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Using VIP *T*-Jump to Distinguish Between Different Folding Mechanisms: Application to BBL and a Trpzip

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Abstract

Protein folding involves a large number of sequential molecular steps or conformational substates. Thus, experimental characterization of the underlying folding energy landscape for any given protein is difficult. Herein, we present a new method that can be used to determine the major characteristics of the folding energy landscape in question, for example, to distinguish between activated and barrierless downhill folding scenarios. This method is based on the idea that the conformational relaxation kinetics of different folding mechanisms at a given final condition will show different dependences on the initial condition. We show, using both simulation and experiment, that it is possible to differentiate between disparate kinetic folding models by comparing temperature-jump (*T*-jump) relaxation traces obtained with a fixed final temperature and varied initial temperatures, which effectively varies the initial potential (VIP) of the system of interest. We apply this method (hereafter refer to as VIP *T*-jump) to two model systems, Trpzip-2c and BBL, and our results show that BBL exhibits characteristics of barrierless downhill folding, whereas Trpzip-2c folding encounters a free energy barrier. In addition, using the *T*-jump data of BBL we are able to provide, via Langevin Dynamics simulations, a realistic estimate of its conformational diffusion coefficient.

INTRODUCTION

Protein folding involves many degrees of freedom or conformational substates and, therefore, represents a hyperdimensional problem.^{1–3} However, in practice the folding free energy landscape is often projected onto a low dimensional space, for example, as a function of a putative ‘folding’ coordinate. As shown (Figure 1), in the case of a one dimensional folding coordinate, various folding scenarios can, in principle, be differentiated by the number, position and magnitude of the free energy barriers that separate the folded state from the unfolded conformational ensemble. For example, two limiting cases become apparent: one contains a single free energy barrier that separates the folded from the unfolded state, i.e., the two-state folding mechanism (Figure 1A), whereas the other involves a continuum of thermally accessible states, i.e., the downhill or one-state folding scenario (Figure 1D).^{2,4–11} Other simple cases involve one or more observable intermediate states, which can be located on either side of the major folding barrier (Figure 1B and 1C). While a one-dimensional representation of the protein folding free energy landscape is informative and practical, for a given protein the existing experimental methods for studying folding kinetics sometimes cannot distinguish between different folding scenarios. For example, it has been shown that both two-state and downhill folding mechanisms can yield folding

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Supporting Information. CD and FTIR spectra, peptide sequences, simulation details, and *T*-jump relaxation times are available free of charge via the Internet at <http://pubs.acs.org>.

kinetics that are essentially indistinguishable by conventional experimental techniques.^{12–13} While these two folding scenarios, which are polar opposites, can be distinguished from each other by whether their folding kinetics^{14,15} and/or thermodynamics¹⁶ depend on the conformational probe, we still lack a more straightforward approach to characterize the underlying nature of the folding free energy surface of the protein in question.

Herein we show, for a given final temperature in a T -jump relaxation experiment,^{17–20} that by varying the initial temperature, which is equivalent to varying the initial potential of the system in question, the difference in the resulting relaxation kinetics reveals the nature of the underlying folding free energy landscape of the target protein. While the strategy of varying T -jump amplitude has been employed before in protein folding studies,^{15,21–25} to the best of our knowledge it has not been used to characterize the underlying folding free energy landscape. Specifically, we apply this VIP T -jump technique to two model systems, Trpzip-2c and BBL, and our results show that the folding kinetics of Trpzip-2c are consistent with an activated folding mechanism, while those of BBL are characteristic of a barrierless downhill folder. In addition, using the free energy surface of BBL determined by Wang and coworkers²⁶ and Langevin Dynamics (LD) simulations we are able to extract the conformational diffusion coefficient of BBL from the experimentally measured conformational relaxation kinetics.

RESULTS AND DISCUSSION

For a two-state folding scenario (Figure 1A), it is easy to show that the population redistribution kinetics, for example in response to a T -jump, only depend on the final temperature and not the initial temperature, whereas the latter determines the amplitude of the probing signal.

Similarly, for folding mechanisms involving an intermediate state, populated at either the left- or right-hand side of the major folding free energy barrier (Figure 1B and 1C), it is easy to show that the population relaxation kinetics of the folded/unfolded state, in response to a T -jump, are biphasic with two relaxation time constants determined by the corresponding microscopic rate constants at the final temperature. Thus, in this case the measurement of relaxation kinetics alone is insufficient to differentiate between the two scenarios. On the other hand, we show that the VIP T -jump technique is able to do so, which complements other methods.^{27,28} The applicability of this method simply stems from the fact that for such reversible 'reaction' systems, the relative amplitudes of the two kinetic phases (for a given final temperature) depend on the initial population distribution or temperature. To further illustrate this point, an example is given below. For the two folding pathways presented in Figure 2, it is easy to show that for pathway A the relative amplitude of the fast phase of the population relaxation kinetics of the folded state (F), at a final temperature (T_f) of 323.0K, is increased from 8.9 to 16.1% when the initial temperature (T_i) is changed from 310.5 K to 298.0 K, whereas the relaxation kinetics of the unfolded state (U) are essentially single-exponential, and that for pathway B it is the relaxation kinetics of U that are sensitive to T_i (e.g., the relative amplitude of the fast phase is decreased from 30.8% to 25% in this case) (Table S2, S3 and Figure S2, Supporting Information). Thus, taken together, this simple numerical analysis illustrates the utility of the VIP T -jump method in distinguishing between folding pathways that conventional kinetics measurements cannot. In principle, the utility of the VIP T -jump technique is not only limited to these classical models, but also can be further applied to the downhill folding scenario.

For a population redistribution process occurring on a free energy surface that does not contain an appreciable barrier between states (Figure 1D), evaluation of the relaxation kinetics is less straightforward. Herein, we employ LD simulations to determine how the

population relaxation kinetics at a given final temperature depend on the initial temperature, assuming that the dynamics occur in the overdamped regime.^{29–31} Specifically, the time-dependent population distribution function, $P(t, q)$, is obtained by numerically solving the following equation for each molecule:³²

$$\frac{k_B T}{D(T)} \frac{dq}{dt} = - \frac{dG(q, T)}{dq} + \Gamma(t) \quad (1),$$

where $G(q, T)$ is the one dimensional free energy surface, q the folding coordinate, $D(T)$ the diffusion coefficient, T the absolute temperature, and k_B the Boltzmann constant. In addition, $\Gamma(t)$ represents the random force arising from the underlying thermal bath with a mean value of 0 and a normally distributed variance that is bounded by the fluctuation-dissipation theorem.³³ In the present study, the variance of $\Gamma(t)$ is set to be $2k_B T^2 \delta(t)/D$, where $\delta(t)$ is the delta function corresponding to a Markovian process.³³ In reality, the diffusion coefficient, D , may show a dependence on q ; for simplicity in the present study we have assumed that it only depends on temperature.

In practice, it is common to choose one of the protein's structural parameters as the folding coordinate q , such as the fraction of native contacts (Q), the radius of gyration (R_g), or the root-mean-square distance (rmsd), which measures the displacement of each atom in a given structure from its native position. While the choice of the folding coordinate and the exact shape of the one dimensional downhill folding free energy surface do not change our conclusions, in the present study we used the folding free energy surface determined by Wang and coworkers²⁶ for BBL as a reference to determine $G(q, T)$. Based on extensive molecular dynamics (MD) simulations and analyses using the Weighted Histogram Analysis Method, Wang and coworkers²⁶ were able to extract an effective folding free energy surface as a function of rmsd at 298 K. To determine $G(q, T)$ at other temperatures, we simply tilted the free energy surface obtained by Wang and coworkers. Specifically, the degree of tilting for any given temperature is determined by the criterion that the average nativeness of the protein at the target temperature matches that estimated from the circular dichroism (CD) temperature melting curve of BBL (see Supporting Information).

To determine whether the kinetics of a T -jump induced population redistribution process on a one dimensional downhill-like folding free energy surface are sensitive to the initial temperature, we carried out two LD simulations that differ only in the initial population distribution. Specifically, we first determined three free energy surfaces using the method discussed above at three temperatures, e.g., 310.15 (T_f), 303.15 (T_{i1}), and 298.15 K (T_{i2}). We then used these free energy surfaces, i.e., $G(q, T_f)$, $G(q, T_{i1})$, $G(q, T_{i2})$, to determine the corresponding equilibrium (or Boltzmann) population distributions, i.e., $P_{eq}(q, T_f)$, $P_{eq}(q, T_{i1})$, and $P_{eq}(q, T_{i2})$. In the next step, we carried out LD simulations to determine the population relaxation dynamics, i.e., $P(t, q)$, using the following conditions: $P(t=0, q) = P_{eq}(q, T_{i1})$ or $P(t=0, q) = P_{eq}(q, T_{i2})$ and $P(t \rightarrow \infty, q) = P_{eq}(q, T_f)$. Finally, following Gruebele and coworkers,²⁹ we converted $P(t, q)$ to a signal, $S(t)$, using the following equation:

$$S(t) = \int H(q - q_{q^*}) P(t, q) dq \quad (2),$$

where H is the Heaviside function, i.e., $H = 1$ when $q \geq q^*$ and $H = 0$ when $q < q^*$. While the exact value of q^* may well depend on the nature and location of the conformational probe used in a specific experiment, without loss of generality in the current study we have assumed that q^* corresponds to an rmsd of 3.36 Å, which is equivalent to a nativeness of 0.7. While this choice is somewhat arbitrary, it does not change the conclusions reached below. In addition, when the amide I band of the protein in question is used to probe conformational relaxation (see below), the use of a Heaviside step function to extract the

signal, $S(t)$, from the time dependent population distribution function is a reasonable approximation.

As shown (Figure 3), the results obtained from LD simulations confirm our expectation that for a T -jump induced population relaxation process occurring on a barrierless free energy surface, the relaxation dynamics depend not only on T_f , but also on T_i , which determines the initial equilibrium population distribution. In addition, consistent with several previous studies^{12–13} the relaxation traces in Figure 3 can be well described by a single-exponential function (Figure S4, Supporting Information), indicating that in order to uncover the true nature of the underlying folding energy landscape of the protein in question, one cannot simply rely on conventional relaxation kinetics measurements. Thus, taken together, these simulation results provide concrete evidence in support of the applicability of the VIP T -jump method in distinguishing between various protein folding mechanisms, especially between two-state and barrierless downhill folding scenarios. Additional simulations indicate that even for a folding free energy barrier as small as $1.5 k_B T$, the population relaxation kinetics show no measurable dependence on initial temperature (Supporting Information), further underscoring the sensitivity of this method.

To further test the utility of the VIP T -jump method, we applied it to two model systems, BBL and Trpzip-2c. We chose BBL because various experimental^{16,34–37} and computational^{13,26,38–43} studies have suggested that its folding encounters a negligible barrier, whereas Trpzip-2 folding has been shown in several studies to involve a free energy barrier.^{44–48} Additionally, BBL folds on a similar time scale to Trpzip-2c,^{48,49} making the comparison more reliable. Specifically we measured the T -jump induced population relaxation kinetics of both systems based on the protocol of the VIP T -jump method, using time-resolved IR spectroscopy and probing frequencies within the amide I band of these polypeptides. The amide I band of proteins/peptides arises predominantly from the stretching vibrations of backbone carbonyls and is a sensitive IR reporter of protein secondary structural contents.⁵⁰

As shown (Figures S1 and S3 in Supporting Information) and consistent with previous studies,^{34,36} the CD thermal unfolding curve of BBL indicates that increasing the temperature from 30 to 65 °C induces a significant change in the secondary structural content of the protein, and that the apparent melting temperature (T_m) is approximately 44 °C. Thus, we carried out a series of T -jump IR measurements on BBL with final temperatures falling within this temperature range. In addition, we used probing frequencies of 1630 and 1668 cm^{-1} . As shown (Figure S5 in Supporting Information), the amide I' band (amide I in D_2O) of BBL decreases in intensity at 1630 cm^{-1} with increasing temperature, corresponding to a loss of helical structures, while there is a concomitant gain in intensity at 1668 cm^{-1} , corresponding to an increase in disordered conformations. Thus, measurements at each of these frequencies provide representative tests of the sensitivity of the VIP T -jump method. As shown (Table S4 in Supporting Information), the T -jump relaxation kinetics obtained with both probing frequencies and at all the final temperatures tested show a certain dependence on the initial temperature. In particular, as indicated (Figures 4 and 5), while these relaxation traces can be fit by a single-exponential function, their overall relaxation kinetics, obtained at a given T_f , show a T_i -dependence with a relationship that a larger T -jump amplitude results in a faster relaxation rate. This is consistent with the LD simulation results for a downhill folder shown above (Figure 3), where a larger T -jump also leads to a faster relaxation, due to a greater force acting on the initial population ensemble. On the other hand, due to the free energy barrier present in its folding pathway, we expect that the relaxation kinetics of Trpzip-2c will not show a similar dependence. Indeed, as shown (Figure 6 and Figure S9), two T -jump IR relaxation traces obtained at the same final temperature (61.8 °C) but with different initial temperatures (46.8 °C and 52.8 °C) are

indistinguishable within our experimental errors, indicating that the population relaxation rate of this peptide depends only on the final temperature, characteristic of an activated folding mechanism. Taken together, these results provide a direct validation of the utility of the VIP T -jump method in revealing the nature of the protein folding energy landscape and, especially, its ability to distinguish between barriercrossing and barrierless folding mechanisms.

Our results provide additional evidence to support the notion that the folding of BBL, at least under our experimental conditions, does not encounter a significant free energy barrier.^{13,16,26,34–37} Thus, its folding process can be treated as a diffusive motion along the folding coordinate and, as such, the T -jump induced relaxation kinetics of BBL can be used, in conjunction with LD simulations, to estimate its conformational diffusion coefficient, as well as the ruggedness of its potential energy surface. Because, as indicated in Eq. 1, the population relaxation kinetics depend on both $G(q, T)$ and $D(T)$, one needs to know $G(q, T)$ in order to accurately extract $D(T)$ from the T -jump experimental data. In principle, one could globally analyze all of the thermodynamic data (e.g., that obtained from CD or IR measurements) and T -jump relaxation kinetics obtained at different temperatures to simultaneously determine, in a selfconsistent manner, $G(q, T)$ and $D(T)$. However this is beyond the scope of the present paper, so instead we use the $G(q, T)$ of Wang and coworkers²⁶ to provide an estimate of the conformational diffusion coefficient of BBL near its T_m . As shown (Figure 7), the T -jump induced relaxation kinetics obtained at 46.8 °C (T_f) can be well described by a population redistribution process via conformational diffusion on the corresponding free energy surface; the diffusion coefficients thus obtained (i.e., 3.3×10^{-5} nm²/ns for $T_i = 38.1$ °C and 2.7×10^{-5} nm²/ns for $T_i = 41.1$ °C) show a dependence on the initial temperature. This T_i dependence is expected, as the conformational diffusion coefficient depends not only on the final temperature but also on the folding coordinate,²⁶ a condition not explicitly considered in the current simulations. Nevertheless, the values of these diffusion coefficients are in quantitative agreement with the diffusion coefficient obtained by Gruebele and coworkers¹⁴ for a mutant of λ repressor (3×10^{-5} nm²/ns). Because λ repressor has approximately twice as many residues as BBL, this agreement suggests that the conformational diffusion of other proteins may have a similar diffusion coefficient. For example, a simple calculation using this value of D suggests that it takes approximately 30 ns to elongate an α -helix by one turn (3.5 residues), via a conformational diffusion search process. This rate of helix propagation is entirely consistent with those estimated from experimental measurements.^{51–53}

CONCLUSIONS

It is apparent that the free energy of a protein system (including solvent) depends on many degrees of freedom, which collectively determine the conformational state of the protein. However, when folding or unfolding is monitored with a specific experimental probe, such as CD, IR, or fluorescence, it often exhibits simple kinetics (e.g., one or two exponentials). Moreover, and perhaps more importantly, different folding scenarios, for example the two-state and downhill mechanisms, can give rise to practically indistinguishable kinetics, making it difficult, if not impossible, to elucidate the nature of the underlying folding energy landscape based on conventional kinetics measurements. Herein, we show, by varying the initial temperature in a T -jump experiment, which essentially varies the initial potential (VIP) of the protein system in question, that it is possible to discriminate between different types of folding mechanisms. This VIP T -jump method is akin to the strategy used in electronic spectroscopy to create different Franck–Condon states on the excited electronic potential energy surface by using different excitation wavelengths. Experimentally, we apply this VIP T -jump method to two model systems, BBL and Trpzip-2c, which have been suggested to follow two different folding mechanisms (i.e., downhill versus activated). We

find that the *T*-jump induced conformational relaxation kinetics of BBL, but not Trpzip-2c, show dependence on the initial temperature at a fixed final temperature, with a larger *T*-jump resulting in a faster relaxation rate. These findings provide additional evidence to support the idea that BBL is a downhill folder. In addition, using LD simulations we are able to extract an apparent conformational diffusion coefficient for BBL, the magnitude of which is in agreement with that determined for another downhill folder, λ repressor. An exciting new direction for this method would be to use VIP *T*-jump to study the free energy landscapes of intrinsically disordered proteins, which currently are relatively unknown.

EXPERIMENTAL SECTION

BBL and Trpzip-2c were synthesized on a PS3 automated peptide synthesizer (Protein Technologies, MA) using standard Fmoc-protocols. Peptide products were further purified by reverse-phase chromatography, and identified by matrix assisted laser desorption ionization mass spectroscopy. Trifluoroacetic acid (TFA) removal and H-D exchange were achieved by multiple rounds of lyophilization.

All peptide samples were prepared in 20 mM phosphate buffer solution (pH 7) and the peptide concentrations were in the range of 45 μ M for CD and 2–4 mM for IR measurements. The details of all spectroscopic measurements, including the *T*-jump IR setup, have been described elsewhere.⁵⁴ Specifically, for the VIP *T*-jump experiments, variation of the *T*-jump magnitude was achieved by appropriately adjusting the pump intensity using a neutral density filter (Schott Glass Technologies, Inc., PA), and the initial temperature. For each *T*-jump relaxation trace obtained, the corresponding final temperature was measured twice, before and after the experiment, using the absorbance change of D₂O in the reference side of the IR cell.⁵⁴ If the final temperature deviated by more than 0.5 °C from the targeted value, the corresponding kinetic trace was discarded.

CD spectra and thermal melting curves were obtained on an Aviv 62A DS spectrometer (Aviv Associates, NJ) with a 1 mm sample holder. Fourier transform infrared (FTIR) spectra were collected on a Magna-IR 860 spectrometer (Nicolet, WI) using a homemade, two-compartment CaF₂ sample cell of 56 μ m pathlength.

Langevin dynamics simulations were performed using Matlab (The MathWorks, MA) and a time step of 35 ns, and integration was performed using Runge-Kutta methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Trpzip	tryptophan zipper
<i>T</i>-jump	temperature jump
IR	infrared
CD	circular dichroism

LD Langevin dynamics

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Figure 1.
Cartoon depiction of representative folding free energy surfaces.

Figure 2.

The relative free energies of U, I, and F in a three-state folding scenario at 298.0 (red), 310.5 (orange), and 323.0 K (blue), respectively, with the intermediate state located at either the left-hand (A) or right-hand (B) side of the major folding free energy barrier.

Figure 3. Simulated relaxation kinetics in response to T -jumps from different initial temperatures to the same final temperature, as indicated, obtained via LD simulations using the free energy surfaces shown in the inset.

Figure 4. Comparison of the normalized conformational relaxation kinetics of BBL obtained with a probing frequency of 1668 cm^{-1} and at a final temperature of $46.8\text{ }^{\circ}\text{C}$, from two different initial temperatures, as indicated. The red lines are fits of the data to a single-exponential function and the resulting time constants are reported in Table S4 (Supporting Information).

Figure 5. Comparison of the normalized conformational relaxation kinetics of BBL obtained with a probing frequency of 1668 cm^{-1} and at a final temperature of $57.0\text{ }^{\circ}\text{C}$, from two different initial temperatures, as indicated. The red lines are fits of the data to a single-exponential function and the resulting time constants are reported in Table S4 (Supporting Information).

Figure 6.

Comparison of the normalized conformational relaxation kinetics of Trpzip-2c obtained at a final temperature of 61.8 °C, from different initial temperatures, as indicated. The probing frequency was 1630 cm^{-1} .

Figure 7.

LD fits (red) of the experimental IR relaxation kinetics of BBL from Figure 4, using the free energy surfaces shown in the inset. The resulting effective conformational diffusion coefficients are given in the text.