Thermodynamic Properties of the Transition State for the Rate-Limiting Step in the Folding of the α Subunit of Tryptophan Synthase†

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ABSTRACT: To gain insight into the physical properties of the transition state for the rate-limiting step in the folding of the α subunit of tryptophan synthase from *Escherichia coli*, the urea dependence of the unfolding reaction was examined as a function of temperature. Consistent with a previous, more limited study [Hurle, M. R., Michelotti, G. A., Crisanti, M. M., & Matthews, C. R. (1987) *Proteins 2*, 54], the activation entropy for unfolding was found to be negative above 4 M urea. The present study extends this finding to show that both the activation entropy and enthalpy decrease with increasing urea concentrations between 4 and 7.5 M. The change in the heat capacity from the native to the transition state is positive and appears to increase with the denaturant concentration. The urea and temperature dependences of the unfolding rates were analyzed in terms of the denaturant-binding model of Tanford [Tanford, C. (1970) *Adv. Protein Chem. 24*, 1]. The values for the activation enthalpy and activation entropy of binding are in good agreement with those obtained from a calorimetric study of urea binding to unfolded proteins [Makhatadze, G. I., & Privalov, P. L. (1992) *J. Mol. Biol. 226*, 491]. These results show that (1) the binding of urea to the transition state of the α subunit has thermodynamic properties which are similar to those for urea binding to unfolded proteins, (2) the transition state is distinct from the unfolded conformation and exposes only a fraction of its urea-binding sites to solvent, and (3) the negative value for the activation entropy for unfolding reflects, in part, the ordering of urea on newly exposed surfaces. The agreement between the thermodynamic properties of urea binding for the transition state and for unfolded proteins demonstrates that similar denaturant-binding sites are available in both states.

Equilibrium studies of protein-folding reactions provide insight into the thermodynamic properties of stable, measurably populated forms, typically the native and unfolded species for many proteins (Tanford, 1970; Pace, 1986; Privalov, 1989). Equally important to the understanding of the mechanism of folding is information on the properties of transient intermediates and transition states which link the various species in the kinetic mechanism. Site-directed mutagenesis has proven to be a valuable probe of the amino acids which play key roles in such intermediates and transition states (Matthews, 1987; Goldenberg et al., 1989; Jennings et al., 1991; Matouschek & Fersht, 1991). However, mutagenesis *per se* does not yield essential thermodynamic properties such as enthalpy, entropy, or heat capacity (or their quasi-thermodynamic counterparts for transition states); thermal studies are required. To determine these parameters for a transition state, it is usually assumed that the relationship between, for example, the unfolding rate constant, k_u, and the temperature is described by the Eyring equation (Atkins, 1978):

$$k_u = (k_B T/h) \exp(-\Delta G_u^{0\ddagger}/RT) \quad (1)$$

where k_B, h, and R are the Boltzmann, Planck, and gas constants, $\Delta G_u^{0\ddagger}$ is the activation free energy for unfolding, and T is the absolute temperature. The assumption that the transition state behaves as an actual thermodynamic state, while widely made (Matthews, 1987; Jennings et al., 1991; Matouschek & Fersht, 1991), has never been experimentally verified.

A previous investigation of the temperature dependence of the single unfolding reaction observed for the α subunit of tryptophan synthase (Hurle et al., 1987) found a negative value for the activation entropy, −47 cal/mol/K, at 5.5 M urea. This surprising result was attributed to the ordering of water on newly exposed surfaces in the transition state, the magnitude of which must be sufficient to dominate the expected positive contribution from disordering the polypeptide. This explanation can be tested by measuring the difference in heat capacity between the native and transition states, i.e., by examining the temperature dependence of the unfolding rate constant. Calorimetric studies of many proteins (Privalov & Potekhin, 1986; Privalov, 1990; Privalov & Makhatadze, 199) have shown that a positive heat capacity difference exists between the native and unfolded forms. The positive sign has been attributed to the ordering of water on exposed, nonpolar surfaces which are buried in the native conformation (Privalov, 1989, 1990). If the factors responsible for the heat capacity differences between the native and unfolded states are also operative for the transition state, then the heat capacity difference between the native and transition states of the α subunit should be positive.

In this paper, an investigation of the unfolding reaction of the α subunit at a series of temperatures *and* urea concentrations is reported. The advantage of investigating unfolding at multiple denaturant concentrations is that the dependence of the activation enthalpy and activation entropy on the urea concentration becomes apparent. A positive heat capacity difference between the native and the transition state was also detected, supporting the interpretation that the negative activation entropy reflects the ordering of solvent and cosolvent

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on freshly exposed surfaces. When the dependence of the activation free energy on the urea concentration was analyzed in terms of the denaturant-binding model of Tanford (1970), the enthalpy and entropy of binding of urea to the transition state were found to be equal, within experimental error, to the values observed for urea binding to denatured proteins (Makhatadze & Privalov, 1992). The demonstration of similar binding environments for the denaturant in both states shows that the transition state has chemical and thermodynamic properties which resemble those of a well-defined thermodynamic state.

MATERIALS AND METHODS

Protein Production and Purification. The gene for the α subunit of tryptophan synthase from *E. coli* was cloned into a commercially available plasmid/phage system, pTZ18, which can be used for both site-directed mutagenesis and protein expression (Pharmacia Biotech, Inc., Piscataway, NJ). The resulting plasmid, designated as pXH, places the expression of the α subunit under the control of the T7 phage promoter. The expression of the α subunit is high in an *E. coli* host (CB149, courtesy of Dr. E. W. Miles), and 40 mg or more of purified protein are produced per liter of culture. The protein appears in both soluble and insoluble forms. The α subunit was purified independently from both forms as described previously (Tsuji et al., 1993) and estimated to be greater than 95% pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The α subunit produced by this plasmid has folding properties and enzymatic activity (4000 units/mg) which are very similar to the properties of protein purified from a previous expression system (Hurle et al., 1986).

Experiments. Chemical reagents and experimental conditions were as described previously (Finn et al., 1991; Chrunyk & Matthews, 1990b). A buffer of 10 mM potassium phosphate, 0.2 mM Na_2EDTA,[1] and 1 mM 2-mercaptoethanol, pH 7.8, was used in all experiments. The kinetic experiments on the unfolding of the α subunit were done at eight different temperatures ranging from 10 to 35 °C. For manual mixing unfolding experiments, a predetermined amount of 9 M urea stock solution first was mixed with the appropriate amount of buffer to give a total volume of 800 μL. The unfolding reaction was then started by manually adding 100 μL of a concentrated protein stock solution to the urea/buffer solution to yield a final urea concentration which varied from 4.0 to 5.5 M. For stopped-flow unfolding experiments, the total volume was 400 μL and the ratios of the buffer, urea stock solution, and protein stock solution were adjusted so that the final urea concentration varied from 6.0 to 7.5 M. The final protein concentration was constant at any particular temperature and varied from 0.6 to 1.2 mg/mL; the folding kinetics of the α subunit have been shown to be independent of the protein concentration in this range (Crisanti & Matthews, 1981).

For reactions with half times longer than 10 s, the unfolding reactions were initiated by manual mixing techniques and the change in absorbance was monitored on an AVIV 118CX spectrometer (Chen et al., 1992). For half times less than 10 s, a Biologic SFM 3 stopped-flow drive train interfaced to an absorbance spectrometer was employed (Bio-logical Instruments de laboratoires, Claix, France). The dead time of this instrument was measured by the method of Tonomura et al. (1978) and found to be 5 ms. The unfolding reaction was detected in both cases by monitoring the change in absorbance at 287 nm, reflecting the exposure of buried tyrosine residues to solvent. Cuvettes with a 1-cm path length were used in both techniques. Water baths were used to maintain the cuvette temperature within ±0.2 °C of the desired value. All unfolding kinetic traces were recorded on an IBM-compatible PC for quantitative analysis. More details on the experimental procedure can be found elsewhere (Finn et al., 1991).

Data Analysis. The kinetic unfolding traces were fit to the sum of a single exponential and a constant term (Matthews & Crisanti, 1981) with a nonlinear least-squares statistical program licensed from SAS Institute (Cary, NC). The relaxation time and amplitude for unfolding at each urea concentration and temperature were obtained from the fits.

RESULTS

The urea-induced unfolding reaction of the α subunit of tryptophan synthase has previously been found to follow a single-exponential decay which accounts for >95% of the change in absorbance at 287 nm expected from equilibrium measurements (Matthews & Crisanti, 1981). This result implies that the rate-limiting step in unfolding involves the disruption of the native conformation, N, and that the properties of the transition state can be compared with those of the native conformation by studying the dependence of the unfolding rate constant on environmental parameters. This same transition state has been shown to be rate limiting in refolding (Hurle et al., 1987; Chrunyk & Matthews, 1990b; Saab-Rincón et al., 1993); however, in this direction, the energy barrier separates a stable folding intermediate, I_1, from the native form. In terms of a simplified folding model,

$$N \underset{k_{I_1N}}{\overset{k_{NI_1}}{\leftrightarrow}} I_1 \underset{k_{I_2I_1}}{\overset{k_{I_1I_2}}{\leftrightarrow}} I_2 \underset{k_{UI_2}}{\overset{k_{I_2U}}{\leftrightarrow}} U$$

rate-limiting step; fast; fast

where k_{XY} represents the microscopic rate constant for the appropriate transition from species X to species Y. The absence of high-resolution structures for the intermediates and the complications with other slow folding reactions at low denaturant concentrations (Hurle & Matthews, 1987; Chen et al., unpublished results) led to the decision to examine only the unfolding reaction of the α subunit.

The dependence of the unfolding relaxation time for the α subunit on the final urea concentration at a series of temperatures is shown in Figure 1. Between 10 and 35 °C and above 4 M urea, the log of the relaxation time decreases linearly with increasing denatuant concentration. To extract the thermodynamic parameters for the transition state, it is necessary to relate the unfolding relaxation time to a microscopic rate constant. Under strongly unfolding conditions, the reciprocal of the relaxation time, τ, can be shown to be equal to the unfolding rate constant, k_{NI_1}, for the α subunit (Matthews, 1987). This requirement is satisfied for the α subunit at and above 4 M urea over the temperature range investigated because $K = [I_1]/[N] = k_{NI_1}/k_{I_1N} > 100$ (Tweedy et al., 1990).

By expanding the activation free energy, $\Delta G^{0\ddagger}$, in terms of activation enthalpy, $\Delta H^{0\ddagger}$, activation entropy, $\Delta S^{0\ddagger}$, and activation heat capacity, $\Delta C_p^{\ddagger}$ (Chen et al., 1989), one can derive the following equation from eq 1:

$$\ln(k/T) = A + B(T_0/T) + C\ln(T_0/T) \qquad (2)$$

where

[1] Abbreviation: EDTA, ethylenediaminetetraacetic acid.

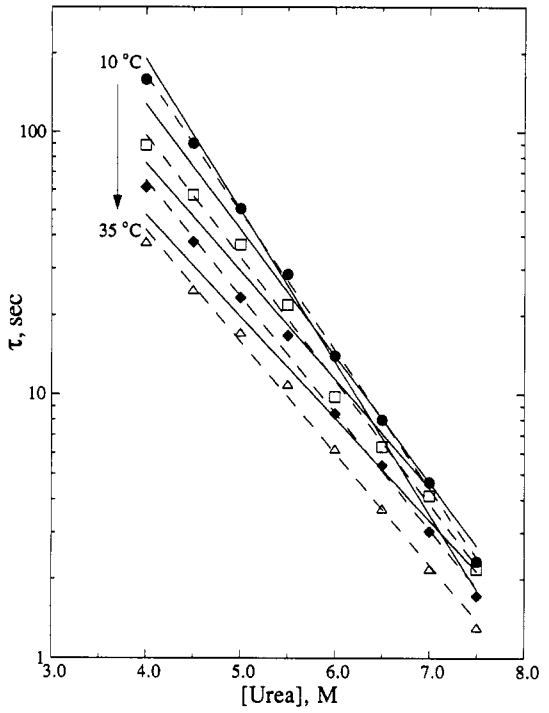

FIGURE 1: Plots of the logarithm of the unfolding relaxation time as a function of the urea concentration at various temperatures and pH 7.8. Data points connected by dashed lines are shown at 15 °C (●), 25 °C (□), 30 °C (◆), and 35 °C (△). Only solid lines are shown for 10, 20, 27.4, and 32.5 °C; the data points have been omitted for clarity. Straight lines are drawn through the data points at each temperature to aid the eye and highlight the apparent linear dependence on the urea concentration.

$$A = [\Delta S^{0\ddagger}(T_0) - \Delta C_p^{\ddagger}]/R + \ln(k_B/h)$$

$$B = [\Delta C_p^{\ddagger} - \Delta H^{0\ddagger}(T_0)/T_0]/R$$

$$C = -\Delta C_p^{\ddagger}/R$$

and T_0 is the reference temperature, which is taken to be 25 °C in this analysis. These equations assume that $\Delta C_p^{\ddagger}$ is itself independent of temperature. This assumption seems reasonable, given that its analog for the native and unfolded states of a number of proteins, ΔC_p, has been found to be temperature independent over the range used in present study, 10–35 °C (Privalov, 1979).

A plot of $\ln(k/T)$ versus $1/T$ at a series of urea concentrations from 4.0 to 7.5 M is shown in Figure 2. The significant curvature observed at all urea concentrations is consistent with a positive change in the activation heat capacity, $\Delta C_p^{\ddagger}$, upon unfolding from the native conformation to the transition state (Kuwajima et al., 1983; Segawa & Sugihara, 1984; Chen et al., 1989; Jackson & Fersht, 1991). If the native and transition states had identical heat capacities, these plots would have been linear because the coefficient C in eq 2 would be zero. The results of independently fitting each of the data sets to eq 2 are also shown in Figure 2. The good agreement between the predicted and observed dependence of $\ln(k/T)$ on $1/T$ shows that a simple two-state kinetic model is sufficient to describe the unfolding reaction of the α subunit if the activation heat capacity is positive.

Although the activation free energy for unfolding has been observed to decrease with increasing denatuant concentration (Matthews, 1987), the behavior of the activation enthalpy and activation entropy has been far less studied. The dependences of the thermodynamic parameters for the

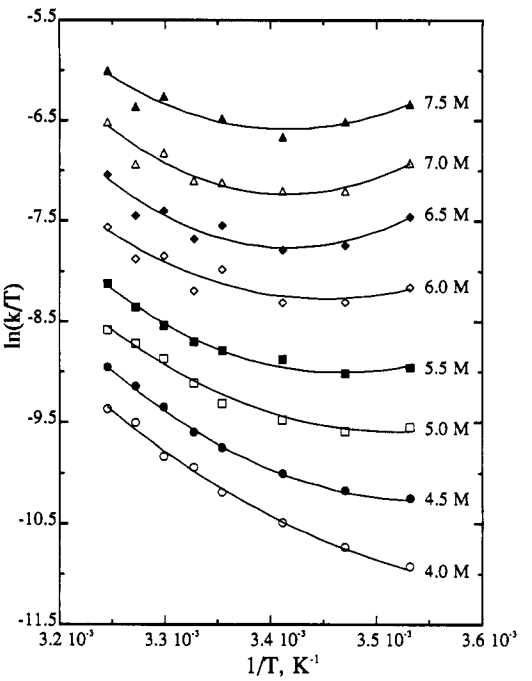

FIGURE 2: Eyring plots for the rate-limiting unfolding reaction of the α subunit at pH 7.8 and various urea concentrations. Lines are theoretical fits according to eq 2. The molar urea concentrations are indicated by each curve.

unfolding transition state of the α subunit on the urea concentration are shown in Figure 3A–D. The activation enthalpy, entropy, and free energy all decrease as the denaturant concentration increases from 4.0 to 7.5 M urea. Although the errors are relatively large, the activation heat capacity is positive and appears to increase with increasing urea concentration in this range.

The apparent linear decreases in the activation enthalpy and activation entropy at increasing denaturant concentrations resemble those for the enthalpy and entropy of unfolding of ribonuclease and lysozyme at various urea concentrations observed by Makhatadze and Privalov in a differential scanning calorimetry study [see Table 4 in Makhatadze and Privalov (1992)]. A quantitative comparison can be made by fitting the transition-state parameter of the α subunit to the following equations which are adapted from the denaturant-binding model of Tanford (1970) and eq 4 in Makhatadze and Privalov (1992):

$$\Delta G^{0\ddagger} = \Delta G^{0\ddagger}(H_2O) - \Delta n^{\ddagger} RT \ln(1 + Ka) \quad (3)$$

$$\Delta H^{0\ddagger} = \Delta H^{0\ddagger}(H_2O) + \Delta n^{\ddagger} \Delta H_B^{0\ddagger} Ka/(1 + Ka) \quad (4)$$

$$\Delta S^{0\ddagger} = (\Delta H^{0\ddagger} - \Delta G^{0\ddagger})/T = \Delta S^{0\ddagger}(H_2O) + [\Delta n^{\ddagger} \Delta H_B^{0\ddagger} Ka/(1 + Ka) + \Delta n^{\ddagger} RT \ln(1 + Ka)]/T \quad (5)$$

where $\Delta n^{\ddagger}$ represents the additional number of urea-binding sites available in the transition state compared to in the native state, K is the binding constant of urea to independent and identical sites on the protein, a is the activity of urea, and $\Delta H_B^{0\ddagger}$ is the molar heat released or absorbed per site upon binding urea. Because the mathematical form of eq 3 is such that $\Delta G^{0\ddagger}$ is not very sensitive to the value of K at urea concentrations greater than 4.0 M (Figure 3A), the average value of K for urea binding to unfolded ribonuclease, lysozyme, and cytochrome c, 0.061 M^{-1}, was assumed to apply to the transition state of the α subunit (Makhatadze & Privalov,

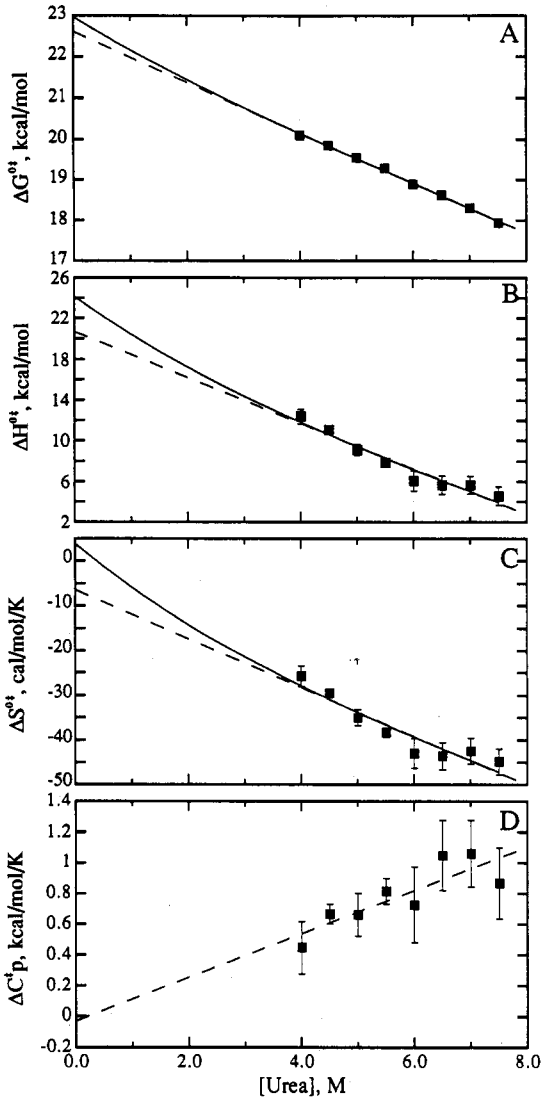

FIGURE 3: Dependence of unfolding activation parameters on the urea concentration at 25 °C and pH 7.8. Errors are standard deviations and, in some cases, comparable to the size of the symbols. Solid lines in panels A–C are fits of the data according to the denaturant-binding model using eqs 3–5, respectively. For comparison, dashed lines are fits of the data to simple linear dependencies.

1992). The free energy of binding of urea to the transition state, $\Delta G_B^{0\ddagger}$, which can be calculated from $\Delta G_B^{0\ddagger} = -RT \ln K$, is 1.7 kcal/mol. The activity of urea was calculated from the molarity using the equation proposed by Pace (1986): $a = 0.9815[\text{urea}] - 0.02978[\text{urea}]^2 + 0.00308[\text{urea}]^3$.

Equation 3 provides an excellent fit of the denaturant dependence of the activation free energy (Figure 3A) and yields values of $\Delta G^{0\ddagger}(H_2O) = 23.0$ kcal/mol and $\Delta n^{\ddagger} = 23.7$ at 25 °C. Equations 4 and 5 also fit the observed dependence of the activation enthalpy and activation entropy, respectively, on urea (Figure 3B,C) and give values of $\Delta H^{0\ddagger}(H_2O) = 24.1$ kcal/mol, $\Delta H_B^{0\ddagger} = -2.9 \pm 0.3$ kcal/mol, and $\Delta S^{0\ddagger}(H_2O) = 4.0$ cal/mol/K. The activation entropy of binding to the newly exposed sites in the transition state, $\Delta S_B^{0\ddagger}$, can be calculated from $(\Delta H_B^{0\ddagger} - \Delta G_B^{0\ddagger})/T$ and has a value of -15.4 ± 1.7 cal/mol/K per mole of binding sites. These values for the activation enthalpy and entropy of binding of urea to the transition state of the α subunit compare closely with those obtained from mixing calorimetry experiments [Table 5 in Makhatadze and Privalov (1992)] on unfolded proteins: $\Delta H_B^0 = -2.2 \pm 0.5$ kcal/mol, and $\Delta S_B^0 = -12.7 \pm 1.9$ cal/mol/K.

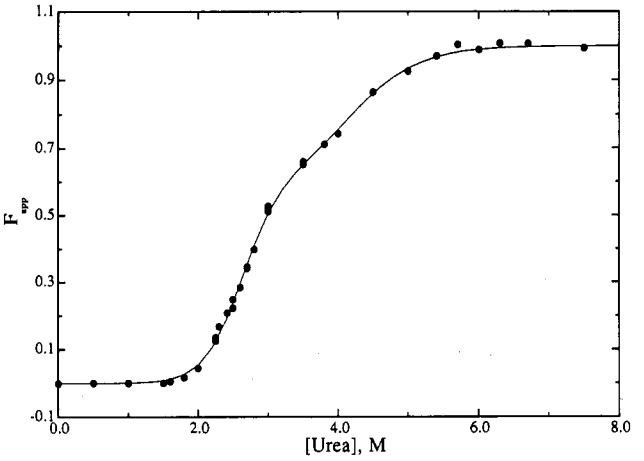

FIGURE 4: Apparent fraction of unfolded α subunit, F_{app}, as a function of the urea concentration at 25 °C and pH 7.8. The ordinate was calculated from the absorbance data at 287 nm and the equation $F_{app} = (\epsilon_O - \epsilon_N)/(\epsilon_U - \epsilon_N)$. In this equation, ϵ_O is the observed extinction coefficient and ϵ_N and ϵ_U are the extinction coefficients of the native and unfolded forms at the same urea concentration. The latter values were obtained by linear extrapolation from the appropriate base-line region. The filled symbols represent the data; the line is the theoretical fit to a three-state model assuming that the urea dependence of the apparent free energy of folding is described by the denaturant-binding model.

To compare the increase in the number of urea-binding sites for the transition state to that for the fully unfolded α subunit, the urea-induced equilibrium unfolding of the α subunit was examined by both tyrosine absorbance and histidine proton NMR spectroscopy (Saab-Rincón et al., 1993). The inflection in the absorbance data at 3 M urea (Figure 4) reflects the presence of a stable intermediate, I_1. Although the tyrosine residues are essentially completely exposed to solvent at 5.0 M urea, a further cooperative unfolding transition between 5.0 and 7.5 M urea can be detected in a proton NMR study of the histidine residues (Saab-Rincón et al., 1993). The stable intermediate which is highly populated at 5.0 M urea is designated as I_2. The absorbance data are well described by a three-state model, $N \leftrightarrow I_1 \leftrightarrow I_2$ (Figure 4), and the NMR data by a two-state model, $I_2 \leftrightarrow U$ (data not shown), assuming that the free-energy changes follow the denaturant-binding model of Tanford (1970). The increase in the number of urea-binding sites for the $N \leftrightarrow I_1$, $I_1 \leftrightarrow I_2$, and $I_2 \leftrightarrow U$ transitions are $\Delta n_{NI_1} = 78$, $\Delta n_{I_1I_2} = 45$, and $\Delta n_{I_2U} = 69$, respectively. The total increase increase in binding sites for the $N \leftrightarrow U$ transition, Δn_{NU}, is, therefore, 192.

DISCUSSION

The systematic investigation of the temperature and urea dependences of the unfolding rate constant for the α subunit of tryptophan synthase described in this paper has provided several important insights into the transition state for the rate-limiting step in unfolding.

Activation Heat Capacity. As inferred from the negative value for the activation entropy for unfolding observed in a previous study (Hurle et al., 1987), the heat capacity difference between the native and transition states above 4 M urea is found to be positive. This result could be taken as support for the original hypothesis that additional solvent must be organized about nonpolar surfaces in the transition state. However, the observed dependences of the activation heat capacity and the activation entropy on the denaturant concentration suggest a more complex picture.

Makhatadze and Privalov (1992) have found that the heat capacities of several unfolded proteins increase with increasing denaturant concentration. The result was attributed to the effect of the changing solvent composition, i.e., the urea/water mixture, on the thermodynamic properties of the unfolded polypeptides. Therefore, it seems quite possible that the observed increase in the activation heat capacity for the unfolding of the α subunit with increasing urea concentration (Figure 3D) reflects the solvation by both water and urea of the partially unfolded protein in the transition state. The contribution of urea to the activation heat capacity can be removed by extrapolation to 0 M denaturant; however, the form of such an extrapolation is not clear from the limited data available. If a linear extrapolation is made, $\Delta C_p^{\ddagger}(H_2O) = -0.03 \pm 0.22$ cal/mol/K (Figure 3D); if there is no further change below 4 M urea, the value of $\Delta C_p^{\ddagger}(H_2O)$ is 0.45 ± 0.17 cal/mol/K. In either case, there is a relatively small difference between the heat capacities of the native and transition states in the absence of denaturant.

If $\Delta C_p^{\ddagger}(H_2O)$ is small, then either (A) there is no significant exposure of additional nonpolar surface in the transition state in the absence of denaturant or (B) the exposure of nonpolar surface is compensated by exposure of polar groups. Measurements of the heat capacity change upon the dissolution of polar groups in water show that these values are negative and of a sufficient magnitude to potentially offset the positive values expected for nonpolar compounds (Privalov & Makhatadze, 1990; Murphy & Gill, 1991; Spolar et al., 1992). The second explanation seems more likely on the basis that the activation enthalpy for the α subunit has a large, positive value in water at 25 °C, $\Delta H^{0\ddagger}(H_2O) = 24.1$ kcal/mol (Figure 3A). Because the enthalpy changes for the transfer of nonpolar compounds to water have negative or small positive values at 25 °C (Gill et al., 1976), the large positive value for $\Delta H^{0\ddagger}(H_2O)$ implies that hydrogen bonds are disrupted in order to reach the transition state. This supposition is consistent with the binding of urea to hydrogen-bond donors and/or acceptors which become exposed in the transition state, e.g., in the polypeptide backbone. Thus, a significant exposure of polar groups to solvent in the transition state seems quite possible. The expected negative contribution to the heat capacity difference between the native and transition states could nearly cancel the positive contribution from nonpolar groups, resulting in a small, net activation heat capacity.

Analysis of the kinetics of unfolding and refolding of hen lysozyme (Segawa & Sugihara, 1984) and chymotrypsin inhibitor (Jackson & Fersht, 1991) at various temperatures revealed that, similar to the α subunit, the activation heat capacity for unfolding is near zero. Chen et al. (1989) found that the activation heat capacity for the unfolding to the cold denatured state of T4 lysozyme is about 25% of the heat capacity difference between the native and unfolded forms. However, their analysis assumed that the activation heat capacity did not depend upon the presence of 3 M guanidine HCl in the final, unfolding conditions. Given that the heat capacities of unfolded proteins also increase with increasing concentrations of guanidine HCl (Makhatadze & Privalov, 1992), it is likely that the activation heat capacity of T4 lysozyme in the absence of denaturant would be less than the reported value. Because the small or negligible values for the activation heat capacities for the unfolding of all four proteins are accompanied by activation enthalpies in excess of 15 kcal/mol, compensating contributions of polar and nonpolar surfaces in a transition state to the activation heat capacity appear to be a common phenomenon.

The source of the negative value for $\Delta S^{0\ddagger}$ for the α subunit at high urea concentration (Hurle et al., 1987) becomes apparent from an examination of the dependence of the activation entropy on the denaturant concentration (Figure 3C). In the absence of urea, the activation entropy of unfolding is predicted to have a relatively small positive value, 4.0 cal/mol/K. However, the binding of urea to the transition state leads to a significant reduction in this value; by 6 M urea, $\Delta S^{0\ddagger}$ decreases to −44 cal/mol/K. This decrease may reflect the immobilization of urea when bound to the protein and/or a restriction in the polypeptide conformational space caused by the binding of urea to multiple hydrogen-bond-donor/acceptor groups on the α subunit (Makhatadze & Privalov, 1992). The small positive value for $\Delta S^{0\ddagger}(H_2O)$ suggests that the increase expected for disordering the α subunit in the transition state slightly exceeds the decrease expected for exposing nonpolar surfaces to water.

Solvent Exposure of the Transition State. The transition state for the unfolding reaction only exposes a fraction fo the buried polar and nonpolar groups which are ultimately exposed to solvent in the fully unfolded protein. If the number of additional urea-binding sites is taken as a measure, then only 12% ($\Delta n^{\ddagger}/\Delta n_{NU}$) of the potential sites is exposed in the transition state. An X-ray study of urea binding to α-chymotrypsin (Hibbard & Tulinsky, 1978) shows that these sites are likely to include both the polypeptide backbone and nonpolar side chains. If the heat capacity changes in the absence of denaturant are considered as an indication, the fractional exposure must be less than 21%. This estimate is based upon the maximal value of 0.45 kcal/mol/K for $\Delta C_p^{\ddagger}$ at 4 M urea and the observed heat capacity change measured for the complete thermal unfolding of the α subunit in the absence of denaturant, 2.1 ± 0.4 kcal/mol/K (Matthews et al., 1980) and 2.6 ± 0.6 kcal/mol/K (Lim et al., 1992). Note that the use of $\Delta C_p^{\ddagger}$ as an estimate of exposure of previously buried surface area is complicated by the counterbalancing contributions of polar and nonpolar groups to the activation heat capacity and should thus be regarded as approximate.

Denaturant-Binding Parameters for the Transition State. The activation enthalpy and entropy for urea binding to the transition state, $\Delta H_B^{0\ddagger} = -2.9 \pm 0.3$ kcal/mol and $\Delta S_B^{0\ddagger} = -15.4 \pm 1.7$ cal/mol/K, are very similar to the thermodynamic parameters for urea binding to several unfolded proteins and a tetrapeptide. Makhatadze and Privalov (1992) employed isothermal mixing calorimetry to examine the binding of urea to unfolded ribonuclease, lysozyme, and cytochrome *c* and found that $\Delta H_B^0 = -2.2 \pm 0.5$ kcal/mol and $\Delta S_B^0 = -12.7 \pm 1.9$ cal/mol/K. Robinson and Jencks (1965), investigating the solubility of *N*-acetyltetraglycine ethyl ester in urea/water mixtures at various temperatures, reported that $\Delta H_B^0 = -2.8$ kcal/mol and $\Delta S_B^0 = -11.8$ cal/mol/K. The excellent agreement between the parameters generated by the kinetic and thermodynamic experiments shows that similar binding sites are accessible in all three systems. The fact that the tetraglycine peptide only offers backbone carbonyl oxygen- and amide hydrogen-binding sites strongly suggests that urea binding to the backbone is principally responsible for the observed thermodynamic effects in unfolded proteins and the transition state for the α subunit.

Assuming the validity of the denaturant-binding model, the activation free energy of the α subunit in the absence of urea is dominated by the activation enthalpy at 25 °C: $\Delta H^{0\ddagger}(H_2O) = 24.1$ kcal/mol and $-T\Delta S^{0\ddagger}(H_2O) = -1.1$ kcal/mol. The very small value for the unfolding activation entropy

implies that the maximum value for $\Delta G^{0\ddagger}(H_2O)$, i.e., the temperature at which $d\Delta G^{0\ddagger}(H_2O)/dT = -\Delta S^{0\ddagger}(H_2O) = 0$, is near room temperature. The precise value depends upon $\Delta C_p^{\ddagger}(H_2O)$. A similar situation holds for the equilibrium unfolding transition of the α subunit where the temperature of maximum stability is predicted to be 16 °C on the basis of the thermodynamic parameters generated in a differential scanning calorimetric study (Matthews et al., 1980). Similar temperatures at which the free energy and the unfolding activation free energy reach maxima have also been found for the cold denaturation of T4 lysozyme (Chen et al., 1989) but not for the acid-induced unfolding and refolding of chymotrypsin inhibition [calculated from the data in Jackson and Fersht (1991)]. Thus, these kinetic and thermodynamic properties do not necessarily track with each other. These limited data suggest that the transition state may not be simply a miniature version of the unfolded state, at least with regards to the structural features implied by the entropy changes (Chen et al., 1989).

Transition-State Model. A model for the rate-limiting transition state of the α subunit of tryptophan synthase can be proposed on the basis of the present results and those from hydrodynamic (Chrunyk & Matthews, 1990b), amide-proton-exchange (Beasty & Matthews, 1985), and mutagenic studies (Tsuji et al., 1993; Chrunyk & Matthews, 1990a; Tweedy et al., 1990). The native conformation has been determined to a resolution of 2.5 Å (Hyde et al., 1988) and found to be a member of the α/β barrel class of proteins (Faber & Petsko, 1990). Eight parallel β strands form the hydrophobic core of the α subunit. Each strand is hydrogen bonded to the preceding and succeeding strand in the sequence; hydrogen bonds between strands 1 and 8 complete the barrel structure. A corresponding set of amphiphatic α helices alternates in sequence with the strands and docks on the surface of the barrel. An additional helix precedes the first strand and covers the end of the barrel defined by the amino termini of the strands.

Mutagenic analysis suggests that the stable intermediate which is linked to the native form by the rate-limiting transition state is an opened form of this barrel (Tsuji et al., 1993). Strands 1 and 8 are not yet in contact; however, several of the side chains in strands 6 and 7 are. The packing of at least one of the side chains in strand 2 (Glu 49) is looser than that in the native form but is not yet fully exposed to solvent (Chrunyk & Matthews, 1990a). Amide-proton-exchange experiments reveal that strands 1–6 form a hydrogen-bonded cooperative unit which behaves independently from a second unit consisting of strands 7 and 8 (Beasty & Matthews, 1985).

The rate constant for the unfolding reaction of the α subunit is known to vary inversely with solvent viscosity (Chrunyk & Matthews, 1990b), demonstrating that access to the transition state requires diffusion through the solvent. Mutational analysis (Jennings et al., 1991; Matouschek & Fersht, 1991; Goldenberg et al., 1989; Matthews, 1987) provides some insight into the nature of the partial unfolding reaction involved. Studies of the effects of single and double mutations on the kinetic and equilibrium properties of the $N \leftrightarrow I_1$ reaction (Tsuji et al., 1993) show that (1) side chains in the interior of the barrel in strands 1 and 8 alter their packing to reach the transition state and (2) these side chains continue to interact in the transition state. As noted above, side chains in strands 6 and 7 have been shown to interact in the native and intermediate, I_1, forms of the α subunit; kinetic data show that they also interact in the transition state linking these forms. The integrity of the β barrel at strand 2, an interior strand in the amino folding unit, is better maintained in the transition state. Replacement of Glu 49 in strand 2 with either Met or Gln does not significantly alter the rate of unfolding, demonstrating that the transition state must be very nativelike in this region (Beasty et al., 1986).

These data and those presented in this report suggest a model for the transition state in which the side chains in the interior of the barrel belonging to strands 1 and 8 and those for strands 6 and 7 interact but with altered contacts. The hydrogen-bonding network that links the two folding units together is disrupted. These two sets of hydrogen bonds are at least some of those that rupture in the native form to reach the transition state and are sites for urea binding. The swelling of the α subunit required to break these β-strand hydrogen bonds and alter the packing of the side chains results in the observed dependence of the rate of unfolding on solvent viscosity (Chrunyk & Matthews, 1990b). Compensating solvent exposure of polar and nonpolar surfaces results in a small, net positive activation heat capacity in the absence of denaturant. For the reverse direction, i.e., refolding, this interpretation implies that this step corresponds to the docking and limited rearrangement of well-organized amino and carboxyl folding units. The subdomain-folding model of Staley and Kim (1990) most closely describes the last event in the folding of the α subunit.

Application of the Denaturant-Binding Model to the Transition State. Several models have been developed to describe the relationship between the free energy of folding and the concentration of a chemical denaturant: the linear dependence model, the free energy of transfer model, and the denaturant-binding model (Pace, 1975). An evaluation of the individual strengths and weaknesses of these three models led to the choice of the denaturant-binding model for the present application. Although a thermodynamic justification for the linear dependence model has been provided by Schellman (1978), this model does not offer molecular insight into the mechanism of denaturation by urea. The free energy of transfer model (Tanford, 1964) focuses on the increased solubility of the various components of a protein in higher concentrations of chemical denaturants. Because the application of this model requires a detailed structure of the transition state, it is not a fruitful approach for the analysis of kinetic data. The denaturant-binding model (Tanford, 1970) does not suffer from these deficiencies; however, its own limitations include the assumption of identical and independent binding sites whose molecular structures are unspecified. Furthermore, this model ignores the fact that the binding of urea to unfolded proteins in sufficiently weak that water as an alternative ligand must be considered in a rigorous analysis (Schellman, 1990; Timsaheff, 1992, 1993). Unfortunately, these more rigorous analyses have not yet led to a workable method for determining the stability of a protein.

Although the assumptions made in the denaturant-binding model may render these parameters to be more apparent than actual, the simplifications that limit their accuracy for thermodynamic measurements should similarly affect their accuracy in the description of transition states for time-dependent processes. Thus, it seems valid to conclude that the excellent agreement between the enthalpies and entropies of urea binding to unfolded proteins and to the transition state for the rate-limiting step in the folding of the α subunit of tryptophan synthase implies similar binding opportunities for urea in both cases. The transition state appears to have chemical and thermodynamic properties which are virtually

identical to those of a stable, well-defined thermodynamic state.

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