Multiple Folding Pathways of the SH3 domain

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Abstract

Experimental observations suggest that proteins follow different pathways under different environmental conditions. We perform molecular dynamics simulations of a model of the SH3 domain over a broad range of temperatures, and identify distinct pathways in the folding transition. We determine the kinetic partition temperature —the temperature for which the SH3 domain undergoes a rapid folding transition with minimal kinetic barriers— and observe that below this temperature the model protein may undergo a folding transition via multiple folding pathways. The folding kinetics is characterized by slow and fast pathways and the presence of only one or two intermediates. Our findings suggest the hypothesis that the SH3 domain, a protein for which only two-state folding kinetics was observed in previous experiments, may exhibit intermediates states under extreme experimental conditions, such as very low temperatures. A very recent report (Viguera et al., Proc. Natl. Acad. Sci. USA, 100:5730–5735, 2003) of an intermediate in the folding transition of the Bergerac mutant of the α-spectrin SH3 domain protein supports this hypothesis.

Keywords: intermediate, molecular dynamics, folding pathways, SH3, kinetic partition.
INTRODUCTION

Recent experimental studies indicate that several proteins exhibit simultaneously a variety of intermediates and folding pathways. Kiefhaber\(^1\) identified at low denaturant concentration a fast pathway (50 ms) in the folding of lysozyme with no intermediates and a slow phase (420 ms) with well-populated intermediates. Choe et al.\(^2\) observed the formation of a kinetic intermediate in the folding of villin 14T upon decreasing the temperature, and Silverman et al.\(^3\) observed the extinction of a slow phase in the folding of the P4-P6 domain upon changes in ion concentration. Kitahara et al.\(^4\) studied a pressure-stabilized intermediate of ubiquitin, identified as an off-pathway intermediate in previous kinetics experiments at basic conditions\(^5\). All these studies suggest that environmental conditions favor some folding pathways over others.

Major theoretical efforts in the study of protein folding\(^6\)–\(^16\) have focused on small, single domain proteins\(^17\). It is found in experiments\(^17\)–\(^18\) that these proteins undergo folding transition with no accumulation of kinetic intermediates in the accessible range of experimental conditions. However, other kinetics studies of two-state proteins\(^19\)–\(^22\) suggest the presence of short-lived intermediates that cannot be directly detected experimentally. Recently, Sánchez et al.\(^23\) explained the curved Chevron plots — the nonlinear dependence of folding and unfolding rates on denaturant concentration\(^24\)–\(^26\) — of 17 selected proteins by assuming the presence of an intermediate state. Led by these studies, we hypothesize that single domain proteins may exhibit intermediates in the folding transition under suitable environmental conditions.

To test our hypothesis, we perform a molecular dynamics study of the folding pathways of the c-Crk SH3 domain revealed only two stable states at equilibrium conditions: folded and unfolded. Both states coexist with equal probability at the folding transition temperature, \(T_F = 0.626\), at which the temperature dependence of the potential energy has a sharp change, and the specific heat has a maximum (experimentally\(^18\), this temperature corresponds to 67°C). Thus, our model reproduces the experimentally-determined thermodynamics of the SH3 domain\(^18\)–\(^31\).

Initial Unfolded Ensemble

Our initially unfolded ensemble consists of 1100 protein conformations that we sample from a long equilibrium simulation at a high temperature \(T_0 = 1.0\) at equal time intervals of \(10^4\) time units (t.u.). This time separation is long enough to ensure that the sampled conformations have low structural similarity among themselves. We calculate the frequency map — the plot of the probability of any two amino acids forming a contact — of this unfolded ensemble (lower triangle of Fig. 1a). At \(T = 1.0\), only nearest and next nearest contacts have high frequency, and the frequency decreases rapidly with the sequence separation between the amino acids.

When we quench the system from \(T = 1.0\) to a target temperature, \(T_{\text{target}}\) (see Methods), the system relaxes in approximately 1500 t.u. Due to the finite size of our heat bath, the heat released by the protein upon folding increases the final temperature of the system by 0.03 units above \(T_{\text{target}}\). After relaxation, the protein stays for a certain time in the unfolded state, then undergoes a folding transition. During this time interval, the protein explores unfolded conformations, and we calculate the frequency map of the unfolded state for different target temperatures.

At \(T_{\text{target}} = T_F\), the secondary structure is unstable (Fig. 1b), with average frequency \(\bar{f} = 0.24\) (see Methods). Successful folding requires the cooperative formation of contacts throughout the protein in a nucleation process\(^6\)–\(^7\). At \(T_{\text{target}} = 0.54\), the secondary structure is more stable (Fig. 1c, \(\bar{f} = 0.50\)). Thus, the conformational search for the native state (NS) is optimized by limiting the search to the formation of a sufficient number of long range contacts. At \(T_{\text{target}} = 0.33\), the lowest temperature studied, secondary structure elements form during the rapid collapse of the model protein in the first 1500 t.u.
(Fig. 1d, $f = 0.73$). During collapse, some tertiary contacts — contacts between secondary elements — may also form. The formation of these contacts prior to the proper arrangement of secondary structure elements may lead the protein model to a kinetic trap. Finally, folding proceeds at this temperature through a thermally activated search for the NS.

**Kinetic Partition Temperature**

In order to determine the temperature below which we can distinguish fast and slow folding pathways, we compute the distribution of folding times $p(t_F, T)$ (Fig. 2a–e), as well as the average $\langle t_F \rangle$ (Fig. 2f) and standard deviation $\sigma_F$. The ratio $r(T) = \langle t_F \rangle / \sigma_F$ measures the average folding time in units of the standard deviation $\sigma_F$. This quantity is particularly useful when the value of the standard deviation correlates with the value of the average as we change $T_{\text{target}}$. For instance, single-exponential distributions $e^{-t_F / (\langle t_F \rangle / \langle t_F \rangle)}$ have $r = 1$.

We expect $r \to 1$ for $T_{\text{target}} > T_F$, because at these high temperatures the folding transitions become rare events and are single-exponential distributed. As we decrease $T_{\text{target}}$, we expect $r > 1$ just below $T_F$, because the folded state becomes more stable than the unfolded state, and the folding transitions are favored. Distributions with $r > 1$ indicate a narrow distribution centered in $\langle t_F \rangle$, so that most of the simulations undergo a folding transition for times of the order of the average folding time. However, if we continue decreasing $T_{\text{target}}$, we expect some folding transitions to be kinetically trapped, and the folding time distribution will spread over several orders of magnitudes. Such distributions have $r < 1$. Thus, there is a temperature below $T_F$ where the maximum of $r(T)$ occurs, and which signals the onset of slow folding pathways. We use the maximum of $r(T)$ to calculate $T_{KP}$.

Fig. 2g suggests that $T_{KP} = 0.54$, which corresponds to a maximally compact distribution of folding times* (Fig. 2d). We find that the ratio approaches one as we increase the temperature above $T_{KP}$, and the distribution of folding times approximates a single-exponential distribution. In particular, the distribution of folding times fits the single-exponential distribution $e^{-t_F / (\langle t_F \rangle / \langle t_F \rangle)}$ for $T = 0.64$, the closest temperature to $T_F$ that we study. The ratio $r(T)$ decreases monotonically below $T_{KP}$, indicating that the distribution of folding times spreads over several orders of magnitude. This is the consequence of an increasing fraction of folding simulations kinetically trapped (Fig. 2a-b). The average folding time $\langle t_F \rangle$ is minimal not at $T_{KP}$, but at a lower temperature $T_{\langle t_F \rangle} = 0.49$ (Fig. 2f). At this temperature, we find that the protein becomes temporarily trapped in approximately 7% of the folding transitions. On the other hand, the remaining simulations undergo a folding transition much faster, thus minimizing $\langle t_F \rangle$. Interestingly, $r(T_{\langle t_F \rangle}) \approx 1.0$, even though the distribution of folding times at this temperature is non-exponential.

**Folding Pathways**

Below $T_{KP}$, an increasing fraction of the simulations undergo folding transitions that take a time up to three orders of magnitude above the minimal $\langle t_F \rangle$. In addition, $\langle t_F \rangle$ increases dramatically (Fig. 2f). At the lowest temperatures studied, we distinguish between the majority of simulations that undergo a fast folding transition (the fast pathway) and the rest of the simulations that undergo folding transitions with folding times spanning three orders of magnitude (the slow pathways). At the low temperature $T = 0.33$, the potential energy of the fast pathway has on average the same time evolution of all the simulations at $T_{KP} = 0.54$, indicating that there are no kinetic traps in the fast pathway.

For each folding simulation that belongs to the slow pathways, we sample the potential energy at equal time intervals of 100 t.u. until folding is finished (see Methods). Then, we collect all potential energy values and construct a distribution of potential energies. We find that below $T = 0.43$, the distribution is markedly bimodal (Fig. 3a). The positions of the two peaks along the energy coordinate do not correspond to the equilibrium potential energy value of the folded state (Fig. 3b). Therefore we hypothesize the existence of two intermediates in the slow pathways. We denote the two putative intermediates as $I_1$ and $I_2$ for the high energy and low energy peaks, respectively. As temperature decreases, the peaks shift to lower energies, but the energy difference between the two peaks, approximately six energy units, remains constant (Fig. 3b). A constant energy difference implies that the two putative intermediates differ by a specific set of native contacts. As temperature decreases, other contacts not belonging to this set become more stable and are responsible for the overall energy decrease. At $T = 0.33$, we record the distribution of survival times for both intermediates and find that they fit a single-exponential distribution, supporting the hypothesis that each intermediate is a local free energy minima and has a major free energy barrier (Fig. 3c).

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*Assuming a linear relation between experimental and simulated temperatures and taking into account$^{18}$ that $T_F = 67^\circ$C, we estimate $T_{KP} \approx 20^\circ$C
To further test the single free energy barrier hypothesis, we select a typical conformation representing intermediate \( I_2 \) and perform 200 folding simulations, each with a different set of initial velocities for a set of temperatures in the range 0.33 \( \leq T \leq 0.52 \). For each simulation, we record the time that the protein stays in the intermediate and find that the average survival time fits the Arrhenius law for temperatures below \( T = 0.44 \) (Fig. 3d). This upper bound temperature roughly coincides with the temperature \( T = 0.43 \) below which \( I_2 \) becomes noticeable in the histogram of potential energies (Fig. 3a). This result indicates that the free energy barrier to overcome intermediate \( I_2 \) becomes independent of temperature for low temperatures, or analogously, that the same set of native contacts must form (or break) to overcome the intermediate.

Next, we determine the structure of the two intermediates. For each intermediate, we randomly select three conformations and find that they are structurally similar. Conformations belonging to intermediate \( I_1 \) have a set of long-range contacts (\( C_1 \)) with high occupancy and a set of long-range contacts (\( C_2 \)) with no occupancy at all (Fig. 3c). Contacts in \( C_1 \) represent a \( \beta \)-sheet made by three strands: the two termini and the strand following the RT-loop, which we name strand “A” (see \( I_1 \) in Fig. 4). Contacts in \( C_2 \) represent the base of the n-Src loop and the contacts between the RT-loop and the distal hairpin (see \( I_2 \) in Fig. 4). In addition, \( I_1 \) has a set of medium-range contacts (\( C_3 \)) with high occupancy (Fig. 3e) representing the distal hairpin and a part of the n-Src loop. For a slow folding transition, the \( \beta \)-sheet (\( C_1 \) contacts) forms in the early events and strand “A” can no longer move freely. This constrained motion prevents strand “A” from forming contacts with one of the strands of the distal hairpin, which we name as strand “B” (see \( I_2 \) in Fig. 4). Similarly, strand “B” cannot move freely because it is a part of \( C_3 \). The missing contacts between strand “A” and strand “B” are the contacts that form the base of the n-Src loop.

Conformational changes leading the protein away from intermediate \( I_1 \) involve either dissociation of the \( \beta \)-sheet, thus breaking some contacts of \( C_1 \), or dissociation of the distal hairpin, thus breaking some contacts of \( C_3 \). We find that the latter dissociation may lead the protein conformation to intermediate \( I_2 \). Intermediate \( I_2 \) has contacts of \( C_1 \), but lacks the set of contacts (\( C_4 \)) that form the base of the distal hairpin (see NS in Fig. 4).

Once we identify the structure of the intermediates, we investigate whether intermediate \( I_1 \) is present at larger temperatures when no distinction can be made concerning fast and slow folding pathways. To test this hypothesis, we sample the protein conformation during the folding transition at equal time intervals of 60 t.u. for each of the 1100 simulations, and compare these conformations to intermediate \( I_1 \) with a similarity score function (see Methods). For each folding transition, we record only the highest value of the similarity score, thus obtaining 1100 highest score values. At \( T_{KP} \), the histogram of the highest scores is bimodal, with 25\% of the folding simulations passing through intermediate \( I_1 \) (Fig. 3f). We find that at \( T_{KP} \), simulations that undergo the folding transition through \( I_1 \) show kinetics of folding no different than those of the rest of simulations.

**DISCUSSION**

It was shown\(^6\) that the simplified protein model and interaction potentials that we use here reproduced in a certain range of temperatures the experimentally-determined two-state thermodynamics of the SH3 domain\(^18\). The qualitative predictive power of the model encouraged us to study the folding kinetics in a wider range of temperatures. From our relaxation studies of the initial unfolded ensemble, we observe that the structure of the unfolded state is highly sensitive to \( T_{target} \). The role of the unfolded state in determining the folding kinetics has already been pointed out in recent experimental and theoretical studies\(^39–42\). We observe nucleation\(^6\), folding with minimal kinetic barriers, and thermally activated mechanisms for the different observed unfolded states.

In previous studies, various methods have been developed to determine the temperature that signals the onset of slow folding pathways. Socci et al.\(^43,44\) determined a glass transition temperature, \( T_g \), at which the average folding time is half way between \( t_{min} \) and \( t_{max} \), where \( t_{min} \) is the minimum average folding time and \( t_{max} \) is the total simulation time. This method is sensitive to the \( a \ priori \) selected \( t_{max} \). The authors varied \( t_{max} \) in the range \( 0.27 \times 10^6 < t_{max} < 0.960 \times 10^6 \), and they found a 10\% error in the calculation of \( T_g \). Also, Gutin et al.\(^45\) estimated a critical temperature, \( T_c \), at which the temperature dependence of the equilibrium potential energy leveled off. From their results, one can evaluate a 20\% error in their calculation of \( T_c \). Both \( T_g \) and \( T_c \) are temperatures that authors use to characterize the onset of multiple folding pathways. In our study we use \( T_{KP} \), which signals the breaking of time translational invariance of equilibrium measurements for temperatures below this value\(^46\). We estimate a 2\% error in our calculation of \( T_{KP} \) from uncertainties in the location of \( T_{KP} \) in Fig. 2g.
At $T_{KP}$, secondary structure elements are partially stable, and the search for the NS reduces to the formation of tertiary contacts. Furthermore, $T_{KP}$ is a relatively high temperature that prevents the stabilization of improper arrangements of the protein conformation, thus minimizing the occurrence of kinetic traps. Below $T_{KP}$, the model protein exhibits two intermediates with well-defined structural characteristics. This modest number of intermediates is a direct consequence of the prevention of non-native contacts. This prevention reduces dramatically the number of protein conformations. Furthermore, since a low energy value implies that most of the native interactions have formed, there are few conformations having both low energy and structural differences with the NS$^{11}$.

It is found experimentally$^{13,47–54}$ that proteins exhibit only a discrete set of intermediates. Even though in real proteins amino acids that do not form a native contact may still attract each other, experimental and theoretical studies confirm that native contacts have a leading role in the folding transition. Protein engineering experiments$^{33,55–58}$ show that transition states in two-state globular proteins are mostly stabilized by native interactions. To quantitatively determine the importance of native interactions in the folding transition, Paci et al.$^{59}$ studied the transition states of three two-state proteins with a full-atom model. They found that on average, native interactions accounted for approximately 83% of the total energy of the transition states. Of relevance to our studies of the SH3 domain are the full-atom study$^{60}$ and the protein engineering experiments$^{33,36}$ showing that the transition state of the src-SH3 domain protein is determined by the NS. On the other hand, evidence exists that in some proteins, non-native contacts are responsible for the presence of intermediates. In their study of the homologous Im7 and Im9 proteins, Capaldi et al.$^{61}$ identified a set of non-native interactions responsible for a intermediate state in the folding transition of Im7 protein. Mirny et al.$^{62}$ performed Monte Carlo simulations of two different sequences with the same NS in the $3 \times 3$ lattice. One sequence presented a series of pathways with misfolded states due to non-native interactions.

We investigate the kinetics of formation of the two intermediates in a wide range of temperatures. At low temperatures, simulations that undergo folding through intermediate $I_1$ reveal that contacts between the two termini form earlier than the contacts belonging to the folding nucleus$^{6,7}$. This result coincides with an off-lattice study$^{53}$ of a 36-monomer protein by Abkevich et al. In this study, the authors found an intermediate in the folding transition of their model protein. Inspection of the intermediate revealed no nucleus contacts, but a different set of long-range contacts already formed. In addition, we learned of the work by Viguera et al.$^{64}$ after completion of our study. They reported that a mutant of the $\alpha$-spectrin SH3 domain undergoes a folding transition through one intermediate. The authors observed that the newly-introduced long-range contacts had already been formed in the denaturated state, preceding the formation of the transition state of the protein. Thus, environmental conditions that favor stabilization of long-range contacts other than the nucleus contacts may induce intermediates in the folding transition.

Alternatively, short-range contacts in key positions of the protein structure may also be responsible for slow folding pathways. After completion of our study, Karanicolas et al.$^{65,66}$ reported their studies on the G6 model of the forming binding protein WW domain. The authors found a slow folding pathway in the model protein, and a cluster of four short-range native contacts that are responsible for this pathway. However, the authors observed that it was the absence, not the presence, of these native contacts in the unfolded state that generated bi-phasic folding kinetics. Thus, environmental conditions that favor destabilization of short range contacts may promote the formation of intermediate states in the folding transition.

We also investigate the survival time of intermediate $I_2$, and find that the free energy barrier separating $I_2$ from the NS is independent of temperature. Thus, the average survival time follows Arrhenius kinetics. The value of the free energy barrier is approximately 5.85 energy units, indicating that about six native contacts break when the protein conformation reaches the transition state that separates $I_2$ from the NS. At the low temperatures where intermediates $I_1$ and $I_2$ are noticeable, thermal fluctuations are still large enough so that the observed survival times of $I_2$ should be much smaller, if only any six native contacts were to break. Thus we hypothesize that it is always the same set of native contact that must break in the transition $I_2 \rightarrow NS$. Our observations of the transition $I_1 \rightarrow I_2$ support this hypothesis. In this transition, we find that the set of contacts $C_4$ always breaks.

At $T_{KP}$, we do not detect the intermediates from kinetics measurements of the average folding time, or analogously, from the folding rate. Thus we analyze the folding transition with the similarity score function that tests the presence of intermediate $I_1$. Then we find that this intermediate is populated in 25% of the folding transitions. In a recent study$^{67}$, Gorski et al. reported the existence of an interme-
We determine the kinetic partition temperature state, root mean square distance with respect to the native as we decrease the temperature, parameters monitoring whether the folded and unfolded states are populated. However, below the folding transition temperature, we observe that only the domain is in a broad range of temperatures. At the folding transition of the Gō model of the c-Crk SH3 domain are highly sensitive to temperature, suggesting that Im9 has an intermediate at normal conditions (pH = 7.0), but it is too unstable to be detected with current kinetic experimental techniques. Interestingly, the homologous protein Im7 (60% sequence identity) undergoes folding through an intermediate in all tested experimental conditions, supporting the authors' hypothesis. Thus, changes in both the environmental conditions and the amino acid sequence may uncover hidden intermediates in the folding transition of a two-state protein. In addition, a detailed study at $T_{KP}$ may reveal the intermediates. This is particularly useful for computer simulations, because simulations at low temperatures when intermediates are easily identifiable may require several orders of magnitude longer than simulations at $T_{KP}$.

**Conclusion**

We perform molecular dynamics analysis of the folding transition of the Gō model of the c-Crk SH3 domain in a broad range of temperatures. At the folding transition temperature, we observe that only the folded and unfolded states are populated. However, as we decrease the temperature, parameters monitoring the folding process such as potential energy and root mean square distance with respect to the native state, $rmsd$, suggest the presence of intermediates. We determine the kinetic partition temperature $T_{KP}$ below which we observe two folding intermediates, I₁ and I₂, and above which we do not observe accumulation of intermediates. Below $T_{KP}$, intermediate I₁ forms when the two termini and the strand following the RT-loop form a β-sheet, prior to the formation of the folding nucleus. This intermediate effectively splits the folding transition into fast and slow folding pathways. Dissociation of part of the β-sheet leads to the native state. We also find that stabilization of this β-sheet and subsequent dissociation of the distal hairpin may lead to intermediate I₂.

The key structural characteristics of intermediate I₁ allow us to define a similarity score function that probes the presence of the intermediate in a folding transition. We find that I₁ is populated even at $T_{KP}$. This result suggests that one can obtain information regarding the existence of putative intermediates by studying the folding trajectories at $T_{KP}$. However, at this temperature, no intermediates are noticeable if one limits the analysis only to the distribution of folding times.

We observe that the folding pathways of the model SH3 domain are highly sensitive to temperature, suggesting the important role of the environmental conditions in determining the folding mechanism. Our findings suggest that the SH3 domain, a two-state folder, may exhibit stable intermediates under extreme experimental conditions, such as very low temperatures.

**MATERIALS AND METHODS**

**Model Protein and Interactions**

We adopt a coarse-grained description of the protein by which each amino acid is reduced to its Cβ atom (Cα in case of Gly). Details of the model, the surrounding heat bath, and the selection of structural parameters are discussed in detail in a previous study. The selection of the set of interaction parameters among amino acids is of crucial importance for the resulting folding kinetics of the model protein. Experimental and theoretical studies of globular proteins suggest that native topology is the principal determinant of the folding mechanism. Thus, we employ a variant of the Gō model of interactions — a model based solely on the native topology — in which we prevent formation of non-native interactions, since we are solely interested in the role that native topology and native interactions may have in the formation of intermediates. We perform simulations and monitor the time evolution of the protein and the heat bath with the discrete molecular dynamics algorithm. The higher performance of this algorithm over conventional molecular dynamics allows one to increase the computational speed up to three orders of magnitude.

**Frequencies and Folding Simulations**

To calculate the frequency map at $T = 1.0$, we probe the presence of the native contacts in each of the 1100 initially unfolded conformations. Then, we compute the probability of each native contact to be present. To calculate the frequency map at $T_{target}$, we select one particular folding transition and we probe the presence of the native contacts during the time interval that spans after the initial relaxation and before the simulation reaches the folding time $t_F$. To compute $t_F$, we stop the folding simulation when 90% of the native contacts form. Then, we trace back the folding trajectory and record $t_F$ when the root mean square distance with respect to the native state, $rmsd$, becomes smaller than 3Å. We consider all protein conformations occurring for $t > t_F$ as belonging to the folded state and of no relevance to the folding.
transition.

**Similarity Score Function**

We introduce the similarity score function, \( S = \frac{(a/23)(15 - b)/15} \), where \( a \) is the number of native contacts belonging to the set of contacts \( C_1 \), and \( b \) is the number of native contacts belonging to set \( C_2 \) (Fig. 3e). \( C_1 \) has 23 contacts and \( C_2 \) has 15 contacts. If the protein is unfolded, then \( a \approx b \approx 0 \), thus \( S \approx 0 \). Similarly, if the protein is folded, then \( a \approx 23 \) and \( b \approx 15 \), thus \( S \approx 0 \) again. Finally, if the protein adopts the intermediate \( I_1 \) structure, then \( a \approx 23 \) and \( b \approx 0 \), thus \( S \approx 1 \).

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Figure 1: (a) Upper triangle: c–Crk SH3 domain contact map with 160 native contacts. The secondary structure elements are the clusters of contacts that are organized perpendicularly to the map diagonal. Long-range contacts between the two termini are enclosed in the circle, and long-range contacts between the RT-loop and the distal hairpin are enclosed in the square. Lower triangle: the frequency map for the initial set of 1100 unfolded conformations at $T = 1.0$. (b) Frequency map of the unfolded state at $T = T_F = 0.626$. We compute the frequencies for a particular folding transition, whose potential energy trajectory we show in the inset (see Methods). Same for (c) folding transition, whose potential energy trajectory $0$ is collected for $T = 0$. (d) Frequency map of the unfolded state at $T = T_F = 0.33$. We estimate the existence of putative intermediates. At $T = 0$, the distribution of folding times is unimodal, with the average survival time of 1500 t.u. (dashed line). We estimate the errors of the histogram bars as the square root of $f_i = n_i / 4$, where $n_i$ is the number of contact maps $\{ I_i \}$ with $i$ contacts “C$^1$” and 15 contacts in “C$^2$”. (f) Average folding time versus temperature. Each dot represents the folding time for a particular folding transition. (g) Ratio $r$ of the average and the standard deviation, $r = \langle t_F \rangle / \sigma_F$, for the distribution of folding times. The ratio approaches one above $T_{KP}$ and zero below $T_{KP}$. The ratio is maximal at $T_{KP}$, indicating a compact distribution of folding times at this temperature.

Figure 2: (a–e) Histograms of folding times for selected temperatures. At $T = 0.33$ and $T = 0.36$, the two lowest temperatures studied, histograms have a maximum for long folding times ($\downarrow^+$), which suggests the existence of putative intermediates. At $T = 0.33$, a maximum in the histogram ($\downarrow^+$), not present at $T = 0.36$, corresponds to short lived kinetic traps. The distributions of folding times are unimodal at higher temperatures. At $T = 0.54$, the histogram is compact, and has no tail of long folding times. At $T = 0.64$, the histogram fits a single-exponential distribution $e^{-t_F/(\langle t_F \rangle)}$ for times larger than the relaxation time of 1500 t.u. (dashed line). We estimate the errors of the histogram bars as the square root of each bar. (f) Average folding time versus temperature. Each dot represents the folding time for a particular folding transition. (g) Ratio $r$ of the average and the standard deviation, $r = \langle t_F \rangle / \sigma_F$, for the distribution of folding times. The ratio approaches one above $T_{KP}$ and zero below $T_{KP}$. The ratio is maximal at $T_{KP}$, indicating a compact distribution of folding times at this temperature.

Figure 3: (a) Distributions of the potential energies of the slow folding pathways for temperatures below $T = 0.44$. This upper bound temperature coincides with the temperature below which the distribution of the potential energies (Fig. 3a) of the slow folding pathways becomes bimodal. (c) Upper triangle: Absent contacts (filled squares) and present contacts (crosses, “C$^1$”) in intermediate $I_1$. Upon the transition $I_1 \rightarrow I_2$, these contacts reverse their presence (so that the filled squares are the present contacts and the crosses are the absent contacts). There are five more squares than crosses, which roughly accounts for the difference of six energy units between the two intermediates. Lower triangle: long-range contacts “C$^1$” are present in intermediate $I_1$, and long-range contacts “C$^2$” are absent. There are 23 contacts in “C$^1$” and 15 contacts in “C$^2$”. (f) Probability that a folding transition at $T = T_{KP} = 0.54$ contains a protein conformation with similarity $S$ to intermediate $I_1$ (see Methods).
Figure 1:
Figure 2:
Figure 3:
Figure 4: