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Differential Scanning Calorimetric Studies on the Thermal Denaturation of Ribonuclease A in Aqueous 2-Methyl-2,4-pentanediol

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The thermal denaturation of ribonuclease A in aqueous solutions of 2-methyl-2,4-pentanediol(MPD) was investigated by differential scanning calorimetry at pH 5.8, *i.e.*, conditions similar to those used to crystallize the protein, and also at pH 3. A two-state reversible denaturation occurred in aqueous MPD up to 50%(v/v) MPD. Under both conditions the denaturation temperature, T_d, decreased almost linearly with increasing MPD concentration; there was a slightly greater effect at pH 5.8. The calorimetric enthalpy, ΔH_d, first increased with increasing MPD concentration and then decreased slightly at MPD concentrations above 20—30%. This behavior is analogous to that observed for organic additives, such as ethanol and dimethyl sulfoxide, with denaturing agents. The plot of ΔH_d against ΔS_d, the denaturation entropy at T_d, gave a linear correlation between these parameters, indicating a close relationship between the MPD-induced unstabilization of the protein and the water structure around the protein molecule. The standard thermodynamic parameters for denaturation, $\Delta G°$, $\Delta H°$, and $\Delta S°$, were calculated from T_d and ΔH_d, assuming a constant heat capacity change. The unstabilizing effect of MPD on the thermal stability of the protein results from that the increase in $\Delta H°$, induced by MPD, is compensated by a large increase in $\Delta S°$, with a resulting decrease in $\Delta G°$; it is in accord with an effect on solvent ordering around the exposed nonpolar groups of the protein. It also indicates a temperature dependence of the MPD effect on the protein stability.

Differential scanning calorimetry(DSC)** is a useful tool for measuring the thermally induced conformational transition of biopolymers such as proteins and nucleic acids. Application of DSC to the study of protein denaturation has increased the available thermodynamic information on the unfolding process of protein; this has been recently reviewed by Biltonen and Freire[1] and Privalov.[2] The thermogram can be used to calculate the van't Hoff enthalpy[3] or an effective enthalpy[4] for comparison with the calorimetric enthalpy to determine the cooperativity and nature of the denaturation. Furthermore, the other thermodynamic parameters for denaturation, as well as the denaturation temperature and enthalpy, can be calculated without any ambiguous assumptions.[2,5] These studies may lead to an understanding of the nature and magnitude of the forces that stabilize the native conformation of proteins, which has been a major focus of protein research.

Organic solvents such as alcohols and sulfoxides alter the conformation of proteins when added to aqueous protein solutions as result of solvent effects on the local Gibbs energy of conformational stability of the amino acid residues exposed to the surface.[6,7] 2-Methyl-2,4-pentanediol(MPD) is well known as a crystallizing reagent for proteins. For the purpose of X-ray crystal-structure analysis, bovine pancreatic ribonuclease A(RNase A) was crystallized from a mixture of 55% MPD and 45%(v/v) water and the crystals equilibrated with 75% MPD were used.[8] The water–MPD solvent system has a partially hydrophobic character, since the MPD molecule contains six methylene groups, and one might expect it to unstabilize the native structure of a protein. Indeed, monohydric alcohols depress the denaturation temperature of RNase A, a reflection of their unstabilizing action on the native structure, and the unstabilizing effect becomes more effective with an increase in their hydrophobic character.[9] We have observed that ethylene glycol also lowers the denaturation temperature of RNase A at pH 5.[10] In addition, solubility measurements of amino acids in 55% MPD have shown a favorable Gibbs energy of transfer of most amino acid side chains to this medium.[11] This suggests that MPD has a denaturing rather than a crystallizing action.

On the other hand, Bello and Nowoswiat[12,13] have reported that RNase A crystals in 75% MPD possess enzymatic activity and that the same histidine residues are carboxymethylated in the crystal as in aqueous solution, suggesting that the structure of crystalline RNase A appears to be similar to that in aqueous solution. They have further shown from the solubility data of amino acids that the unfolding of RNase A in 55% MPD would have a positive Gibbs energy change at 25 °C.[11] The CD spectra of RNase A in water and 50% MPD are indistinguishable in the far-and near-UV regions.[11,14] Pittz and Timasheff[14] have reported that RNase A is preferentially hydrated in mixtures of water and MPD. According to Bull and Breese,[15] any substance which trends to maintain or increase the hydration of proteins stablize a protein. Indeed, sugars and polyhydric alcohols which induce the preferential hydration of proteins stabilize them against thermal denaturation.[16–19] Therefore, it is of interest to study the effect of MPD on the thermal stability of RNase A.

In this paper, the thermal denaturation of ribonuclease A in aqueous MPD solutions at MPD concentrations up to 50%(v/v) was investigated by DSC at pH 5.8, *i.e.*, conditions similar to those used to crystallize the protein, and at pH 3 and the results are reported.

** Abbreviations used: DSC,differential scanning calorimetry; MPD, 2-methyl-2,4-pentanediol; RNase A, ribonuclease A(bovine pancreatic, EC 3.1.4.22).

Experimental

Materials. Bovine pancreatic ribonuclease A (EC 3.1.4.22) was purchased from Sigma Chemical Co.(type I-A,five-times recrystallized). 2-Methyl-2,4-pentanediol was purified by the method of Bello and Nowoswiat.[12] Other chemicals were reagent grade products from Wako Pure Chemical Industries. The experiments were carried out in either 0.05 mol dm^{-3} acetate or glycine buffer adjusted respectively to pH 5.8 and 3 with 1 mol dm^{-3} HCl. Protein concentrations were determined spectrophotometrically on a Hitachi 323 automatic recording spectrophotometer. The extinction coefficient was assumed to be 7.1 at 277 nm for a 10 mg cm^{-3} RNase A solution.[20] The same value was used at all solvent compositions, since it has been shown that variation of solvent composition from water to 50% MPD does not significantly affect the position of the absorption maximum nor the extinction coefficient of this protein.[21]

DSC Measurements. The thermal denaturation of RNase A was measured on a Seiko Instruments & Electronics SSC-560U differential scanning calorimeter(conduction type) at a heating rate of 1 K min^{-1} and at protein concentrations of 5—10 mg cm^{-3}. The sample solutions were extensively dialyzed at 4 °C against the solvent which was used as reference material, and then hermetically sealed in a silver vessel (0.06 cm^3). The DSC apparatus was calibrated using gallium, benzophenone, palmitic acid, and naphthalene. The area between the transition peak and a base line fitted by inspection was measured with a planimeter and converted to the calorimetric enthalpy of denaturation, ΔH_d, using the calibration constant, 2.29 mJ $mV^{-1}s^{-1}$, and the molecular weight of 13700 for RNase A. The denaturation temperature, T_d, was estimated from the temperature at which the area of the transition peak was divided into halves by assuming that the area under the peak was proportional to the amount denatured. The difference between the obtained T_d and the temperature of maximal heat flow, T_p, did not exceed 0.5 K in every measurement because of the nearly symmetrical nature of the transition peak. Privalov and Khechinashivili[4] have reported that T_d was lower than T_p by approximately 0.7 K. For each solvent composition, these thermodynamic parameters were evaluated as the averaged value of five or more experiments. Temperatures have experiment errors of ±0.1 °C. The ΔH_d values have maximum expected errors of ±5% including errors in sample preparation, calibration constant, and reproduciblity.

Results and Discussion

Representative DSC scans for the thermal denaturation of RNase A in aqueous MPD solutions at pH 5.8 are shown in Fig. 1. The DSC curves shown correspond to denaturation of a protein solution of 8 mg cm^{-3} and MPD concentrations of 0, 20, and 50%(v/v). In every measurement, a reproducible endothermic peak was observed occurring over a temperature width of 19—22 K. The increase in MPD content leads to a shift of the transition peak towards lower temperature and variation of the peak area. The endothermal slope of the base line in the pre- and post-transition regions which was observed in the DSC curves obtained at MPD concentrations below 40% were typical features of globular proteins, indicating that the temperature coefficients of the heat capacities of the native and denatured states are positive. As can be seen from this figure, the DSC curve obtained at 50% MPD exhibited only a slightly exothermal slope

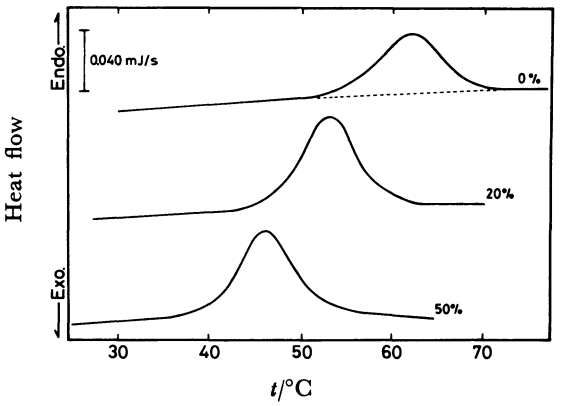

Fig. 1. DSC curves for the thermal denaturation of RNase A in aqueous MPD solutions at pH 5.8. Protein concentration is a 8 mg cm^{-3} and MPD concentrations are 0, 20, and 50% (v/v). The dotted line in the top figure shows the base line assumed in the transition region for the determination of the thermodynamic parameters of denaturation.

of the base line in the post-transition region; this was probably a reflection of partial aggregation of the denatured protein. A further addition of MPD brought about a remarkable increase in the exothermal slope of the base line and an extreme decrease in the solubility of the protein. At MPD contents more than 50%, therefore, the thermodynamic parameters for denaturation could not be estimated exactly.

The reversibility of the thermal denaturation of RNase A in MPD-water mixtures was confirmed by reheating the protein solution after rapidly cooling from the first scan, although the reversible nature of the denaturation of RNase A in aqueous solution has been well established.[4,22-25] The thermal denaturation was almost completely reversible, 98±5%, in aqueous MPD solutions at MPD concentrations up to 50%. The denaturation temperature was scarcely influenced by heating rates slower than 1 K min^{-1}, allowing equilibrium analysis of the data. The ΔH_d values obtained in aqueous buffer solutions were 370±9 kJ mol^{-1} at pH 3 and 428±17 kJ mol^{-1} at pH 5.8. These values are in very good agreement with those reported by Tsong *et al.*[24] and Privalov *et al.*[25] from calorimetric experiments at similar pH values. However, the T_d value in glycine buffer at pH 3, 53.5±0.1 °C, is considerably higher compared to the data of Tsong *et al.*[24] and Privalov *et al.*[25] The value obtained in acetate buffer at pH 5.8, 61.5±0.1 °C, is in good accord with those obtained by Tsong *et al.*,[24] Crescenzi and Delben[26] from DSC measurements, and Konishi and Scheraga[27] from spectrophotometric measurements at similar pH, but it is slightly lower than those reported by Privalov *et al.*[25] at pH 5.5 and Jacobson and Turner[28] from DSC measurements at pH 5. Although the reason for the differences in T_d is not clear, a number of factors such as protein concentration, ionic strength, specific buffer effect, purity of the protein sample (contamination), and heating rate could contribute to the differences. For example, Tsong *et al.*[24] have shown that the T_d of RNase A in aqueous buffer at pH 2.8 increases by 4.2 K when the protein con-

centration is increased from 0.1 to 2.7%(w/w).

The calorimetric transition profile permits assessment of the validity of the two-state theory of denaturation for this protein. If the transition does not involve the formation of thermodynamically stable intermediates, the shape of the transition profile is dictated by the van't Hoff equation. In this case, the effective enthalpy of denaturation, ΔH_{eff}, was evaluated by applying an approximated expression,[25]

$$\Delta H_{eff} = 4RT_d^2/\Delta T_{1/2}$$

where $\Delta T_{1/2}$ is the half-width of the transition peak. The ratio of calorimetric to effective enthalpies of denaturation, $\Delta H_d/\Delta H_{eff}$, showed near-unity value, 1.02±0.04, meaning that the thermal denaturation of RNase A in aqueous MPD solutions is very nearly a two-state process.

Figure 2 shows the dependence of the denaturation temperature on the MPD concentration at pH 3 and at pH 5.8. Under both conditions the T_d decreases monotonously with an increase in the MPD concentration. It is evident that MPD unstabilizes RNase A against thermal denaturation. This is consistant with the expectation based on the hydrophobic character of MPD and on the results of our investigation with ethylene glycol.[10] Therefore, the thermal unstabilization of RNase A by MPD appears to be responsible for the hydrophobic character of solvent induced by the addition of MPD. Needless to say, the T_d values at pH 5.8 which is the pH near the isoelectric point of this protein are higher than those at pH 3. The lowering of T_d by MPD is slightly larger at pH 5.8 than at pH 3, suggesting the temperature dependence of the thermally unstabilizing effect of MPD on the protein. The dependence of the calorimetric enthalpy of denaturation on the MPD concentration is shown in Fig. 3. The error bar shown in the figure is the standard deviation of the data. The denaturation enthalpy exhibits a maximum, that is, the ΔH_d first increases with increasing the concentration of MPD and then decreases slightly at MPD concentrations above approximately 30%(v/v), corresponding to about 2.3 mol dm^{-3}. A similar trend of ΔH_d has been observed for the thermal denaturation of RNase A with dimethylsulfoxide[28] and lysozyme with monohydric alcohols[3,29] and with sulfoxides.[30] These organic solvents strongly unstabilize these proteins against thermal denaturation.

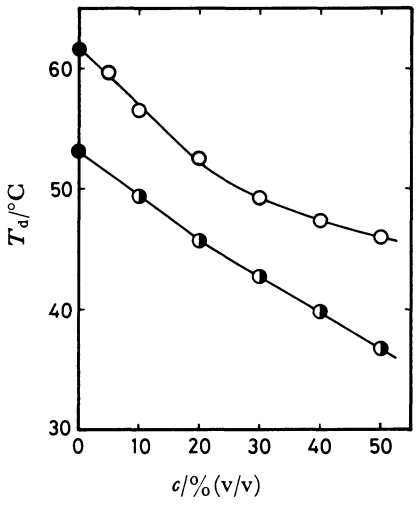

Fig. 2. Variation of the denaturation temperature, T_d, with the concentration of MPD at pH 5.8 (○) and at pH 3 (◑).
The symbol ● refers to the buffer solution without MPD.

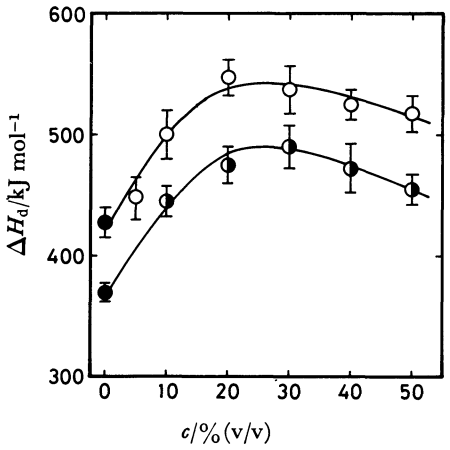

Fig. 3. Variation of the calorimetric enthalpy of denaturation, ΔH_d, with the concentration of MPD. The symbols used are identical with those in Fig. 2. The error bar shows the standard deviation of the data.

As is evident from Figs. 2 and 3, the dependence of ΔH_d on T_d is analogous to each other under both conditions; ΔH_d passes through a maximum with increasing temperature. The similarity of the temperature dependences of ΔH_d under both conditions, pH 5.8 and 3, suggests that the thermal unstabilization of RNase A induced by MPD is not directly dependent on the pH. Furthermore, the dependence of ΔH_d on T_d in the presence of MPD differs drastically from that described by Privalov[2] for variation of T_d induced by variation of the pH and addition of guanidine hydrochloride. He has shown that the denaturation enthalpy is a linear function of the denaturation temperature under all conditions. This means that the mechanism of the MPD-induced unstabilization is different, at least, from that induced by denaturants such as guanidine hydrochloride and urea.

In Fig. 4, the denaturation enthalpy is plotted against the denaturation entropy at the denaturation temperature, $\Delta S_d(=\Delta H_d/T_d)$. A linear correlation between these thermodynamic parameters suggests that the unstabilization by MPD on the thermal stability of RNase A is closely related to the water structure around the protein molecule.[31] The compensation temperature, which is defined as the slope of the graph, is about 300 K. This value is within the temperature range of 250 to 320 K observed for many processes.

The equilibrium constant of denaturation can also be obtained directly from the thermogram, since the area under the transition peak is proportional to the amount of the protein denatured. The equilibrium constant, K, at temperature T may be taken as $Q_T/(Q-Q_T)$, where Q is the total area of the transition peak and Q_T is the partial area up to temperature T. For

the present three-component system, the dependence of the equilibrium constant of denaturation on the solvent variables is ascribed to the difference in the preferential binding of solvent compositions between the native and denatured states.[32,33] The logarithm of K obtained at pH 5.8 thus is plotted against the logarithm of the MPD concentration in Fig. 5. The molarity unit was used instead of activity on the assumption that the activity coefficient is unity, since there is no activity datum for MPD in water. The slope of the lines is a positive, 3.09±0.07, and nearly temperature independent within the experiment error. This indicates that the binding of MPD to the protein increases upon denaturation. In fact, the increase in the protein surface on denaturation is generated by exposing the nonpolar residues buried in the interior of the native protein and by breaking peptide–peptide hydrogen bonds in the native state and replacing them by peptide–water hydrogen bonds. The resultant increase in the surface area of the denatured protein probably contributes to an increase in the extent of the hydrophobic interactions between the nonpolar groups of the protein and the nonpolar moiety of MPD.

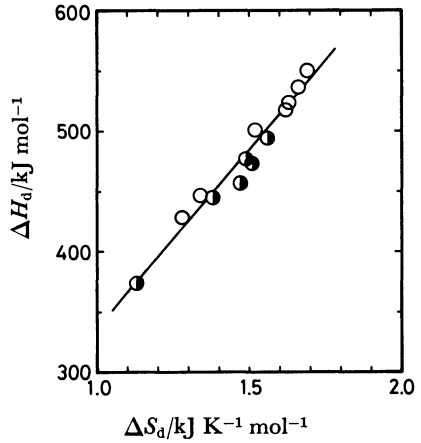

Fig. 4. Enthalpy-entropy compensation plot for the thermal denaturation of RNase A in MPD–water mixtures at pH 5.8 (○) and at pH 3 (◑).

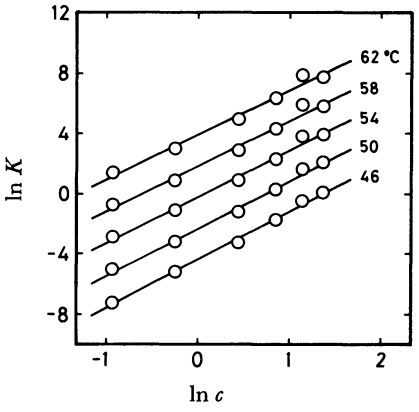

Fig. 5. Wyman plots of the effect of MPD on the thermal denaturation of RNase A at several temperatures.
The number at the right end of each line shows the temperature.

A more quantitative estimation of the MPD effect on the thermal stability of RNase A may be given by comparison of the thermodynamic parameters of denaturation, $\Delta G°$, $\Delta H°$, and $\Delta S°$, at identical temperatures. These have not been reported previously for this solvent system. Assuming that the heat capacity change of denaturation, ΔC_p, is independent of temperature, standard thermodynamic parameters at any temperature, T, can be calculated by the equations.[3,4]

$$\Delta H° = \Delta H_d - \Delta C_p(T_d - T)$$
$$\Delta S° = \Delta H_d/T_d - \Delta C_p \ln(T_d/T)$$
$$\Delta G° = \Delta H° - T\Delta S°$$

For the calculation, ΔC_p in MPD–water mixtures was assumed to be identical with that in aqueous buffer, 5.02 kJ K^{-1} mol^{-1}, since, unfortunately it can not be determined accurately in the present study. This assumption will not cause significant error in the standard thermodynamic parameters when T is close to T_d. Using the data obtained at pH 5.8, therefore, the standard thermodynamic parameters of denaturation were calculated at 53 °C, which is near the midpoint of the range, 46.0 to 61.5 °C, of the T_d obtained at pH 5.8 and were plotted as a function of the MPD concentration in Fig. 6. It is evident that $\Delta G°$ decreases monotonously with increasing MPD concentration. On the other hand, $\Delta H°$ and $\Delta S°$ increase gradually with an increase in the MPD content and tend to flatter at the MPD concentrations above 20%(v/v). In the thermal denaturation of RNase A, the increase in $\Delta H°$ induced by MPD is compensated by a large increase in $\Delta S°$, with a resulting decrease in $\Delta G°$. Therefore, the thermal unstabilization of RNase A by MPD seems to be dominantly produced by an entropic rather than an enthalpic effect, suggesting the possibility of some kind of solvent-disordering effect around the protein. These

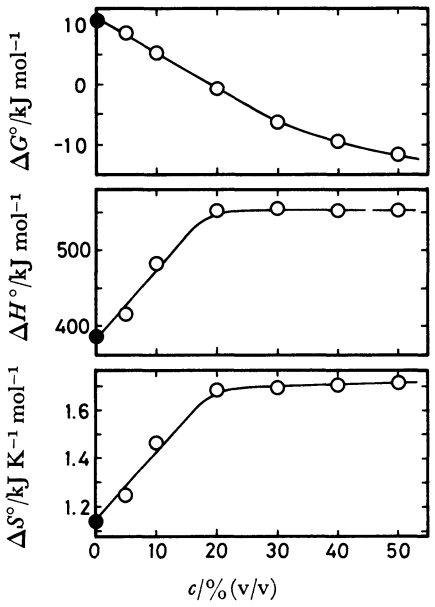

Fig. 6. Variation of the standard thermodynamic parameters of denaturation, $\Delta G°$, $\Delta H°$, and $\Delta S°$, with the concentration of MPD at 53°C.
The symbols ● and ○ refer to the aqueous and the mixed solvents at pH 5.8, respectively.

results are similar to the observation by Brandts[34] that, at a given temperature, $\Delta H°$ and $\Delta S°$ are much larger for the denaturation of chymotrypsinogen in ethanol -water mixtures than in water. Brandts and Hunt[22] have also shown that $\Delta H°$ of the thermal denaturation of RNase in the same solvent system increases with increasing ethanol content, with a maximum around an ethanol concentration of 20%(v/v). At present, the unstabilization of proteins by the addition of alcohols has been explained in terms of their effect on the structure of the water in contact with the nonpolar residues and the direct interaction of alcohol with the protein, which cause weakening the hydrophobic interactions between the nonpolar residue of protein.[7,22] Although RNase A is preferentially hydrated in MPD–water mixtures at low temperature near room temperature,[14] it is not clear at present whether MPD is preferentially bound to the protein or not at elevated temperatures which the thermal denaturation occurs, but, at least, the binding of MPD to the protein increases upon denaturation. A decrease in solvent ordering around the protein and the consequent reduction of the hydrophobic effect when MPD is added to the medium would render more favorable the exposure to solvent of hydrophobic groups buried in the interior of the native protein.

The unstabilizing effect of MPD is consistent with our expectation based on previous studies of the thermal denaturation of lysozyme in aqueous solutions of monohydric alcohols[29] and ethylene glycol[19] and RNase A in aqueous ethylene glycol.[10] However, Pittz and Bello[11] have concluded from the solubility data of amino acids that the unfolding of RNase A in 55% MPD has a positive Gibbs energy change at 25 °C, that is, a stabilizing effect of MPD on the protein. The study of the interactions between RNase A and solvent components in MPD–water mixtures at pH 5.8 by differential refractometry and light scattering has shown that the protein is preferentially hydrated at all solvent compositions up to 50%(v/v) MPD.[14] According to Bull and Breese,[15] any substance which trends to increase the hydration of proteins stabilize a protein. These findings suggests the stabilization of the native protein by MPD at low temperatures near room temperature and the temperature dependence of the MPD effect on solvent ordering around the protein. Thus, in order to examine the temperature dependence of the MPD effect on the thermal stability of RNase A, the $\Delta G°$ at each solvent composition was calculated at several temperatures in the transition region. As expected, it was found that the $\Delta G°$ value increased with a decrease in temperature and further that the decrease in $\Delta G°$ by MPD became reduced with decreasing temperature. Therefore, the temperature dependence of $\Delta G°$ at each MPD content was extrapolated to 25 °C and the resulting value of $\Delta G°$ was plotted against the MPD concentration in Fig. 7. At high temperature, as shown in Fig. 6, MPD is quite effective in decreasing the Gibbs energy change of denaturation. However, at low temperature, MPD acts differently even at a qualitative level, *i.e.*, it behaves as a rather stabilizing agent for native RNase A at low MPD concentrations, almost independent of the pH. Although this conclusion depends to some extent on the validity of the extrapolation, this qualitative

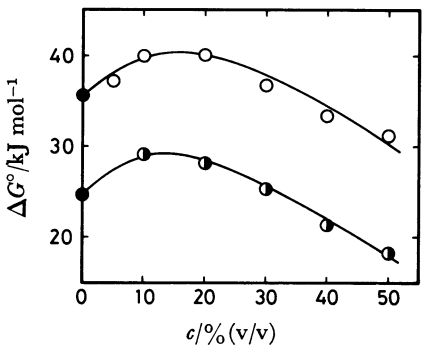

Fig. 7. Variation of $\Delta G°$ with MPD concentration at 25°C.
The symbols used are identical with those in Fig. 2.

conclusion appears to be justified, as the temperature range of extrapolation is short and the effect is large. Moreover, the native protein is too stable at 25 °C and pH 3 to permit direct measurement of the denaturation. Such behavior of $\Delta G°$ has also been found for the denaturation of RNase A in aqueous ethanol solution.[22] This implies that relatively nonpolar organic additives, which have hitherto been considered to be only denaturing agents, might show somewhat the same behavior at low temperature. Indeed, we observed for the thermal denaturation of RNase A in aqueous solutions of ethylene glycol at different pH values that ethylene glycol decreased the denaturation temperature at high temperature, near 60 °C, but increased it at lower temperature, near 40 °C.

These results may be explained on the basis of solvent ordering effect, similarly to that proposed by Brandts and Hunt[22] for the stabilizing effect of ethanol at low temperature and at low ethanol concentration. According to their hypothesis, ethanol replaces ordered water molecules around exposed hydrophobic groups, both solvent components becoming part of the solvent sheath which surrounds the nonpolar side chains of the protein.[34–36] In the case of ethanol, this structure is disrupted at temperatures near room temperature, permitting the formation of hydrophobic contacts between the nonpolar moiety of the alcohol and the nonpolar groups of the protein and leading to the unstabilization of the protein. In the case of MPD, such a solvation sheath around the exposed nonpolar groups of the protein should remain intact up to relatively high temperature, at least, near 25 °C, since the hydrophobicity of MPD appears to be smaller than that of ethanol.

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