# Thermodynamic and Kinetic Examination of Protein Stabilization by Glycerol†

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ABSTRACT: The effect of concentrated glycerol on the thermal transitions of chymotrypsinogen and ribonuclease has been examined by differential spectrophotometry at 293 and 287 nm, respectively. It was found that for both proteins addition of glycerol raises the transition temperature, the increase in $T_m$ being greater for ribonuclease than for chymotrypsinogen. This increase in the free energy of denaturation appears to reflect primarily a decrease in the entropy change. Analysis in terms of the Wyman linkage equation shows that, for both proteins, the exclusion of glycerol from the protein domain increases on denaturation, i.e., the chemical potential of glycerol becomes even more positive when the protein unfolds relative to the native structure. This provides the thermodynamic stabilization free energy. Results of the kinetic examination of the slow unfolding reaction are consistent with the concept that the preferential exclusion of glycerol is related, at least in part, to enhanced solvent ordering.

In the preceding paper (Gekko & Timasheff, 1981), we have shown that a number of proteins are preferentially hydrated in aqueous glycerol solution. This phenomenon, which is a reflection of an increase in the chemical potential of glycerol induced by contact with protein, is thermodynamically unfavorable. We have proposed that it is the tendency of the system to reduce this situation by minimizing the unfavorable contact, i.e., the extent of the protein–solvent interface, which induces stabilization of protein structure by favoring the folded, or compact, state. The same phenomenon has been invoked to explain the boosting of self-association reactions by glycerol (Lee & Timasheff, 1977; Timasheff, 1978). The preferential exclusion of glycerol from the domain of protein molecules appears to be due, at least in part, to enhanced solvent ordering, in agreement with the proposal of Brandts & Hunt (1967) that the driving force for stabilizing protein conformation is the solvation effect. The contribution of the free energy of cavity formation to macromolecule stabilization, proposed by Sinanoglu & Abdulnur (1965), cannot play a significant role in the case of glycerol, since glycerol slightly decreases the surface tension of water.

In order to test the hypothesis formulated in the previous paper, we have carried out an equilibrium thermodynamic and kinetic study of protein denaturation in aqueous glycerol solutions. The dependences on glycerol concentration of the transition temperature, $T_m$, the thermodynamic parameters of the denaturation reaction, $\Delta H°$ and $\Delta S°$, and the apparent kinetic parameters of the unfolding process have been determined. These parameters have not been measured previously for systems containing stabilizing cosolvents. In this study, we have used methods of analysis developed by Brandts & Hunt (1967) and by Pohl (1968a,b), whose results have supported the concept that the effect of ethanol on protein conformation might be related to a change in the solvation of the nonpolar residues with a change in ethanol concentration and temperature. The results of such studies of the thermal denaturation of chymotrypsinogen and ribonuclease in aqueous glycerol solution are presented in this paper. These two proteins have been selected because they differ greatly in hydrophobicity and polarity and their denaturation had been investigated extensively in the past.

## Experimental Procedures

*Materials.* The chymotrypsinogen A used in these experiments was purchased from Sigma (lot 114C-8330, type II from bovine pancreas, recrystallized 6 times). It was deionized completely by dialysis against $10^{-3}$ M HCl and lyophilized before use. Ribonuclease A (RNase A)[1] was also purchased from Sigma (lot 46C-8080, type IIA from bovine pancreas). This protein was further purified by Sephadex G-75 exclusion chromatography in phosphate buffer, following essentially the procedure of Crestfield et al. (1962, 1963). The fractionated protein was subjected to exhaustive dialysis against doubly distilled water at 4 °C, then completely deionized by passage through a mixed-bed ion-exchange resin (Amberlite MG-1), and finally lyophilized. Spectroquality glycerol from Matheson, Coleman & Bell was used without further purification. Fisher standardized 1 N HCl solution was used for the pH adjustment of solutions. The glycine used was the reagent grade product of Sigma.

*Thermodynamics of Thermal Denaturation.* The thermal denaturation was followed by difference-UV spectroscopy at an appropriate wavelength for each protein, 293 nm for chymotrypsinogen A and 287 nm for ribonuclease A. The UV difference spectra were measured on a Cary 14 double-beam spectrophotometer in the chymotrypsinogen study and on a Cary 118 spectrophotometer in the case of RNase, using matched 1-cm quartz-jacketed cells. The temperature of the cell in the sample beam was controlled by circulating water of a given temperature through the cell housing from a Haake circulating bath (Berlin). The reference cell was controlled at 20 °C for chymotrypsinogen and at 18 °C for RNase by using a second water bath. The temperature control was found to be better than ±0.05 °C.

Extreme care was exercised in the preparation of the sample solutions since the experimental results of thermal denaturation are very sensitive to the protein concentration and the pH of the solution (Tsong et al., 1970). In the case of RNase, 1 mg/mL protein solutions were prepared in a 0.04 M glycine

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[1] Abbreviation used: RNase, ribonuclease.

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buffer–glycerol mixture with a final pH of 2.80. In the case of chymotrypsinogen, 5 mL of a native protein stock aqueous solution (5 mg/mL in concentration) and a given amount of glycerol were added to a 25-mL volumetric flask. These were then diluted with distilled water to a total volume of 25 mL. The five solutions obtained in this manner were of identical protein concentration, 1.0 mg/mL, and varying glycerol concentrations, 0, 10, 20, 30, and 40 vol %. These were titrated individually with 1 N HCl to a pH reading of 2.00. The change in protein concentration caused by dilution in the titration process was within 1% of the concentration before titration, and the resulting difference in ionic strength between the various glycerol solutions was less than $3.5 \times 10^{-3}$. These differences may be regarded as negligible for the purpose of the present experiments, in view of the results of Tsong et al. (1970) and Eisenberg & Schwert (1951). All solutions were clarified by filtration through a Millipore filter (GSWP01300) prior to use in order to remove dust or other particulate impurities.

In the temperature studies, two matched cells were filled with aliquots of the same solution and sealed tightly with a Teflon stopper to avoid evaporation during the thermal denaturation experiments. The progress of the reaction and attainment of equilibrium were monitored by the change of the difference in absorbance at 293 nm for chymotrypsinogen and at 287 nm for RNase. After attainment of equilibrium, difference spectra were recorded from 250 to 360 nm to check base-line shifts. The difference in absorbance at 293 nm for chymotrypsinogen and at 287 nm for RNase was then plotted as a function of temperature.

*Kinetics of Thermal Denaturation.* The rate of thermal denaturation was determined from the time dependence of the development of the difference spectra on a Cary 118 spectrophotometer following a slow temperature jump. At each glycerol concentration, a 6 mg/mL chymotrypsinogen solution, adjusted to pH 2.00 with HCl, was placed into a water bath at 30 °C until thermal equilibrium. At the same time, a pH 2.00 HCl solution containing the same amount of glycerol as the protein solution was preheated to a temperature a few degrees higher than the desired denaturation temperature. These preheating temperatures were set by assuming that the heat capacity of the protein solution is identical with that of the acid solution. They were 48.0, 50.4, and 52.8 °C for the denaturation temperatures of 45, 47, and 49 °C, respectively. At zero time, 0.2 mL of the protein solution and 1.0 mL of the acid glycerol solution were delivered simultaneously from automatic pipets (Clay Adams & Gilson) into the jacketed quartz cell controlled at the denaturation temperature. The cell was sealed with a Teflon stopper and quickly agitated to mix the solution. These manipulations were performed as rapidly as possible, usually within 10 s. Simultaneously the recorder on the spectrophotometer was switched on, and the time dependence of the evolution of the difference in absorption at 293 nm was recorded. The reference cell was filled with a protein solution of the same composition as the sample cell (final protein concentration 1 mg/mL) and was held at room temperature. The apparent rate constant of thermal denaturation was evaluated as an average of six to nine sets of data obtained at each denaturation temperature. By use of the same procedures, the rate of thermal denaturation of RNase was determined from the time dependence of the evolution of difference spectra at 287 nm. For this protein, the solution in the native state was thermally equilibrated at 25 °C. Although this method yielded results of sufficient accuracy to assess the effect of glycerol on the rate of denaturation, the experimental deviation being usually within 5%, it was found that in 40% glycerol solution the data were frequently erratic, especially at low denaturation temperatures. This is probably due to the imperfect mixing of the protein and acid solutions and to the persistence of bubbles formed during mixing.

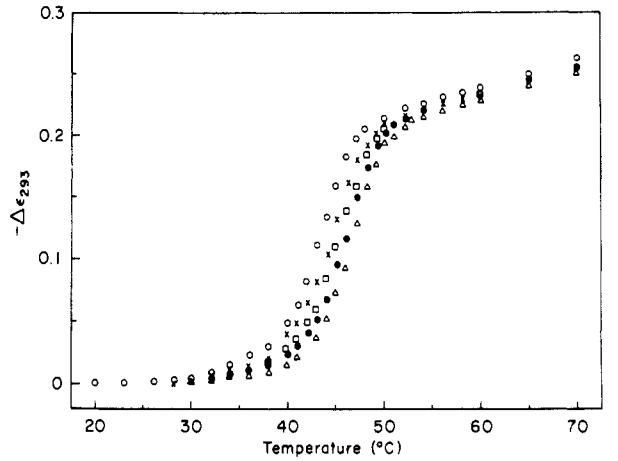


FIGURE 1: Temperature dependence of the differential absorbance of chymotrypsinogen at 293 nm. The glycerol concentrations are (○) 0, (×) 10, (□) 20, (●) 30, (△) 40%.

## Results and Discussion

*Thermodynamics of Thermal Denaturation.* When a reversible thermal denaturation is dominated by the two end states, N (native) $\rightleftharpoons$ D (denatured), the apparent overall equilibrium constant, $K = (D/N) = ([D]/[N])$, can be calculated from the difference spectrophotometric data as

$$K = (\Delta\epsilon - \Delta\epsilon_N)/(\Delta\epsilon_D - \Delta\epsilon) \quad (1)$$

where $\Delta\epsilon_N$ is the difference extinction coefficient between the two temperatures for the native protein, $\Delta\epsilon_D$ is that for the denatured protein, and $\Delta\epsilon$ is the measured extinction coefficient difference of the solution in the transition region. The changes in the standard free energy, $\Delta G°$, standard enthalpy, $\Delta H°$, and standard entropy, $\Delta S°$, of denaturation may then be obtained from eq 2–4.

$$\Delta G° = -RT \ln K \quad (2)$$

$$\Delta H° = -R\mathrm{d}(\ln K)/\mathrm{d}(1/T) \quad (3)$$

$$\Delta S° = (\Delta H° - \Delta G°)/T \quad (4)$$

The reversibility of the thermal denaturation was established by spectroscopic measurements, which showed that for both proteins at all glycerol concentrations used, the difference UV spectra and the CD spectra at room temperature were identical before and after heating.

Figures 1 and 2 show the temperature dependence of the difference spectra of chymotrypsinogen at 293 nm and ribonuclease at 287 nm as a function of glycerol concentration. Both protein systems exhibit similar transition curves whether glycerol is present or not, the curves being translated to higher temperatures with increasing glycerol concentration. This suggests that the thermal denaturation process itself is not affected significantly by the addition of glycerol. The equilibrium constant, $K$, was calculated with the usual assumption that $\Delta\epsilon_N$ and $\Delta\epsilon_D$ have the same temperature coefficients in the transition region as in the pure native and denatured states (Brandts, 1964; Brandts & Hunt, 1967), since the extinction coefficients for both end states were found to vary linearly with temperature at all conditions studied. van't Hoff plots of equilibrium data are shown in Figures 3 and 4. A slight curvature is evident at all glycerol concentrations, the slope

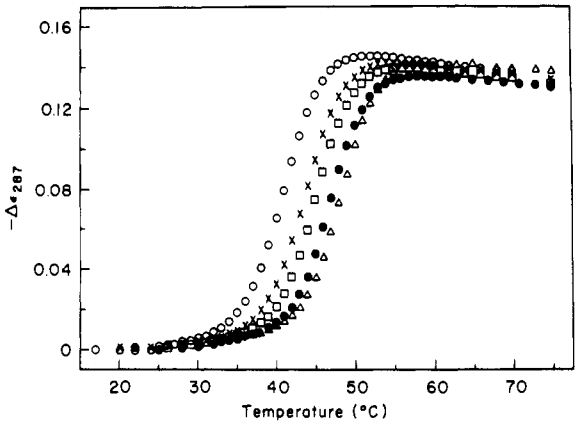


FIGURE 2: Temperature dependence of the differential absorbance of RNase at 287 nm. The glycerol concentrations are (○) 0, (×) 10, (□) 20, (●) 30, (△) 40%.

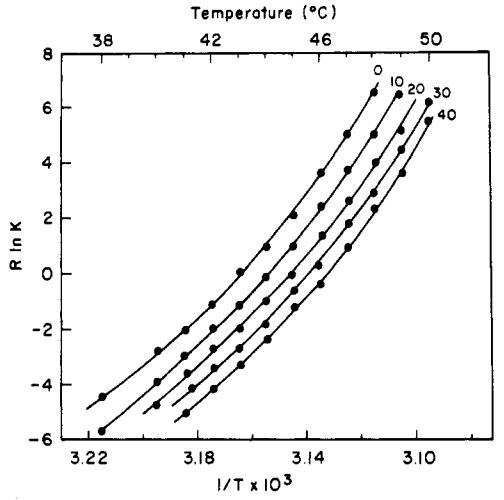


FIGURE 3: van't Hoff plots of the thermal denaturation of chymotrypsinogen. The numbers on the figure mean the volume percent of glycerol in the solution.

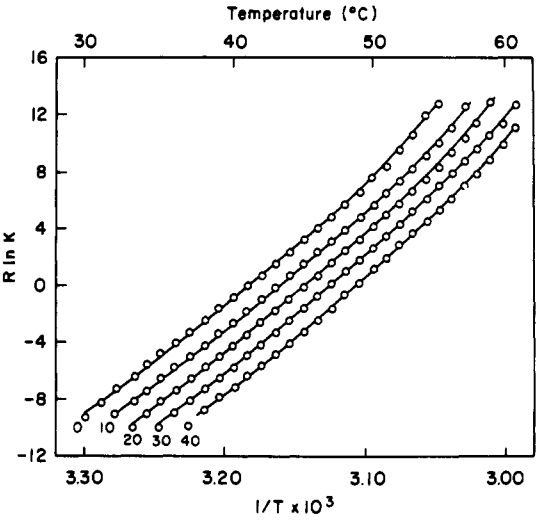


FIGURE 4: van't Hoff plots of the thermal denaturation of RNase. The numbers on the figure mean the volume percent of glycerol in the solution.

Table I: Thermodynamic Parameters of Thermal Denaturation of Proteins in Glycerol-Water Mixtures

| glycerol (% v/v) | $T_m$ (°C) | $\Delta H^\circ_{T_m}$ (kcal/mol) | $\Delta S^\circ_{T_m}$ [cal/(mol·deg)] |
|---|---|---|---|
| | Chymotrypsinogen A [a] | | |
| 0 | 42.9 | 109 | 345 |
| 10 | 44.0 | 112 | 353 |
| 20 | 44.9 | 113 | 355 |
| 30 | 45.7 | 117 | 367 |
| 40 | 46.2 | 126 | 394 |
| | Ribonuclease A [b] | | |
| 0 | 41.1 | 83 | 264 |
| 10 | 43.5 | 82 | 259 |
| 20 | 45.1 | 86 | 270 |
| 30 | 46.8 | 86 | 269 |
| 40 | 48.5 | 88 | 274 |

[a] Protein concentration 1 mg/mL, 0.01 M HCl, pH 2.00. [b] Protein concentration 1 mg/mL, 0.04 M glycine buffer, pH 2.80.

decreasing with an increase in temperature, in agreement with previous reports that protein denaturation is accompanied by a change in heat capacity (Brandts & Hunt, 1967; Brandts, 1964). The curvature for RNase seems to be slightly smaller than for chymotrypsinogen, as should be expected from the difference between the heat capacities of the two proteins (Brandts, 1964; Brandts & Hunt, 1967; Jackson & Brandts, 1970; Tsong et al., 1970). The transition temperature, $T_m$, defined as the temperature at which $K = 1$ ($R \ln K = 0$), is presented in Table I. $T_m$ gradually increases with increasing glycerol contents, by about 3.3 and 7.4 °C between aqueous and 40% glycerol solutions for chymotrypsinogen and RNase, respectively. It is clear from these data that glycerol stabilizes these proteins against thermal denaturation. Similar results on the glycerol-induced change in $T_m$ of RNase have been reported by Gerlsma (1968) and Gerlsma & Sturr (1972, 1974). The value of $T_m$ for chymotrypsinogen in water solution of pH 2.00, 42.9 °C, is identical with the reported value of 43 °C (Gerlsma, 1970; Brandts & Lumry, 1963). Our results, however, differ from those of Gerlsma (1970), who reported that, for chymotrypsinogen, $T_m$ is independent of glycerol content at pH 2 and decreases with increasing glycerol content at pH 3. The reason for this discrepancy is not clear; it may reflect a difference in the experimental procedures. For example, while we realize that a true measurement of pH in concentrated organic solvent–water mixtures is very difficult, we have adopted the generally accepted procedure, (Brandts & Hunt, 1967; Pohl, 1968a,b; Tsong et al., 1970) of adjusting the solution to a pH reading of 2.00 on a pH meter.

A better quantitative description of the glycerol stabilization of these proteins is given by the thermodynamic parameters. These have not been reported previously for this solvent system. A good measure of stabilization is the change in the standard free energy of denaturation, $\Delta(\Delta G^\circ)$, brought about by the addition of a given amount of glycerol

$$\Delta(\Delta G^\circ) = \Delta G^\circ(c) - \Delta G^\circ(0) \quad (5)$$

where $\Delta G^\circ(c)$ and $\Delta G^\circ(0)$ are the standard free energy changes of denaturation in aqueous solution containing $c$% and no glycerol, respectively. The exact value of $\Delta(\Delta G^\circ)$ depends on the reference state used in evaluating $\Delta G^\circ$. We have measured it under conditions of identical pH and temperature, although the standard free energy of the native state cannot be identical in water and aqueous glycerol because of the preferential interaction results (Gekko & Timasheff, 1981) that $(\partial\mu_2/\partial m_3)_{T,P,m_2} > 0$. $\Delta(\Delta G^\circ)$ may be useful, however, in evaluating the relative stabilizing effect of glycerol in a manner similar to what had been done in studies on the effects of ethanol and urea on protein denaturation (Brandts, 1964; Brandts & Hunt, 1967). The resulting values of $\Delta(\Delta G^\circ)$ at 45 °C, plotted against glycerol concentration in Figure 5, are found to be positive. They increase with increasing glycerol contents, the slightly downward curvature indicating a gradual saturation of protein structure stabilization. The stabilization

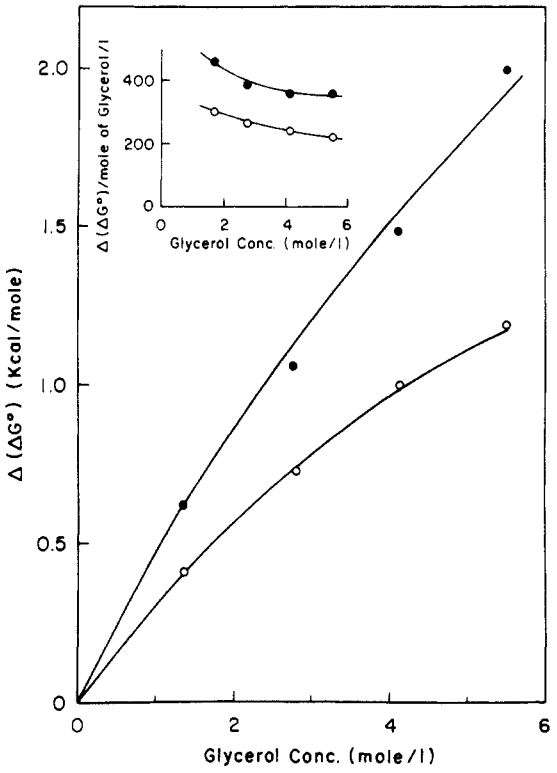


FIGURE 5: Glycerol concentration dependence of $\Delta(\Delta G°)$ of chymotrypsinogen (○) and RNase (●) at 45 °C. The units of the ordinate in the inset are calories per mole of protein per mole of added glycerol per liter.

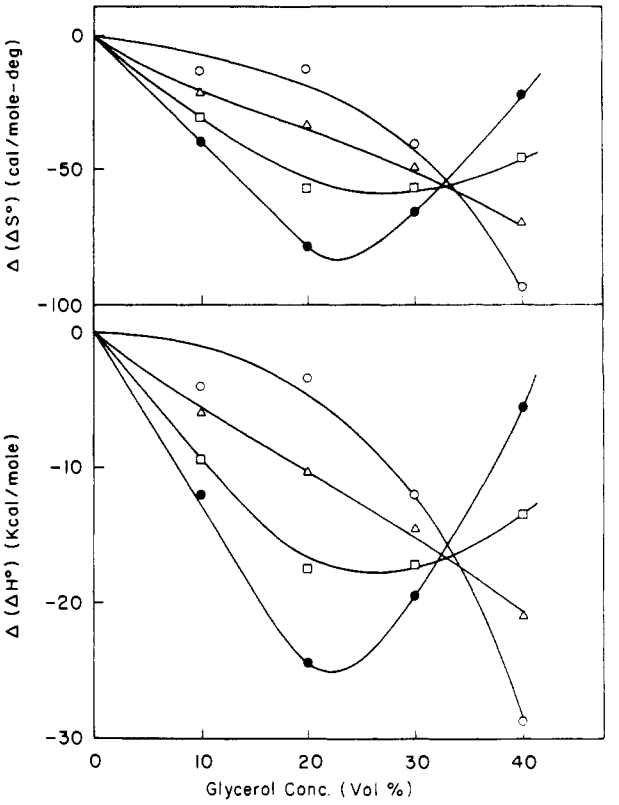


FIGURE 6: Dependence of $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ of chymotrypsinogen on glycerol concentration: (○) 42, (△) 44, (□) 46, (●) 48 °C.

free energy per mole of added glycerol at 45 °C is plotted for both proteins against glycerol concentration in the inset of the same figure. This free-energy change decreases with increasing glycerol content in the case of chymotrypsinogen from 300 cal (mol of protein)$^{-1}$ (mol of added glycerol)$^{-1}$ L$^{-1}$ in a 10% glycerol solution to 220 cal (mol of protein)$^{-1}$ (mol of added glycerol)$^{-1}$ L$^{-1}$ in a 40% glycerol solution. Although not very large, these values of the standard free energy increments are sufficient to stabilize the proteins effectively, since the net free energy of stabilization of proteins is in general small ($\sim$10 kcal/mol). As seen in the figure, it is clear that RNase is stabilized by glycerol better than chymotrypsinogen, the difference in stabilization free energy between the two proteins being 2–2.6 cal (mol of amino acid residue)$^{-1}$ (mol of added glycerol)$^{-1}$ L$^{-1}$.[2] The difference may be related to the greater instability of RNase toward thermal perturbation, since at pH 2.00 RNase exists in a partly unfolded state even at 20 °C while chymotrypsinogen is stable at the same temperature. According to Gerlsma (1968), the degree of change in $T_m$ with glycerol decreases with an increase in pH and the accompanying increase in structural stability. Thus, glycerol should play a more effective stabilizing role at conditions at which a protein is more unstable, such as RNase at pH 2.8, than at conditions where it is very stable, such as chymotrypsinogen at pH 2.0.

The values of $\Delta H°$ were obtained from the van't Hoff plots, shown in Figures 3 and 4. Since these plots are curved for all the systems investigated, an estimate of the variation of $\Delta H°$ with glycerol requires that the slopes at different glycerol concentrations be compared at the same temperature. Extrapolation of the data to a temperature range far from the transition region, however, must be carried out with extreme caution (Brandts, 1964; Brandts & Hunt, 1967). Such an extrapolation is carried out with the assumption that $K$ is a unique uniform function of temperature over the desired temperature range. The higher uncertainty of the values of $K$ at temperatures near the two end points of the transition region can be expected to result in an appreciable error in the evaluation of $\Delta H°$, and this extrapolation may introduce some uncertainty into conclusions on the effect of glycerol on the thermodynamic parameters of denaturation, in particular since this effect is considerably smaller than those of ethanol and urea. In view of these considerations, $\Delta H°$ was estimated only over a small temperature span in the transition region by least squaring the data with the assumption that $\Delta G°$ is a second-order function of temperature. The deduced values of $\Delta H°$ carry an experimental error of $\pm$5 kcal/mol. Furthermore, the observed $\Delta H°$ is an apparent value because of possible small increases in pH due to proteon binding during denaturation and a resulting shift of the denaturation equilibrium, even in buffered solutions (Privalov et al., 1971). The changes in enthalpy, $\Delta(\Delta H°)$, and the entropy, $\Delta(\Delta S°)$ of denaturation brought about the addition of a given amount of glycerol ($c$%) are given by

$$\Delta(\Delta H°) = \Delta H°(c) - \Delta H°(0) \quad (6)$$

$$\Delta(\Delta S°) = \Delta S°(c) - \Delta S°(0) \quad (7)$$

The values of $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ for chymotrypsinogen are plotted in Figure 6 as a function of glycerol concentration at several temperatures. Within the stated errors of this procedure, a gradual decrease in $\Delta H°$ is evident with an increase in glycerol content in the lower temperature range of the transition region. At higher temperatures, this tends to level off in 20–30% glycerol solution and to diminish with a further increase in glycerol content. The change in $\Delta S°$ follows a

[2] As the conditions used for the RNase and chymotrypsinogen denaturations are not identical, this comparison should be regarded with caution. However, the pH difference may have only a small effect on the glycerol stabilization of proteins, since the effect of ethanol on the stability of chymotrypsinogen is known to be independent of pH and ionic strength (Brandts, 1964a,b).

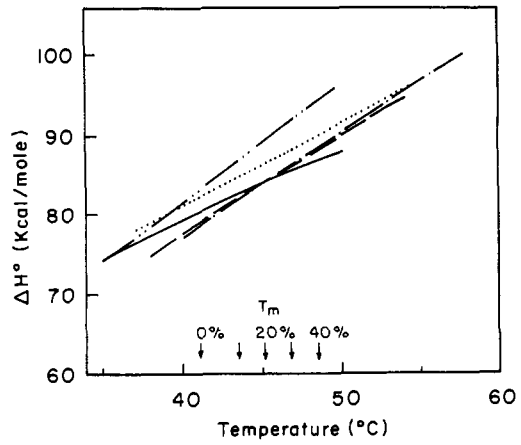


FIGURE 7: Temperature dependence of $\Delta H°$ for RNase in aqueous glycerol solutions. The glycerol concentrations are (—··—) 0, (—) 10, (·····) 20, (— — —) 30, (—·—) 40%. The arrows in the figure represent the melting points.

similar trend, so that in the thermal denaturation of chymotrypsinogen, the decrease in $\Delta H°$ induced by glycerol is compensated by a large decrease in $\Delta S°$, with a resulting small increase in $\Delta G°$. The stabilization of chymotrypsinogen by glycerol seems, therefore, to be the result of an entropic rather than an enthalpic effect. Brandts (1964) has shown 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. Furthermore, $\Delta H°$ of the thermal denaturation of RNase in the same medium increases with increasing ethanol content, with a maximum around an ethanol concentration of 20 vol % (Brandts & Hunt, 1967). In general, it would seem reasonable to regard stabilization and denaturation as a thermodynamically continuous phenomenon. Since protein stabilization by glycerol is a process opposite to the enhancement of protein denaturation by ethanol, one should expect from the work of Brandts & Hunt (1970) that glycerol would induce a decrease in $\Delta H°$ and $\Delta S°$ of denaturation, a prediction borne out by our results.

The values of $\Delta H°$ for RNase in glycerol solutions are presented as a function of temperature in Figure 7. These are found to have little dependence on glycerol concentration, although the values seem, in general, to be lowered by the addition of glycerol, as expected. The observed increase in $\Delta G°$ can, therefore, be attributed to a decrease in $\Delta S°$, which compensates the small change in $\Delta H°$. These results are consistent with the observations by Brandts & Hunt (1967) and of Schrier et al. (1965) that, at these temperatures, $\Delta H°$ of RNase denaturation has either a small dependence or no dependence at all on solvent composition in ethanol–water mixtures.

The enthalpy, $\Delta H°_{T_m}$, and the entropy, $\Delta S°_{T_m}$, changes at the transition temperature, listed in Table I, display small increases in the case of chymotrypsinogen with increase in glycerol concentration but appear to have little such dependence for ribonuclease. As is evident from Figures 3 and 4, the denaturation process is accompanied by a positive change in heat capacity, $\Delta C_p$, for both proteins, suggesting an increase in water ordering. The current experiments, however, cannot detect any effect of glycerol on $\Delta C_p$. Nevertheless, while it is not possible at present to offer a definitive explanation for these observations on the molecular level, it may seem attractive to speculate on the possible stabilizing effect of solvent ordering around protein molecules (Eagland, 1974).

The patterns of preferential interaction between proteins and solvent components in aqueous glycerol medium, described in the preceding paper (Gekko & Timasheff, 1981), have led to the proposal that, in this system, preferential hydration may be explained in terms of an enhancement of the hydrophobic effect and the consequent increase in solvent ordering around the protein when glycerol is added to the medium. These concepts may be extended as well to the mechanism of protein stabilization by glycerol under isothermal conditions. At present, the conformational stability of proteins in aqueous medium is viewed as the result primarily of the pressure which water exerts on nonpolar residues, compressing them into the interior of the molecule. Conversely, the disruption of protein structure by the addition of various denaturing agents, such as organic solvents, urea, or guanidine hydrochloride, is due to the direct interaction of these agents with the protein and their effect on the structure of the water in contact with the nonpolar residues (Kauzmann, 1959; Tanford, 1962; Nemethy & Scheraga, 1962; Timasheff, 1964, 1970; Lee and Timasheff, 1974; Von Hippel & Wong, 1965; Robinson & Jencks, 1965). Aqueous glycerol solutions should belong to the first class of media, since any increase in the hydrophobicity of proteins in aqueous glycerol wound render even more unfavorable the exposure to solvent of hydrophobic groups buried in the interior of native proteins, while those that are exposed would be under even greater pressure to enter into the interior of the protein, even though this may not be possible. Such pressure should result in the stabilization of the native protein conformation. A similar explanation based on solvent ordering has been proposed for the stabilizing effect of ethanol on proteins at low temperatures and low ethanol concentrations (8–15% w/w). According to this 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 (Brandts & Hunt, 1967; Pohl, 1968b; Brandts, 1969; Eagland, 1974). In the case of ethanol, this structure is disrupted at high 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 denaturation of the protein. A similar explanation has been proposed for the denaturation of proteins by 2-chloroethanol (Timasheff, 1970). In the case of aqueous glycerol, such a solvation sheath around the exposed nonpolar groups of a protein should remain intact at relatively high temperatures, since the hydrophobicity of glycerol is considerably smaller than that of ethanol. It should be recalled, furthermore, that the interaction of glycerol 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 glycerol molecules with the water of hydration around the nonpolar groups of the protein. It appears, therefore, that conceptually the glycerol stabilization of proteins against thermal denaturation may be accounted for reasonably in terms of the thermodynamically related phenomena of solvent ordering around a protein and steric exclusion of bulky solvent components from contact with the protein surface.

Let us examine this in a more quantitative manner. In a water–glycerol mixture, the equilibrium constant, $K$, for protein denaturation at constant temperature and pressure is related to solvent composition by

$$\mathrm{d} \ln K = \left(\frac{\partial \ln K}{\partial \ln a_{\mathrm{glyc}}}\right)_{a_{H_2O}} \mathrm{d} \ln a_{\mathrm{glyc}} + \left(\frac{\partial \ln K}{\partial \ln a_{H_2O}}\right)_{a_{\mathrm{glyc}}} \mathrm{d} \ln a_{H_2O} \quad (8)$$

where $a_{\mathrm{glyc}}$ and $a_{H_2O}$ are the activities of glycerol and water,

respectively. When the Wyman equation (164) is used

$$\left(\frac{\partial \ln K}{\partial \ln a_i}\right)_{a_{j\neq i}} = \Delta\bar{\nu}_i \tag{9}$$

rearrangement of eq 8 gives (Aune et al., 1971; Lee & Timasheff, 1977; Timasheff, 1978)

$$\left(\frac{\mathrm{d} \ln K}{\mathrm{d} \ln a_{\mathrm{glyc}}}\right) = \Delta\bar{\nu}_{\mathrm{glyc}} + \Delta\bar{\nu}_{\mathrm{H_2O}}\left(\frac{\mathrm{d} \ln a_{\mathrm{H_2O}}}{\mathrm{d} \ln a_{\mathrm{glyc}}}\right) \tag{10}$$

where $\Delta\bar{\nu}_i$ is the change in the number of molecules of $i$ bound to the macromolecule during the course of the reaction. For multicomponent systems, the quantity d ln $K$/d ln $a_i$ is the difference between the preferential binding of solvent components, $\Delta\xi$, to the two end states of the reaction in question (Tanford, 1969). Then

$$\left(\frac{\mathrm{d} \ln K}{\mathrm{d} \ln a_{\mathrm{glyc}}}\right) = \Delta\xi_{\mathrm{glyc}} = \left(\frac{\partial m_{\mathrm{glyc}}}{\partial m_{\mathrm{p}}}\right)^{\mathrm{D}}_{T,P,\mu_{\mathrm{glyc}}} - \left(\frac{\partial m_{\mathrm{glyc}}}{\partial m_{\mathrm{p}}}\right)^{\mathrm{N}}_{T,P,\mu_{\mathrm{glyc}}} \tag{11}$$

where $m_i$ is molal concentration of component $i$, $\mu_i$ is its chemical potential, and the subscript p refers to protein. Preferential interaction is related to the total interaction of solvent components with protein, $\bar{\nu}_i$, by (Inoue & Timasheff, 1972; Kupke, 1973; Reisler et al., 1977)

$$\left(\frac{\partial m_{\mathrm{glyc}}}{\partial m_{\mathrm{p}}}\right)_{T,P,\mu_{\mathrm{glyc}}} = \bar{\nu}_{\mathrm{glyc}} - \frac{m_{\mathrm{glyc}}}{m_{\mathrm{H_2O}}}\bar{\nu}_{\mathrm{H_2O}} \tag{12}$$

Therefore, $\Delta\xi_{\mathrm{glyc}}$ can be expressed as

$$\Delta\xi_{\mathrm{glyc}} = \bar{\nu}^{\mathrm{D}}_{\mathrm{glyc}} - \bar{\nu}^{\mathrm{N}}_{\mathrm{glyc}} - \frac{m_{\mathrm{glyc}}}{m_{\mathrm{H_2O}}}(\bar{\nu}^{\mathrm{D}}_{\mathrm{H_2O}} - \bar{\nu}^{\mathrm{N}}_{\mathrm{H_2O}}) \tag{13}$$

Plots of the logarithm of the equilibrium constant for the thermal denaturation vs. the logarithm of the glycerol activity give a close to linear relationship at any temperature in the transition region, as shown in Figure 8, where the activity of glycerol, $a_{\mathrm{glyc}}$, was calculated from osmotic coefficient data (Scatchard et al., 1938). For RNase, this plot results in a series of straight lines, with a slope of −0.93 ± 0.05, independent of temperature. On the other hand, $\Delta\xi_{\mathrm{glyc}}$ for chymotrypsinogen becomes increasingly negative with increasing temperature. The requirement for $\Delta\xi_{\mathrm{glyc}}$ to be negative is $(\partial m_{\mathrm{glyc}}/\partial m_{\mathrm{p}})^{\mathrm{N}}_{T,P,\mu_{\mathrm{glyc}}} > (\partial m_{\mathrm{glyc}}/\partial m_{\mathrm{p}})^{\mathrm{D}}_{T,P,\mu_{\mathrm{glyc}}}$. It has been found that all proteins examined (Gekko & Timasheff, 1981; Na & Timasheff, 1981) have negative values of $(\partial m_{\mathrm{glyc}}/\partial m_{\mathrm{p}})_{T,P,\mu_{\mathrm{glyc}}}$ in the native state in aqueous glycerol medium; i.e., the proteins are preferentially hydrated, or glycerol is preferentially excluded from the immediate domain of the protein. For example, the value of this parameter in 30% glycerol solution is −11.9 ± 3.0 for RNase and −34.2 ± 2.8 for chymotrypsinogen at 20 °C. The data of Figure 8 show, therefore, that $(\partial m_{\mathrm{glyc}}/\partial m_{\mathrm{p}})^{\mathrm{D}}_{T,P,\mu_{\mathrm{glyc}}}$ must be more negative than $(\partial m_{\mathrm{glyc}}/\partial m_{\mathrm{p}})^{\mathrm{N}}_{T,P,\mu_{\mathrm{glyc}}}$, i.e., glycerol is more strongly excluded from the denatured protein than from the native one. The calculation of the additional glycerol exclusion brought about by denaturation, namely, $\bar{\nu}^{\mathrm{D}}_{\mathrm{glyc}} - \bar{\nu}^{\mathrm{N}}_{\mathrm{glyc}}$, is precluded by the fact that the glycerol concentration used in the present studies is too high to approximate the term $(m_{\mathrm{glyc}}/m_{\mathrm{H_2O}})$ in eq 13 by zero and by the lack of knowledge of the difference in protein hydration between the native and denatured states.

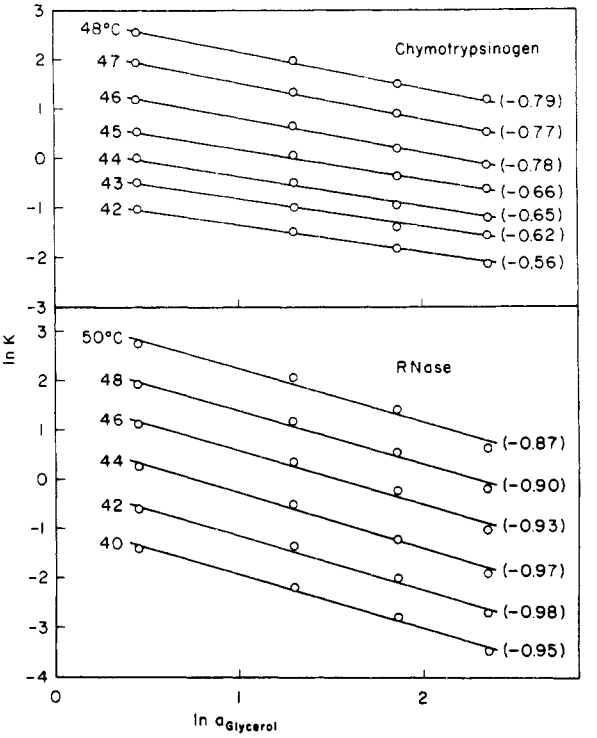


FIGURE 8: Wyman plots of the effect of glycerol on protein unfolding for chymotrypsinogen and RNase at several temperatures. The numbers at the left end of each line show the temperature and the numbers in parentheses at the right end indicate the slopes of the lines, i.e., $\Delta\xi_{\mathrm{glyc}}$, calculated by least squares.

How does the increase in glycerol exclusion bring about structure stabilization? Preferential interaction is strictly an activity coefficient effect:

$$\left(\frac{\partial m_{\mathrm{glyc}}}{\partial m_{\mathrm{p}}}\right)_{T,P,\mu_{\mathrm{glyc}}} = -\left(\frac{\partial \mu_{\mathrm{glyc}}}{\partial m_{\mathrm{p}}}\right)_{T,P,m_{\mathrm{glyc}}} \Big/ \left(\frac{\partial \mu_{\mathrm{glyc}}}{\partial m_{\mathrm{glyc}}}\right)_{T,P,m_{\mathrm{p}}} \tag{14}$$

where $\mu_i$ is the chemical potential of component $i$, $\mu_i = \mu^\circ_i(T,P) + RT \ln m_i + RT \ln \gamma_i$, $\gamma_i$ is the activity coefficient of component $i$, and

$$\left(\frac{\partial \mu_{\mathrm{p}}}{\partial m_{\mathrm{glyc}}}\right)^{\mathrm{D}}_{T,P,m_{\mathrm{p}}} - \left(\frac{\partial \mu_{\mathrm{p}}}{\partial m_{\mathrm{glyc}}}\right)^{\mathrm{N}}_{T,P,m_{\mathrm{p}}} = -RT\,\Delta\xi_{\mathrm{glyc}}\left(\frac{1}{m_{\mathrm{glyc}}} + \frac{\partial \ln \gamma_{\mathrm{glyc}}}{\partial m_{\mathrm{glyc}}}\right) \tag{15}$$

Thus, a negative value of $\Delta\xi_{\mathrm{glyc}}$ means that $(\partial\mu_{\mathrm{p}}/\partial m_{\mathrm{glyc}})^{\mathrm{D}}_{T,P,m_{\mathrm{p}}} > (\partial\mu_{\mathrm{p}}/\partial m_{\mathrm{glyc}})^{\mathrm{N}}_{T,P,m_{\mathrm{p}}}$. Since the latter quantity is known to be positive (Gekko & Timasheff, 1981), the denatured system must be thermodynamically even less favorable than the native one. This unfavorable effect can be reduced by a displacement of the equilibrium toward the native state with the net result that the native conformation of the protein is stabilized.

The increase in the chemical potential of the protein or in the preferential exclusion of glycerol, brought about by protein denaturation, has three principal sources: (1) the increase in steric exclusion of glycerol; (2) the enhanced repulsion of glycerol from the newly exposed hydrophobic groups; and (3) the increased absolute hydration around the newly exposed peptide or hydrophobic groups of the denatured protein (see eq 12). How do the enthalpy and entropy changes contribute to the stabilizing effect of glycerol? As shown above, addition of glycerol renders both $\Delta H^\circ$ and $\Delta S^\circ$ of thermal denaturation of chymotrypsinogen less positive. The stabilizing effect of

glycerol is a consequence, therefore, of the decrease in $\Delta S°$ of denaturation. Brandts (1968) has analyzed the thermodynamic parameters of protein denaturation in terms of four principal contributions: (a) the conformational entropy, $\Delta S_c$, which increases by 2–6 eu per unfolded residue, due to the increased rotational freedom of the polypeptide chain ($\Delta S_c > 0$); (b) the enthalpy change, $\Delta H_h$, involved in breaking a peptide–peptide hydrogen bond in the native state and replacing it by a peptide–water hydrogen bond in the denatured state, which is positive ($\Delta H_h > 0$), and the corresponding entropy change, $\Delta S_h$, which is also positive (Kauzmann, 1959; Schellman, 1955) ($\Delta S_h > 0$); (c) the entropy change, $\Delta S_p$, and the enthalpy change, $\Delta H_p$, involved in exposing the hydrophobic side chains buried in the interior of the native protein to solvent (water) on denaturation, which are both negative ($\Delta H_p < 0$, $\Delta S_p < 0$); (d) the electrostatic free energy of the protein, which decreases with unfolding due to the decrease in charge density ($\Delta G_{el} < 0$). Contribution a, which is unrelated to interactions with solvent, should not be different for solvent systems with and without glycerol. The major contributions to $\Delta(\Delta G°)$ must come from contributions b and c, with a possible minor contribution from (d) (Tanford, 1970). It is reasonable to assume that, in this case, contribution d will be negligible, since the decrease in dielectric constant with addition of glycerol is not sufficient to induce ion-pair formation (Singer, 1961). Thus, we may attribute the observed changes in $\Delta H°$ and $\Delta S°$ mainly to contributions b and c, which indicates that protein–solvent interactions are an important factor in the glycerol-induced stabilization of proteins.

An analysis based on protein–solvent preferential interactions similar to that performed above for the free energy change (chemical potential change) can be applied to the enthalpy and entropy changes as well. The following equations must be satisfied at any given temperature:

$$\Delta H_{tr}^{N} + \Delta H°(c) = \Delta H°(0) + \Delta H_{tr}^{D} \quad (16)$$

$$\Delta S_{tr}^{N} + \Delta S°(c) = \Delta S°(0) + \Delta S_{tr}^{D} \quad (17)$$

where $\Delta H_{tr}$ and $\Delta S_{tr}$ are the enthalpy and entropy changes of transferring a protein in the native (superscript N) and denatured (superscript D) states, respectively, from aqueous medium to glycerol solution of $c$%, and $\Delta H°(c)$ and $\Delta H°(0)$ are the corresponding changes in the enthalpy of denaturation. The difference between $\Delta H_{tr}^{D}$ and $\Delta H_{tr}^{N}$ can be regarded as the difference between the enthalpy changes of the preferential interaction with solvent components of a protein in the native and denatured states. Then from eq 16

$$\Delta(\Delta H°) = \Delta H°(c) - \Delta H°(0) = \Delta H_{tr}^{D} - \Delta H_{tr}^{N} = \Delta\xi_{glyc}\Delta H_{pref} \quad (18)$$

where $\Delta H_{pref}$ is the enthalpy change of preferential solvation for $\Delta\xi_{glyc} = 1$. In similar manner

$$\Delta(\Delta S°) = \Delta S°(c) - \Delta S°(0) = \Delta S_{tr}^{D} - \Delta S_{tr}^{N} = \Delta\xi_{glyc}\Delta S_{pref} \quad (19)$$

Therefore, the values of $\Delta H_{pref}$ and $\Delta S_{pref}$ can be estimated from those of $\Delta(\Delta H°)$, $\Delta(\Delta S°)$, and $\Delta\xi_{glyc}$. The calculated values of $\Delta H_{pref}$ for chymotrypsinogen are plotted as a function of temperature in Figure 9. While the experimental error involved in $\Delta H_{pref}$ is greater than that in $\Delta(\Delta H°)$ due to the addition of the uncertainty in $\Delta\xi_{glyc}$, these results indicate that, for chymotrypsinogen, $\Delta H_{pref}$ is positive in the transition temperature region. It is striking that the glycerol stabilization of chymotrypsinogen is characterized by preferential hydration and positive $\Delta H_{pref}$, while the denaturation of lysozyme is accompanied by the preferential binding of the nonaqueous component and negative $\Delta H_{pref}$ (Pfeil & Privalov, 1976). A

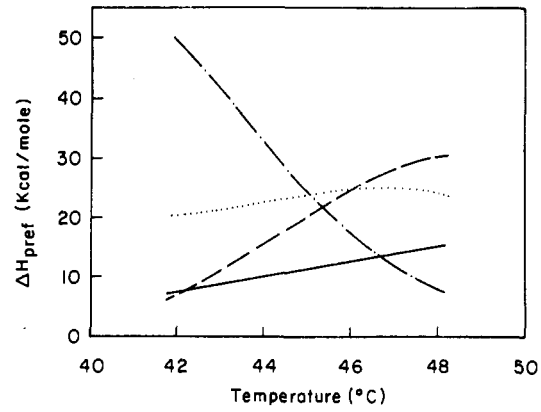


FIGURE 9: Temperature dependence of $\Delta H_{pref}$ of chymotrypsinogen. Glycerol concentrations are (—) 10, (···) 20, (— — —) 30, (—·—) 40%.

positive value of $\Delta H_{pref}$ means that $\Delta H_{tr}^{N} > \Delta H_{tr}^{D}$. Therefore, as shown in eq 19 and the data of Figure 5, $\Delta S_{tr}^{N} > \Delta S_{tr}^{D}$ because $\Delta G_{tr}^{D}$ ($=\Delta H_{tr}^{D} - T\Delta S_{tr}^{D}$) > $\Delta G_{tr}^{N}$ ($=\Delta H_{tr}^{N} - T\Delta S_{tr}^{N}$) > 0. The increase in the chemical potential of the protein on denaturation is brought about, therefore, by the decrease in the entropic contribution of preferential interaction with solvent components. The value of $\Delta H_{pref}$ for RNase was found to be small, even though $\Delta\xi_{glyc}$ is larger for RNase than for chymotrypsinogen, since $\Delta H°$ for RNase is much less affected by the addition of glycerol.

The preferential interaction phenomenon is not a measure of the absolute stoichiometric interactions between the solvent components and the macrosolutes. It is only a measure of the relative affinities of the solvent components for the protein. As proposed in the previous paper, the interaction between glycerol and protein is totally nonspecific and the glycerol molecules may penetrate into the solvation sheath of the protein as a result of the delicate balance between repulsion from nonpolar regions and attraction from polar regions of the protein surface, since glycerol is essentially hydrophilic and can interact strongly with water. Therefore, at present, it would seem difficult to estimate separately the contributions of glycerol to peptide–water interactions (contribution b) and to hydrophobic group–water interactions (contribution c). The hydrophobic effect may constitute an important factor, since solvent molecules should become ordered around the newly exposed nonpolar groups of a denatured protein and the dominant cause of the enhancement of glycerol exclusion from the protein would be repulsion from the hydrophobic regions of the protein. On the other hand, it is not likely that glycerol molecules become bound directly to the peptide groups of proteins. This does not necessarily imply that the effect of glycerol on contribution b is negligible, since replacement of peptide–peptide hydrogen bonds by peptide–water hydrogen bonds may be accompanied by reorganization of hydrogen bonds in the solvation sheath through long-range interactions. Nevertheless, the presently described results are consistent with the concept that the protein stabilization induced by glycerol is totally due to a nonspecific solvent effect.

The same interpretation may be valid for the denaturation of RNase, for which both $\Delta H°$ and $\Delta S°$ have but weak dependence on solvent composition. RNase is one of the most hydrophilic globular proteins. It has an average hydrophobicity, $\Delta H_{oaver}$, of 870 cal/residue and contains 49 nonpolar amino acids per molecule. On the other hand, chymotrypsinogen has a hydrophobicity of 1040 cal/residue and contains 112 nonpolar amino acids per molecule (Bigelow, 1967; Gerlsma, 1970). Even if we take into consideration the dif-

ference in the structural parameter, $p$, which describes the cooperative unit that unfolds during denaturation, i.e., 80% for RNase and 55% for chymotrypsinogen (Brandts & Hunt, 1967), the number of newly exposed hydrophobic groups following denaturation is considerably smaller in RNase than in chymotrypsinogen (Pohl, 1969). Therefore, the hydrophobic contributions, $\Delta S_p$ and $\Delta H_p$, to the thermodynamic parameters should be considerably smaller for RNase than for chymotrypsinogen and may not be measurable by our techniques.

*Kinetics of Thermal Denaturation.* While kinetics offer more useful information than thermodynamics on the mechanism of protein stabilization, analysis of the data is more complex. Until recently, the kinetics of the reversible unfolding of proteins have been analyzed by the two-state model with the assumption that all intermediates were present in negligible amounts (Pohl, 1968a,b, 1969; Lumry & Biltonen, 1969). The experimental basis for the two-state model was the observation that the ratio of the calorimetric enthalpy or heat capacity change to the corresponding van't Hoff quantity was nearly unity. This criterion, of course, would not be valid if any of the reactions in a sequence proceeded with a zero enthalpy change. More recent kinetic studies have shown the existence of detectable intermediate species in the process. Tsong and co-workers (Tsong, 1972; Tsong & Baldwin, 1972a,b; Tsong, 1973) have proposed a nucleation-dependent, sequential model, in which folding starts by a nucleation step, followed by a series of equal propagation steps. Ikai & Tanford (1971), Ikai et al. (1973), and Tanford et al. (1973) have analyzed the kinetic data of protein denaturation in guanidine hydrochloride solutions by applying a three-state model, in which an abortive, or dead-end, intermediate is formed rapidly after the initiation of refolding. Recent detailed kinetic studies (Hagerman & Baldwin, 1976; Nall et al., 1978; Brandts et al., 1975; Garel & Baldwin, 1975) have led to the proposal of a three-state mechanism in which native protein (N) is equilibrated with two forms of unfolded protein ($U_1$, $U_2$) with markedly different rates of refolding. When the notations of Hagerman & Baldwin (1976) and Nall et al. (1978) are used, this process can be expressed as

$$\mathrm{N} \underset{k_{23}}{\overset{k_{32}}{\rightleftarrows}} \mathrm{U_2} \underset{k_{12}}{\overset{k_{21}}{\rightleftarrows}} \mathrm{U_1} \qquad (20)$$

fast slow

According to this model, the concentration of the native protein changes biphasically with two relaxation times, $\tau_2$ (fast phase) and $\tau_1$ (slow phase). At the limiting conditions, $\tau_1/\tau_2 \gg 1$ and $k_{23}, k_{32} \gg k_{12}, k_{21}$:

$$1/\tau_1 = k_{12} + k_{21}/(1 + k_{23}/k_{32}) \qquad (21)$$

$$1/\tau_2 = k_{23} + k_{32} \qquad (22)$$

If the three-species model is as valid a description of protein denaturation in glycerol solution as it is in aqueous solution, a complete understanding of the mechanism of protein stabilization by glycerol should require knowledge of the effect of glycerol on each rate constant.

In the present study we have measured the effect of glycerol only on the slow unfolding reaction, since the time scale in our methods precluded the observation of the fast phase with a time constant of milliseconds. Since before the thermal perturbation the proteins were in their native (N) state, the results were expressed in terms of a pseudo-first-order reaction

$$(\Delta A)_t = (\Delta A)_0 \exp(-k_{21}{}^{\mathrm{app}} t) \qquad (23)$$

where $(\Delta A)_0$ is the difference between the absorptions of the final and initial states, and hence equilibrium concentrations, $(\Delta A)_t$ is the difference between the absorptions, and hence the

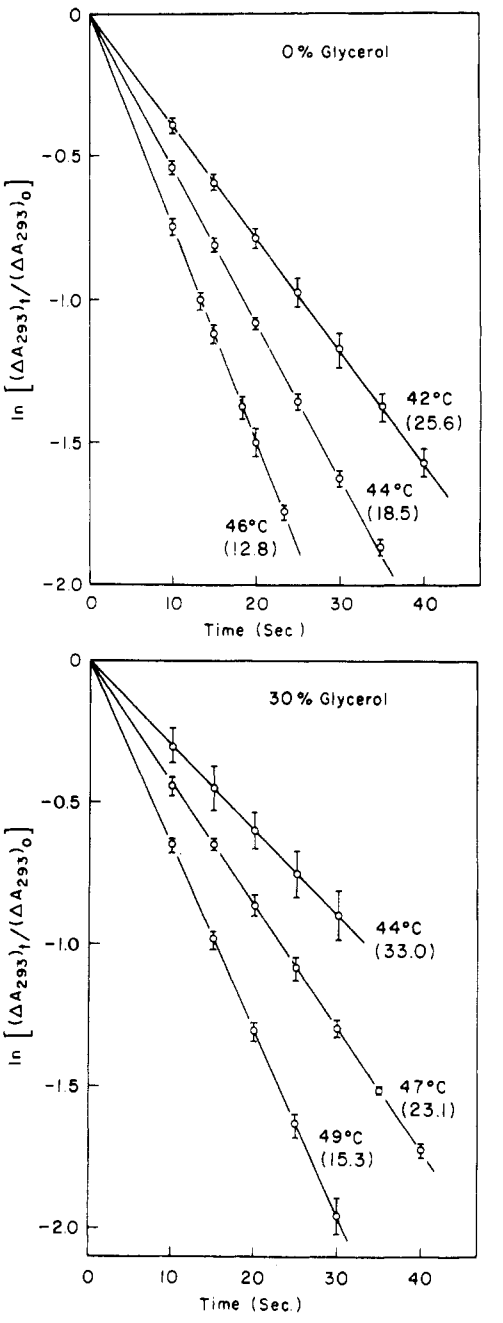


FIGURE 10: First-order plots of the kinetic data for the thermal denaturation of chymotrypsinogen (a, upper) in water of pH 2.00 and (b, lower) in 30% glycerol solution of pH 2.00. The numbers in parentheses under each temperature are $1/k_{21}{}^{\mathrm{app}}$ in seconds.

concentrations of the species at time $t$ and in the initial states, and $k_{21}{}^{\mathrm{app}}$ is the apparent first-order rate constant for the slow unfolding.

Typical first-order plots of the kinetic data for chymotrypsinogen, presented in Figure 10, show a linear time dependence of $\ln (\Delta A_{293})_t/(\Delta A_{293})_0$ at all temperatures and all glycerol concentrations studied. Similar results were observed for RNase. The values of $k_{21}{}^{\mathrm{app}}$ calculated from the slopes of these straight lines are presented in Arrhenius plots in Figure 11. As seen in Figure 11, $(k_{21}{}^{\mathrm{app}})^{-1}$ decreased with increasing temperature in the transition zone in a manner similar to that reported for other proteins (Tsong & Baldwin, 1972a,b; Hagerman & Baldwin, 1976; Tsong, 1973). Our value of $1/k_{21}{}^{\mathrm{app}}$ for RNase appears to be close to the value of $\tau_1$ measured at pH 3.0 by Hagerman & Baldwin (1976). For both proteins, there is a linear relationship between $\ln k_{21}{}^{\mathrm{app}}$ and $1/T$ in the transition zone above the transition tempera-

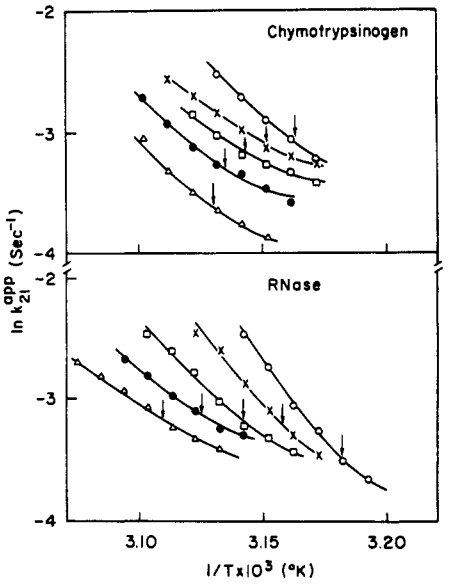


FIGURE 11: Variation of $k_{21}^{app}$ as a function of temperature in the denaturation of chymotrypsinogen and RNase in aqueous solutions with glycerol contents of (○) 0, (×) 10, (□) 20, (●) 30, (△) 40%. The arrow next to each curve represents the transition temperature, $T_m$, in the system.

Table II: Apparent Activation Energy in the Transition Zone above $T_m$

| glycerol (% v/v) | $E_a^{app}$ (kcal/mol) | |
|---|---|---|
| | RNase | chymotrypsinogen |
| 0 | 49 | 37 |
| 10 | 44 | 28 |
| 20 | 38 | 39 |
| 30 | 32 | 42 |
| 40 | 24 | 48 |

ture, $T_m$, permitting the calculation of an apparent activation energy, $E_a^{app}$, since

$$\partial(\ln k_{21}^{app})/\partial(1/T) = E_a^{app}/R \quad (24)$$

The values of $E_a^{app}$ calculated by the least-squares method are listed in Table II. It appears that $E_a^{app}$ for RNase decreases with increasing glycerol contents while for chymotrypsinogen no such trend is seen within our experimental error. These data, however, are not sufficient to identify the process which corresponds to the measured activation energy in the transition zone, since inside this zone the slow $U_2 \rightleftharpoons U_1$ reaction is coupled to the fast $N \rightleftharpoons U_2$ reaction.

A detailed discussion based on the three species model is outside the scope of this study since we have obtained kinetic data for only the slow phase. Nevertheless, it seems of interest to examine the state in the denaturation equilibrium at which glycerol exercises its effect. For both proteins, addition of glycerol decreases the apparent rate of unfolding by a factor of 2–3 at 40% glycerol. Equation 21 shows that this could be due to an effect either on the slow reaction rate constants or on $K_{23} = k_{23}/k_{32}$. Tsong & Baldwin (1978) have shown that, for ribonuclease at pH 7.0, glycerol accelerates the slow refolding reaction but has no effect on $\tau_2$, suggesting that the stabilizing effect is exercised at the stage of the slow reaction. Currently there is general agreement that the slow-folding species is the product of proline isomerization (Brandts et al., 1975; Schmid & Baldwin, 1978). In a careful examination of the process, Cook et al. (1979) have found that there is a linkage between the folding and isomerization reactions such that the folding process actually increases the rate of proline isomerization during folding. This folded state, however, need not be exactly identical with the starting native molecule. Since it is known that proline isomerization is affected neither by guanidine hydrochloride (Nall et al., 1978; Cheng & Bovey, 1977) nor by $(NH_4)_2SO_4$ (Cook et al., 1979), one might expect the same to be true of glycerol. If this is so, then glycerol could, in fact, exercise its effect on the process described by Cook et al. (1979), either accelerating the refolding or retarding the unfolding reaction. Such a thesis is fully consistent with the finding by Cook et al. (1979) that the structure-stabilizing salt, $(NH_4)_2SO_4$, speeds up the tyrosine-detected folding reaction in RNase but has little effect on the kinetics of proline isomerization during folding. A similar relation between structure stabilization and an acceleration of refolding has been found for guanidine sulfate (Tsong & Baldwin, 1978). In this respect, it seems significant that, just like glycerol, $(NH_4)_2SO_4$ and guanidine sulfate are preferentially excluded from the immediate domain of the protein (T. Arakawa and S. N. Timasheff, unpublished results).

What then is the nature of the glycerol effect on the molecular level? Schmid & Baldwin (1979) have shown that guanidine hydrochloride acts by destabilizing hydrogen-bonded intermediates. Glycerol, on the other hand, should have little effect on hydrogen-bonded structures. One possibility it that it acts by stabilizing hydrophobic interactions through solvent ordering as discussed above. This could manifest itself either in an acceleration of the slow folding (Cook et al., 1979) or in an increase in the activation energy of the unfolding, i.e., expansion of the molecule, and a retardation of the slow unfolding reaction. In either case, the stabilized structure would be compact, although the tertiary structure in the first case would not need to correspond exactly to that of the native molecule. This analysis is analogous to the "subtle conformational change" model of Lumry & Biltonen (1965) according to which the number of exposed amino acid residues in the activated complex is similar to that in the folded species, the gross unfolding occurring during the transition from the activated state to the denatured state with the concomitant exposure of large numbers of hydrophobic and peptides groups to solvent. This would be the step affected by glycerol. According to this model, the dependence of $\Delta H°$ of unfolding on solvent composition could be interpreted in terms of a redistribution of solvent components about the newly exposed hydrophobic side chains and peptide groups. In this regard, it seems pertinent to note that in the case of $\beta$-lactoglobulin, addition of glycerol decreases the heat capacity of the protein denatured in urea, while there is no such effect on the native protein (DiPaola & Belleau, 1978).

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