Role of Diffusion in the Folding of the α Subunit of Tryptophan Synthase from *Escherichia coli*†

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ABSTRACT: The rate-limiting step in the folding of the α subunit of tryptophan synthase has been proposed to be the association of two folding units. To probe the role of diffusion in this rate-limiting step, the urea-induced unfolding and refolding of the protein was examined in the presence of a number of viscosity-enhancing agents. The analysis was simplified by studying the effect of these agents on folding unit dissociation, the rate-limiting unfolding reaction, and the reverse of the rate-limiting step in refolding. In the presence of ethylene glycol, the relaxation times for unfolding to the same final conditions increased with increasing concentration of the cosolvent. When the effects of the cosolvent on protein stability were taken into account, the rates were found to show a unitary linear dependence on the viscosity of the solution. Similar results were obtained with glycerol and low concentrations of glucose, demonstrating that the effect is general and not specific to any viscogenic agent. These results clearly demonstrate that the rate-limiting folding unit association/dissociation reaction in the α subunit of tryptophan synthase involves a diffusional process.

The complex conformational change that occurs when proteins fold to their unique, native conformations must involve diffusional motions of the polypeptide through the solvent. An unfolded protein has a larger hydrodynamic radius and is generally devoid of secondary and tertiary structure (Tanford, 1968). Thus, both the condensation of the unfolded protein and the formation of higher order structure during folding require significant reordering of the polypeptide in the solvent. What is less clear is whether such diffusional motion is rate limiting in folding (Karplus & Weaver, 1976) or whether the rate-limiting process is the formation of organized structure (Baldwin, 1980).

An early study on the effects of viscogenic agents on the refolding kinetics of ribonuclease A reported that the fast folding phase was not affected by increased solvent viscosity; curiously, the slow phase was slightly accelerated (Tsong & Baldwin, 1978). The authors concluded that the formation of structure must be the rate-limiting step in folding rather than friction-limited segmental motion. Tsong (1982) found in a temperature jump study on the thermal unfolding of ribonuclease A that a relaxation in the millisecond time range had a linear dependence on the solution viscosity; two slower processes did not. The slope of this linear dependence varied in a monotonic fashion with the molecular volume of the polyhydric alcohol used to alter the viscosity. More recently, Garel and his colleagues (Vaucheret et al., 1987; Teschner et al., 1987) have used monomeric and polymeric polyhydric alcohols to examine the effect of solvent viscosity on the folding of octopine dehydrogenase and aspartokinase–homoserine dehydrogenase. In both cases, complex effects were observed in that some kinetic phases depended upon the viscosity while others did not.

These results suggest that diffusion through solvent can play an important role in protein folding. However, the complexities involved in each case suggest that it would be advantageous to examine a simpler system in order to obtain a quantitative description of the role of diffusion in the rate-limiting steps in folding.

A particularly interesting and relatively simple system in which the effects of solvent viscosity on the kinetics of folding can be studied is the α subunit of tryptophan synthase from *Escherichia coli*. A stable folding intermediate has been detected for both urea (Matthews & Crisanti, 1981) and guanidine hydrochloride induced unfolding (Yutani et al., 1979). This intermediate appears to have a folded amino-terminal region, residues 1–188, and an unfolded carboxy-terminal region, residues 189–268 (Higgins et al., 1979; Miles et al., 1981). The X-ray structure (Hyde et al., 1988) shows that the amino region corresponds to the first six strands and five helices of this α/β barrel protein while the carboxy region corresponds to the last two strands and three helices. For simplicity, these two regions will be designated as the amino and carboxy folding units. The term domain has usually been associated with independent structural units, a situation which does not appear to be the case for the α subunit.

Kinetic studies on the reversible folding reaction for wild-type and mutant α subunits have demonstrated that replacements in either folding unit can affect the rate-limiting step in folding (Beasty et al., 1986a). Thus, it was proposed that the rate-limiting step involves the association/dissociation of the amino and carboxy folding units. Because the two segments of the polypeptide must diffuse through solvent in order to associate, this system offers a direct test of the role of diffusion in limiting the folding of proteins.

In a limited study on the α subunit, Hurle et al. (1987) found that the presence of 0.58 M sucrose slowed both unfolding and refolding reactions. However, the dependence was less than first order, leading the authors to speculate that the folding unit association reaction might be more complex than a simple diffusion process. The present study involves a systematic investigation of this problem, monitoring the dissociation reaction, with the use of several different viscogenic agents. A common transition state for the refolding/unfolding reaction justifies the study of the unfolding reaction. The results show that the folding unit association/dissociation

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reaction follows the behavior expected for friction-limited polymer diffusion.

Materials and Methods

α Subunit of Tryptophan Synthase. The α subunit of tryptophan synthase from *Escherichia coli* (EC 4.2.1.20) was isolated from strain W3110 (ΔtonB-trp)BA17 *his* containing plasmid pBN55 (a gift from Brian Nichols) as discussed previously (Beasty et al., 1986a). The purity of the protein used in these studies was verified by the presence of a single band in both native and sodium dodecyl sulfate (NaDodSO$_4$)[1]-polyacrylamide gel electrophoresis. The activity of the α subunit was determined by measuring its ability to enhance the activity of the β_2 subunit in the condensation of indole and serine to form tryptophan; the maximum specific activity in this assay is 5500 units/mg (Yanofsky et al., 1967). The specific activity of the protein was determined to be 3500 ± 500 units/mg. Protein concentrations were determined from the optical spectrum by using the specific absorption $E^{278}_{1\%} = 4.4$ (Adachi et al., 1974); the molar extinction coefficient is calculated to be 12600 M^{-1} cm^{-1}.

Chemicals. Ultrapure urea was purchased from Schwarz/Mann and used without further purification. Ethylene glycol was certified Fisher grade, glycerol was Baker analyzed anhydrous, and glucose was Fisher reagent grade. Poly(ethylene glycol) (PEG) (approximate molecular weight 8000) was obtained from Sigma. All cosolvents were used without further purification.

Methods. The equilibrium unfolding of the protein in the presence of the cosolvents was performed by using the tandem cell technique of Herskovits (1967). Samples were prepared in the presence of cosolvent and varying amounts of urea, and the absorbance spectrum was scanned from 350 to 250 nm following equilibration. The difference in the absorbance at 287 nm was used to determine the change in the extinction coefficient as a function of the urea concentration.

Unfolding jumps were initiated by injection of protein stock solution in a microliter syringe to the preincubated buffer/urea solutions, followed by manual mixing. Refolding jumps were performed similarly, with the exception that the protein was first unfolded in 6 M urea in the presence of the cosolvent to reduce schlieren effects upon mixing. The kinetics of unfolding (refolding) were followed by observing the time-dependent changes in absorbance at 287 nm on an AVIV 118DS spectrophotometer. Data from both kinetic and equilibrium experiments were fit by using methods described previously (Beasty et al., 1986a; Hurle et al., 1987). To correct a previous typographical error we note that the equilibrium data were fit to the equation

$$F_{app} = \frac{K_{NI}(Z + K_{IU})}{1 + K_{NI} + K_{NI}K_{IU}}$$

where F_{app} is the fraction of unfolded protein, K_{XY} is the equilibrium constant between species X and Y, and Z is the fractional change in the total extinction coefficient of the N ↔ I transition. The final concentration of protein ranged from 0.6 to 1.2 mg/mL, a range in which the folding of the α subunit shows no concentration dependence. All experiments were performed in 10 mM potassium phosphate, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol buffer, pH 7.8, at 25 °C.

Viscosities were measured by using a Cannon-Fenske viscometer size 50 (Induchem Lab Glass Co.) calibrated with distilled, deionized water. The instrument constant was found to be (4.25 ± 0.10) × 10^{-3} s^{-2}, based on the mean of 10 measurements. The efflux times for the cosolvent/buffer mixtures were the average of a minimum of three measurements. The temperature was 25 ± 0.5 °C.

Results

The use of polyols as viscogenic agents is complicated by the effect of these cosolvents on the stability of proteins. Timasheff and his colleagues (Arakawa & Timasheff, 1982; Gekko & Timasheff, 1981a,b; Lee & Timasheff, 1981) have investigated the thermodynamic aspects of this problem and attributed the effect to preferential hydration of hydrophobic surfaces and/or changes in the surface tension of water. The complication with regard to the evaluation of folding kinetics comes from the fact that changes in stability, i.e., the equilibrium constant, imply changes in the rate constants for the folding reaction. Thus, to determine whether the kinetic aspects of folding actually depend on viscosity, one must account for the effect on stability.

Equilibrium Studies. The effects of increasing concentrations of ethylene glycol on the urea-induced equilibrium unfolding transition are shown in Figure 1a; these data are based upon changes in the molar extinction coefficient at 287 nm and reflect changes in solvent exposure of buried tyrosines. In the absence of ethylene glycol, the apparent fraction of unfolded protein, F_{app}, is zero and remains unchanged up to ~1.5 M urea, indicating that the native form is stable under these conditions. Between 1.5 and 6 M urea, F_{app} increases in a biphasic fashion with an inflection near 3 M, indicating that unfolding is occurring via a stable intermediate (Crisanti & Matthews, 1981). Above 6 M urea, the changes in F_{app} are complete, indicating that the unfolded form is populated at high urea concentrations. The changes in F_{app} between 1.5 and 3 M urea monitor the N ↔ I reaction while the changes between 3 and 6 M monitor the I ↔ U reaction.

For the N ↔ I transition, the presence of 1 M ethylene glycol increases the cooperativity, i.e., the slope, but causes no significant change in the midpoint. However, the progressive increase in glycol concentration from 1 to 4 M results in a uniform increase in the midpoint of the transition. When the data are fit to the appropriate three-state model (Beasty et al., 1986a), a progressive increase in the free energy difference between N and I calculated at the standard state defined by the urea concentration at the midpoint in the absence of polyol, 2.62 M urea, is also observed (Table I). Comparisons of stability changes in this manner appear to be more precise than those made by extrapolation to zero denaturant (Cupo & Pace, 1983). In contrast, the changes in the transition curve for the I ↔ U reaction are not a monotonic function of the glycol concentration. The midpoints for 1 and 2 M glycol are less than that in its absence while those for 3 and 4 M glycol are higher. This nonmonotonic trend for the I ↔ U midpoints suggests that glycol is having a complex effect on the relative free energies of the I and U conformations. Because the focus of this study is the effect of altered viscosity on the kinetics of the N ↔ I reaction, these complexities will not be further considered.

To test whether the increase in stability for the N ↔ I transition is a general phenomenon or is specific to ethylene glycol, the effects of glycerol, glucose, and poly(ethylene glycol)(PEG) were also studied; the results for glycerol and PEG are shown in Figure 1b; the glucose results are shown in Figure 1c. In all cases, an inflection in the F_{app} curves indicated that the intermediate is significantly populated and that the three-state folding model is still appropriate. The

[1] Abbreviations: EG, ethylene glycol; PEG, poly(ethylene glycol); NaDodSO$_4$, sodium dodecyl sulfate; F_{app}, apparent fraction of unfolded protein; cP, centipoise; s, seconds.

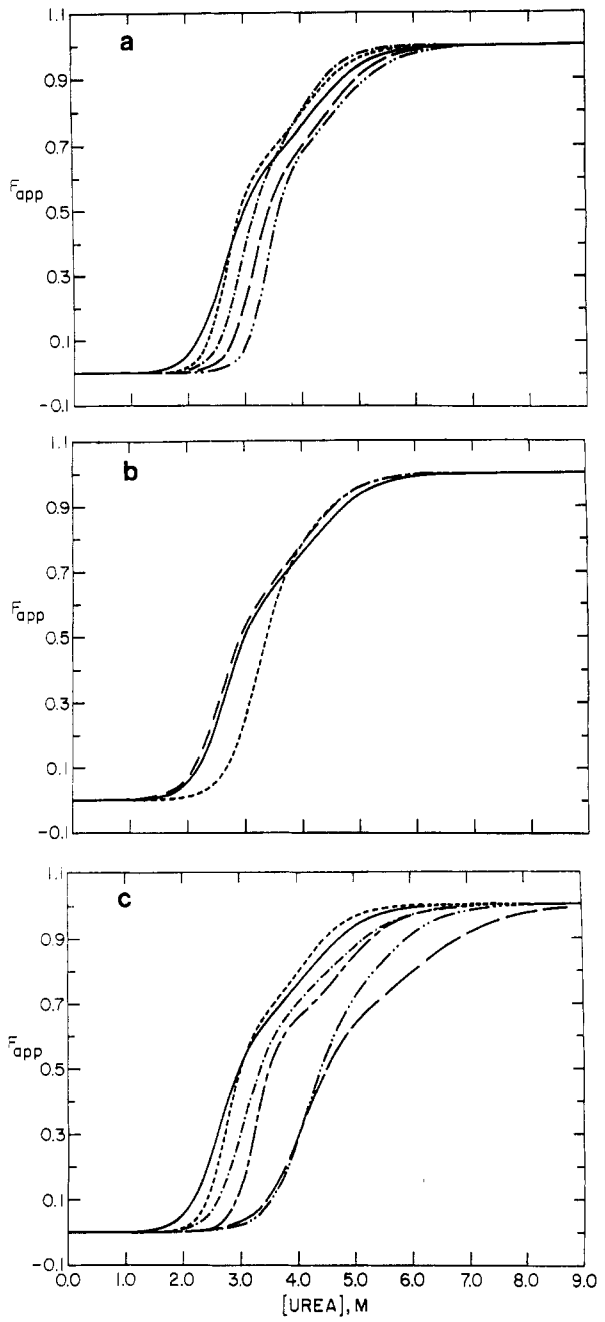

FIGURE 1: Fraction of unfolded protein, F_{app}, as a function of the urea concentration in the presence of solvent additives in 10 mM potassium phosphate, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol buffer, pH 7.8, 25 °C. (a) No additive (—) and 1 M (- - -), 2 M (-·-), 3 M (– –), and 4 M (-··-) ethylene glycol. (b) No additive (—), 3% PEG (– –), and 1.63 M glycerol (- - -). (c) No additive (—) and 0.25 M (- - -), 0.52 M (-·-) 0.75 M (– –), 1.1 M (-··-), and 1.4 M (– –) glucose. Data points were omitted for clarity. Lines represent computer fits to the data [see Beasty et al. (1986a) and Matthews (1987)].

presence of 1.63 M glycerol or glucose concentrations between 0.25 and 0.75 M again increase the midpoint of the N ↔ I transition, i.e., the stability, in a progressive fashion (Table I). However, glucose concentrations above 1.1 M have no additional effect on stability even though the bulk viscosity increases. PEG, at concentrations which result in significant bulk viscosity changes (1.7% and 3% w/v), has no measurable effect on the stability of the subunit (Figure 1b). Taken together, these results clearly demonstrate that the correct interpretation of the effects of ethylene glycol, glycerol, and glucose on the folding kinetics must account for the effects on stability.

Kinetic Studies. The kinetics of folding of the α subunit of tryptophan synthase from *E. coli* are consistent with a model which includes three slow folding phases whose relaxation times have been designated τ_1, τ_2, and τ_3 (Beasty et al., 1986b). The τ_1 and τ_2 reactions reflect the folding of corresponding intermediates I_1 and I_2, and are thought to involve cis/trans proline or other slow isomerization reactions (Hurle & Matthews, 1987). The τ_3 phase has been assigned to the reversible folding unit association/dissociation reaction N ↔ I_3 (Beasty et al., 1986b). The relaxation time for this latter reaction has a characteristic dependence on the final urea concentration (Matthews, 1987) and resembles an inverted V or chevron shape; the maximum occurs very near the midpoint of the corresponding equilibrium transition, N ↔ I.

The effects of increasing concentrations of ethylene glycol on the urea dependence of the τ_3 phase are shown in Figure 2a. The most striking features are (1) a progressive increase in the urea concentration at the maximum and (2) an increase in the value of the maximum relaxation time between 1 and 4 M glycol. The value of τ_{max} increases from 282 s in the absence of glycol to 650 s in 4 M glycol. The refolding, when compared at the same value of urea, appears to be slightly accelerated in the presence of ethylene glycol, an effect which will be discussed later.

When the other viscogenic agents were examined, glycerol was found to have the same type of effect on the relaxation times as ethylene glycol (Figure 2b). At 1.63 M glycerol, the τ_{max} shifts to a higher urea concentration and increases in magnitude. The effect of glucose (Figure 2c) is complex in that between 0.25 and 0.75 M, τ_{max} shows a uniform shift to higher urea concentration and a progressive increase in value, similar to ethylene glycol. However, increasing the glucose concentration to 1.40 M causes no further increase in the urea concentration at τ_{max}. This change in behavior occurs at the same glucose concentration as for the equilibrium transition (Figure 1c) and supports the previously observed close correspondence between the kinetic and equilibrium data (Beasty et al., 1986a). Interestingly, the magnitude of τ_{max} increases between 1.11 and 1.40 M glucose, from 450 to 600 s. PEG is again unique in that 1.7% and 3.0% (w/v) have no discernible effect on the relaxation times. The data are quite similar to those obtained in the absence of viscogenic agent (Figure 2b), although the viscosity is increased 2–3-fold.

Data Analysis. The equilibrium data (Figure 1 and Table I) clearly shows that ethylene glycol, glycerol, and glucose (up to 1.11 M) alter the stability of the α subunit to urea-induced unfolding. Thus, the analysis of the effects of these viscogenic agents on folding kinetics must account for the effects on stability. Hurle et al. (1987) have previously analyzed the effect of 0.58 M sucrose on this folding reaction. They adopted the approach of comparing the relaxation times at the urea concentrations where the equilibrium constants for the N ↔ I transitions are equal to a value of 100. Under these conditions, the relaxation time principally depends on the unfolding rate constant (Matthews, 1987). Note that, as stated previously, the reversibility of the N ↔ I_3 reaction, and the involvement of a common transition state for the rate-limiting step in both folding and unfolding, justifies the measurement of the effect of viscosity on the unfolding rate constant as a means of ascertaining the effect on the refolding reaction.

A complication with the previous approach is that the equilibrium data contain contributions from the native form, N, and two intermediates, I_2 and I_3 (which together comprise the equilibrium intermediate I), while the τ_3 relaxation time

Table I: Thermodynamic Parameters for the Urea-Induced Unfolding Transition in the Presence of Various Cosolvents at pH 7.8, 25 °C[a]

cosolvent	$\Delta G_{NI}^{H_2O}$ [b]	A_{NI} [c]	$\Delta\Delta G_{NI}$ [d]	$\Delta G_{IU}^{H_2O}$ [b]	A_{IU} [c]	$\Delta\Delta G_{IU}$ [e]	$C_{m_{NI}}$ [f]	$C_{m_{IU}}$ [f]
0 M	5.7 (0.4)	2.2 (0.2)		5.0 (0.6)	1.2 (0.2)		2.62 (0.02)	4.18 (0.06)
1 M EG	8.4 (2.0)	3.1 (0.8)	0.34	5.2 (1.3)	1.3 (0.3)	−0.21	2.68 (0.12)	4.00 (0.11)
2 M EG	7.9 (2.3)	2.7 (0.8)	0.89	6.2 (2.3)	1.6 (0.6)	−0.47	2.92 (0.08)	3.97 (0.13)
3 M EG	8.5 (2.6)	2.7 (0.9)	1.26	5.7 (2.2)	1.3 (0.5)	0.28	3.10 (0.13)	4.15 (0.22)
4 M EG	10.1 (3.8)	3.0 (1.1)	2.30	5.1 (1.9)	1.1 (0.4)	0.52	3.39 (0.09)	4.56 (0.50)
0.25 M glucose	8.1 (2.0)	2.9 (0.8)	0.57	5.4 (1.6)	1.4 (0.4)	−0.44	2.75 (0.06)	3.98 (0.12)
0.52 M glucose	7.0 (1.1)	2.3 (0.4)	1.04	4.5 (1.0)	1.0 (0.2)	1.90	3.00 (0.05)	4.36 (0.10)
0.75 M glucose	10.2 (1.6)	3.1 (0.5)	2.08	5.7 (1.2)	1.2 (0.3)	2.57	3.23 (0.04)	4.93 (0.12)
1.1 M glucose	7.3 (2.6)	1.8 (0.7)	2.65	5.5 (2.7)	1.1 (0.5)	2.63	4.09 (0.16)	5.28 (0.27)
1.4 M glucose	6.5 (1.0)	1.6 (0.3)	2.37	4.8 (1.2)	0.8 (0.2)	2.72	4.08 (0.08)	5.98 (0.13)
1.63 M glycerol	6.6 (1.3)	2.1 (0.5)	1.16	4.7 (1.8)	1.2 (0.4)	1.59	3.15 (0.11)	3.94 (0.18)
1.7% PEG	5.4 (1.3)	2.3 (0.6)	−0.56	5.3 (1.7)	1.5 (0.4)	1.39	2.35 (0.07)	3.64 (0.11)
3.0% PEG	5.8 (2.7)	2.2 (1.1)	0.10	5.3 (3.2)	1.3 (0.8)	1.91	2.63 (0.16)	4.00 (0.25)

[a] Units are the following: ΔG, kilocalories per mole; A, kilocalories per mole per molar (urea); C_m, (midpoint) molar urea; $\Delta\Delta G$, kilocalories per mole. Numbers in parentheses represent 95% confidence limits. [b] $\Delta G_{xy}^{H_2O}$, the free energy difference between species x and y in the absence of denaturant. [c] A_{xy}, urea dependence of the free energy difference between species x and y. [d] $\Delta\Delta G_{NI}$, the difference in the ΔG_{NI} values between the wild-type protein (no cosolvent) and mutant (cosolvent), calculated at the midpoint of the wild-type protein (no cosolvent) (2.62 M urea); $\Delta\Delta G_{NI} = \Delta G_{NI}^{co}(2.62\ M) - \Delta G_{NI}^{wt}(2.62\ M)$. [e] $\Delta\Delta G_{IU}$, the difference in the ΔG_{IU} values between wild-type protein (no cosolvent) and mutant (cosolvent), calculated at the midpoint for the wild-type protein (4.18 M urea). [f] $C_{m_{xy}}$, urea concentration at the midpoint of the transition between x and y.

only reflects the behavior of N and I_3.

An alternative approach which appears to have greater internal consistency is to compare the relaxation times at urea concentrations which are displaced by the identical amounts from the urea concentration at τ_{max} in the log τ versus [urea] plots. If the slopes of the log τ plots do not change significantly in the presence of the polyols, this procedure allows the comparison of relaxation times at the same value of the equilibrium constant for the N ↔ I_3 reaction.[2] The assumption for common slopes is supported by the data shown in Figure 2; the slopes vary from 0.54 to 0.61 s M^{-1} in a random manner. At 2 M urea above the value at τ_{max}, the equilibrium constant for the N ↔ I_3 reaction is ~48 000, ensuring that the relaxation time principally reflects the unfolding rate constant ($\tau^{-1} = k_{unf} + k_{ref}$ and $k_{unf}/k_{ref} = 48\,000$).

The relaxation time for a diffusion-controlled reaction is expected to be directly proportional to the viscosity (von Smoluchowski, 1917). A log–log plot of the unfolding relaxation time (relative to the value in the absence of polyol) versus the solvent viscosity (relative to that in the absence of polyol) for a series of ethylene glycol concentrations is shown in Figure 3. The values for the relaxation times and viscosities used in this analysis are shown in Table II. The data clearly demonstrate that the relaxation time increases linearly with the solvent viscosity; the slope of a linear fit is 0.96 ± 0.13 with a correlation coefficient of 0.96. Likewise, a linear fit of the glucose data from 0.25 to 0.75 M glucose gives a line with slope of 1.08 ± 0.15. Thus, both cosolvents show a linear and unitary correlation between relaxation time and viscosity. Though only a single concentration of glycerol was examined, the resulting value of relaxation time as a function of viscosity falls on the line of unit slope (Figure 3). However, 1.11 and 1.40 M glucose have a smaller effect on the relaxation time than would be expected from a unitary dependence on solvent viscosity. PEG has no measurable effect on the relaxation time, although it causes a significant increase in bulk viscosity.

Table II: Relaxation Times and Viscosities in the Presence of Cosolvents at 2 M Urea Offset from τ_{max} at 25 °C

cosolvent	viscosity (cP)	relaxation time (s)
0 M	1.16	52.0
1 M EG	1.41	67.8
2 M EG	1.67	83.5
3 M EG	2.02	109.2
4 M EG	2.56	117.2
0.25 M glucose	1.34	60.6
0.52 M glucose	1.65	87.5
0.75 M glucose	1.96	90.0
1.11 M glucose	2.79	99.9
1.40 M glucose	3.56	127.5
1.63 M glycerol	1.77	81.4
1.7% PEG	1.73	50.1
3.0% PEG	2.22	48.0

Discussion

When the effects of polyols on the stability of the α subunit of tryptophan synthase are considered, it is clear that the presence of ethylene glycol, glycerol, and low concentrations of glucose slows the rate-limiting step in the unfolding/refolding reaction in direct proportion to their effect on the bulk solvent viscosity. Therefore, friction-limited segmental motion of the polypeptide plays a key role in this process.

In a previous study of the temperature dependence of the unfolding rate constant (Hurle et al., 1987), it was found that the unfolding reaction is entropy-controlled. At 25 °C, $\Delta G^{\ddagger}$ = 19.4 kcal/mol, $\Delta H^{\ddagger}$ = 5.4 kcal/mol, and $-T\Delta S^{\ddagger}$ = 14.0 dcal/mol; the activation entropy was −47 cal/(mol·K). The negative value for the activation entropy was interpreted in terms of the ordering of solvent at newly exposed hydrophobic surfaces. Combining this hypothesis with the present finding, one is led to the proposal that the rate-limiting step in unfolding involves the concerted dissociation of the amino and carboxy folding units and hydration of hydrophobic surfaces. Since the reverse reaction is rate limiting at low urea concentration,[3] this implies that refolding is limited by the association of the folding units and the simultaneous dehydration of the docking surfaces.

Estimates of the decrease in entropy per square angstrom due to the ordering of water on hydrophobic surfaces (Chothia, 1975; Richards, 1977) permit one to calculate that an addi-

[2] The equilibrium constant can be calculated by fitting the observed relaxation time, τ, to a first-order kinetic process, $\tau^{-1} = k_{unf} + k_{ref}$. Assuming that the rate constants depend exponentially on the urea concentration (Matthews, 1987), the unfolding, k_{unf}, and refolding, k_{ref}, rate constants can be calculated at any urea concentration. The ratio gives the equilibrium constant, $K_{eq} = k_{unf}/k_{ref}$.

[3] At urea concentrations in the transition zone (~3 M urea), the relaxation times of this phase observed for unfolding and refolding are equal (Matthews, 1987). Therefore, the same transition state is accessed from either direction. The excellent agreement between the equilibrium unfolding curve predicted from these kinetic data (Hurle et al., 1987) and that observed by direct measurement shows that this same reaction is rate-limiting over a range of urea concentrations.

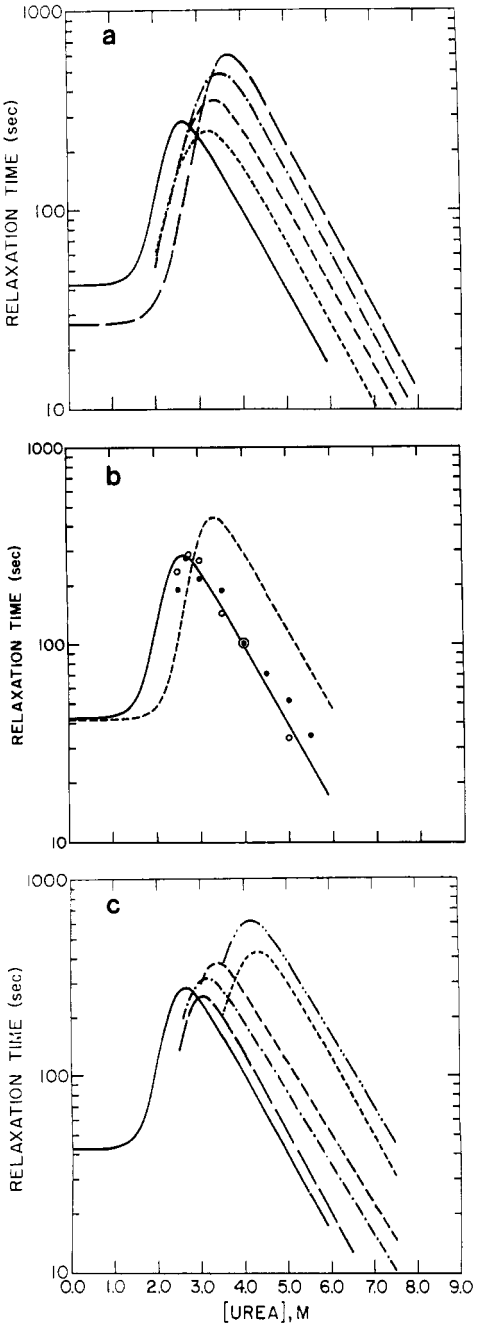

FIGURE 2: Semi-log plot of the τ_3 relaxation time as a function of urea concentration in the presence of solvent additives. (a) No additive (—) and 1 M (---), 2 M (--), 3 M (-·-), and 4 M (— —) ethylene glycol. (b) No additive (—), 1.63 M glycerol (---), and 1.7 (●) and 3% PEG (○). (c) No additive (—) and 0.25 M (— —), 0.52 M (-·-), 0.75 M (--), 1.11 M (---), and 1.4 M (-·-) glucose. Lines represent computer fits to the data. Symbols for the PEG are actual data showing close correspondence of the PEG data to those in the absence of additive.

tional 560–700 Å^2 of hydrophobic surface are exposed in the transition state. This value is considerably less than the folding unit interface computed from the X-ray structure using Connolly's algorithm and a spherical solvent molecule of 1.5-Å radius: 3000 Å^2 (C. Hyde, personal communication). Thus, the transition state appears to involve only a fraction of the folding unit interface. This conclusion is supported by studies on the effects of single amino acid replacements at the folding unit interface on the folding kinetics. Replacements at some positions at the interface have a selective effect on the transition state while others do not (Beasty et al., 1986a; Hurle et al., 1986). Taken together, these results show that the

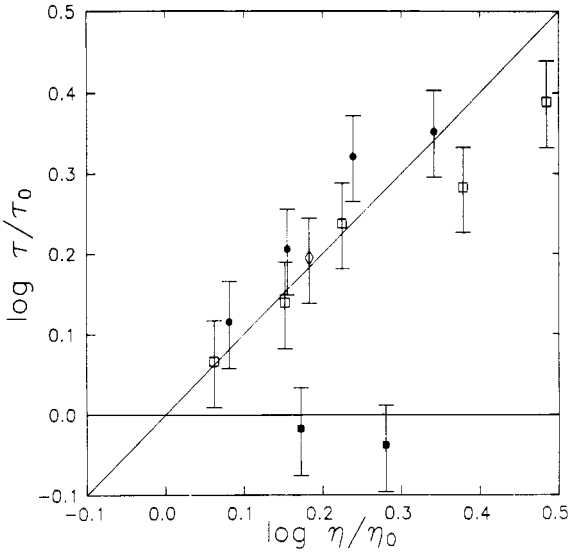

FIGURE 3: Log–log plot of the τ_3 relaxation time as a function of viscosity both normalized to the values in the absence of additive. (●) Ethylene glycol; (□) glucose; (◇) glycerol; (■) PEG. Line through the data represents a slope of unity.

association/dissociation reaction is controlled by the formation/disruption of a limited number of hydrophobic interactions at the interface. Further mutagenic studies are required to identify all of the residues involved in the transition state.

The large, negative value of ΔS^* is also consistent with electrostatic interactions in the transition state, since they, like hydrophobic interactions, are principally entropy controlled (Cantor & Schimmel, 1980). In this case, the above estimate of 560–700 Å^2 surface for the transition state would represent an upper limit; electrostatic interactions would imply an even smaller hydrophobic surface exposed. Several potential electrostatic interactions between side chains in the amino and carboxy folding units have been observed in the X-ray structure. For example, arginine-70 and glutamic acid-242 are within 4 Å, and arginine-256 and aspartic acid-42 are within 3 Å.

The magnitude of the activation enthalpy for unfolding, 5.4 kcal/mol, is comparable to that expected for diffusion-limited processes (Moore, 1972). Thus, the dissociation of the two folding units must contribute rather little to the activation enthalpy. If hydrogen bonds between β strands are disrupted in the transition state, they must be replaced in a concerted fashion by hydrogen bonds with the solvent.

The absence of an effect of PEG on the folding kinetics of the α subunit illustrates the difference between the micro- and the macroviscosity (Blacklow et al., 1988). The former is a measure of the friction experienced by the polypeptide during diffusion while the latter affects bulk flow through a capillary. Presumably, the monomeric additives such as glycerol and ethylene glycol are able to closely approach the polypeptide and alter the structure of water at and near its surface. Because PEG is excluded from this region for steric reasons (Lauffer & Schachman, 1949; Arakawa & Timasheff, 1985), PEG has no effect on the folding kinetics. The absence of an effect of PEG on the stability of the α subunit is consistent with an inability to cause preferential hydration. Teschner et al. (1987) also reported a similar result in that PEG, unlike glucose or glycerol, did not alter the rate of reactivation of octopine dehydrogenase, and Tsong (1982), as well, found no dependence with the polymeric sugar Ficoll.

The effect of glucose on the folding and stability of the α subunit is complex in that there is an abrupt change in behavior between 1.11 and 1.40 M glycerol. Below this point, glucose

acts like glycerol and ethylene glycol, causing progressive changes in the transition midpoint and relaxation time for the $N \leftrightarrow I_3$ reaction. However, the increase in glucose concentration from 1.11 to 1.40 M does not alter the transition midpoint or the urea concentration of τ_{max}. The only significant change is an increase in the value of τ_{max} from 450 to 600 s. The change in behavior may reflect a decrease in preferential hydration at high glucose concentrations observed by Arakawa and Timasheff (1982). The less than linear effect of 0.58 M sucrose on the relaxation time reported by Hurle (Hurle et al., 1987) may have a similar explanation.

The simple linear dependence of the relaxation time for the folding unit association/dissociation step for the α subunit of tryptophan synthase on solvent viscosity demonstrates the importance of diffusive motion in folding. This observation depends critically on a quantitative assessment of the effect of the viscogenic agents on the stability. The omission of such an assessment in previous studies may account for (1) the apparent absence of an effect of glycerol or sucrose on the fast folding phase of ribonuclease A (Tsong & Baldwin, 1978), (2) the nonunitary dependence of relaxation times on viscosity with different polyols for thermal unfolding of ribonuclease A (Tsong, 1982), and (3) the complex effects observed on the folding of octopine dehydrogenase and aspartokinase–homoserine dehydrogenase (Teschner et al., 1987).

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