 4.1 to 5.0 (Table I). A 1.5 °C increase in T_d was also observed. Privalov et al. (1973) reported an increase of 4 kcal/mol in ΔH_{cal} and a 6 °C increase in T_d when the pH was changed from 4.0 to 5.4. The agreement in enthalpy changes with pH is good. The differences in T_d are probably due to differential heating rates. Variation in the protein samples (contamination) could also contribute to differences in T_d (Ginsberg & Carroll, 1965) as well as protein concentration differences (Tsong et al., 1970).

The effect of organic solvent on transition temperature is shown in Figure 3. T_d is lowered as the concentration of organic solvent is raised. At equivalent mole fractions, *p*-dioxane produces a considerably greater decrease in T_d than Me_2SO does. The change in T_d is relatively small with Me_2SO. Enthalpy values are also shown in Figure 3. With Me_2SO an increase in ΔH_{cal} is observed. A maximum value was obtained at 0.15 mole fraction of Me_2SO. A large decrease in ΔH_{cal} is observed with *p*-dioxane at low mole fractions of organic solvent.

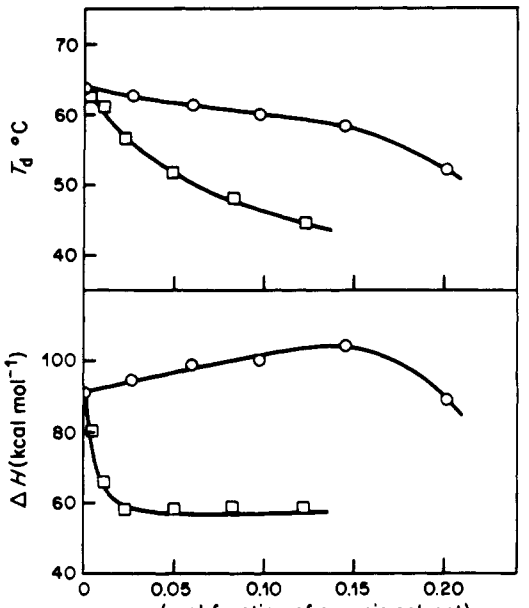

FIGURE 3: Calorimetric enthalpy and melting temperature of ribonuclease as a function of organic solvent composition: O, aqueous dimethyl sulfoxide; □, aqueous *p*-dioxane.

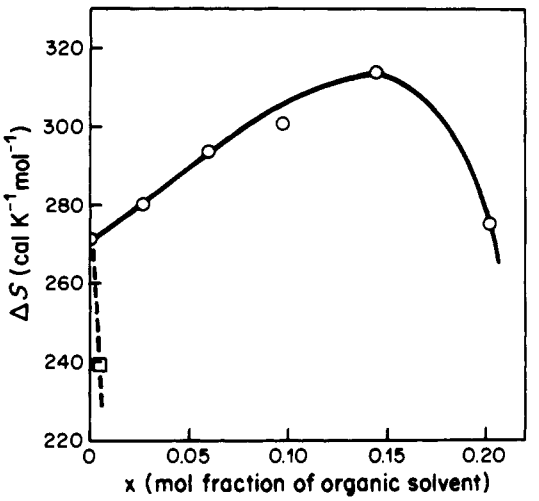

FIGURE 4: Entropy of the denaturation calculated at the transition temperature as a function of mole fraction of dimethyl sulfoxide (solid line). The entropy change has been estimated at a low mole fraction of *p*-dioxane where the transition is 80% reversible (□, dashed line) and is shown only to indicate types of changes.

The ratio of the observed to the effective enthalpy ($\Delta H_{cal}/\Delta H_{eff}$) was calculated as suggested by Privalov et al. (1973). In agreement with Privalov's data, this ratio for ribonuclease in glycine buffer at pH 4.1 is 1.0. There was no change in this ratio with Me_2SO. A ratio of 1 indicates that a two-state reversible transition occurs with Me_2SO as well as in aqueous solution.

The entropy of the thermal denaturation (ΔS) was calculated for the reversible denaturations by assuming that the free energy change at the midpoint of the transition is zero. The protein is assumed to be half-denatured at T_d and $\Delta H_{cal} = T_d\Delta S$. This is equivalent to assuming that an equilibrium exists when the protein is half-denatured. This is a common assumption and a usual method of obtaining entropy values [reviewed by Biltonin & Freire (1978)]. ΔS values for ribonuclease with Me_2SO are shown in Figure 4. ΔS also passes through a maximum at 0.15 mole fraction of Me_2SO. The equilibrium assumption is not valid for the irreversible denaturation of ribonuclease with *p*-dioxane. An estimated value of ΔS for 0.005 mole fraction of *p*-dioxane (80% reversibility) is shown in Figure 4 only to suggest that the trend of the change in ΔS with organic solvent is probably dependent on the specific organic solvent.

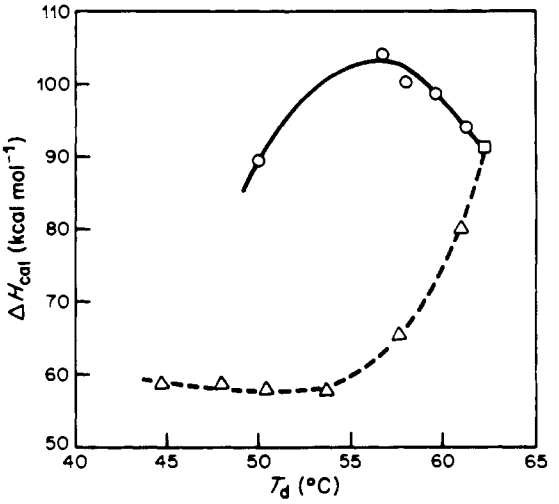

FIGURE 5: Variation in the calorimetric measured enthalpy with transition temperature: □, aqueous solution; O, aqueous dimethyl sulfoxide; Δ, aqueous *p*-dioxane.

The change in ΔH_{cal} with T_d is shown in Figure 5. The change in ΔH_{cal} shows a maximum with Me_2SO and a minimum with *p*-dioxane. A linear correlation is not obtained with either solvent.

Addition of an organic solvent to a protein solution usually causes pH changes due to p*K* changes in both the protein and the buffer. Since Privalov et al. (1973) have shown that both ΔH_{cal} and T_d of ribonuclease are larger at higher pH, the pH changes caused by the organic solvent were measured. Standard HCl solutions containing the organic solvents were used to calibrate the pH meter. Calorimetric measurements were also made as a function of pH. These results are shown in Table I. Only a small part of the changes in enthalpy in the aqueous–organic solvent can be attributed to the pH change caused by the organic solvent. Organic solvents decrease the transition temperature while the pH effect is in the opposite direction. Values of ΔH_{cal} and T_d corrected to pH 4.1 are also shown in Table I. In this calculation it was assumed that the portion of the change due to pH could be obtained from a linear interpolation of values in aqueous solution of pH 4.1 and 5.0. Me_2SO causes an increase in ΔH_{cal} and a small decrease in T_d. *p*-Dioxane causes large decreases in both ΔH_{cal} and T_d.

In theory it is possible to obtain the difference in heat capacity between the native and the denatured form (ΔC_p^d) from the thermograms. The raw data are in the form of specific heat as a function of temperature. In practice these measurements are very difficult to make with the commercially available scanning calorimeters. The measurements must be taken far enough away from the transition temperature to obtain the heat capacity as a function of temperature. Slow denaturation in protein solutions even at temperatures far from T_d is well-known. In addition, the solvent base here must be subtracted from the thermogram. In practice a linear extrapolation of C_p (after subtraction of solvent base line) to T_d is usually performed (Privalov et al., 1973; Velicelebi & Sturtevant, 1979). Large errors are inherent in this calculation. The values of ΔC_p^d as a function of the mole fraction of Me_2SO are shown in Figure 6. The line obtained from a least-squares fitting of the data is also shown. The average

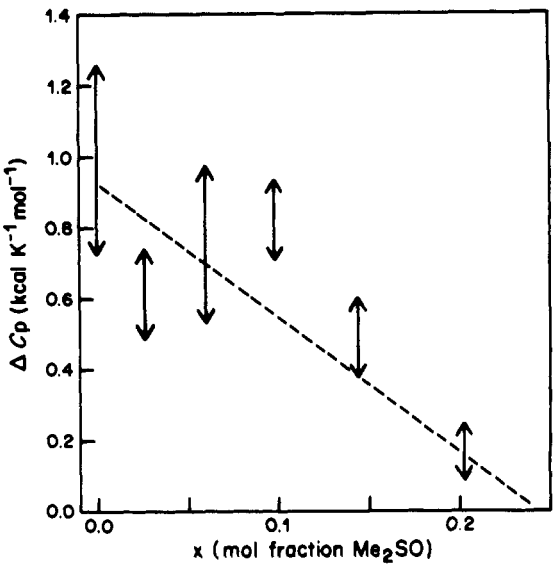

FIGURE 6: Variation in heat capacity with mole fraction of dimethyl sulfoxide. The dashed line is the least-squares fitting of data.

value for ΔC_p^d in buffer is 1.00 ± 0.26 kcal K^{-1} (mol of protein)$^{-1}$, and the value obtained from the least-squares fit is 0.93 kcal K^{-1} (mol of protein)$^{-1}$. Since ΔC_p^d has been reported to be independent of pH (Brandts & Hunt, 1967; Tsong et al., 1970; Privalov & Khechinashvili, 1974), comparisons can be made to values obtained by a number of workers. A range of values has been reported: Beck et al. (1965), 0.66; Brandts & Hunt (1967), 2.01; Reeg (1969), 1.5; Tsong et al. (1970), 2.0; Privalov et al. (1973), 1.04; Privalov & Khechinashvili (1974), 1.2 kcal K^{-1} (mol of protein)$^{-1}$. This wide range of values can be attributed to the inherent difficulties of the measurement as well as to possible differences in protein purity. Our value most closely corresponds to the value obtained by Privalov et al. (1973).

If it is assumed the ΔC_p^d is independent of temperature, values of $\Delta H°$, $\Delta G°$, and $\Delta S°$ at any temperature can be obtained from ΔC_p^d and T_d (Gibbs equation). If the least-squares values for ΔC_p^d are used, broad maximums are obtained in plots of $\Delta H°$, $\Delta G°$, and $\Delta S°$ as a function of the mole fraction of Me_2SO (Figure 7). $\Delta G°$ decreases with increasing temperature. These trends indicate that the organic solvent increased stabilization of the denatured state relative to the native state.

Discussion

The maximum in ΔH_{cal} and ΔS for the denaturation of ribonuclease with Me_2SO is similar to behavior reported with ethanol (Brandts & Hunt, 1967) and to the behavior of lysozyme with ethanol (Velicelebi & Sturtevant, 1979). The effect of the organic solvent on the structure of water should be expected to be a major factor in determining the effect of organic solvent on the state of the protein. A number of properties of water–Me_2SO, water–alcohol, and water–*p*-dioxane mixtures show maxima or minima as a function of solvent composition. This behavior reflects the shift in the tendency of water molecules from a highly structured self-associated state to a more unstable or free state (Lumry & Rajender, 1970). This occurs with water–Me_2SO at ~0.3 mole fraction (Macdonald & Hyne, 1970), at 0.15 mole fraction with water–ethanol (Brandts & Hunt, 1967), and between 0.1 and 0.2 mole fraction with water–*p*-dioxane (Malcolm & Rowlinson, 1957; Brandts & Hunt, 1967). The maximum in ΔH_{cal} for the denaturation of the proteins in

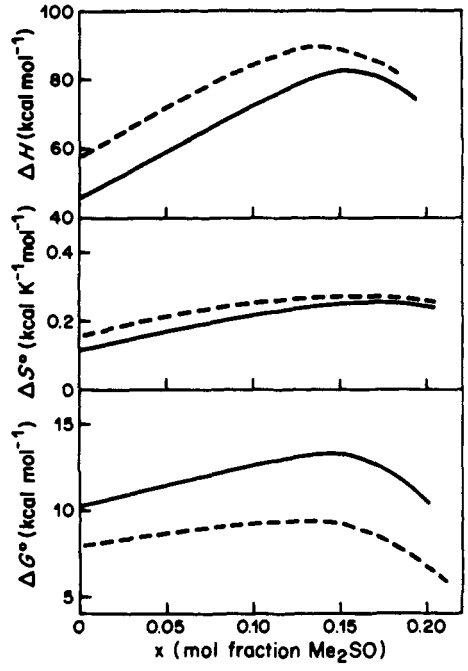

FIGURE 7: Free energy, entropy, and enthalpy calculated at 25 °C (dashed line) and at 10 °C (solid line) from heat capacity changes between native and denatured forms and the measured transition temperatures and enthalpies.

Me_2SO and in ethanol appears to correlate with the behavior of the water–organic solvent system. However, if the major effect of the organic solvent is on the properties of water, a maximum in ΔH_{cal} for the thermal denaturation of ribonuclease with *p*-dioxane would be predicted. In this solvent system a decrease in ΔH_{cal} of ribonuclease is observed. Hence, the relative changes cannot be correlated with the behavior of the solvent in water. The difference in behavior must be due to specific solvent effects. Strong hydrogen bonds between Me_2SO and the protein could contribute to the stability of the protein in both the native and denatured state.

The interpretation of the thermodynamic parameters found for the denaturation is complicated since both initial and final states will be altered by the organic solvents. Optical rotation studies by Aune et al. (1967) have shown that when proteins such as ribonuclease are thermally denatured, they contain ordered structures which can be disrupted by addition of guanidine hydrochloride. Proton magnetic resonance studies (Roberts & Benz, 1973) have also shown similar differences in the final thermally unfolded state of ribonuclease. The final thermally denatured state of the proteins in the presence of solvents such as Me_2SO and *p*-dioxane would be expected to be different but may be closer to the final state observed in the guanidine hydrochloride denaturation rather than to the thermally denatured state found in aqueous solution.

Changes in the initial state of ribonuclease have been shown by UV difference spectra and optical activity with Me_2SO (Herskovits & Laskowski, 1968) and dioxane (Bigelow, 1964). IR absorption spectroscopy (Jacobson & Krueger, 1975) shows conformational changes in these solvents. A decrease in the total amount of secondary structure is observed in the *p*-dioxane mixtures, while the total amount of secondary structure in the Me_2SO mixtures remains high. Steinschneider & Druck (1972) suggested that there were no gross conformational changes with Me_2SO from intrinsic viscosity and hyperchromicity measurements. It appears likely that *p*-dioxane has a greater effect on the initial state of ribonuclease than Me_2SO does. The changes in T_d for ribonuclease with Me_2SO and

p-dioxane are in agreement with this conclusion.

The lower values of enthalpy and transition temperature with *p*-dioxane are very similar to values reported by Brandts & Hunt (1967), Delben et al. (1969), and Cresenzi & Delben (1971) for the denaturation of ribonuclease at low concentrations of urea. With urea ΔH_{cal} appears to be a linear function of T_d (Delben et al., 1969), while this linearity is not observed with *p*-dioxane.

In summary, specific solvent effects are observed in the thermal denaturation of ribonuclease with Me_2SO and with *p*-dioxane. The decrease in T_d can probably be related to destabilization of the native state relative to the denatured state. The increase in ΔH_{cal} with Me_2SO and the decrease in ΔH with *p*-dioxane cannot be explained by the effect of the solvent on the water and must be due to specific changes in both native and denatured states. Differing mechanisms of denaturation may occur in the two solvent systems.

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