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A Survey of λ Repressor Fragments from Two-State to Downhill Folding

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Abstract

We survey the two-state to downhill folding transition by examining 20 λ_{6-85}^* mutants that cover a wide range of stabilities and folding rates. We investigated four new λ_{6-85}^* mutants designed to fold especially rapidly. Two were engineered using the core remodeling of Lim and Sauer, and two were engineered using Ferreiro *et al.*'s frustratometer. These proteins have probe-dependent melting temperatures as high as 80 °C and exhibit a fast molecular phase with the characteristic temperature dependence of the amplitude expected for downhill folding. The survey reveals a correlation between melting temperature and downhill folding previously observed for the β -sheet protein WW domain. A simple model explains this correlation and predicts the melting temperature at which downhill folding becomes possible. An X-ray crystal structure with a 1.64-Å resolution of a fast-folding mutant fragment shows regions of enhanced rigidity compared to the full wild-type protein.

Keywords

heat denaturation; cold denaturation; CD; fluorescence; temperature jump

Introduction

The free-energy landscape model has shown that the decreasing configurational entropy of a folding polypeptide is largely compensated for by the buildup of contact energy, resulting in low activation barriers compared to other chemical reactions.¹ The most extreme case of compensation, downhill folding, has been detected by kinetic and thermodynamic signatures: a microsecond 'molecular phase' that disappears when a protein is destabilized by heating or denaturant,^{2,3} or probe dependence upon thermal denaturation.^{4,5} Nonexponential kinetics and probe dependence are also observed when metastable folding intermediates are populated.^{6,7} Downhill folding is the limit when barriers between metastable states are reduced to about $k_B T$, allowing folding to occur at the 'speed limit'.⁸

A large number of λ repressor fragment mutants (λ_{6-85}^*) have been investigated by NMR lineshape analysis^{9–15} and laser temperature jump (T-jump).^{3,16–21} This five-helix bundle can fold by many mechanisms such as two-state folding,⁹ helical intermediates,²² or downhill folding,¹⁶ depending on sequence and solvent conditions. Mechanistic malleability

indicates that competing pathways have similar and low activation energies.²² λ_{6-85}^* is thus an ideal candidate for a survey correlating protein stability and downhill folding.

We survey 20 λ_{6-85}^* sequences, including four new ones engineered either according to a core repacking designed by Lim *et al.*²³ or according to side-chain substitutions suggested by the frustratometer of Ferreiro *et al.*²⁴ All four new proteins have probe-dependent thermodynamics and a molecular phase. (Seven of 20 of the most stable sequences have a molecular phase.) We analyze the data with a model that we recently developed to account for the onset of downhill folding.²⁵ The model uses the balance between the heat denaturation and the cold denaturation of a protein to predict at what temperature, if at all, a given protein can fold downhill. Only the most stable mutants with the highest melting temperature can fold downhill.

What is the source of engineered stability? Natural proteins evolve some flexibility in order to function, so we hypothesize that downhill folding correlates with an increased rigidity of the native state compared to the wild-type protein. We obtained a 1.64-Å-resolution crystal structure of the stable mutant upon which our four engineered mutants are based and compare it to the wild-type 1.80-Å structure. The *B*-factor of the mutant is significantly smaller than that of the wild type in the region of the particularly stabilizing Q33Y mutation.

Results

Protein mutants

We investigated the folding of four new mutants of the λ repressor fragment. They were based on the fast-folding λ_{YA} mutant,^{18,22} which contains the mutations Y22W/Q33Y/G46A/G48A. The new mutant λ_W contained the additional mutations A37K/G43S. This double mutant was based on the frustratometer of Ferreiro *et al.*, a code that pinpoints potential nonnative interactions that could lead to trapping and suggests alternative side chains.²⁴ λ_S contained the additional mutations V36L/M40L/V47 L. This triple mutant was based on the core redesign by Lim *et al.*, which leads to improved packing and stability.²³ The λ_{YA} mutant is already a very stable incipient downhill folder (barrier estimated at <3 $k_B T$ at room temperature based on fluorescence and NMR measurements).^{18,19,22,26} We expected both new mutants to have high melting points and to show a fast molecular phase and probe-dependent heat denaturation if the redesign was completely successful.

In addition, we made the D14A mutants of the two proteins, as this mutation has also been shown to accelerate folding.¹⁴ To have more proteins for comparison, we studied 16 additional mutants whose kinetic and thermodynamic parameters are published by Myers and Oas,¹⁴ Ghaemmaghami *et al.*,¹⁵ Dumont *et al.*,¹⁷ Ma and Gruebele,¹⁸ Yang and Gruebele,¹⁹ and Huang and Oas,²⁷ and discussed in Methods.

Kinetics

All four new mutants exhibited nonexponential relaxation kinetics below their melting points. Figure 1 shows the data for λ_S after T-jumps to 65, 70, and 75 °C, obtained at a protein concentration of 10 μ M. The data at a final temperature of 65 °C were fitted to an equation shown to approximately account for both two-state and downhill dynamics:²⁸

$$\text{Signal}(t) = A_m e^{-t/\tau_m} + (1 - A_m) e^{-t/\tau_a} \quad (1)$$

The molecular phase had a time constant $\tau_m = 1 \pm 0.5$ μ s, and the activated phase had a time constant $\tau_a = 9 \pm 2$ μ s. The red residual in Fig. 1 shows that a single-exponential fit was not quite as good. As the temperature is increased towards the melting point, the fast molecular

phase gradually disappears; at a final temperature of 75 °C, λ_S behaves as an apparent two-state folder that can be fitted by a single-exponential curve.

The model discussed in detail below and in Methods²⁵ was used to estimate the temperature $T_{\max}$ at which the minimum activated relaxation time would occur. Table 1 shows that the model agrees well with the actual $T_{\max}$ measured, and that the λ_S mutant had the largest activated rate coefficient. Below T_m , all mutants showed a molecular phase with $\tau_m \approx 1\text{--}2\ \mu\text{s}$ at $T_{\max}$, indicating that they were at least incipient downhill folders. In the case of λ_S , the ‘activated’ rate $(9\ \mu\text{s})^{-1}$ is so fast compared to the molecular rate that the estimated barrier $\Delta G^\ddagger = RT \ln(9/1) \approx 2.2RT$ is comparable to thermal energy.

As reported previously,²¹ λ repressor downhill folders are prone to aggregation, probably because no large activation barrier prevents the molecules from reaching partly unfolded states along the reaction coordinate. For example, λ_{YA} showed a slow aggregation phase at concentrations above 100 μM . Each of the four new mutants had a slow kinetic phase ($\tau_{\text{agg}} \approx 100\ \mu\text{s}$) whose amplitude increased at higher protein concentration and higher temperature (see Methods). An upper concentration limit was determined for each mutant below which the aggregation phase could not be observed. Only the kinetic measurements below this threshold were used to extract τ_a and to estimate $T_{\max}$. When the temperature was above 65 °C, the limits were as follows: λ_W , 90 μM ; λ_W D14A and λ_S D14A, 20 μM ; and λ_S , 10 μM .

Transient aggregation propensity is most strongly correlated with $\tau_a(T_{\max})$: the faster the protein folds, the greater is the transient aggregation propensity.

Thermal stability

To assess whether the λ_W and λ_S mutants have probe-dependent thermal denaturation transitions, we measured thermal unfolding titrations by CD and fluorescence intensity. Figure 2 shows the signal for both proteins and their D14A mutants over the range from 4 to 98 °C. The signal $S_i(T)$ was fitted to a model with a linear dependence of free energy on temperature ($\Delta G_i^{(1)}$), a melting temperature T_{mi} , and linear signal baselines S_{Ni} and S_{Di} for the native and denatured states:

$$S_i(T) = \frac{S_{Di}(T) + S_{Ni}(T)K_i}{1 + K_i} \quad (2)$$

$$K_i = e^{-\Delta G_i/RT}$$

$$\Delta G_i = \Delta G_i^{(1)}(T - T_{mi})$$

The subscript “i” refers to either CD or fluorescence probe. Table 2 summarizes the results. Even with the uncertainty in CD baselines at high temperature, it is clear from Fig. 2 and from the table that fluorescence transition midpoints occur at a lower temperature than CD midpoints. The two signals could not be fitted together within measurement uncertainty by the same melting temperature T_{mi} . The D14A mutants have a lower cooperativity $\Delta G_i^{(1)}$ and a much larger discrepancy between CD and fluorescence-derived T_m than λ_S and λ_W .

We also carried out a thermal denaturation titration probed by fluorescence lifetime for λ_S . It yielded a melting temperature very close to the one obtained by CD. Furthermore, adding a quadratic temperature dependence to ΔG_i or using a heat capacity model (see Methods) did not make the CD and fluorescence intensity fits more coincident.

Downhill folding model

Only the most stable proteins can fold downhill. The reason is shown in Fig. 3a. Proteins heat denature at a temperature T_m and cold denature at a temperature T_{cd} . If, at some temperatures between T_m and T_{cd} , the bias towards the native state is strong enough, the

activation barrier will go to zero, and the protein will fold downhill (Fig. 3a, green zone).²⁵ T_{downhill} is the temperature below T_m where folding, if at all, begins to go downhill.

To model T_{downhill} quantitatively, we need to obtain $G(x, T, T_m)$, the free energy along a reaction coordinate x at temperature T for a protein that melts at T_m . For a given protein with melting temperature T_m , we can then determine at what temperature, if any, the barrier of G disappears.²⁵

First, we assumed that at T_m , free energy is represented by a double well along a coordinate x ($\approx \pm 1$ for the denatured and native states):

$$G_m(x, T_m) = \Delta G^\ddagger(T_m) x^4 - 2\Delta G^\ddagger(T_m) x^2 \quad (3)$$

$\Delta G^\ddagger(T_m)$ is the activation free energy at T_m . We expanded $\Delta G^\ddagger(T_m)$ in a Taylor series in T_m that fits all the proteins of interest here (see Methods). We further assumed that there is a linear bias of the free energy as a function of x , so the free energy depends on temperature as:

$$G(x, T, T_m) = G_m(x, T_m) + \Delta G_f(T) x/2 \quad (4)$$

$\Delta G_f(T)$ is the folding free energy near T_m or T_{cd} . It has the concave shape shown in Fig. 3a because of heat denaturation and cold denaturation, and was modeled by a commonly used constant heat capacity of folding model (see Methods).²⁹ We can now solve Eq. (4) for the temperature $T = T_{\text{downhill}}$ where the barrier near $x=0$ vanishes.

Figure 3b plots the resulting T_{downhill} versus T_m for barriers of 0, 1, and 2 $k_B T_0$ ($T_0 = 287.5$ K; see Methods). Downhill folding (barrier $< 1 k_B T$) requires a melting temperature T_m 65 °C. For $T_m = 65$ °C, downhill folding is predicted to occur at 20 °C, 45 °C below the melting point. This would be difficult to observe experimentally by a laser T-jump: so far, below the melting point, the equilibrium constant strongly favors the native state, and a small T-jump produces no measurable unfolding reaction. $T_m - 20$ °C is usually the lowest temperature where the cooperativity of λ repressor fragments still allows detection of a population change. With a barrier less than 1 $k_B T_0$ as our criterion for downhill folding, it should be quite easy to observe downhill folders: a protein melting at 71 °C or above would have T_{downhill} , at most, 20 °C below T_m .

Two-state to downhill survey

Figure 4 shows that this is indeed the case. All four mutants with melting points 71 °C had a molecular phase (triangles). All λ repressor mutants melting below 63 °C could be fitted by a single slow-activated phase (circles). Between 63 and 71 °C, some proteins had both phases, whereas some only the activated phase. One protein (λ_{HA} from Liu and Gruebele¹⁶) folded so rapidly that its molecular and activated phases could not be distinguished.

Protein structure and rigidity

To see how the structure and flexibility of an incipient downhill folder differ from those of the wild-type protein, we solved the 1.64-Å-resolution X-ray crystal structure of λ_{YA} , the basis for our four mutants (see Methods). The wild-type full protein [Protein Data Bank (PDB) file 1LMB]³⁰ was previously determined at a similar resolution of 1.8 Å.³¹ The two structures, aligned in Fig. 5, are almost identical where the λ repressor fragment overlaps the wild type. The stacking interaction between the Y33 and the W22 side chains is clearly resolved. Formation of this interaction in the native state is responsible for the strong fluorescence signal changes observed upon unfolding for all Y22W/Q33Y mutants (e.g., Fig. 2) but are absent in other mutants of the fragment.

The Debye–Waller factor B characterizes the thermal motions of atoms in a crystal structure.^{33,34} Results for the wild type and fast-folding mutant are aligned in Fig. 6. The B -values of mutant loops 1 and 2, as well as in helix 2, are significantly smaller than those of the wild type. The mutant has a structure more rigid than that of the wild type in residues 22 through 42 containing Y and W engineered residues. The two alanines at positions 46 and 48 contribute to stability, but there was no local increase in the rigidity of helix 3. Only helix 5 of the fragment, known to be less stable than any other helices of the fragment,³⁵ has larger B -factors than the wild type, where this helix does not terminate the sequence.

Discussion

Thus far, faster-folding λ repressor fragments were selected by considering the tradeoff between evolution and the physics of folding:³⁶ functionally conserved residues that may reduce native-state stability or increase denatured-state entropy (e.g., glycines in helices), or functional polar/charged residues (e.g., Q33) were replaced by more stabilizing or hydrophobic residues. In contrast, the λ_S and λ_W mutations were based on rational core design or computational design. Lim and Sauer redesigned the core of λ repressor to improve packing and to lower free energy;³⁷ in Fig. 3a, this would increase the temperature range between T_{cd} and T_m , increasing the probability of downhill folding. As discussed by Lazar *et al.*, such redesign is more likely to be successful for helix bundles than for β -sheet proteins.³⁸ The ‘frustratometer’ of Ferreiro *et al.* analyzes side chains for the likelihood that they will make nonnative contacts.²⁴ Indeed, in many cases, they find that the most frustrated residues are those in active catalytic or binding sites, in agreement with our general prediction.³⁶ D14A is a mutation designed to remove nonnative interactions (a salt bridge between the N terminus and the C terminus). It shows that mutations are no longer additive when the speed limit is approached: in Table 1, the D14A mutant is still somewhat faster than its λ_W parent, but the D14A mutant of the faster λ_S is not. It becomes harder to speed proteins as they approach the speed limit.

The four mutants engineered here display all the behaviors postulated for proteins that switch from two-state to downhill kinetics as they are brought near their temperature of maximal stability: the proteins are aggregation-prone because no barrier prevents partial unfolding;^{21,36} thermal denaturation becomes probe-dependent;^{4,39} and slower two-state kinetics with activated relaxation time τ_a turn into much faster biphasic kinetics with an additional molecular relaxation time τ_m when the temperature is lowered below the melting temperature T_m of the protein.^{2,40} The molecular phase results from large observable protein populations diffusing across the transition region because the activation barrier is very low.²⁰

We have postulated that increased thermodynamic stability and extremely rapid folding could go hand in hand with increased structural rigidity.³⁶ For λ_{YA} , the overall reduced B -factors support this idea, while the structure of the mutant remains very close to that of the wild type. The polypeptide chain between mutations Y22W and Q33Y is more rigid, as is the nearby helix 2 (Fig. 6). On the other hand, the mutations G46,48A in helix 3 do not improve rigidity. As shown by Burton *et al.*, these mutations act by destabilizing the denatured state, not by stabilizing the native state.¹¹ In fact, the more flexible glycine residues may allow slightly better packing in the native state, as seems to be the case in Fig. 6. Given the large B -value of loop 4–5 and helix 5 (Fig. 6), we predict that one should be able to clip residues 76–85 from λ repressor fragment 6–85, making a mini version λ_{6-75} without much loss of stability. This prediction is also supported by an experiment–simulation study,³⁵ which shows that helices 1 and 4 are the folding core and that helix 5 is very unstable.

The survey of λ repressor fragments in Fig. 4 shows the correlation between the activated folding rate, protein stability (T_m), and the appearance of the molecular phase. A $2 k_B T$ barrier can be achieved within 15 °C of the melting temperature by proteins melting above 63 °C. That region is easily accessible to T-jump experiments. A barrier below $1 k_B T$ can be achieved within 20 °C of the melting temperature by all proteins melting above 71 °C. At $2 k_B T$, the activated population is about 15%; at $1 k_B T$, it is about 40%. Given the signal-to-noise ratio of kinetic measurements, this explains why three of five proteins melting between 63 and 71 °C have observable downhill folding and why 100% of proteins melting above 71 °C do. In principle, tiny molecular phases should exist even for less stable proteins,²⁸ but they simply cannot be observed with signal-to-noise levels as shown in Fig. 1.

The general conclusions of the model are similar for the WW domain,²⁵ an all- β -sheet protein that we studied previously. Thus, a model postulating a temperature of maximal stability between cold denaturation and heat denaturation, coupled with a simple double-well activation energy at the melting temperature of a protein, is sufficient to predict quantitatively the two-state to downhill folding transition of these two structurally very different proteins.

The molecular relaxation time measured for the λ repressor five-helix bundles (1–2 μ s; Fig. 4, triangles) is slower by about a factor of 10 than measurements of helix formation times for Ala-based peptides.⁴¹ One possible explanation is that assembly of the helices into tertiary structure requires some trial and error (i.e., the protein gets stuck in shallow traps during the assembly of tertiary structure). Another possibility is that helical secondary structure in natural peptides forms more slowly than in Ala-based model peptides. Mukherjee *et al.* recently observed a folding relaxation time of 2 μ s for a natural helical peptide from protein L9.⁴² Thus, it is possible that secondary structure formation defines the speed limit of small helix bundles. In the diffusion–collision fit of λ repressor by Burton *et al.*, both tertiary-structure and secondary-structure formations play comparable roles.¹²

The triangles in Fig. 4 set a firm limit on the timescale that must be reached by single-molecule experiments in order for them to resolve the actual folding process: unless time resolution approaches 1–2 μ s, only the dwelling in the folded and denatured wells, not the actual transitions, will be resolved.²⁸ The current single-molecule technology is at the 250- μ s level,⁴³ so small proteins such as the λ repressor fragment or the WW domain are out of range. Larger proteins with slower ‘speed limits’ (e.g., PGK, 10 μ s)² are the best targets for such studies.

Such an effort will be worthwhile because a complex transition behavior may be observed. Although we fitted the molecular phase here by a single exponential in equation, the diffusional kinetics between the native state and the denatured state are more complex: for λ_{YA} , we observed different kinetics with different probes, as well as a nonexponential molecular phase,¹⁸ indicating multidimensional diffusion on a rough energy landscape. Recent continuous-flow studies of several λ repressor fragments (including 1 and 2 in Table 3) by DeCamp *et al.* suggest an unfolded state much more complex than a single unfolded well that Fig. 3 would suggest, as well as extremely fast phases associated with a denaturant-sensitive and temperature-sensitive denatured state.⁴⁴

Methods

Proteins

A complete list of the proteins plotted in Figs. 4 and 7, from Liu and Gruebele¹⁶ and Yang and Gruebele,¹⁹ is given in Table 3. The four new mutants were made by site-directed

mutagenesis (Stratagene Quickchange kit), starting with the λ_{YA} plasmid from Liu and Gruebele, based on a plasmid donated by T. Oas.¹⁶ Genes were overexpressed in *Escherichia coli* BL21 cells, and protein was purified as described in detail by Dumont *et al.*¹⁷ Purity was confirmed by matrix-assisted laser desorption ionization mass spectrometry and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Concentrations were measured by the aromatic amino acid absorbance method.⁴⁵

Thermodynamics

CD spectra and thermal titrations in the range of 4–98 °C were measured in two steps on a JASCO J-715 spectropolarimeter equipped with a Peltier temperature controller. Protein samples used in the measurement had concentrations of 2–3 μ M. Integrated fluorescence data were collected simultaneously with CD measurements so that differences in T_m could be determined reliably.

Fluorescence lifetime profiles were measured on our T-jump apparatus with a time resolution of 500 ps.⁴⁶ Lifetime profiles were obtained in two steps over the temperature range of 26–94 °C and analyzed by singular value decomposition. No significant shift in T_m with respect to CD data at 222 nm was found.

All thermodynamic data were normalized in the range of 0–1 and fitted by Eq. (2). In addition to the linear free-energy model, we also tested a quadratic term $\Delta G = \Delta G_1(T - T_m) + \Delta G_2(T - T_m)^2$.² Fitting results were slightly better in some cases when ΔG_2 was floated but—as can be seen from the good linear model fits in Fig. 2—not sufficient to warrant inclusion of a quadratic parameter.

T-jump kinetics

All the protein samples were prepared in 40 mM aqueous phosphate buffer at pH 7. T-jump-induced relaxation kinetics was measured with 5–10 °C jumps on our laser T-jump instrument.⁴⁶ Protein fluorescence was excited with 280-nm UV pulses. Fluorescence light was filtered with a Hoya B370 filter, collected with a photomultiplier tube, and digitized every 500 ps. Protein folding kinetics were monitored by the evolution of the fluorescence lifetime profile $f(t)$ upon T-jump by linear analysis on the fluorescence traces detected:⁴⁶ $f(t) = \chi(t)f_1 + (1 - \chi(t))f_2$, where f_1 and f_2 are the fluorescence lifetime profiles 70 ns after T-jump and 500 μ s after T-jump, respectively. For two-state folding kinetics, $\chi(t)$ can be fitted to a slow single exponential. For incipient downhill folding kinetics, $\chi(t)$ needs to be fitted to a double exponential, as shown in Eq. (1). For downhill kinetics (λ_{HA}), $\chi(t)$ can be fitted approximately to a fast single exponential.

When transient aggregation kinetics were observed for downhill folders at high protein concentration and high temperature, a triple exponential was used to fit the data: adding $\tau_{agg} \approx 30$ –200 μ s (depending on the concentration) to the molecular time τ_m , and activated folding time τ_a . The bottom panel of Fig. 7 shows 65–70 °C jump kinetics for λ_S at protein concentrations of 10 and 100 μ M. As discussed in the main text, the concentration below which no slowing of the kinetics was observed varied from protein to protein.

In the tables and in Fig. 4, we report $\tau_a(T)K_{eq}(T)/[1 + K_{eq}(T)]$ as the activated folding time. $\tau_a(T)$ is the fitted activated folding time at temperature T from Eq. (1). We apply the equilibrium constant correction because the temperature of the maximum folding rate did not occur at the same K for every protein. For a two-state folder, the equilibrium correction to $\tau_a(T)$ yields the forward reaction rate. For consistency, we used the equilibrium constant determined from CD and corrected the observed relaxation rate for all proteins in the same way. For each protein, at least five data points of folding kinetics at different temperatures were measured to estimate the T_{max} reported in the tables. When a clear minimum in the

curve $\tau_a(T)K_{eq}(T)/[1+K_{eq}(T)]$ was not reached, we report the temperature where the highest corrected folding rate was measured.

Two-state to downhill folding model

To use the model outlined in the main text, we need formulas for $\Delta G_f(T)$ and $\Delta G^\ddagger(T_m)$ that work well for all the proteins.

For $\Delta G_f(T)$, we used a heat capacity model of the protein free energy:

$$\Delta G_f(T) = -\Delta C_p T (1 - \ln [T/T_0]) + \Delta C_p T_m (1 - \ln [T_m/T_0]) \quad (5)$$

with ΔC_p constant. In this equation, ΔC_p is the unfolding heat capacity change, and T_0 is the temperature where the protein free energy goes through a minimum. T_m was taken from Tables 2 and 3 for each protein. We used average values $\Delta C_p = 0.752 k_B T_0/(\text{mol K})$ and $T_0 = 287.5 \text{ K}$ that provided a good thermodynamic fit to available heat denaturation^{19,20} and cold denaturation^{10,47} data for all 20 proteins. T is expressed in Kelvin in the logarithm.

For $\Delta G^\ddagger(T_m)$, we fitted the available kinetic data at the melting temperature of the 20 proteins and obtained the best correlation:

$$\Delta G^\ddagger(T_m) = -0.816 + 0.164 T_m - 0.0017 T_m^2 \quad (6)$$

where $\Delta G^\ddagger(T_m)$ is expressed in units of $k_B T_0$, and T_m is expressed in degrees Celsius. Equations (5) and (6) describe all proteins in this study with only T_m as input, with all other parameters fixed. Inserting Eqs. (5) and (6) into Eq. (4), we then computed at what T_m the barrier vanishes, when it equals $1 k_B T_0$ and when it equals $2 k_B T_0$, as shown in Fig. 3.

We also fitted the relationship between the maximal activated (τ_a phase) folding rate and the temperature $T_{\max}$ at which it occurs for proteins other than the four mutants that we measured here. We obtained:

$$T_{\max} = 0.606 T_m + 18 \text{ K} \quad (7)$$

This relation predicted the temperatures of the maximal folding rates for the four new mutants accurately (Table 1). Figure 7 shows a plot of the fits for $\Delta G^\ddagger(T_m)$ and $T_{\max}$.

Protein crystallization and structure

The λ_{YA} mutant fragment 6–85 (mutations P6S Y22W Q33Y G46A G48A Y85R from wild type) was grown and purified as described above, then lyophilized after dialysis into pure water. Lyophilized protein was redissolved in a range of buffer conditions to yield diffracting crystals of sufficient size. Crystals were grown by the vapor-suspended drop method from polyethylene glycol solution (pH 6.5). Crystals were flash-frozen in liquid nitrogen for collection of data sets at the Brookhaven National Laboratory. Like the wild type, the mutant crystallized in the $P2_1$ space group, so two chains were available to calculate average B -values in Fig. 6. The structure was solved by molecular replacement using the coordinates of PDB file 1LMB in the MOLREP program. Refinement data are summarized in Table 4.

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Abbreviations used

T-jump	temperature jump
PDB	Protein Data Bank

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Fig. 1.

Relaxation kinetics of the λ_S mutant at 13, 8, and 3 °C below its CD-detected melting temperature. Top: The blue curve is the best double-exponential fit with $\tau_m=1\pm0.5$ μ s, $l_m=0.4\pm0.1$, and $\tau_a=9\pm2$ μ s; the red curve is the best exponential fit with $\tau_a=4.5\pm0.5$ μ s (constrained to fit the data at $t=0$). As T_m is approached in the lower panels, the fast phase disappears, and the exponential fit becomes as good as the double-exponential fit. The protein concentration was 10 μ M in 40 mM aqueous phosphate buffer at pH 7.

Fig. 2.

Thermal denaturation data by CD and fluorescence. The parameters of a linear free-energy model $\Delta G = \Delta G_1(T - T_m)$ with linear baselines are presented in Table 2. The CD melting midpoint temperatures T_m are much higher, hence the CD baseline is not reached at high temperature, while the fluorescence baseline is fully reached.

Fig. 3.

Model correlating the onset of downhill folding with protein stability. The orange/light green/green zones indicate two-state folding, incipient downhill folding, and downhill folding. (a) The folding free energy $\Delta G(T)$ goes through a minimum at T_0 . Only if this minimum is low enough will the free-energy surface shown at the bottom be biased all the way to downhill folding. (b) Using only thermodynamic input parameters, the model estimates at what melting temperature T_m the barrier reaches 0–2 $k_B T$.

Fig. 4.

Measured folding time *versus* melting temperature T_m . The orange/light green/green zones indicate the range for two-state folding, incipient downhill folding, and downhill folding. A fast molecular phase τ_m is observed for mutants with T_m 63 °C, whose activated folding time τ_a is in the incipient downhill folding zone. The black circle is for the mutant λ_{HA} from Liu and Gruebele,¹⁶ whose τ_a and τ_m could not be distinguished due to very fast relaxation. K_{eq} is the equilibrium constant from thermal denaturation CD data (e.g., Fig. 2) and corrects τ_a for the backward (unfolding) reaction (see Methods). T_{max} is the estimated temperature of the fastest folding (e.g., Table 1). Sigmoid fit shows the trend of the observed activated folding times with melting temperature.

Fig. 5.

X-ray crystal structure (blue) of the fast-folding λ_{6-85}^* Q33Y/G46A/G48G mutant on which the mutants made here are based. The wild type from PDB^{30,31} file 1LMB is colored with the structure alignment Q score per residue.³² Most of the wild type is shown in green, indicating a high Q score and structural identity between the mutant and the wild type. The interaction between the Y33 and the W22 residues is shown in orange.

Fig. 6. Debye–Waller factors of the λ_{6-85}^* Q33Y/G46A/G48G mutant, averaged over the two proteins in the unit cell. Compare wild-type 1LMB, whose helices are shown in purple, and loops in red.

Fig. 7.

Top: Effect of protein concentration on the relaxation kinetics of λ_S at 70 $^\circ$ C. Middle: Best fit of $\Delta G^\ddagger(T_m)$ to a quadratic function. Bottom: Best fit of $T_{max}(T_m)$ to a straight line.

Table 1

Predicted and measured temperatures of the maximal activated folding rate and the smallest τ_a

Proteins	Model $T_{\max}$ [°C]	Measured $T_{\max}$ [°C]	$\tau_a(T_{\max})K/(K+1)$ [μs]
λ_W	59	55±2	30±3
λ_S	65	65±5	9±2
λ_W D14A	56	61±5 ^a	20±2
λ_S D14A	66	60±5	12±2

The model is $T_{\max}=0.606T_m+18$ °C (see Methods). τ_m was in the range 1.5±0.5 μs for all four mutants (e.g., Fig. 1).

^aThe uncertainty quoted is the 5 °C measurement interval at which T-jumps were carried out.

Table 2

Melting temperature T_m and cooperativity ΔG_1 parameters obtained by sigmoidal fits to the thermal titration data showing a large difference between CD and fluorescence intensity detection

Protein	CD at 222 nm		Fluorescence at 280 nm	
	T_m [°C]	ΔG_1 [J/(mol K)]	T_m [°C]	ΔG_1 [J/(mol K)]
λ_w	68±1	503±21	58±1	340±32
λ_w D14A	63±2	317±28	47±1	357±29
λ_s	78±1	519±27	67±1 ^a	324±24
λ_s D14A	80±8	240±26	57±2	241±18

^aWith inclusion of ΔG_2 .

Table 3

Sixteen remaining λ repressor fragments used in the fitting model (Fig. 4 and 7)

Mutations	T_m [°C]	T_{max} [°C]	$\tau_a(T_{max})K_{eq}/$ $(K_{eq}+1)$ [μ s]
Wild type ^a	61	58	34
A81G	47.5	46	76
A37G	54.5	51	51.5
A63V	60	59	51.5
Q33Y	61.5	53	38
A37GA49G (1)	47	46	82
Q33YA37G	59.5	55	44
A37GG46AG48AA49G	56	55	47.5
G46AG48A	67.5	57	24
Q33YG46AG48A	71	61	20
Q33HG46AG48A	68	45	2.5
Q33YG46AS45AG48A	69.5	61	23
D14AQ33YG46AG48A (2)	73.5	80	10
Q33YG46AS45AG48AS79A	70.5	61	23
Q33YM42S45AG46AG48AS79G	59	50	42
D14AQ33YA37GG46AG48AA49G	62.5	55	28

^a All sequences, including wild type, have the mutation Y22W. All values are rounded to the nearest 0.5. The equilibrium constant K was calculated at T_{max} from CD data.

Table 4Data collection and refinement of λ_{YA} compared to wild type

	Mutant	PDB file 1LMB
<i>Crystallographic data</i>		
Space group	$P2_1$	$P2_1$
Unit cell parameters	32.630	37.220
$^\circ a$ (Å)	58.710	68.720
$^\circ b$ (Å)	42.850	57.030
$^\circ c$ (Å)	98.180	92.200
$^\circ \beta$ (°)		
<i>Data collection statistics</i>		
Wavelength (Å)	1.1	
Resolution (Å)	1.64	1.80
Total number of reflections	142,936	
Number of unique reflections	19,544	
R_{sym} or R_{merge} (%)	6.7 (16.7)	
Redundancy	7.3 (6.6)	
Completeness (%)	99.1 (90.9)	
<i>Refinement statistics</i>		
R_{all} (%) (4-s cutoff)	18.57	18.90
R_{work} (%) (4-s cutoff)	18.43	
R_{free} (%) (4-s cutoff)	21.871	
RMS bond lengths (Å)	0.007	0.020
RMS bond angles (°)	1.885	2.10
Total number of atoms	1238	2348
Number of water molecules	282	140
Average B -factor (protein) (Å ²)	23.52	25.2
Average B -factor (water) (Å ²)	33.01	34.2