SHORT COMMUNICATION

Exponential Decay Kinetics in "Downhill" Protein Folding

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ABSTRACT **The observation of single-exponential kinetic phases in early stages of protein folding is often interpreted as evidence that these phases are rate limited by significant energy or entropy barriers. However, although the existence of large barriers reliably implies exponential kinetics, the reverse is not necessarily true. A simple model for the hydrophobic collapse of a chain molecule demonstrates that a barrierless or "downhill" diffusional relaxation can give rise to kinetics that are practically indistinguishable from a pure exponential. Within this model, even a highly nonlinear experimental probe such as resonance energy transfer (Förster transfer) could exhibit a large amplitude decay (greater than 90% in fluorescence) that deviates from a simple exponential by less than 0.5%. Only a detailed analysis of the dynamics is likely to reveal that a free energy barrier is absent. Proteins 2003;50:1–4.** © 2002 Wiley-Liss, Inc.

Key words: fluorescence; exponential; diffusion; tryptophan; cytochrome *c*; energy landscape

INTRODUCTION

Recent years have seen tremendous experimental, theoretical, and computational advances in the study of protein folding dynamics, and especially in the study of fast phenomena that take place during the early stages of folding. One conclusion arising from such studies is that even the fastest kinetic phases of folding may be barrier limited: The single-exponential kinetics observed in the early (microsecond) collapse or folding of a number of small, single domain proteins has led a number of authors to conclude that significant energy or entropy barriers divide the free energy landscapes of such molecules into distinct regions.[1–6] This is the logical inverse of the principle that fast nonexponential (or at least multiexponential) kinetics in the folding of certain other proteins implies that these macromolecules diffuse unimpeded down a relatively smooth—or barrierless—free energy surface, toward their native state.[5–8]

The argument that exponential decay implies barrier-limited kinetics is technically valid in the ideal case of perfectly precise measurements,[9] and it may seem obvious in cases where experimental folding data are derived from a spectroscopic probe such as resonance energy transfer (Förster transfer), for which the relationship between the intrachain distances and the detected signal is strongly nonlinear. Nevertheless, its validity as a practical basis for interpreting real experimental data—and therefore the existence of such barriers—has been questioned.[10–13] The aim of this article is to demonstrate that experimental observations of exponential kinetics need not imply the presence of a free energy barrier. Even in a simple model for the hydrophobic collapse of a polypeptide chain, where the molecule diffuses freely downhill in free energy, the time course of contraction may be virtually indistinguishable from a pure exponential decay. This can remain true even for fairly large amplitude relaxations where Förster transfer provides the experimental probe. This result contradicts expectations based on simpler analyses[4] and shows that the observation of a highly exponential decay in a folding or collapse experiment does not by itself prove that a single free energy barrier controls these kinetics.

METHODS

Figure 1 illustrates a simple model for the diffusional collapse of a polymer. The equilibrium probability distribution for the distance r between the endpoints of a randomly coiled chain is the Gaussian distribution,[14]

$$P_0(r, R)r^2\,dr = \left(\frac{3}{2\pi R^2}\right)^{3/2} 4\pi r^2\,dr\,\exp\left(-\frac{3r^2}{2R^2}\right).$$

Here, $R = \sqrt{\langle r^2\rangle}$, that is, R^2 is the ensemble average of r^2. A nonspecific contraction of the polymer, like that triggered by a rapid solvent or temperature change, reduces R from an initial value R_i to a smaller value R_f. One simple approach to modeling the response to this perturbation is to treat the system as an ensemble of particles diffusing on a free energy surface in r.[15] The particles are assumed to move with a position-independent diffusion constant D on the surface $G(r) = -k_BT\,\log(P_0(r, R_f))$, for which the equilibrium Boltzmann distribution is $P_0(r, R_f)$. If the initial distribution of particles is $P_0(r, R_i)$, one may then

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Received 22 May 2002; Accepted 29 July 2002

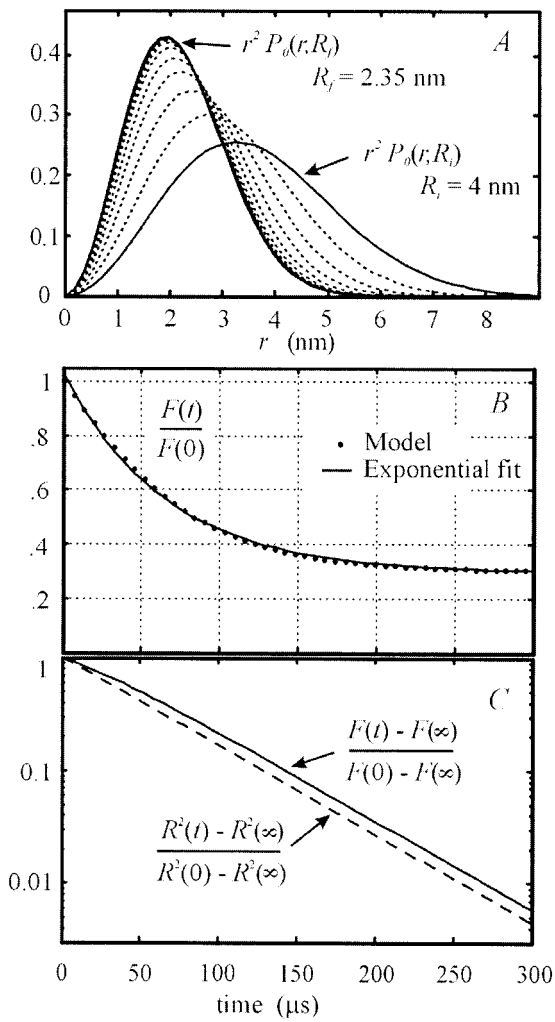

Fig. 1. Relaxation in diffusive model. (**A**) Initial and final end-to-end distance distributions for a Gaussian-distributed random coil collapsing from $R^2 = R_i^2$ to $R^2 = R_f^2$ (solid curves) and intermediate distributions (dotted curves). (**B**) Calculated fluorescence signal during the above relaxation, based on fluorescence of a Förster donor at one chain terminus, transferring energy to the other terminus. The solid curve shows best fit to a single exponential decay plus constant. Model uses R_0 = 3.4 nm, R_f = 2.35 nm, R_i = 4 nm, and D = 16.3 nm²/ms. (**C**) Predicted decay of donor fluorescence (solid curve) and R^2 (broken line) toward their equilibrium values during the modeled relaxation.

numerically solve the diffusion equation to find the time-dependent distribution $P(r,t)$ that describes the relaxation toward $P_0(r, R_f)$. This relaxation, and the resulting time dependence of properties like average chain dimension and fluorescence, were obtained here by numerically solving a discrete approximation to the diffusion equation on $G(r)$ in spherical coordinates, using Matlab (Mathworks, Natick, MA).

The distribution-averaged end-to-end separations $\langle r \rangle$ and R^2 ($\langle r^2 \rangle$) are then both functions of time, obtained from the numerical solutions for $P(r,t)$. To model a Förster transfer experiment, r should specifically represent the distance between the fluorescence donor and acceptor. Because the chain dynamics are much slower than the donor fluorescence lifetime, the average fluorescence of the system (relative to the free donor) is

$$F(t) = \int P(r,t) r^2 dr \, \frac{r^6}{r^6 + R_o^6}$$

where R_0 is the Förster distance, typically a few nanometers.[16] The decay amplitude is the overall fractional change in $F(t)$, given by $\Delta F/F(0) = (F(0) - F(\infty))/F(0)$.

RESULTS AND DISCUSSION

In a precise comparison, the relaxation kinetics of the fluorescence $F(t)$, chain dimension R^2, etc. in this model deviate from a single exponential. However, they can deviate so slightly that—given the experimental uncertainties of instrument dead time, noise, normalization of the initial signal, etc.—they may appear nearly indistinguishable from much of the laboratory data that is typically identified as single exponential. This is illustrated by the example of the initial collapse of horse ferricytochrome c.[2,4,10] The reduction in molecular dimensions during the collapse of this polypeptide can be detected by the quenching of the fluorescence of Trp59, through resonance energy transfer to the heme group that is covalently attached to Cys14, His18, and Cys17, with a Förster distance $R_0 \approx$ 3.2–3.4 nm. Shastry and Roder showed that mixing guanidine-denatured cytochrome c into a refolding buffer triggers a fast, highly exponential fluorescence decay, with a relaxation time $\tau \approx 65$ μs and a decay amplitude as large as 70% under strongly refolding conditions (0.5 M GdnHCl at pH 7).[2] Although these exponential kinetics have been interpreted as evidence for a free energy barrier separating the unfolded and collapsed regions of the energy surface, Figure 1 shows that virtually exponential kinetics can also be obtained in the barrierless diffusive model. For R_0 = 3.4 nm and a final R_f = 2.35 nm (which gives the experimentally observed fluorescence yield for the resulting compact ensemble), the model generates a 70% fluorescence decay amplitude if $R_i \approx$ 4 nm. Figure 2 shows that this decay never deviates more than ≈4% from the best-fit single exponential and that this maximum deviation occurs well within the dead time (≈45 μs) of the mixing instrument used in ref. 2. The later deviations are not more than 1–2%, and they can be substantially smaller when the total fluorescence change is less than 70%. Other average properties of the distribution, such as R^2, show even less deviation from an exponential time course.

Although the authors of ref. 2 actually interpret the fluorescence decay in cytochrome c as a 100% loss of fluorescence by 70% of the population, rather than the reverse, a larger-amplitude decay is still consistent with diffusive collapse. The contour plots of Figure 3 show how the deviation from exponential kinetics varies with both R_0 and the extent of hydrophobic collapse, $(R_i - R_f)/R_f$, in resonance energy transfer studies of the collapsing chain modeled here. The degree of exponentiality of a fluorescence decay in the model depends on both the initial condition (R_i) and the nature of the probe (R_0). Surprisingly, there exist conditions in this model for which the

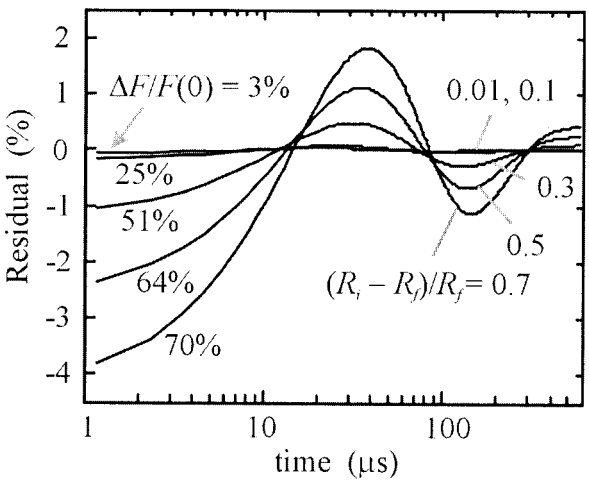

Fig. 2. Residuals from fit of model-generated fluorescence decays to single exponential (plus constant). For each curve, the degree of collapse $(R_i - R_f)/R_f$ is indicated, as is the resulting total fluorescence decay amplitude, $\Delta F/F(0)$. Fixed parameters are R_f = 2.35 nm, D = 16.3 nm^2/ms, and R_0 = 3.4 nm. Residual is given as a percentage of $F(0)$.

average fluorescence of the sample drops by nearly 95% as the chain contracts, but with a time course that does not deviate by more than 0.5% from a pure exponential.

How then would the experimentalist determine that no barrier exists? A more detailed study of the kinetics can reveal their diffusional origin because the relaxation times in this model will depend on the extent of collapse. Moreover, they may vary dramatically with the choice of experimental probe. Figure 4 shows that deeper quenches [i.e. larger values of $(R_i - R_f)/R_f$] may either accelerate or slow the kinetics, depending on the experimental probe. In fact, measurements of $\langle r \rangle$, R^2, and fluorescence (in Förster transfer) would all generate different values for the decay time constant τ, and changes in R_0 generate additional variation in τ. In the limit of small perturbations ($R_i \approx R_f$), all the decay rates converge to a universal value, $\tau \approx 0.159\ R_f^2/D$. Unfortunately (from an experimental perspective), the cytochrome c conditions described above [R_f = 2.35 nm, R_i = 4 nm, R_0 = 3.4 nm, R_0/R_f = 1.45, $(R_i - R_f)/R_f$ = 0.70] will result in these three observable relaxation rates agreeing to within 20%, a difference that probably lies within experimental uncertainty. Further, Figure 4 shows that the dependence on the initial conditions (via R_i) becomes weak for values of R_0/R_f near 1.5–2, at least for moderate perturbations of the coil size. Thus, even the absence of initial condition dependence or probe dependence in experimentally observed rates may fail as a test of barrier-controlled relaxation.

Quantitative properties of the dynamics may provide a better means of discriminating barrier-limited from barrierless kinetics. The barrierless decay curve of Figure 1, which reproduces the character and timescale (τ = 65 μs) of cytochrome c collapse data, is generated by D = 16.3 nm^2/ms (= 1.63 × 10^{-10} cm^2/s) in this model. Further, those collapse data show an Arrhenius behavior in the collapse rate, which implies (within a downhill diffusion model) that D must have an activation energy, roughly

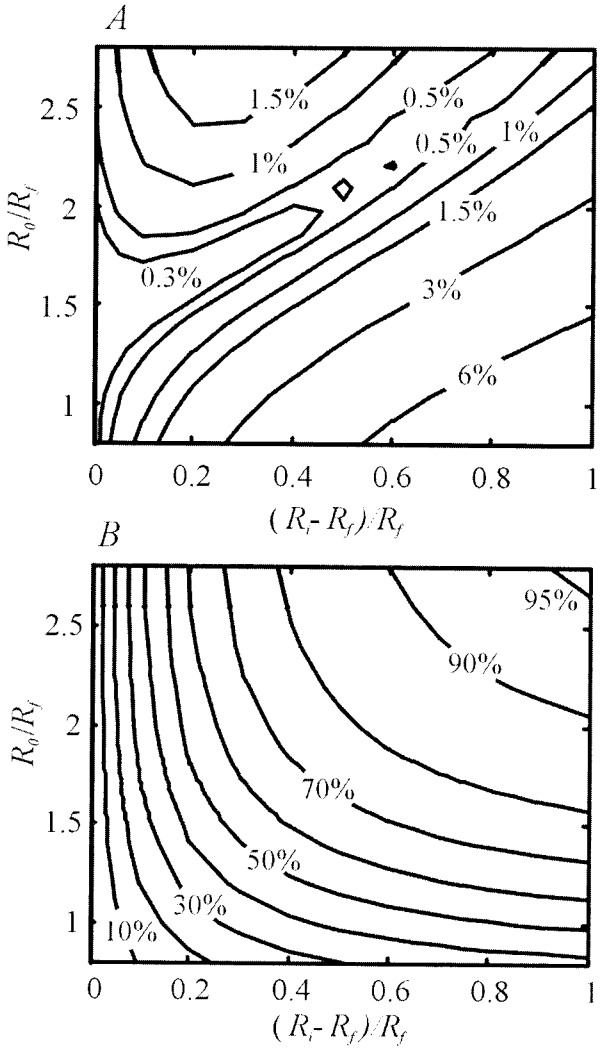

Fig. 3. Contour plots characterizing fluorescence decay during chain compaction in the diffusive model, assuming observation of Förster donor fluorescence. (**A**) Maximum residual of fluorescence decay compared to best-fit exponential. (**B**) Total fluorescence decay amplitude. Figure 1 corresponds to R_0/R_f = 1.45, $(R_i - R_f)/R_f$ = 0.70.

≈30–40 kJ/mol.[2,4] One may compare these numbers to independent measurements of the speed of end-to-end diffusion of the unfolded cytochrome c chain. When analyzed in a diffusive model[15] similar to that presented here, those data suggest that the rate of relative diffusion between two internal points on the polypeptide chain exhibits a thermal activation energy of 30–70 kJ/mol at low denaturant concentrations.[17,18] Thus, both the collapse experiments, as interpreted within the diffusional model, and other studies of cytochrome c diffusion indicate a similar activation energy for D. This implies that no additional enthalpic barrier affects the collapse. However, the magnitude of the diffusion constant $D \approx 4 \times 10^{-7}$ cm^2/s observed in the relative diffusion studies[17,18] is much larger (by a factor of ≈2 × 10^3) than that derived from the collapse data in the present diffusive model. This suggests either that the diffusive model greatly overestimates the collapse rate of a chain (see below) or that an

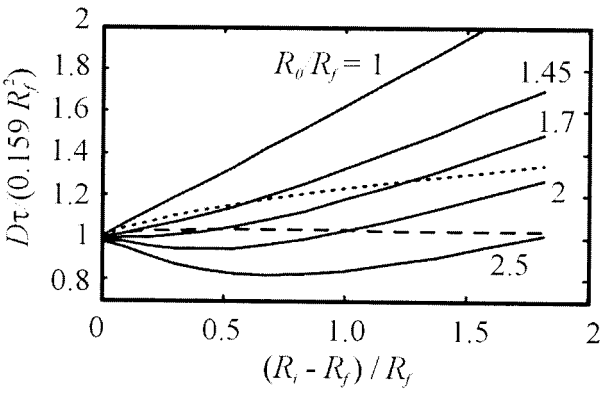

Fig. 4. Dependence of apparent relaxation times τ (i.e., from fit to exponential) on initial chain dimension R_i for various probes: (solid curves) decay times for donor fluorescence at indicated values of R_0/R_f and decay times for average chain dimension $\langle r \rangle$ (dotted curve) and R^2 (broken curve).

entropic barrier exists and slows the collapse process. To explore the second possibility, we can introduce a barrier of height ΔS and width δr in the model at $r = R_f$ and solve the diffusion equation again. Unless $\delta r/R_f$ is extremely small, the relaxation time becomes $\tau \approx (R_f \delta r/4D)\, e^{\Delta S/N_A k_B}$. Then, $D \approx 4 \times 10^{-7}$ cm^2/s and $\tau \approx 65$ μs would require $\delta r e^{\Delta S/N_A k_B} \approx 4 \times 10^{-4}$ cm, or $\Delta S \geq 8.4\, N_A k_B \approx 70$ J mol^{-1} K^{-1} for $\delta r \leq 1$ nm.

The example of cytochrome *c* illustrates that observations of exponential kinetics (even in the absence of probe dependence or initial condition dependence in the measured decay rates) are not sufficient evidence that polypeptide collapse or folding processes are rate limited by free energy barriers. Only a more careful investigation is likely to shed light on the dynamics behind these events. However, although the discussion above finds evidence for an entropic barrier in cytochrome *c*, this conclusion is subject to an important caveat: If the diffusive model significantly overestimates the speed of compaction, the evidence for a barrier becomes much weaker. This remains a possibility because the model employs a minimal description of the polymer dynamics and cytochrome *c* does not necessarily collapse to a Gaussian-distributed coil as the model assumes.[19] These uncertainties highlight the need for more experimental studies of chain collapse in a variety of systems, especially those where barriers are likely to be absent.

ACKNOWLEDGMENTS

The author thanks Dr. Adrian Roitberg for helpful discussions. Funding support has been provided by the National Science Foundation, MCB Award 0077907.

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