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The Effect of Ethylene Glycol on the Thermal Denaturation of Ribonuclease A and Chymotrypsinogen A as Measured by Differential Scanning Calorimetry

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The thermal denaturation of ribonuclease A(RNase A) and chymotrypsinogen A in aqueous solutions of ethylene glycol (EG) was investigated by differential scanning calorimetry at several pH values in the acidic region. The calorimetric enthalpies of denaturation, ΔH_d, of these proteins increased monotonously with increasing the EG concentration under all the conditions studied. These proteins, however, differed from one another in the variation of the denaturation temperature, T_d, with the addition of EG. The T_d of chemotrypsinogen decreased slightly with an increase in EG concentration under all the conditions, producing a greater effect at high pH. The T_d of RNase A, on the other hand, was almost independent of the EG content at pH 3.8, while at pH 2 and 3, the T_d increased slightly with increasing the EG concentration and decreased slightly at pH 5. That is, EG stabilized or unstabilized these proteins against thermal denaturation, in a way which seemed to be dependent on temperature rather than the pH. The standard thermodynamic parameters for denaturation, $\Delta G°$, $\Delta H°$, and $\Delta S°$, which were calculated using T_d and ΔH_d and assuming a constant heat capacity change, suggested that the slight increase or decrease in $\Delta G°$ induced by EG is attributed to whether $\Delta H°$ increases as much as $\Delta S°$ does or not. These results may be explained on the basis of the effect of EG on the solvation around hydrophobic groups of the protein. The EG effect may depends on the structural properties of proteins such as hydrophobicity and polarity, as well as on temperature.

The structural stability of proteins is extensively controlled by the interactions between the protein and surrounding solvent molecules. Various substances stabilize or unstabilize the native structure of proteins when added to aqueous protein solutions as a reflection of their effect on the water structure around the protein.[1-4] Polyhydric alcohols such as glycerol and sorbitol prevent the lose of enzymatic activities[5,6] and raise the thermal transition temperature of proteins. This stabilizing effect had been attributed to a lessened hydrogen bond-rupturing capacities of the medium.[7] Recently, Gekko and coinvestigators[10-13] concluded from extensive studies that the protein stabilization by polyhydric alcohols is due to a preferential hydration effect which strengthens the hydrophobic interactions of the protein. The preferential hydration of proteins in the presence of polyhydric alcohols has been directly shown from equilibrium dialysis[14] and densimetric experiments.[12] On the other hand, monohydric alcohols unstabilize the native structure of proteins. This may be attributed to the perturbation of the characteristic water structure around the protein molecule and to the direct interactions with the protein.[1-3] These alcohols are preferentially bound to protein in alcohol-water mixtures, which is accompanied by a protein dehydration.[14,15] These studies imply that any substance which trends to maintain or increase the hydration of proteins stabilizes a protein and, to the contrary, any substance which is bound to and dehydrates the protein unstabilizes the native structure of proteins.

According to this hypothesis, ethylene glycol (EG)** would have a stabilizing effect on the native structure of proteins, since a number of proteins are preferentially hydrated in EG–water mixtures.[14,16] Actually there have been many observations which show the protein stabilization induced by EG. For example, we reported previously that the denaturation temperature and enthalpy of lysozyme are higher in aqueous EG solutions at pH 2 than in aqueous solution at the same pH.[17] The stabilization of ribonuclease A (RNase A) and lysozyme by the addition of EG has been also shown by Gerlsma and Stuur.[8] However, there have been some contradictory observations concerning the EG effect on the thermal stability of proteins. The thermal transition temperatures of chymotrypsinogen and ovalbumin were depressed by the addition of EG.[7,9] Furthermore, a lowering of the denaturation temperatures of RNase A and lysozyme by EG has been shown at high pH in the acidic region,[2,10] which is in contrast with Gerlsma and Stuur's and our observations for the same proteins. These observations attracted our attention to a systematic examination of the EG effect on the thermal stability of proteins.

Differential scanning calorimetry (DSC) is a useful tool for measuring the thermally induced conformational transition of biological macromolecules such as proteins and nucleic acids. An application of DSC to the study of protein denaturation provides the available thermodynamic information on the unfolding process of proteins. This may contribute greatly to an understanding of the nature and magnitude of the forces that stabilize the native structure of proteins. Recent studies have been reviewed by Biltonen and Freire[18] and by Privalov.[19] We have been investigating the effect of various substances on the thermal stability of globular proteins by means of DSC.

A study of the thermal denaturation of ribonuclease A (RNase A) and chymotrypsinogen A in aqueous EG solutions at several acidic pH values was carried out by DSC; this paper reports the results. These proteins were chosen because they differ greatly in hydrophobicity and polarity and because their thermal denaturation had been investigated extensively in the past.

Experimental

Materials. Bovine pancreatic ribonuclease A (five-times recrystallized, type I-A) and chymotrypsinogen A (six-

** Abbreviations used: DSC, differential scanning calorimetry; EG, ethylene glycol; RNase A, ribonuclease A (bovine pancreatic, EC 3.1.4.22).

times recrystallized, type II) were purchased from Sigma Chemical Co. Ethylene glycol was purified by distillation under reduced pressure. Other chemicals were reagent grade products from Wako Pure Chemical Industries. In the case of RNase A, the experiments were carried out in either 0.05 mol dm^{-3} glycine buffer–EG mixtures adjusted to pH 2, 3, and 3.8 or 0.05 mol dm^{-3} acetate buffer–EG mixtures adjusted to pH 5 with 1 mol dm^{-3} HCl as measured by a Horiba f-7ss pH meter standardized at pH 4 in aqueous solution. In the case of chymotrypsinogen A, either 0.01 mol dm^{-3} glycine (at pH 2 and 3) or 0.01 mol dm^{-3} acetate buffer (at pH 4) was used to minimize aggregation of the denatured protein. The protein solutions were subjected to extensive dialysis against the solvent at 4 °C prior to making DSC measurements. Protein concentrations were determined spectrophotometrically on a Hitachi 323 automatic spectrophotometer. The extinction coefficients were assumed to be 7.38 at 277 nm[20] and 20.3 at 282 nm[21] for a 10 mg cm^{-3} aueous solution of RNase A and chymotrypsinogen A, respectively. The coefficients were corrected for each mixed solvent by the method of Lee *et al.*[22]

DSC measurements. The thermal denaturation of these proteins 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 8—12 mg cm^{-3} for RNase A and 15—19 mg cm^{-3} for chymotrypsinogen A. The sample solutions (0.06 cm^3) were hermetically sealed in a silver vessel. An equal amount of the solvent was used as reference material. 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 13 700 for RNase A and 25 700 for chymotrypsinogen A. The denaturation temperature, T_d, was estimated from the temperature at which the area under the transition peak was divided into halves, since the area is proportional to the amount denatured. The difference between the T_d obtained and the temperature of maximal heat flow did not exceed 0.5 K in every measurement because of the symmetrical nature of the transition peak.

Results and Discussion

Representative DSC scans for the thermal denaturation of RNase A in aqueous EG solutions at pH 3 are shown in Fig. 1. The DSC curves shown correspond to denaturation of a protein solution of 10 mg cm^{-3} in EG concentrations of 0 and 4.64 mol dm^{-3}. At pH 3, the addition of EG to a aqueous solution of RNase A induces a slight shift of the transition peak toward high temperature and an increase of the peak area. In the case of RNase A, although the temperature width of the transition peak, 20—22 K, was hardly influenced by the variation of pH or by the addition of EG, the shift of the transition peak induced by EG was dependent on the pH. On the other hand, the transition peak of chymotrypsinogen A was slightly shifted a toward low temperature by the addition of EG and the peak area slightly increased under all the conditions studied. Moreover, the temperature width of the transition peak became smaller in going from low pH to high pH, consistent with the observation by Brandts[23] in the spectrophotometric experiments; the temperature width of 22—24 K at pH 2 decreased to 16—19 K at pH 4.

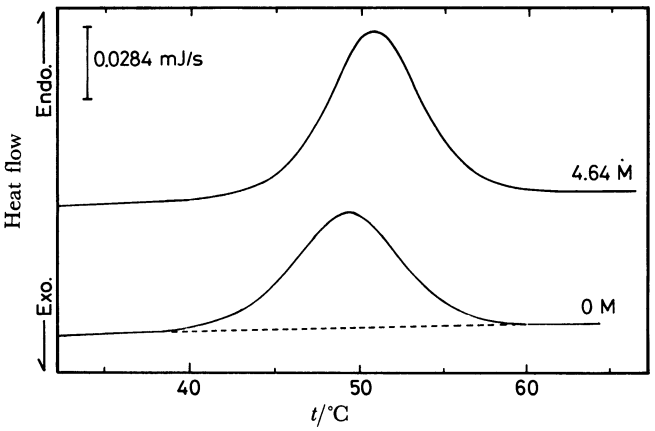

Fig. 1. DSC curves for the thermal denaturation of RNase A in aqueous EG solutions at pH 3. Protein concentration is a 10 mg cm^{-3} and EG concentrations are 0 and 4.64 mol dm^{-3}. The dotted line shows the base line assumed in the transition region for the determination of the thermodynamic parameters of denaturation.

As seen in the figure, the DSC curves obtained exhibit that there is a difference in the heat capacity between the native and denatured states of these proteins; this has been observed for many proteins including the proteins.[19] Therefore, one of the major problem for estimating exactly the thermodynamic parameters of denaturation is how to determine a suitable base line in the transition region. Some estimations of ΔH_d were made by using the base line obtained by extrapolation of the base lines for native and denatured states to the denaturation temperature. However, the ΔH_d obtained by such a procedure was not signifiently different from that estimated using a linear base line fitted by inspection. Under our conditions, relatively low protein concentration, the exact heat capacity change upon denaturation seems to be considerably difficult for the DSC apparatus used to determine directly in the individual scan. In this experiment, therefore, we employed a linear base line fitted to the difference of the base lines before and after the transition. Although the thermal denaturation of these proteins has been shown to be a reversible two-state process in aqueous solution,[23-26] the reversibility for the denaturation in EG–water mixtures was checked by reheating the protein solution after rapidly cooling from the first scan. In each solvent composition, there were no significant variations in the position of the transition peak and in the peak area on reheating, indicating that the denaturation of these proteins is almost reversible in EG–water mixtures. In addition, the denaturation temperature was scarcely influenced by heating rates slower than 1 K min^{-1}, allowing equilibrium analysis of the data.

The values of T_d and ΔH_d obtained in EG–water mixtures at different pH values are listed in Table 1. These thermodynamic parameters were evaluated as the average value of five and more experiments. The T_d values have experimental errors of ± 0.1 °C and the ΔH_d values have maximum expected errors of $\pm 5\%$ including errors in sample preparation, calibration constant, and reproducibility. For RNase A, the ΔH_d values in aqueous buffers at different pH in the range

TABLE 1. THERMODYNAMIC PARAMETERS OF THERMAL DENATURATION OF PROTEINS IN AQUEOUS EG SOLUTIONS AT DIFFERENT pH VALUES

pH	c_{EG} / mol dm^{-3}	T_d / °C	ΔH_d / kJ mol^{-1}	ΔS_d / kJ K^{-1} mol^{-1}
		RNase A		
2.0	0	38.0	348	1.12
	1.07	38.9	377	1.21
	2.45	39.5	410	1.31
	3.94	40.6	425	1.35
3.0	0	49.3	390	1.21
	1.01	49.5	401	1.24
	2.13	49.8	431	1.33
	3.43	50.2	465	1.44
	4.64	50.4	487	1.51
3.8	0	56.1	423	1.28
	0.99	56.1	428	1.30
	2.14	56.2	470	1.43
	3.43	56.1	485	1.47
	4.68	56.0	500	1.52
5.0	0	61.0	453	1.36
	0.99	60.7	472	1.41
	2.14	60.5	477	1.43
	3.42	60.2	506	1.52
	4.70	59.9	528	1.59
		Chymotrypsinogen A		
2.0	0	41.5	351	1.12
	1.06	41.0	367	1.17
	2.46	40.2	380	1.21
	4.03	39.6	406	1.30
3.0	0	51.4	498	1.53
	0.97	50.9	509	1.57
	2.24	50.2	520	1.61
	3.55	49.6	533	1.65
	4.70	49.0	547	1.70
4.0	0	61.9	637	1.90
	1.07	61.2	652	1.95
	2.44	60.2	660	1.98
	3.98	59.1	670	2.02

2 to 5 are in agreement with those obtained by Tsong *et al.*[24] and Privalov *et al.*[25] from calorimetric experiments at similar pH values, while the T_d values at low pH are higher than the data of theirs and those at high pH are slightly lower. However, Jacobson and Turner[26] have recently reported considerably higher T_d values from DSC measurements at pH 4.1 and 5.0. Although the reason for the differences in T_d is not clear at present, a number of factors such as protein concentration, ionic strength, buffer, purity of the protein sample (contamination), or heating rate could contribute to the differences. For example, the heating rate used by Jacobson and Turner[26] is considerably higher than that used in this experiment. In addition, Tsong *et al.*[24] have reported that the T_d of RNase A increases with an increase in protein concentration. The T_d and ΔH_d values of chymotrypsinogen A in aqueous buffer solutions at pH 2, 3, and 4 are in accord with previous values obtained by Jackson and Brandts[27] and Privalov *et al.*[28] at similar pH values. Figure 2 represents the temperature dependence of the denaturation enthalpy in aqueous buffer without EG. As has been pointed out by Privalov,[19] ΔH_d is a linear function of T_d, with slope equal to the heat capacity change, ΔC_p^d, on denaturation. The values of ΔC_p^d obtained from the slope of the graph, 4.57 kJ K^{-1} mol^{-1} for RNase A and 14.0 kJ K^{-1} mol^{-1} for chymotrypsinogen A, are in very good agreement with those calculated using the data of Privalov *et al.*[24,27] for the thermal denaturation of these proteins in aqueous solutions of varying the pH. A similar value for chymotrypsinogen has been obtained by Biltonen *et al.*[29] from the heat capacities of the native and denatured states of the protein.

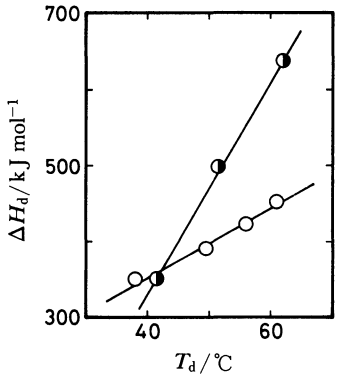

Fig. 2. Dependence of the denaturation enthalpies of RNase A(○) and chymotrypsinogen A(◑) in aqueous buffer without EG on the denaturation temperature.

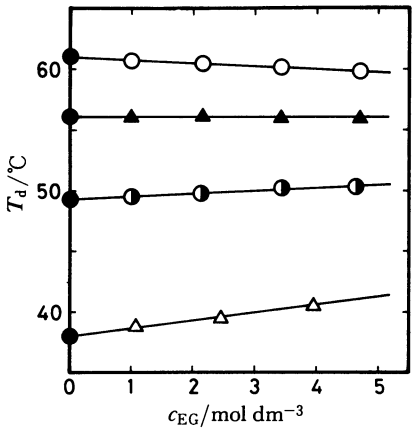

Fig. 3. Variation of the denaturation temperature of RNase A with the EG concentration at pH 2(△), 3(◑), 3.8(▲), and 5(○).
The symbol ● refers to the buffer solution without EG.

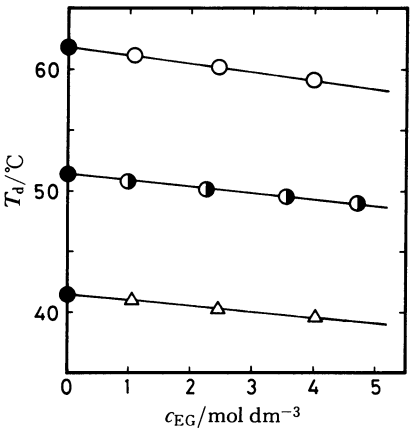

Fig. 4. Variation of the denaturation temperature of chymotrypsinogen A with the EG concentration at pH 2(△), 3(◑), and 4(○).

Figures 3 and 4 show the dependence of the denaturation temperature of RNase A and chymotrypsinogen A on the EG concentration, respectively. It is obvious from Fig. 3 that at pH 3.8 the T_d of RNase is almost independent of the EG content, while the T_d increases slightly with increasing the EG concentration at pH 2 and 3 and decreases slightly at pH 5. Similar results on the EG-induced change in T_d at low pH have been reported by Gerlsma and Stuur. EG stabilizes RNase A against thermal denaturation at low pH but unstabilizes or denatures the protein at high pH in the pH region studied. As show in Fig. 4, on the other hand, the T_d of chymotrypsinogen A is depressed by the addition of EG under all the conditions and the lowering of T_d appears to become more pronounced with increasing the pH and the corresponding T_d. That is, EG has a thermally unstabilizing effect on the protein in the pH 2 to 4 region. These results suggest that the EG-induced effect on the thermal stability of these proteins is dependent on the pH or temperature. However, it is unlikely that the EG effect depends directly on the pH, since the effect on the two proteins differs from each other at identical

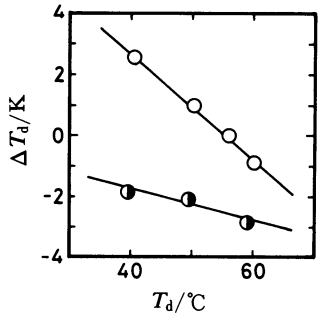

Fig. 5. Dependence of the variation of T_d induced by addition of 4 mol dm^{-3} EG, ΔT_d, of RNase A(○) and chymotrypsinogen A(◑) on the denaturation temperature.

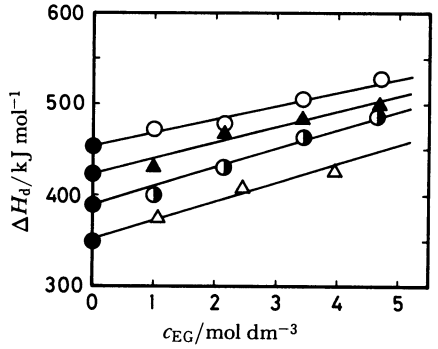

Fig. 6. Variation of the denaturation enthalpy of RNase A with the EG concentration at pH 2(△), 3(◑), 3.8(▲), and 5(○).

pH of 2 and 3 and the T_d of these proteins increases monotonously with increasing the pH in the acidic region. Therefore, the variation of T_d with the addition of EG of 4 mol dm^{-3}, ΔT_d, which is evaluated from a linear dependence of T_d on EG concentration, is plotted as a function of the corresponding temperature in Fig. 5. For both proteins, the ΔT_d appears to be a linear function of temperature, meaning that the EG-induced effect may be directly dependent on temperature.

The dependence of the denaturation enthalpy of RNase A on the EG concentration is shown in Fig. 6. Under all the conditions, ΔT_d increases almost linearly with increasing the EG concentration. Such behavior of ΔH_d was also observed for chymotrypsinogen A, though the degree of the change in ΔH_d with EG was slightly smaller for the protein than for RNase A. As shown in Table 1, the denaturation entropies at the denaturation temperature, $\Delta S_d(=\Delta H_d/T_d)$, of these proteins also increase monotonously with an increase in the concentration of EG. This means that the observed increase (or decrease) in T_d occurs because ΔH_d increases (or does not increase) as much as ΔS_d does upon the addition of EG.

The plot of ΔH_d as a function of ΔS_d gives a linear relationship, presented in Fig. 7, indicating that the EG-induced effect on the thermal stability of these proteins is closely related to the water structure around the protein molecule.[30] The compensation temperatures, which are defined as the slopes of the graph, are 375 and 364 K for RNase A and chymotrypsinogen A, respectively. Although these values are consider-

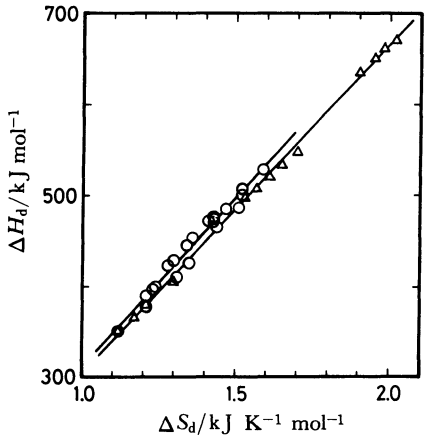

Fig. 7. Enthalpy-entropy compensation plots for the thermal denaturation of RNase A(O) and chymotrypsinogen A(Δ) in EG-water mixtures.

ably higher than the temperature range of 250 to 320 K observed for many processes, the value for RNase is in good agreement with the compensation temperature deduced from the results obtained for denaturants such as urea and guanidine hydrochloride.[31]

A more definite estimation of the effect of EG on the protein stability may be given by the comparison of the thermodynamic functions of denaturation, $\Delta G°$, $\Delta H°$, and $\Delta S°$, at identical temperatures. Assuming that the heat capacity change, ΔC_p^d, on denaturation is independent of temperature, standard thermodynamic parameters of denaturation at any temperature, T, can be calculated with T_d and ΔH_d by the equations:[32,33]

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

Unfortunately, ΔC_p^d in each solvent composition can not be accurately determined in the present study. Thus the ΔC_p^d values of these proteins in mixed solvents were assumed to be identical with those in aqueous buffer, 4.57 kJ K^{-1} mol^{-1} for RNase A and 14.0 kJ K^{-1} mol^{-1} for chymotrypsinogen A, which were estimated from the linear temperature dependence of the ΔH_d in this experiment. This assumption will not cause significant error in standard thermodynamic parameters of denaturation when T is close to T_d, since ΔC_p^d does not seem to be largely affected by additions of EG. Therefore, these parameters were calculated at several temperatures in the transition region. Figure 8 represents the variation of $\Delta G°$, $\Delta H°$, and $\Delta S°$ calculated at 48 °C with the EG concentration at pH 3. It is evident that the $\Delta H°$ and $\Delta S°$ for both proteins are increasing functions of EG concentration, indicating that the enthalpy and entropy of transfer of denatured protein from water to aqueous EG are larger than those of native protein. On the other hand, although the variation induced by addition of EG is very small, the $\Delta G°$ for RNase increases obviously with increasing EG concentration, but that for chymotrypsinogen decreases. These findings suggest that the thermal stabilization of RNase by EG is dominantly produced by an enthalpic rather than an entropic effect. However, the unstabilization of chymotrypsinogen by EG may be attributed to the fact

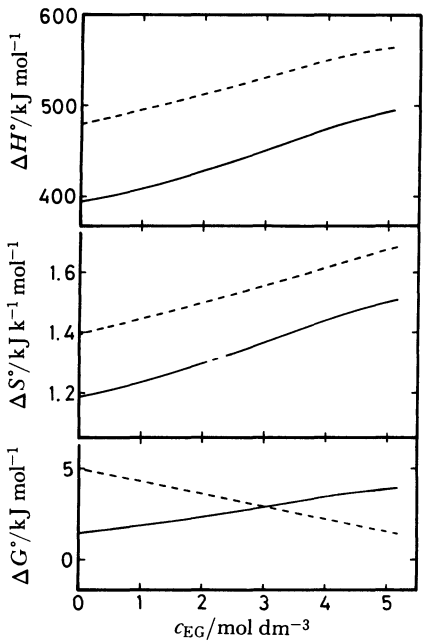

Fig. 8. Variation of the standard thermodynamic parameters of denaturation calculated at 48°C of RNase A (——) and chymotrypsinogen A (----) with the EG concentration.

that $\Delta H°$ does not increase as much as $\Delta S°$ does upon addition of EG. Therefore, the slight increase or decrease in $\Delta G°$ upon addition of EG is produced from the delicate balance between the increased $\Delta H°$ and $\Delta S°$, which may be affected by numerous factors such as temperature and the nature of the protein surface. These findings seem to offer direct evidence for regarding protein stabilization and denaturation (unstabilization) as a thermodynamically continuous phenomenon. The observed increase in $\Delta H°$ and $\Delta S°$ is similar to the observations by Brandts[23] 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. Such a trend of $\Delta H°$ has been also found for the thermal denaturation of RNase A in the same solvent system.[34]

The $\Delta G°$ values for RNase A and chymotrypsinogen A in water and aqueous EG are presented as a function of temperature in the transition region in Figs. 9 and 10, respectively. In the case of RNase, as can be seen from Fig. 9, the $\Delta G°$-temperature curves for these solutions cross at about 55 °C, corresponding roughly to the denaturation temperature of the protein at pH 3.8, which is almost independent of the pH. This means that, at temperatures lower than about 55 °C, EG is quite effective in increasing $\Delta G°$, that is, it stabilizes RNase against thermal denaturation while, at higher temperatures, it behaves as a rather unstabilizing (denaturing) agent for the protein. In the case of chymotrypsinogen, on the other hand, although the dotted line shown in Fig. 10 is the extrapolation of the curve, the crossing of the $\Delta G°$-temperature curves appears to occur at about 35 °C, which is considerably lower than that for RNase. The protein stabilization induced by polyhydric alcohols has been explained as a strengthening of the intramolecular hydrophobic

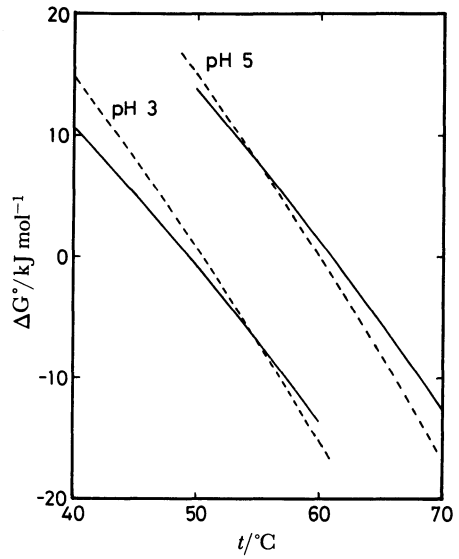

Fig. 9. Variation of $\Delta G°$ of RNase A in water (——) and aqueous EG (----) at pH 3 and 5 with temperature in the transition region.
EG concentrations are 4.64 mol dm^{-3} at pH 3 and 4.70 mol dm^{-3} at pH 5.

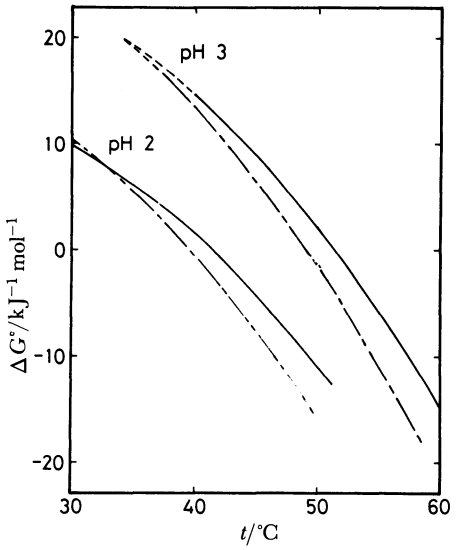

Fig. 10. Variation of $\Delta G°$ of chymotrypsinogen A in water (——) and aqueous EG (—) at pH 2 and 3 with temperature in the transition region.
EG concentrations are 4.03 mol dm^{-3} at pH 2 and 4.70 mol dm^{-3} at pH 3. The dotted line shows the extrapolation of the curve.

interaction of the protein.[10-13] The conformational stability of proteins in aqueous medium is viewed, at present, as a result primarily of the pressure which water exerts on nonpolar residues, compressing them into the interior of the molecule. An enhancement of the hydrophobic effect in medium by addition of EG at low temperature would render more unfavorable the exposure to solvent of nonpolar groups buried in the interior of native protein. The pronounced relationship between temperature and the EG-induced effect on the thermal stability of these proteins may be explained on the basis of a solvent ordering effect, similar to that proposed by Brandts and Hunt[34] for the stabilizing effect of ethanol at low temperatures and low ethanol concentrations. According to their hypothesis, the mechanism is closely connected with the solvation which forms about exposed nonpolar groups at low temperature and with the disruption of the solvation structure which occurs as the temperature is increased. Ethanol or EG added may replace ordered water molecules around exposed hydrophobic groups, both solvent components becoming part of the solvation which surrounds the nonpolar side chains of the protein. In the case of ethanol, this structure is disrupted at low temperatures, permitting the formation of hydrophobic contacts between the nonpolar moiety of the alcohol and the nonpolar groups of the protein and leading to the denaturation of the protein. In the case of EG, however, such a solvation around the exposed nonpolar groups of a protein should remain intact up to relatively high temperature, at least near 55 °C for RNase and near 35 °C for chymotrypsinogen, since the hydrophobicity of EG is considerably smaller than that of ethanol. Furthermore, the interaction of the EG with the exposed nonpolar groups of proteins is not direct, as would be in the case of hydrophobic interactions, but it occurs indirectly through the strong interaction of EG molecule with the water of hydration around the nonpolar groups of the protein.

There is an important difference between RNase A and chymotrypsinogen A in the observed effect of EG on the thermal denaturation. These proteins differ greatly in the molecular parameters. The average hydrophobicity and polarity are 870 cal (1 cal=4.184 J) per residue and 1.73 for RNase A and 1040 cal per residue and 0.83 for chymotrypsinogen A.[35] RNase A is one of the most hydrophilic globular proteins and the content of nonpolar amino acid residues is very low, 49 residues per molecule. On the other hand, chymotrypsinogen A has high hydrophobicity and contains 112 nonpolar amino acids per molecule. Although it has been shown by Brandts and Hunt[34] that the fraction of the total number of residues which unfold during denaturation is smaller for chymotrypsinogen than for RNase, the number of newly exposed nonpolar groups following denaturation would be considerably larger in chymotrypsinogen than in RNase. Addition of EG seems to give rise to a decrease in the heat capacity of solvation of nonpolar groups.[34,36] Such a effect should be greater for chymotrypsinogen A than for RNase A. This may be partially responsible for the result that the observed decrease in $\Delta G°$ for chymotrypsinogen A upon addition of EG occurs because $\Delta H°$ does not increase as much as $\Delta S°$ does.

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