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Internal friction in the ultrafast folding of the tryptophan cage ☆

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Abstract

Protein folding is a diffusional process, and the speed of folding is controlled by the frictional forces that act on the polypeptide chain. Several previous studies have suggested that the bulk viscosity of the solvent is the only important source of friction in folding reactions. By contrast, our studies of the folding dynamics of the Tryptophan Cage, a small, ultrafast-folding protein, show clear evidence for additional frictional forces that are independent of the solvent viscosity. The nature of this internal friction appears qualitatively different for the folding vs. unfolding reactions. We expect that this phenomenon could significantly influence the rate of many folding processes that proceed on sufficiently fast (∼μs) time scales. In particular, it may limit folding speeds to values substantially slower than the diffusion limits estimated from simple contact-formation studies in polypeptides.
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1. Introduction

The increasing numbers of proteins that are discovered to fold at "ultrafast" rates (folding rate > (100 μs)$^{-1}$) has drawn attention to the question of what physical phenomena impose upper limits to the speed of protein folding [1]. Given the microscopic size of an unfolded protein molecule and the finite viscosity of its solvent, friction and diffusion are certainly at the center of this problem. The dynamics of protein comformational changes and folding are both driven and damped by dissipative interactions with the solvent environment. Many experimental investigations of the upper limits to folding speed have therefore focused on the rate of bulk diffusional reconfigurations of disordered polypeptides in aqueous solvent. However, the nature of the frictional

forces that actually control these dynamics has received much less attention.

It is not clear, for example, how the compaction of a polypeptide chain during folding may affect the speed of its conformational diffusion. The polymer dynamics literature clearly suggests that interactions within a chain may generate internal friction forces [2,3], which may slow the search for the folded state. While several authors have shown that the frictional drag that controls chain diffusion in "slow" protein folding is dominated by the viscosity of the surrounding solvent [4–8], here we examine the nature of frictional forces that govern ultrafast folding. Our experimental studies of the folding of the Tryptophan Cage, one of the very fastest folding proteins, suggest that solvent viscosity alone does not control these dynamics. We find evidence that, for a sufficiently fast folding reaction, solvent friction may be less important than dissipative intrachain interactions in controlling the speed of folding.

2. Background – Kramers theory and protein folding

Even the fastest protein folding reactions proceed on time scales (∼μs) far slower than the velocity relaxation

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time (<ps) of any part of the polypeptide chain. This strong frictional damping, together with the typically two-state character of many folding reactions, implies that the simple one-dimensional Kramers' theory for an overdamped reaction should capture many essential properties of protein folding reactions [9–11]. In its simplest form, Kramers' theory describes the rate at which Brownian forces drive a hypothetical particle across a one-dimensional barrier of height ΔE. In the high-friction limit, the net rate is

$$k = (\omega_a \omega_b / 2\pi\gamma) \exp(-\Delta E / RT), \tag{1}$$

where ω_a and ω_b are the curvature of the potential energy surface at the bottom and top of the barrier, respectively, and γ is the frictional damping parameter appearing in the Langevin equation.

Because the one-dimensional Langevin equation that underlies Eq. (1) is only a phenomenological description of the folding reaction, its connection to physically measureable parameters is not obvious. However, a reasonable and simple interpretation of the friction γ is that it arises from the dynamic viscosity η_s of the solvent, $\gamma \propto \eta_s$. Eq. (1) then predicts that folding rates will scale as $k_f \propto \eta_s^{-1}$. Many authors have tested this prediction by studying the folding rates k_f of small proteins as a function of solvent viscosity [4–8,12–15]. The viscosity of the folding buffer cannot readily be reduced below that of water ($\eta_{H_2O} = 1$ mPa s = 1 centipoise at 20 °C), but it can be raised by the addition of cosolutes, such as sugars or glycerol. In those studies where the thermodynamic stability of the native state was maintained constant as the viscogens were added, the anticipated linear behavior $k_f \propto \eta_s^{-1}$ was in fact observed [4,5,7,8]. For example, Plaxco and Baker [5] found that the folding time $1/k_f$ of protein L is quite linear in η_s, over the range $1 \leqslant \eta_s \leqslant 3.5$ mPa s. A linear extrapolation gives $k_f^{-1} = 0$ at $\eta_s = -0.1 \pm 0.2$ mPa s. Jacob et al. [8] found similar results for the folding of CspB. These results support the simple view that solvent drag provides the reaction friction that controls folding speed.

In fact, this result was not necessarily expected, because a previous study had found that conformational relaxations of a folded protein deviate from the simple $k \propto \eta_s^{-1}$ behavior. Ansari et al. [16] studied the rapid internal relaxation that occurs in myoglobin when a CO ligand at the heme iron is photodissociated by a laser pulse. They found that the relaxation rate k obeys $k \propto \eta_s^{-1}$ only for η_s much larger than 1 mPa s. At low viscosities the rate k approaches a limiting value. Thus $k(\eta_s)$ can be fit to a modified Kramers form, $k \propto 1/(\eta_s + \sigma)$, where $\sigma \approx 4.1 \pm 1.3$ mPa s. This analysis suggests that the total friction γ for the relaxation is the sum of the solvent friction η_s and a "protein friction" σ. This concept of a protein friction or "internal viscosity", resulting from dissipative interactions internal to a chain molecule, in fact has a long history in the polymer

dynamics literature [2,3]. Although the likely experimental signature of the effect remains an unresolved question, the departure from $k \propto \eta_s^{-1}$ in myoglobin appears to confirm that internal friction can influence protein conformational dynamics. However, the large σ in myoglobin (larger than η_s) raises the question of why $\sigma = 0$ in the folding of both protein L and CspB. This is especially puzzling given that both proteins fold through highly compact transition states [5], where one might expect pronounced internal friction if the effect exists at all.

We suggest that these previous results are not necessarily in contradiction. One could imagine that internal friction is best described, not in terms of a viscosity σ, but in terms of a time scale τ_{int} for chain dynamics in the absence of strong solvent damping. That is, conformational dynamics in a viscous solvent are controlled primarily by the solvent viscosity, but as η_s tends toward zero, interactions within the molecule define a characteristic time scale τ_{int} for reconfigurations. These prevent the protein relaxation rate from becoming larger than $1/\tau_{int}$. Although this model is still consistent with the Ansari data (see Section 4), it implies that the impact of internal friction on protein dynamics depends on the time scale of those dynamics. One expects the rate of protein folding to deviate from simple Kramers behavior ($k_f \propto 1/\eta_s$) only when $k_f \sim 1/\tau_{int}$. Folding of protein L and CspB is relatively slow ($1/k_f \sim$ ms), whereas the Ansari data suggest $\tau_{int} \sim$ ns. The simple Kramers behavior of protein L and CspB folding is then less surprising. This argument suggests that internal friction could influence the very fastest protein folding reactions: In order to observe this influence, one must study a protein that folds at rates comparable to $1/\tau_{int}$.

We therefore studied the relationship between solvent viscosity and folding rate for the Tryptophan Cage TC5b ("TrpCage"). TrpCage is a 20-residue designed miniprotein [17] that folds through a two-state transition at a rate ($k_f > 250,000$ s^{-1} at 23 °C) that is among the fastest ever directly measured [18]. The native structure (pdb 1L2Y – see Fig. 1) comprises an α-helical N-terminal region, a short 3_{10} helix, and a polyproline II helix at the C-terminus, which wraps around to pack the single Trp residue within a compact hydrophobic core. Our studies of the rapid folding of TrpCage appear to confirm that internal friction can impose a significant constraint on fast folding dynamics.

3. Methods

We used laser temperature-jump spectroscopy to trigger and probe the folding of the TrpCage. Our T-jump instrument uses a H$_2$ Raman cell to shift a 5–7 ns Nd:YAG laser pulse from 1.06 to 1.89 μm wavelength. Beam splitting and steering optics then separate the IR
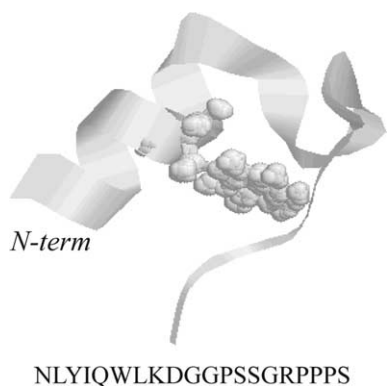
Fig. 1. Ribbon diagram and sequence of the Tryptophan Cage TC5b designed by Neidigh et al. [17]. The single tryptophan residue in the hydrophobic core is shown in detail.

pulse into two counterpropagating pulses and focus these onto the sample flow cell. The two IR pulses enter the aqueous sample within an interval ∼30 ns, causing an immediate rise of 5 °C in the solvent temperature. This triggers a re-equilibration between folded and unfolded states of the protein. Unfolding of the TrpCage causes a weak increase in the fluorescence of Trp-6. We therefore detect the folding kinetics by exciting the Trp fluorescence with a 266 nm pulse laser (Nd:YAG 4th harmonic) and collecting the emission on a photomultiplier. After the T-jump, the sample cools and returns to its initial state on a time scale ($\sim 10^{-1}$ s) that is much slower than the folding kinetics.

TrpCage TC5b was synthesized in the University of Florida Protein Chemistry Core facility by solid phase (FMOC) chemistry and purified by reverse-phase HPLC on a C-18 column. The lyophilized protein was dissolved in 50 mM phosphate buffer, pH 7. In this solvent the protein unfolds at $T_f \approx 41$ °C. We varied the dynamic viscosity η_s of the solvent as much as 3–4× (depending on T) by adding glucose at concentrations as high as 2 M. Because glucose has a molecular weight (180.2 g/mol) substantially smaller than that of the TrpCage, we do not expect that the folding dynamics are significantly affected by the microviscosity and molecular crowding effects that affect the diffusion of a smaller molecule in a solvent containing high molecular weight viscogen (e.g., PEG). However, the glucose does tend to stabilize the folded state, which could generate spurious effects on the kinetics. Therefore we compensate stability shifts so that the ΔG of unfolding remains constant as η_s rises [5,8]. We accomplished this by adding GdnHCl simultaneously with the glucose. We used far-UV circular dichroism to measure the stability shifts of the TrpCage that are induced by both GdnHCl and glucose. These studies showed that the unfolding free energy of the TrpCage is well-described by

$$\Delta G \approx \Delta G_0 + m_{\text{GdnHCl}}[\text{GdnHCl}] + m_{\text{glucose}}[\text{glucose}],$$

where $\Delta G_0 \approx 2.5$ kJ/mol, $m_{\text{GdnHCl}} \approx -3.11 \pm 0.24$ kJ/mol/M, and $m_{\text{glucose}} \approx +1.90 \pm 0.53$ kJ/mol/M at 25 °C in pH 7 phosphate. Although these parameters are all temperature-dependent, we found the ratio of the GdnHCl and glucose m-values to be nearly independent of T for 15–35 °C. Therefore, within this temperature range the viscosity of the solvent can be raised while the stability (ΔG) remains constant if GdnHCl and glucose are added in a fixed molar ratio [GdnHCl]/[glucose] $= m_{\text{glucose}}/m_{\text{GdnHCl}} \approx 0.61$. We directly measured the kinematic viscosity of these buffer mixtures at each experimental temperature, using a calibrated Cannon–Fenske viscometer immersed in a water bath, controlled to ± 0.1 °C. This technique routinely gives viscosity values accurate to ± 1% or better.

4. Results

The rapid T-jump triggers a relaxation in the tryptophan fluorescence as the TrpCage re-equilibrates between its folded and unfolded states. This relaxation follows an exponential time course with a rate $k \sim 10^5$ s^{-1}. When the thermodynamic stability of the native fold is maintained constant, we find the relaxation rate slows as the dynamic viscosity η_s of the solvent rises, consistent with the general expectations from a Kramers description of protein folding. However, Fig. 2A shows that the data do not closely obey $k^{-1} \propto \eta_s$. The relaxation time k^{-1} has a weaker dependence on η_s, implying that the reaction friction is more complex than $\gamma \propto \eta_s$.

Fig. 2A shows that the data can be fit to a linear model,

$$k^{-1} = a + b\eta_s. \tag{2}$$

The TrpCage data clearly imply $a \approx 700$ ns, while simple Kramers behavior corresponds to $a = 0$. That is, the dynamics accelerate as solvent viscosity declines, but instead of growing without bound the rate tends toward a finite limiting value ($k \rightarrow a^{-1}$). Eq. (2) is virtually equivalent to the Ansari et al. [16] model $k^{-1} \propto \sigma + \eta_s$ for the viscosity-dependent relaxation of myoglobin, except that a is the y-intercept of the dashed lines in Fig. 2A whereas σ is the (negative) x-intercept.

Of course, one can parameterize the departure from simple Kramers behavior in numerous other ways. Fig. 2D also shows a fit to a power law,

$$k^{-1} \propto \eta_s^\alpha, \tag{3}$$

as suggested in some previous studies of protein conformational dynamics [14,19]. The TrpCage data are consistent with $\alpha \approx 0.8-0.9$ (Fig. 2F). It is evident from Fig. 2 that the models of Eqs. (2) and (3) both fit the data. However, they describe very different physical pictures of the role of solvent friction in relaxation dynamics. Eq. (3) implies that the relaxation accelerates
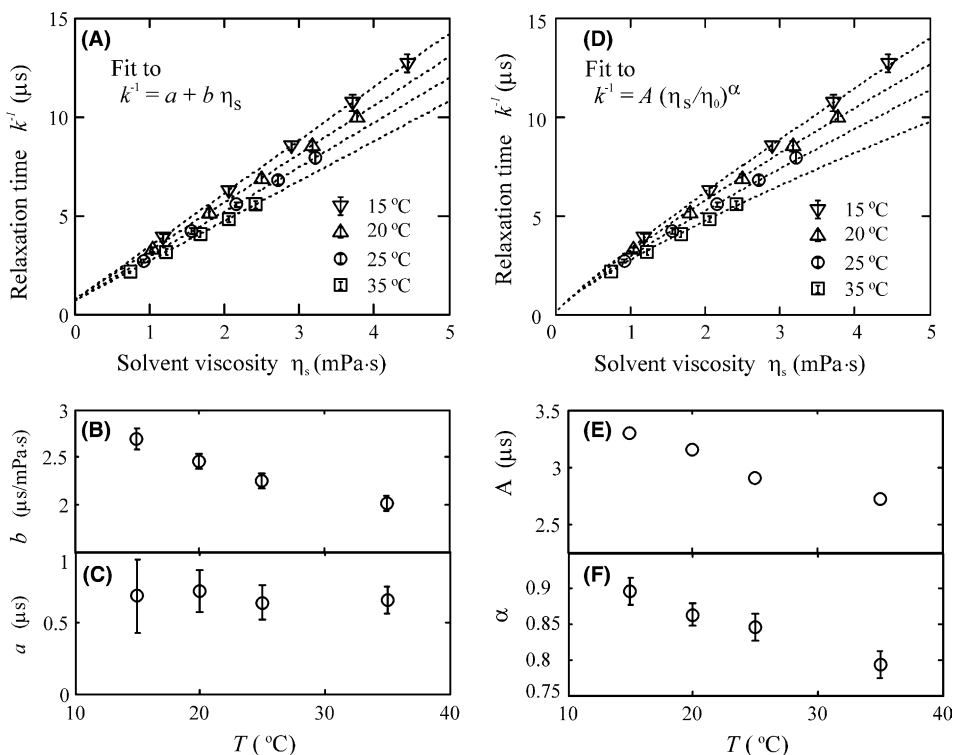
Fig. 2. Temperature and viscosity dependence of relaxations observed by T-jump fluorescence spectroscopy of TrpCage. (A) Relaxation time k^{-1} versus solvent viscosity η_s, with dotted lines indicating linear fits to data at each T. (B) Slope of k^{-1} obtained from linear fits. (C) y-intercept from linear fits. (D) Relaxation time versus solvent viscosity, with dotted lines indicating fits to power law (Eq. (3)) at each T. (E) Prefactor from power law fit. (F) Exponent α from power law fit. Solvent viscosities indicated on the x-axis of panels A and D were directly measured at each temperature indicated, so the temperature dependence of the solvent viscosity itself (which can be characterized by an activation energy $\sim$17 kJ/mol) does not produce any additional effects on the rates.

without limit ($k^{-1} \to 0$) at low η_s, which requires the fits in Fig. 2D to curve downward at low η_s. Eq. (2) does not require the relaxation rate to diverge at low η_s – rather it suggests that another damping mechanism intercedes to control the folding rate when solvent friction declines.

We argue that Eq. (2) represents a more appropriate analysis of the data than does Eq. (3). First, if during folding the protein passes through a compact configuration from which solvent is largely excluded, one may expect these reconfigurations to depend less strongly on solvent friction. Thus the folding rate should (at low η_s) tend toward a value that is insensitive to η_s, rather than continuing to depend on η_s. This argument favors the linear model (Eq. (2)) over the power law (Eq. (3)). Second, Eq. (3) yields no insight into why the TrpCage data of Fig. 2 and the myoglobin relaxation data of Ansari et al. deviate from simple Kramers behavior, while the folding rates for protein L and CspB do not: this requires the exponent α to vary greatly from one protein to another. In fact, for the TrpCage alone, this fit requires that α is strongly temperature-dependent (Fig. 2F). By contrast, Eq. (2) leads to the simpler conclusion that, for protein conformational changes, the value of a corresponds to a fast (e.g., nanoseconds) time scale. Therefore only microsecond (or faster) conformational

dynamics deviate noticeably from simple Kramers behavior. For these reasons we use the linear model of Eq. (2) to analyze the viscosity dependence of the folding and unfolding rates of the TrpCage.

Within a two-state model for folding, the relaxation rate k obtained from a T-jump experiment is the sum of the folding rate k_f and the unfolding rate k_u: $k = k_f + k_u = k_f(1 + K_{eq}^{-1})$, where $K_{eq} = \exp(\Delta G/RT) = k_f/k_u$ is the equilibrium constant for folding. Since the experimental data at each temperature fall (by design) along a line of constant K_{eq}, both k_u and k_f are simply proportional to k at each temperature. Therefore the nonzero y-intercept of k^{-1} at $\eta_s \to 0$ (Fig. 2A) requires that the unfolding and folding times k_u^{-1} and k_f^{-1} are also linear in η_s and tend toward finite limits at low η_s.

Figs. 3 and 4 illustrate this behavior of k_u^{-1} and k_f^{-1} and permit several interesting observations about the folding dynamics of the TrpCage. First, the folding time $1/k_f$ is insensitive to T at fixed solvent viscosity; much of the T-dependence of k_f near 1 mPa s (i.e., in water) evidently arises from changes in the solvent viscosity [20]: thus the kinetic barrier to TrpCage folding is largely entropic, rather than energetic. By contrast, the unfolding rate of the TrpCage depends strongly on T (Fig. 4A), implying a substantial enthalpic cost in passing over the
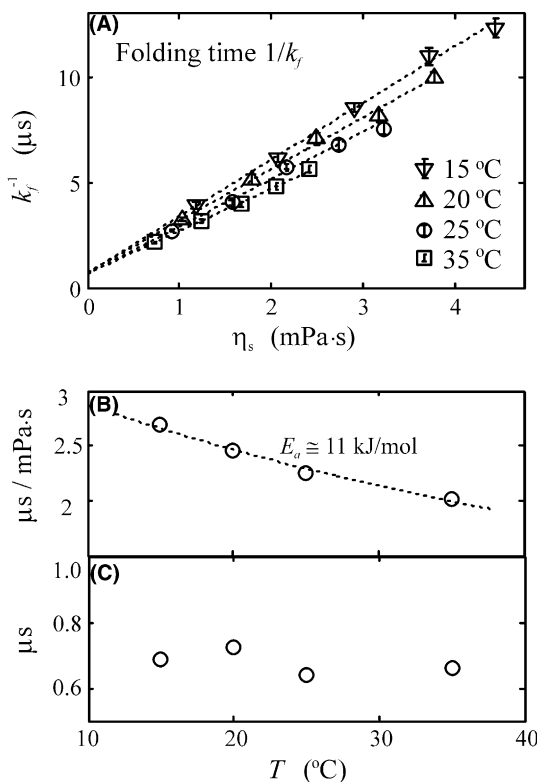
Fig. 3. (A) TrpCage folding time k_f^{-1} versus solvent viscosity η_s, with dotted lines indicating linear fits to the data at each T. (B) Slope obtained from linear fits, with dotted line indicating Arrhenius fit. (C) Intercept (or limiting value of k_f^{-1} at low viscosity) obtained from linear fits.

Fig. 4. (A) TrpCage unfolding time k_u^{-1} versus solvent viscosity η_s, with dotted lines indicating linear fits to the data at each T. (B) Slope obtained from linear fits, with dotted line indicating Arrhenius fit. (C) Intercept (or limiting value of k_u^{-1} at low viscosity) obtained from linear fits, and dotted line indicating Arrhenius fit.

primary unfolding barrier. Second, the folding time tends toward a limiting value ($\sim$0.6–0.7 μs) at low η_s that is virtually independent of temperature (Fig. 3C). By contrast, the unfolding time tends towards a limiting value (1–4 μs) that varies strongly with T. This can be characterized by a fairly large activation enthalpy $E_a \approx 51$ kJ/mol (Fig. 4C). Therefore, the folding and unfolding rates not only show a significant deviation from simple Kramers behavior, but they also show that the deviations can differ qualitatively (entropic or energetically controlled) for the forward (folding) and reverse (unfolding) directions in the folding reaction. This implies a different physical mechanism for the internal friction of folding vs. unfolding.

5. Discussion

The present data show that, for this ultrafast protein folding reaction, the folding and unfolding rates do not simply scale inversely with the bulk viscosity of the solvent. The solvent friction alone does not control the reaction rate, and for this reason it is useful to consider internal friction within the polypeptide chain. The concept of internal friction arises because, in progressing
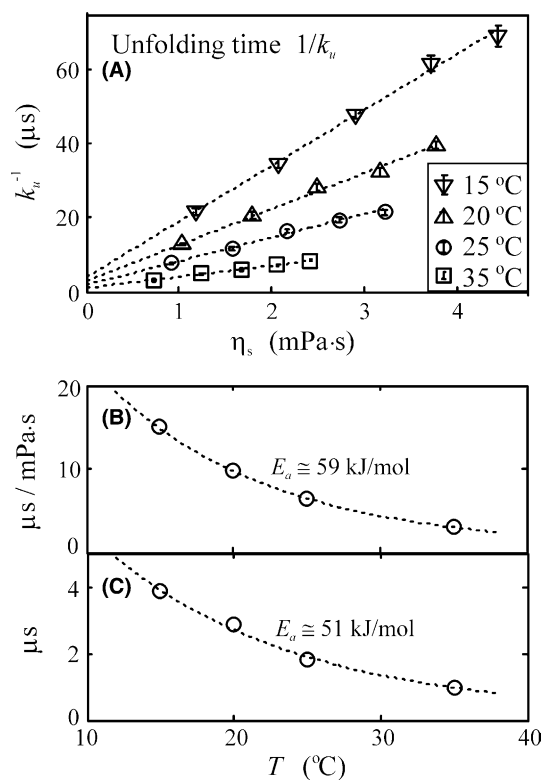
from a microscopic physical description (atomic and granular) to a macroscopic description (chain in a continuous medium), the dynamics of a polymer chain interacting with its solvent do not neatly separate into (1) conservative forces acting between parts of the chain and (2) a frictional force arising solely from the bulk viscosity η_s of the solvent. Many velocity-dependent (i.e., dissipative) forces have been proposed that arise from the physical structure and connectivity of the chain – and not (or not directly) from the bulk solvent viscosity. Some of these potential mechanisms of internal friction may act to multiply the solvent friction, while others may act additively with η_s. They may include local energetic barriers to the rotation of backbone (ϕ,ψ) angles, the energetics of actual physical contact at points where the chain interacts with itself, and "free volume" effects associated with the discrete structure of the solvent at microscopic length scales [2,3]. However, although the theoretical community appears to agree that internal friction effects could play a role in protein folding dynamics [21,22], it is not clear exactly what experimental signatures are anticipated.

From an empirical perspective, the linearity of the k_f^{-1} and k_u^{-1} data of Figs. 3 and 4 suggests an additive model for internal friction effects on folding. We suggest that

the folding (or unfolding) time is the sum of two time scales,

$$k_f^{-1} = \tau_s + \tau_{int}$$

as proposed in *Background*. That is, two different types of dynamics contribute to the folding: solvent-controlled reconfigurations of the polypeptide occur on a time scale τ_s, which scales in a simple Kramers-like fashion, $\tau_s \propto \eta_s$. Internal friction controls the rate of physically distinct solvent-independent dynamics, giving an internal time scale τ_{int}. The data do not support the existence of both a solvent-dependent and a solvent-independent pathway to the folded state: the additivity of the time scales, rather than the rates, implies that the two types of dynamics are sequential, rather than parallel.

As an example, one may imagine sequential dynamics,

$$\text{Unfolded} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Compact} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Native}$$

where the first step comprises solvent-controlled bulk motion of the chain ($k_1, k_{-1} \propto \eta_s$) while the second step requires reorganization of the compact molecule, characterized by solvent-independent rates k_2 and k_{-2}. The equilibrium constant for this model is viscosity independent and, if k_2 and k_{-2} are sufficiently rapid compared to k_1 and k_{-1} in aqueous buffer, the overall kinetics may be indistinguishable from normal 2-state folding with a single characteristic rate. For such a model, however, the viscosity dependence of the kinetics can deviate from the simple $k_f \sim \eta_s^{-1}$, even if the population of the compact intermediate never exceeds 5–10%.

The qualitative difference in the temperature dependence of τ_{int} for folding *vs.* unfolding is particularly interesting. The strong enthalpy of activation of τ_{int} for unfolding (Fig. 4C) indicates that the internal friction impeding escape from the folded state arises from fairly strong energetic interactions, such as the breaking of tertiary contacts in the native structure. For the folding process, however, the intercept in Fig. 3C shows virtually no temperature dependence, implying that this internal friction is not associated with any large energy scale. One entropic mechanism for internal friction may be the free volume effect, where the elements of the polymer backbone cannot undergo any displacement until random thermal motion creates a sufficiently large opening among the solvent molecules. If, for example, the folding of the TrpCage requires the exclusion of individual water molecules from the tertiary core, the time scale for folding will depend on the kinetics of that exclusion. One expects this effect to depend only weakly on either temperature or the bulk solvent viscosity.

Recent molecular dynamics (MD) simulations may shed some light on the origin of the internal friction in TrpCage. Simulation offers an interesting comparison to laboratory studies, because while η_s cannot be reduced
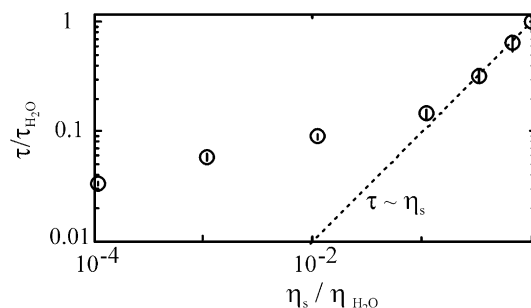

Fig. 5. Molecular dynamics folding times τ for TrpCage at 300 K, obtained by distributed computing simulation in implicit solvent. The data are from Zagrovic and Pande [23]. Solvent viscosity is scaled with respect to the viscosity of water at 300 K, and folding times (vertical axis) are scaled with respect to folding time in water. Dotted line indicates linear $\tau \sim \eta_s$ behavior.

much below 1 mPa s in the laboratory, it is a readily adjustable parameter in silico. Zagrovic and Pande [23] used distributed computing methods to simulate the folding of the TrpCage (TC5b) in implicit solvent at 300 K. That is, their simulations treated the solvent as a viscous continuum with variable viscosity. They simulated multiple folding trajectories at different values of the solvent friction γ ranging from $\gamma = 91$ ps^{-1}, corresponding to water, down to $\gamma = 0.01$ ps^{-1}, or $\sim 10^4$ less viscous than water. Fig. 5 shows that the simulated folding times (for $\eta_s \leqslant \eta_{H_2O}$) obey $k_f \sim \eta_s^{-1}$ at near-aqueous viscosities, and deviate from that behavior only at very low friction, below roughly 0.1 η_{H_2O}. Although these simulations provide intriguing evidence for an internal friction effect in TrpCage folding at very low η_s, they do not match up with the viscosity behavior observed in the laboratory ($\eta_s \geqslant \eta_{H_2O}$). Because the composition of the solvent is the primary difference between the experiment and the simulation model, free volume effects may potentially explain the disagreement. Thus the MD results provide some support for a free-volume effect as the origin of the internal friction in TrpCage folding.

6. Conclusions

In summary, we find that, at low solvent viscosities, the folding and unfolding rates of the TrpCage do not diverge, but rather tend toward finite limiting values. Limits are imposed on the configurational dynamics by frictional forces that are independent of the bulk solvent. A simple interpretation of the data suggests that these internal friction effects are characterized by a fast ($\sim$ns) time scale, so that even if they are typical in protein dynamics they may become rate-limiting only for the fastest folding reactions. It will be quite interesting to see how these internal friction effects vary among proteins of different chain length or native structure.

Finally, these findings may have implications for the physical origin of the "speed limit" for protein folding. The rate k_{equilb} at which an unfolded protein diffusionally

explores its various denatured states represents a natural upper limit on folding speed, but k_{equilb} is not well understood in general. One may imagine that k_{equilb} could be estimated from the known timescales for simple diffusional reconfigurations of unfolded polypeptide chains. The relaxation time of a 100-residue random coil, for example, is predicted ($\eta_s R^3 / k_B T$ [24]) and measured [25] to be of order 100 ns; end-to-end diffusion of an unfolded polypeptide of $\sim$10 residues occurs within $\sim$100 ns [26–28]. However, while this ''free chain'' diffusion occurs at nanosecond rates, real folding phenomena are generally much slower: the fastest observed protein folding rates [18,29], the best estimates for k_{equilb} [30], the collapse of a polypeptide to a molten globule [31], and the extrapolation of contact order graphs to their upper ranges [32], all point toward microsecond time scales as the practical upper limit for folding speed. Given the present data, it is quite conceivable that internal friction within the compact configurations of a folding peptide slows k_{equilb} from the nanosecond time scale of the freely diffusing chain to the microsecond timescale of real folding events. Thus internal friction phenomena may define the real practical limits on protein folding speed.

Acknowledgements

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