Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 10636–10640, September 1997
Biophysics

Protein folding kinetics exhibit an Arrhenius temperature dependence when corrected for the temperature dependence of protein stability

MICHELLE L. SCALLEY AND DAVID BAKER*

Department of Biochemistry 357350, University of Washington, Seattle, WA 98195

Communicated by Robert L. Baldwin, Stanford Medical Center, Stanford, CA, August 1, 1997 (received for review May 28, 1997)

ABSTRACT **The anomalous temperature dependence of protein folding has received considerable attention. Here we show that the temperature dependence of the folding of protein L becomes extremely simple when the effects of temperature on protein stability are corrected for; the logarithm of the folding rate is a linear function of $1/T$ on constant stability contours in the temperature–denaturant plane. This convincingly demonstrates that the anomalous temperature dependence of folding derives from the temperature dependence of the interactions that stabilize proteins, rather than from the super Arrhenius temperature dependence predicted for the configurational diffusion constant on a rough energy landscape. However, because of the limited temperature range accessible to experiment, the results do not rule out models with higher order temperature dependences. The significance of the slope of the stability-corrected Arrhenius plots is discussed.**

The temperature dependence of protein refolding exhibits strongly non-Arrhenius behavior (1–4). Two explanations for the curvature observed in plots of ln *rate* versus $1/T$ have been proposed. The first explanation is based on the argument that the rate of escape from local minima on a rough energy landscape has a super Arrhenius temperature dependence. The argument may be summarized as follows (5): assume that the energy distribution for conformations with a particular similarity to the native conformation is Gaussian with mean E_{av} and variance ΔE^2. The most probable energy in a Boltzman distribution of such conformations is that for which $\exp-[E/kT + \{(E - E_{av})^2/2\Delta E^2\}]$ has a maximum; this is $E_{m.p.} = E_{av} - \Delta E^2/kT$. With the further assumption that these probable conformations are surrounded by conformations with energy close to E_{av}, the simple transition-state theory estimate for the rate of transitions out of probable conformations is rate $\sim \exp-(E_{av} - E_{m.p.})/kT = \exp-(\Delta E/kT)^2$ (a more detailed derivation (6) yields an expression with an additional linear term at intermediate temperatures). The temperature dependence of the rate of transitions between conformations is thus strongly non-Arrhenius because the argument of the exponential is quadratic in $1/T$ rather than linear. Protein folding may be modeled as a process of diffusion in configurational space (5, 7, 8); in such a treatment, the configurational diffusion constant is simply the rate of transitions between configurations. The strongly non-Arrhenius temperature dependence of the configurational diffusion constant leads directly to a non-Arrhenius temperature dependence for overall folding (see Eq. **3** below).

The second explanation traces the non-Arrhenius temperature dependence of folding to the unusual temperature dependence of the hydrophobic interaction (1–4, 9–11). The burial of hydrophobic residues involves a large change in heat capacity and, thus, the associated free energy changes are strongly dependent on temperature. To account for this temperature dependence, refolding kinetic data are generally fit by experimentalists using a simple transition-state theory-based model that allows for a change in heat capacity in going from the unfolded state to the folding transition state. The large heat capacity changes indicated by such fits are thought to reflect a large decrease in solvent-accessible surface area in the transition state relative to the unfolded state.

Which of these two explanations accounts for the majority of the curvature in Arrhenius plots for protein folding? There is general agreement that the temperature dependence of the hydrophobic interaction contributes at least in part to the non-Arrhenius temperature dependence of folding, and experimental data can be fit quite well by models that incorporate a large heat capacity change in the rate limiting step in folding without a non-Arrhenius temperature dependence in the prefactor. However, because there are three free parameters in such models (ΔH^{TS}, ΔS^{TS}, ΔCp^{TS}; see below), the quality of the fits probably does not exclude a nonlinear dependence of the prefactor on temperature.

In this paper we deconvolute the intrinsic temperature dependence of folding from the temperature dependence of protein stability by considering the temperature dependence of folding for conditions in which the overall stability is kept constant by manipulating the denaturant concentration. The model system is the 62 residue IgG binding domain of protein L, which like many other very small proteins folds in a highly cooperative two-state reaction (12).

METHODS

Throughout this paper protein L refers to the Y43W point mutant of protein L (12). Protein and buffers were prepared as described previously (12). The folding and unfolding kinetics of protein L between 277 K and 331 K were obtained with a BioLogic SFM/QFM4 as described (12). Kinetic measurements below 277 K were obtained using a Spex Fluorolog2 fluorimeter. Protein and buffer conditions were identical to those used in stopped-flow experiments and either refolding or unfolding was initiated by manual mixing of the protein solution with the appropriate buffer. Thermal control to within ± 0.5 K was achieved in all experiments using a circulating water bath; in the low temperature experiments the temperature within the cuvette was measured directly using a calibrated thermistor. The kinetic traces were fit to single exponentials using the KALEIDOGRAPH software package or the Biokine analysis software.

Activation parameters for folding and unfolding were obtained from rate constants determined at temperatures ranging from 277 K to 331 K and guanidine concentrations ranging from 0.3 to 6.5 M by fitting the data with the simple model

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*e-mail: baker@ben.bchem.washington.edu.

$$k(T,[gnd]) = D\Bigg[\exp -\frac{1}{RT}\Bigg(\Delta H_{U-TS} - T\Delta S_{U-TS} + \Delta C_{p:U-TS}\Bigg(T - T_0 - T\ln\Bigg(\frac{T}{T_0}\Bigg)\Bigg) - m_{U-TS}[gnd]\Bigg) + \exp -\frac{1}{RT}\Bigg(\Delta H_{N-TS} - T\Delta S_{N-TS} + \Delta C_{p:N-TS}\Bigg(T - T_0 - T\ln\Bigg(\frac{T}{T_0}\Bigg)\Bigg) - m_{N-TS}[gnd]\Bigg)\Bigg], \quad [1]$$

where $U-TS$ denotes parameters for the folding reaction and $N-TS$ refers to parameters for the unfolding reaction. Here and throughout the paper, ΔH and ΔS indicate standard state (295 K, 0 M denaturant) quantities and m and ΔC_p are assumed for simplicity to be independent of temperature and denaturant concentration.

RESULTS AND DISCUSSION

The temperature and denaturant dependences of the thermodynamics of folding of protein L were determined in an earlier study from thermal denaturation melts monitored by circular dichroism at nine different guanidine concentrations (13). A simple functional form for the free energy of folding,

$$\Delta G(T,den) = \Delta G(T) - m[den] = \Delta H - T\Delta S + \Delta C_p[T - T_0 - T\ln T/T_0] - m[den] \quad [2]$$

fit the data quite well. The values of the thermodynamic parameters are listed in Table 1.

The temperature and denaturant dependence of the kinetics of folding of protein L were determined in kinetic folding and unfolding experiments at temperatures ranging from 267K to 331K and guanidine concentrations ranging from 0.3 to 6.5 M. Rate constants were obtained from single exponential fits of these data; the fits were excellent at all temperature and denaturant concentrations.

How is such kinetic data best modeled? Recent work has demonstrated that for simple computational models of folding where the folding rate and ΔG^{TS} can be determined independently, a very simple transition-state theory expression

$$rate = D\exp(-\Delta G^{TS}/RT) \quad [3]$$

provides an excellent approximation of the folding rate (8, 14). Thus, the features that distinguish protein folding from the simple chemical reactions in which Eq. **3** is most often applied, notably the large change in configurational entropy, do not reduce the utility of the transition state concept (unlike simpler reactions, however, the transition state in folding reactions is likely to be a large ensemble of possibly quite disparate conformations). A temperature dependence consistent with Eq. **3** has been observed in lattice simulations (15).

The temperature and denaturant dependences of folding kinetics can be modeled by combining Eqs. **2** and **3** (see Eq. **1**). The kinetic data on protein L are well fit by such a model; the data points and the surface corresponding to the best global fit of the data are shown in Fig. 1*A*. This model assumes that the denaturant dependences (m-values) for the folding and unfolding reactions are temperature independent. A justification for this assumption is illustrated in Fig. 1*B*; the folding and unfolding sections of kinetic "v" curves for different temperatures have very similar slopes. In the kinetic modeling, D was taken to be $10^{10}\ s^{-1}$ (16); the rate estimated for the elementary step of adding a residue at the end of a helix. More plausible estimates of D may be obtainable from measurements of the magnitude of fluctuations in partially folded states (8, 17).

The temperature dependence of the folding and unfolding of protein L resembles that of other proteins. Plots of $\ln k$ versus $1/T$ are strongly curved for refolding, but almost linear for unfolding (Fig. 1*C*). This is reflected in the parameter estimates obtained from fits of Eq. **1** to the data: ΔC_p is considerably larger for refolding than for unfolding (Table 1). In a simple two-state model, the kinetic and thermodynamic parameters are not independent; the parameters in the third column should be close to the N → TS values minus the U → TS values. The agreement between the kinetic and thermodynamic parameters is reasonable, perfect agreement is not expected because of the partial breakdown of Eq. **2** for protein L (13).

To deconvolute the indirect effects of temperature on the forces driving folding from the intrinsic temperature dependence of the folding reaction, we considered the temperature dependence of the folding rate for sets of conditions in which the overall stability of the protein was constant. Fig. 2 shows the logarithm of the folding rate constant as a function of $1/T$ on constant stability contours through the temperature-denaturant plane. The guanidine concentration for each temperature was chosen such that the overall free energy of folding divided by the temperature was that of the appropriate contour. For example, the data indicated by the solid circles were collected under conditions where $\Delta G/T$ was close to 0.0136. For clarity, the dependence of the folding rate on temperature and denaturant concentration along this contour is provided in detail in Table 2.

The striking result is that the plots of $\ln k$ versus $1/T$ in Fig. 2 are linear; the temperature dependence of folding now appears to be similar to that of simple chemical reactions. This simple dependence suggests that the non-Arrhenius behavior observed for folding kinetics is entirely a result of the temperature dependence of the forces driving folding. The simplicity of the curves in Fig. 2 is encouraging for the theoretical understanding of protein folding.

Is the linearity of the $\ln k$ versus $1/T$ plots shown in Fig. 2 a general feature of protein folding reactions? Answering this question requires data on the temperature and denaturant dependence of both the kinetics and thermodynamics of folding. The only other protein for which such data are available in the literature is the *Bacillus* cold-shock protein, CspB (3). The individual rate constants at different temperatures and guanidine concentrations have not been published; instead, activation parameters for the folding of CspB analogous to those in Table 1 for protein L have been reported. Fig. 3 shows the dependence of $\ln k$ on $1/T$ plots for CspB along

Table 1. Thermodynamic and kinetic parameters for protein L refolding

Parameter		U → TS	N → TS	N → U
m	(kcal mol^{-1} M^{-1})	−1.4 ± 0.02	0.6 ± 0.01	2.4 ± 0.1
ΔC_p	(kcal mol^{-1} K^{-1})	−0.32 ± 0.02	0.19 ± 0.03	0.77 ± 0.02
ΔS	(kcal mol^{-1} K^{-1})	−0.01 ± 0.001	0.03 ± 0.001	0.05 ± 0.001
ΔH	(kcal mol^{-1})	9.4 ± 0.2	26.8 ± 0.2	20.1 ± 0.4

Activation parameters and standard deviations were determined from the nonlinear least-squares fit of the data to Eq. **1**. The reference temperature (T_0) was 295 K. True errors may be larger than the errors reported here; in particular, the estimates of ΔH and ΔS are highly correlated. Thermodynamic parameters were obtained as described in Yi *et al.* (13).

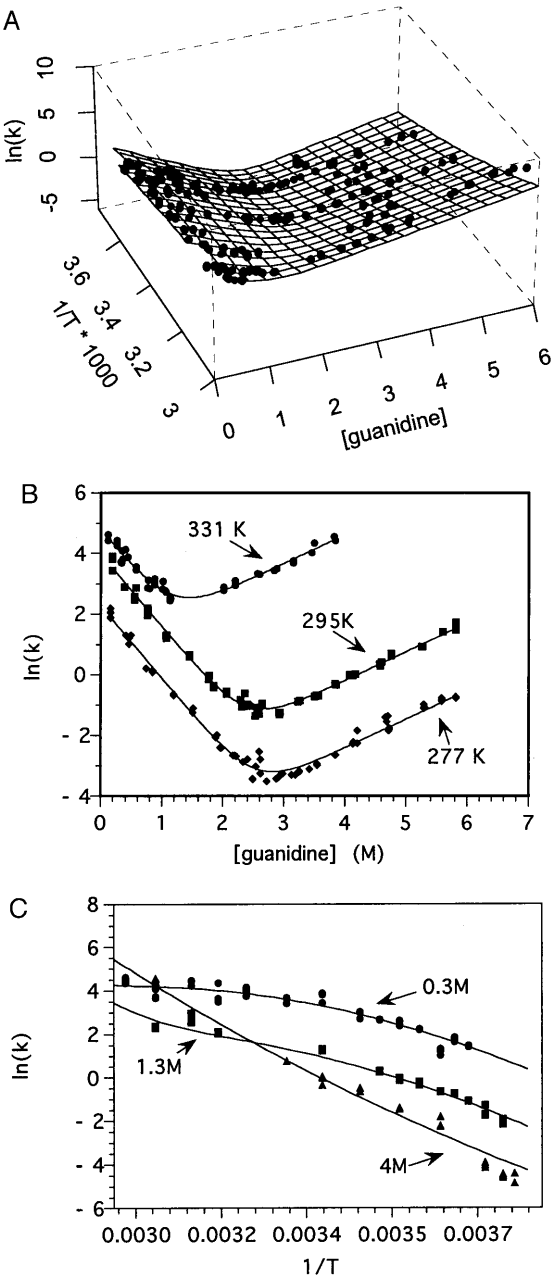

FIG. 1. Temperature and denaturant dependence of protein L folding kinetics. (*A*) Folding rate versus temperature and denaturant concentration. Data were fit using Eq. **1** with the nonlinear least-squares function in the S plus statistical package (Statistical Science, Seattle, WA). (*B*) Guanidine dependence of folding and unfolding rates at 277 K (◆), 295 K (■), and 318 K (●). (*C*) Temperature dependence of folding and unfolding. The observed relaxation rate is dominated by folding at 0.3 M (●) and 1.3 M guanidine (■), and by unfolding at 4.0 M (▲) guanidine. Solid lines are the best fit of the data to Eq. **1**. The upward curvature at small values of $1/T$ in the 1.3 M curve is due to the increasing contribution of the unfolding reaction to the observed relaxation rate (the melting temperature under these conditions is ≈330 K).

constant stability contours; the rates shown are estimated using the published activation parameters rather than the actually measured rates as in Fig. 2 for protein L. Again, the plots of ln k versus $1/T$ along constant stability contours are close to linear.

Although the manipulations leading to Fig. 2 are independent of any particular kinetic model, the interpretation of the slope of the constant stability contours requires additional assumptions. Two very different interpretations are of particular interest.

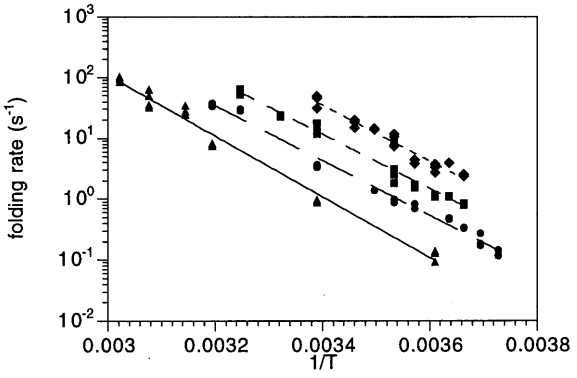

FIG. 2. Temperature dependence of protein L folding on constant stability contours. (◆), $\Delta G/T$ = 0.02 ± 0.0008 kcal mol^{-1} K^{-1}; (■), 0.0168 ± 0.0008 kcal mol^{-1} K^{-1}; (●), 0.0135 ± 0.0008 kcal mol^{-1} K^{-1}; (▲), 0.0084 ± 0.0008 kcal mol^{-1} K^{-1}. The guanidine concentration for each temperature was chosen such that the overall free energy of folding divided by the temperature was that of the appropriate contour. The lines are simple linear fits of the data. For clarity, the guanidine dependence is not shown explicitly in Figs. 2–4.

Model I

Assume that all properties of the folding landscape remain unchanged upon changes in the denaturant concentration and temperature provided that $\Delta G/T$ is kept constant. This amounts to assuming that if the relative population of the unfolded state remains constant upon a given change in conditions, then so do the populations of all partially folded species. Support for this assumption is found in recent experimental (18, 19) and theoretical (20, 21) work, which suggests that the rate of folding is closely tied to the stability of the native state. The folding of two domains of fibronectin provides a dramatic example; in the absence of denaturant, one domain folded several hundred times faster than the other, but when the stabilities of the two proteins were matched by adding denaturant to the more stable domain, the folding rates of the two proteins were found to be very similar. Such a strong correlation between stability and the folding rate is expected if the interactions that stabilize the native state also stabilize the transition state.

The assumption that the critical features of the folding free energy landscape are unchanged by changes in conditions that leave $\Delta G/T$ unchanged can be implemented using a simple extension of Eq. **3** in which the configurational diffusion constant has an Arrhenius temperature dependence with activation energy ΔE_a,

$$\ln(rate) = \ln D_0 - \Delta E_a/RT - \Delta G^{TS}/RT. \qquad [4]$$

Since by assumption $\Delta G^{TS}/RT$ is independent of temperature as long as $\Delta G/T$ is constant, the slope of a plot of ln k versus $1/T$ on a constant stability contour is $-\Delta E_a/R$. Thus, the slope provides a direct measure of a fundamental quantity, the activation energy associated with configurational diffusion. It should be noted that this analysis is independent of the transition state approximation used in Eq. **3**; the same result is obtained with the exact double integral solution to the diffusion equation (7, 8) provided that D_0 is invariant.

Whereas this model is attractive because it relates the slope of the plots to the activation barrier for configurational diffusion, a fundamental physical quantity, there are problems. First, the slopes of the lines in Figs. 2 and 3 are close to 10,600 K, which corresponds to activation barriers of ≈22 kcal mol^{-1} or ≈36 kT. This is considerably larger than what might be expected for the barriers to configurational diffusion; Wolynes *et al.* (21) suggested a value of ≈7 kT. Second, it can be shown (E. Thayer, personal communication) that if the temperature and denaturant depen-

Table 2. Temperature and guanidine conditions for a protein L contour with $\Delta G/T = 0.0135 \pm 0.0008$ kcal mol^{-1} K^{-1}

Temperature, K	[Guanidine], M	$\Delta G/T$, kcal mol^{-1} K^{-1}	ΔG, kcal mol^{-1}	Folding rate, s^{-1}
268.3	1.26	0.014	3.80	0.12
268.3	1.28	0.014	3.75	0.14
270.7	1.28	0.014	3.69	0.18
270.7	1.28	0.014	3.69	0.27
270.7	1.28	0.014	3.69	0.17
273.0	1.29	0.014	3.76	0.34
273.0	1.27	0.014	3.81	0.34
275.0	1.29	0.014	3.78	0.49
275.0	1.23	0.014	3.93	0.47
280.0	1.34	0.013	3.67	0.83
280.0	1.36	0.013	3.62	0.70
283.0	1.32	0.013	3.68	0.98
283.0	1.30	0.013	3.73	0.88
286.0	1.33	0.013	3.61	1.37
295.0	1.07	0.013	3.92	3.35
295.0	1.06	0.013	3.95	3.60
308.0	0.55	0.014	4.36	28.09
308.0	0.55	0.014	4.36	30.22
313.0	0.42	0.014	4.25	36.25
313.0	0.44	0.013	4.19	35.31

These data are indicated by circles in Fig. 2.

dences of ΔG^{TS} and ΔG can be expressed as in Eq. **2**, the assumption underlying the model requires that ΔH^{TS}, $\Delta C_p{}^{TS}$, and m^{TS} are proportional to the corresponding thermodynamic parameters; this is problematic because m^{TS} and m (and $\Delta C_p{}^{TS}$ and ΔC_p) have opposite signs, whereas ΔG^{TS} and ΔG and probably ΔH^{TS} and ΔH (if the above estimate for the barrier to configurational diffusion is accurate) have the same sign under native conditions.

Model II

Because of the problems with Model I, it is useful to also consider a model based only on the assumption that the temperature and denaturant dependences of folding kinetics can be described using Eqs. **2** and **3**. Unlike Model I, no additional relationships are assumed between the activation parameters ($\Delta C_p{}^{TS}$, ΔH^{TS}, ΔS^{TS}, and m^{TS}) and the corresponding overall thermodynamic parameters. Using $[den] = [\Delta G(T) - \Delta G(T, den)]/m$ leads to

$$\ln(rate) = \ln D_0 - \Delta E_a/RT - \frac{m^{TS}}{m}[\Delta G(T, den)]/RT - \left[\Delta G^{TS}(T) - \frac{m^{TS}}{m}\Delta G(T)\right]/RT. \quad [5]$$

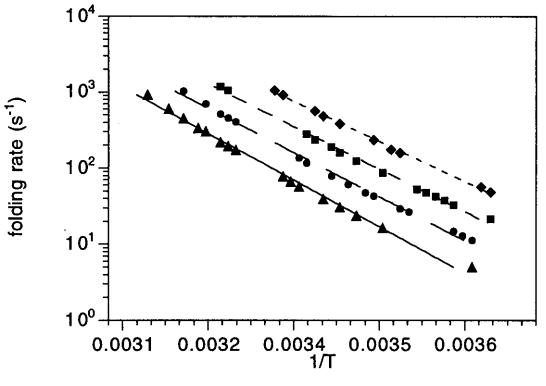

FIG. 3. Temperature dependence of CspB folding on constant stability contours. (◆), $\Delta G/T = 0.026$ kcal mol^{-1} K^{-1}; (■), 0.020 kcal mol^{-1} K^{-1}; (●), 0.014 kcal mol^{-1} K^{-1}; (▲), 0.0085 kcal mol^{-1} K^{-1}. Lines are simple linear fits of the data.

Since by construction $\Delta G(T, den)/T$ is constant along the contours in Figs. 2 and 3, excluding the preexponential factor the temperature dependence is determined by $[\Delta G^{TS}(T) - (m^{TS}/m)\Delta G(T)]$, which can be further expanded using Eq. **2** for both $\Delta G^{TS}(T)$ and $\Delta G(T)$:

$$\left[\Delta G^{TS}(T) - \frac{m^{TS}}{m}\Delta G(T)\right] = \Delta H^{TS} - \frac{m^{TS}}{m}\Delta H - T\left(\Delta S^{TS} - \frac{m^{TS}}{m}\Delta S\right) + \left(\Delta C_p^{TS} - \frac{m^{TS}}{m}\Delta C_p\right) \cdot \left[T - T_0 - T\ln\left(\frac{T}{T_0}\right)\right]. \quad [6]$$

The observation that the logarithm of the rate is a nearly linear function of $1/T$ implies in this model that $[\Delta C_p{}^{TS} - (m^{TS}/m)\Delta C_p]$ is close to zero. This is the case for protein L and the other small proteins which have been studied (2, 3); similar values of $\Delta C_p{}^{TS}/\Delta C_p$ and m^{TS}/m are usually thought to reflect the dependence of both ratios on the fractional solvent accessibility of the transition state relative to the unfolded state. With the cancellation of this term,

$$\ln(rate) = \ln D_0 - \Delta E_a/RT - \frac{m^{TS}}{m}[\Delta G(T, den)]/RT - \left[\Delta S^{TS} - \frac{m^{TS}}{m}\Delta S\right]/R - \left[\Delta H^{TS} - \frac{m^{TS}}{m}\Delta H\right]/RT. \quad [7]$$

Thus, with this model and cancellation of heat capacity containing term, the slope of the contours in Figs. 2 and 3 is $[-\Delta E_a - \Delta H^{TS} + (m^{TS}/m)\Delta H]/R$.

The estimate of the quantity within the brackets from the slope of the lines in Figs. 2 and 3 is $\approx$22 kcal mol^{-1}. The third term in this expression (the product of the ratio of m values with the equilibrium enthalpy change) accounts for 13 kcal mol^{-1}, the remaining 9 kcal mol^{-1} may have contributions from both barriers to configurational diffusion (ΔE_a) and more discrete barriers associated with specific conformational transitions (ΔH^{TS}), such as the breaking of enthalpically favorable interactions present in the unfolded state and the desolvation

of the protein core (22, 23). It is difficult to separate activation barriers associated with configurational diffusion from discrete enthalpic barriers to folding; toward this end we are currently trying to determine what contributes to the observed activation enthalpy via mutational studies.

In Eq. **7**, the rate is expressed as a function of two independent variables, $\Delta G(T, den)/T$ and T. Whereas ln(*rate*) depends on both variables, the quantity $[\ln(rate) + \Delta G(T, den)m^{TS}/mRT]$ is predicted to be a function of temperature alone. As shown in Fig. 4*A*, the addition of $\Delta G(T, den)m^{TS}/mRT$ to each of the points in Figs. 2 and 3 collapses the different stability contours for protein L and CspB to single lines. The slope of the composite contours for the two proteins are strikingly similar (Fig. 4*A*, the slopes of the best fit lines are 10,800 K and 11,000 K for protein L and CspB, respectively). From the point of view of Model II, the small amount of curvature in the CspB "data" reflects the lack of cancellation of the nonlinear $[\Delta C_p^{TS} - (m^{TS}/m)\Delta C_p]$ term in Eq. **6**.

Does the similarity in the slopes of the protein L and CspB composite contours (Fig. 4*A*), despite the very different topologies of the proteins, reflect a deeper similarity in the folding of small proteins? Data on the temperature and denaturant dependence of the thermodynamics and kinetics of several other small proteins will be necessary to answer this important question.

Thus, protein folding exhibits an Arrhenius temperature dependence similar to that of simple chemical reactions when corrected for the anomalous effects of temperature on stability. This suggests that the configurational diffusion constant has an Arrhenius temperature dependence. We note, however, that because of the relatively small temperature range over which protein folding kinetics can be measured, the kinetic data are also well fit by the super Arrhenius expression suggested by the argument summarized in the first paragraph [compare Figs. 4 *A* and *B*; a similar ambiguity was noted previously in lattice simulation studies (8)]. Unlike the raw kinetic data, the stability-corrected kinetic data are consistent with a simple Arrhenius temperature dependence, but more complex models are clearly not excluded.

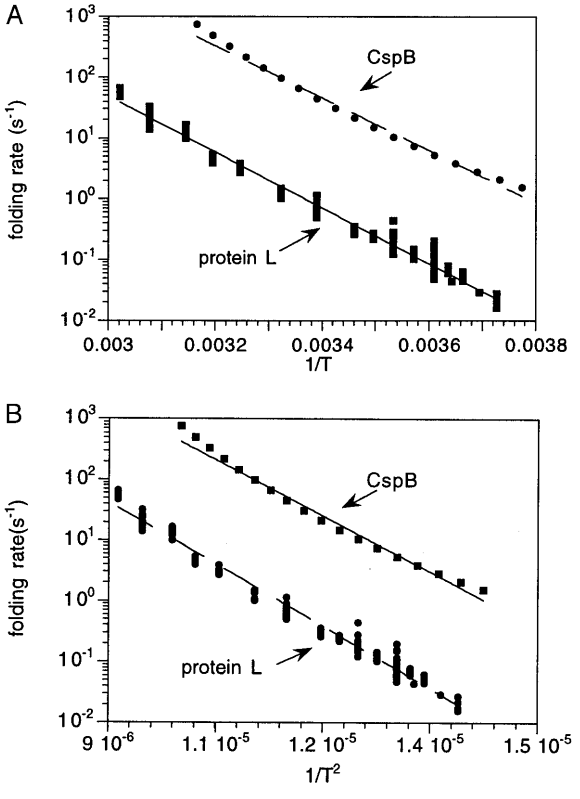

FIG. 4. The slopes of constant stability contours for protein L and CspB are very similar. The contours for protein L (■) and CspB (●) in Figs. 2 and 3 were collapsed to single curves corresponding to a ΔG of 2 kcal mol^{-1} at 295 K by adding $(\Delta G/T - 0.0067)m^{TS}/mR$ to each data point. (*A*) Folding rate versus $1/T$. (*B*) Folding rate versus $1/T^2$.

The major conclusion of this paper is that the pronounced curvature observed in Arrhenius plots for protein folding reflects the temperature dependence of protein stability. The interpretation of the slope of the plots of ln k versus $1/T$ on constant stability contours is a challenge for future theoretical work in this area. Conversely, accurate computational models of protein folding should exhibit an Arrhenius temperature dependence under constant stability conditions similar to that observed experimentally.

We thank Ed Thayer for exploring the assumptions underlying Model I; Jose Oñuchic for suggesting the constant stability plots; Robert Baldwin, Hue Sun Chan, Jose Oñuchic, Jon Goldberg, and Eugene Shakhnovich for helpful comments on the manuscript; and Peter Wolynes, Ken Dill, Hue Sun Chan, Jose Oñuchic, and Eugene Shakhnovich for preprints prior to publication. This work was supported by the National Institutes of Health and by Young Investigator awards to D.B. from the National Science Foundation and the Packard Foundation.

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