Supporting Text

General Properties of the 1D Surface

The default surface has $\Delta G_f = 0$ at T_m. The adjustable parameters thus are the separation between the wells, the width of the wells, the strength of the native bias, the barrier height, and the width of the wells. The well separation and temperature/glucose-dependent thermodynamic bias is discussed in *Methods*. The denatured well is set wider than the native well, assuming that the ensemble of unfolded conformations should be broader than that of native conformations (1) and in agreement with recent molecular dynamics simulations for λ_{Q33Y} (2). The setting of the remaining parameters to match the kinetic data is discussed in the discussion section.

The equation used to generate the free energy surfaces is given by

$$V = h*(-\exp(-A*(x-x1)^2) - \exp(-B*(x-x2)^2) + 1)*H(x-x1)H(x2-x) \\ +C*(x-x1)^2*H(x1-x) + D*(x-x2)^2*H(x-x2) + k*x \qquad \textbf{[S1]}$$

In Eq. **S1**, $H(x)$ is the Heaviside function (= 0 if $x \leq 0$; = 1 if $x > 0$). The numerical parameters for the free energy surface are shown in Table S1.

General Properties of the 2D Surface

The equation used to generate the 2D free energy surface in Fig. 6 is given by

$$\begin{aligned} V = {} & h*(-\exp(-A*((x-x1)^2 + (y-x1)^2)) - \\ & \exp(-B*((x-x2)^2 + (y-y2)^2)) + 1) + \\ & C*((x-x1)^2*H(x1-x) + (y-y1)^2*H(y1-y)) + \\ & D*((x-x2)^2*H(x-x2) + (y-y2)^2) + \\ & k*(x+y-4) \end{aligned} \qquad \textbf{[S2]}$$

The numerical parameters for the surface and other constants used in the Langevin simulation are shown in Table 4.

Probe Signal Switching Functions

When the surface in **S1** is switched upon T-jump, the protein ensemble relaxes. This produces an observable signal because probe signals such as IR or fluorescence depend on the distribution of the ensemble along the reaction coordinate. For example, the IR signal peaks more strongly near 1,650 cm^{-1} once helices have formed. During two-state folding, only two states, the denatured and the folded well, have appreciable population and contribute to the probe signal. During downhill folding, other positions along the reaction coordinate contribute, and if different probes switch in different positions, their respective kinetics differ.

The simulated kinetics was calculated by using switching functions shown at the top of Fig. 7. To match the large time-scale difference between IR and fluorescence detected kinetics, as well as the signs of the amplitudes of the decays with the model, the switch for both IR and fluorescence needs to occur on the native side of the energy barrier. We also used more realistic sigmoidal switching functions, which yielded results similar to those shown in the main text.

Other placements of the switching function were used to test whether the probes can be positioned on the unfolded side of the energy barrier (Fig. 7 *Middle*, dotted lines). The calculated relaxation kinetics cannot reproduce the time-scale differences between IR and fluorescence probes (Fig. 8, bottom). The reason is that at 63°C, the folded fraction is >90% of the total populations (Fig. 2), so that the population on the unfolded side contributes very little to the signal. Thus, the IR signal in our model must switch at lower free energy than the fluorescence, and also at a more native position along the reaction coordinate.

Thermodynamic Fitting

In general, faster-folding $\lambda_{6\text{-}85}$ mutants have larger "native" baselines than slower-folding mutants during heat denaturation (3). Such discrepancies are usually neglected in two-state fits, where arbitrary linear or higher order baselines are fitted, and no explanation is

given why the native or denatured states should have temperature-dependent probe signals. (In some cases, such temperature dependences may indeed be "trivial" variations of the probe itself with temperature, but this is generally not tested with model compounds or otherwise verified.)

To fit the thermodynamic data with the model free energy surface in Fig. 5, the model's native/denatured populations were not adjusted any further, and the same dividing surfaces as for the kinetic fits were used. Because the model switches from a type 0 to a type 1 scenario to account for the merging of the IR and fluorescence kinetic time scales, it also correctly predicts the cooperativity and small variation of melting temperatures, T_m, determined by different techniques. The discrepancy between the melting temperature of the IR spectrum and fluorescence decay lifetime observables remains small, $\Delta T_m = 1°C$. This is to be contrasted with a 0.5°C observed in the steady-state FTIR, fluorescence intensity, and circular dichroism data.

Such close T_m and sigmoidal shape are usually taken to imply a two-state transition, even at low temperatures where the native state is more stable than the denatured state. The free energy surface for 55°C (≈16°C below the melting midpoint) in Fig. 5*C* shows that such an extrapolation is not warranted unless the nature of the baselines is fully understood. On the other hand, a purely type 0 fit, as used for the thermodynamics of protein BBL (4, 5), does not yield the observed merging of IR and fluorescence kinetic time scales. Our thermodynamic and kinetic results are thus best compatible with a type 1 scenario above the denaturation midpoint, and a type 0 scenario only below it.

Model Surfaces with Explicit Traps

We previously reported that model surfaces with small explicit traps along the reaction coordinate equally well fit the experimental data, compared with smooth surfaces where the traps are subsumed in a smaller value of the diffusion coefficient (3, 6). Fig. 8 schematically summarizes our other attempts to fit different smooth or multiwell potentials to the kinetic and thermodynamic data.

Case A (two-state, barrier > 3 kT) cannot reproduce the different IR and fluorescence time scales and thermodynamics, no matter where the dividing surfaces are placed. When

the barrier exceeds 3 *kT*, the "IR" and "fluorescence" signals coincide, unless the dividing surfaces are placed deep within one of the wells (in which case the thermodynamics cannot be fitted).

Case B, the classic scenario of a rapidly formed intermediate, cannot reproduce the relative amplitudes of the fast and slow phases and their temperature dependence, no matter where the dividing surfaces are placed. We previously showed (3) that the fast phase takes $\lambda_{6\text{-}85}$ mutants to the native state under particularly strong bias, so variations where state "I" in scenario B is deeper than state "D" (so "D" becomes an off-pathway intermediate) are also ruled out. Finally, cases that produce sufficiently large additional phases require an intermediate so deep that it shows up in the thermodynamics simulations. This is incompatible with Fig. 2. Fig. 9 shows an example of "classic intermediate" surfaces before and after the T-jump, with the computed "IR" (red) and "fluorescence" (blue) transients.

Case C, a small trap following the largest barrier, was shown in ref. 6 to yield a molecular phase of the correct size and temperature dependence. Simulations done here (Fig. 9) show that it can also reproduce the difference between the IR and fluorescence kinetic data, if the dividing surfaces are placed on the first D → T barrier (fluorescence) and T → N barrier (IR). The key is that the T → N barrier must be *lower* in energy than the D → T barrier to yield the correct kinetic amplitudes and signs, whereas in the "classic" intermediate model the final barrier is the largest one. Traps following the largest barrier have also be discussed for conventional two state folding by Englander, Bai, Sosnick, and coworkers (*7*). Surface C cannot reproduce the single+stretched exponential data in Fig. 4, because it yields only two well defined rate eigenvalues.

Case D yields a triple-exponential fast phase. if the switching function for the fluorescence is carefully placed. Careful adjustment of the barriers is required to yield a triple exponential that fits the observed data as well as a single+stretched exponential. The two barrier heights compatible with experiment at 63°C are 1.4-1.8 *kT* when a diffusion constant of 0.05 nm^2/ns is used (a much larger value than the one used in the main text, and close to the diffusion constant of a free helix through water). This still places the explicit trap surface D in the type 0 limit at low temperature. The only serious problem with this model is that it places the fluorescence dividing surface rather late

along the reaction coordinate to get the correct fluorescence decay, leaving the infrared dividing surface in a place where it becomes difficult to simulate the observed two-state thermodynamics near T_m. For this reason we favor the 2D model discussed in the main text, i.e., at least some of the roughness responsible for the stretched fast phase is "transverse." The relative amount is impossible to ascertain with the current set of data.

Case E corresponds to the smooth downhill surface (barrier < 3 *kT*) used in this work. It provides correct phases, differences between "IR" and "fluorescence" irrespective of the exact shape of the potential, as long as the residual barrier lies between 0.3 and 1.2 *kT*, and the dividing surfaces are placed roughly in the right position along the reaction coordinate (Fig. 5) This model yields thermodynamic unfolding simulations fully compatible with experiment as long as the barrier is picked near 1 *kT*.

As discussed above, our data does not fully distinguish between the 2D version of model E and model D with two explicit traps; we favor the 2D model because it requires less fine-tuning and because model D yields less two-state-like than observed in Fig. 2. The only significant difference between models D and E is that D lumps all the free energy roughness into "longitudinal" roughness along a 1D reaction coordinate and attempts to fit the data with a minimal number of traps (two required for λ_{Q33Y}), whereas E lumps all the free energy roughness into a diffusion constant (or invokes additional coordinates = "transverse roughness" in the 2D version of Fig. 6). Molecular dynamics simulations and kinetics experiments on the trpzip 2 peptide (8, 9) and molecular dynamics simulations on λ_{Q33Y} indicate that it may be best not to distinguish the denatured state D and traps T_i for downhill folders but to consider all of them as different partially structured nonnative ensembles accessible to the protein during folding and unfolding (2). The property shared by all the workable models and existing simulations for λ_{Q33Y} folding is that they are downhill ("barriers" < 2 *kT*) and do not involve an intermediate followed by a late transition state.

1. Cavalli, A., Haberthur, U., Paci, E. & Caflisch, A. (2003) *Protein Sci.* **12,** 1801–1803.
2. Pogorelov, T. V. & Luthey-Schulten, Z. (2004) *Biophys. J.* **87,** 207–214.
3. Yang, W. & Gruebele, M. (2004) *Biophys. J.* **87,** 596–608.

4. Garcia-Mira, M. M., Sadqi, M., Fischer, N., Sanchez-Ruiz, J. M. & Muñoz, V. (2002) *Science* **298,** 2191–2195.

5. Muñoz, V. (2002) *Int. J. Quantum Chem.* **90,** 1522–1528.

6. Yang, W. Y. & Gruebele, M. (2003) *Nature* **423,** 193–197.

7. Englander, S. W., Sosnick, T. R., Mayne, L. C., Shtilerman, M., Qi, P. X. & Bai, Y. (1998) *Acc. Chem. Res.* **31,** 767–744.

8. Yang, W. Y. & Gruebele, M. (2004) *J. Am. Chem. Soc.* **126,** 7758–7759.

9. Yang, W. Y., Pitera, J., Swopes, W. & Gruebele, M. (2004) *J. Mol. Biol.* **336,** 241–251.