Single-molecule fluorescence probes dynamics of barrier crossing

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Kramers developed the theory on how chemical reaction rates are influenced by the viscosity of the medium1,2. At the viscosity of water, the kinetics of unimolecular reactions are described by diffusion of a Brownian particle over a free-energy barrier separating reactants and products. For reactions in solution this famous theory extended Eyring’s transition state theory, and is widely applied in physics, chemistry and biology, including to reactions as complex as protein folding3,4. Because the diffusion coefficient of Kramers’ theory is determined by the dynamics in the sparsely populated region of the barrier top, its properties have not been directly measured for any molecular system. Here we show that the Kramers diffusion coefficient and free-energy barrier can be characterized by measuring the temperature- and viscosity-dependence of the transition path time for protein folding. The transition path is the small fraction of an equilibrium trajectory for a single molecule when the free-energy barrier separating two states is actually crossed. Its duration, the transition path time, can now be determined from photon trajectories for single protein molecules undergoing folding/unfolding transitions5. Our finding of a long transition path time with an unusually small solvent viscosity dependence suggests that internal friction as well as solvent friction determine the Kramers diffusion coefficient for α-helical proteins, as opposed to a breakdown of his theory, which occurs for many small-molecule reactions6. It is noteworthy that the new and fundamental information concerning Kramers’ theory and the dynamics of barrier crossings obtained here come from experiments on a protein rather than a much simpler chemical or physical system.

The molecule studied in this work is the all-α-helical, 73-residue, designed protein known as α3D (Fig. 1b and Extended Data Fig. 1). In single-molecule experiments7, as well as in all-atom molecular dynamics simulations8, α3D is a two-state protein (Fig. 1a) at neutral pH (Extended Data Figs 3 and 4), that is, only two states are observable at equilibrium and at all times in kinetic experiments. In the single-molecule studies reported here we use the maximum likelihood method of Gopich and Szabo6 in a photon-by-photon analysis of the fluorescence trajectories to obtain both the average transition path time (tTP) and, from the residence time) with numerous unsuccessful attempts at crossing the free-energy barrier to the folded state. The diagram indicates that the ‘jump’ in the energy barrier height (Fig. 1a). β = 1/kBT, where kB is the Boltzmann constant, T is the absolute temperature and ω is Euler’s constant (0.577…).

Equation (1) is from Kramers’ and equation (2) from Szabo9,11, which makes the same assumptions and approximations as Kramers concerning the underlying physics. (The major difference between Kramers and the transition state theory is that the pre-exponential factor of the latter does not contain a diffusion coefficient, and is simply 2πωω0 (ref. 1), D* (= kBT/ω, the Einstein relation) is determined by the friction, ω*, that damps the motion across the barrier top, which in the simplest case is due entirely to solvent viscosity. Two critical assumptions in these equations are that a one-dimensional free-energy surface is sufficient to accurately describe the dynamics and that the dynamics are Brownian. That a one-dimensional free-energy surface is adequate has been validated for protein folding by lattice simulations12, off-lattice simulations13,14, and by the agreement of experiment and predictions of theoretical models15,16.

An important property of equation (2) for the following development is that the transition path time is predicted to be insensitive to the barrier top and the unfolded well, respectively, and ΔGf is the free-energy barrier height (Fig. 1a). β = 1/kBT, where kB is the Boltzmann constant, T is the absolute temperature and ω is Euler’s constant (0.577…).

\[
t_{TP} = \frac{1}{\beta D^* \omega^2} \ln \left( \frac{2e^\omega \Delta G_f}{\omega^*} \right)
\]

(2)

in which D* is the diffusion coefficient at the free-energy barrier top, (ω*)2 and (ω0)2 are the curvatures of the free-energy surface at the barrier top and the unfolded well, respectively, and ΔGf is the free-energy barrier height (Fig. 1a). β = 1/kBT, where kB is the Boltzmann constant, T is the absolute temperature and ω is Euler’s constant (0.577…). Equation (1) is from Kramers’ and equation (2) from Szabo9,11, which makes the same assumptions and approximations as Kramers concerning the underlying physics. (The major difference between Kramers and the transition state theory is that the pre-exponential factor of the latter does not contain a diffusion coefficient, and is simply 2πωω0 (ref. 1), D* (= kBT/ω*, the Einstein relation) is determined by the friction, ω*, that damps the motion across the barrier top, which in the simplest case is due entirely to solvent viscosity. Two critical assumptions in these equations are that a one-dimensional free-energy surface is sufficient to accurately describe the dynamics and that the dynamics are Brownian. That a one-dimensional free-energy surface is adequate has been validated for protein folding by lattice simulations12, off-lattice simulations13,14, and by the agreement of experiment and predictions of theoretical models15,16.

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Figure 1 | Schematics of α3D structure and a one-dimensional free-energy surface for a two-state protein. a, Free energy as a function of reaction coordinate (q), a segment of a FRET efficiency trajectory, and a segment of a photon trajectory (red, acceptor; green, donor) indicating the transition path time (tTP) in the idealized case in which there is no noise in the FRET efficiency in either the unfolded or folded states because they are exactly 0 and 1, respectively. In the folding reaction, the vast majority of the time is spent exploring the configurations of the unfolded state (called the waiting or residence time) with numerous unsuccessful attempts at crossing the free-energy barrier to the folded state. The diagram indicates that the ‘jump’ in the FRET efficiency corresponds to the transition path. The brown trajectory on a transition path region is due entirely to solvent viscosity. Two critical assumptions in these equations are that a one-dimensional free-energy surface is sufficient to accurately describe the dynamics and that the dynamics are Brownian. That a one-dimensional free-energy surface is adequate has been validated for protein folding by lattice simulations12, off-lattice simulations13,14, and by the agreement of experiment and predictions of theoretical models15,16.

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barrier height, in contrast to the folding time, which is greatly affected by a small change of the barrier height that may be caused by viscogens and/or chemical denaturants even when the equilibrium population is unchanged\(^1\). This insensitivity of the transition path time to barrier height has very recently been observed in our single-molecule fluorescence experiments for two proteins with folding times that differ by \(\sim 10^4\)-fold\(^5\). Consequently, the properties of the transition path time are expected to be determined by \(D^*(\omega \alpha^*)^2\).

Figure 2 shows how the transition path times are determined using the Gopich–Szabo maximum likelihood method (see also Methods); Figs 3 and 4 show how the transition path and folding times depend on temperature and viscosity. One immediate result from these experiments is that the height of the free-energy barrier can be simply obtained from the ratio of the folding time to the transition path time, \(t_f/t_{TP}\) (equations (1) and (2)), if \(\alpha^*/\omega^*\) is known. (Although energy barrier heights for reactions are routinely determined from the temperature dependence of the rate, albeit most often without consideration of the temperature-dependence of the pre-exponential factor (equation (1)), the free-energy barrier height is much more difficult to determine.) Because \(\alpha^*/\omega^*\) cannot be obtained from our experiments, we use the value of 1.3 calculated from the potential of mean force in all-atom molecular dynamics simulations given in ref. 7 for \(\alpha^*/\omega^*\). With our measurements of \(t_f/t_{TP}\) yields a \(\Delta G^*_f\) of 4.2 ± 1.0 \(k_BT\) at 22 °C (Fig. 3d and Methods). With a barrier height of 4.2 \(k_BT\), the pre-exponential factor (equation (1)) is \(\sim 40 \mu s\), much larger than previous estimates of \(\sim 1 \mu s\). However, the larger pre-exponential factor is consistent with the longer transition path time of 10–20 \(\mu s\) for this protein compared with our previously measured transition path times for an all-\(-\ell\) (2 \(\mu s\)) and an all-\(-\beta\) (10–20 \(\mu s\)) protein\(^9\). For very low free-energy barriers (\(\leq 2 \kappa_BT\)), heights have previously been estimated from ensemble kinetic experiments by Gruebele and co-workers\(^17\). In this case there is a detectable population of partially folded molecules at the barrier top. \(\Delta G^*_F\) was then obtained from an approximate relation: \(t_f/t_{RW} = \exp(\beta/\Delta G^*_F)\), in which \(t_{RW}\) is a relaxation time corresponding to a ‘molecular phase’, interpreted as resulting from a change in population at the barrier top produced by a temperature jump\(^17\). Barrier heights have also been estimated from single-molecule force experiments\(^4\), and, for very low barriers, from calorimetric measurements of the excess heat capacity\(^9\). One caveat to our measurements is that Förster resonance energy transfer (FRET) measures the transition path time for compaction of the polypeptide chain, which would underestimate the transition path time if collapse and folding are not simultaneous. For example, a twice-longer transition path time that could result from additional time for side-chain annealing within a compact structure would lower the calculated barrier, but only by 0.7 \(k_BT\).

As the transition path time is insensitive to the barrier height, the more interesting result is that our measurements characterize \(D^*(\omega \alpha^*)^2\), the pre-logarithmic factor in equation (2). The temperature-dependent data suggest that the variation of the transition path time results from variation of \(D^*\) and not from \(\alpha^*\). First, within the errors of our experiment, the small variation of the ratio \(t_f/t_{TP}\), which does not depend on \(D^*\), is consistent with both a constant \(\Delta G^*_F\) and \(\alpha^*/\omega^*\) over this temperature range (Fig. 3c). Furthermore, the equilibrium constant does not change with temperature (Extended Data Fig. 3 and Extended Data Table 1), suggesting that the curvatures in neither the unfolded, \(\eta^*/\omega^*\), nor folded, \(\omega^*/\alpha^*\), wells have changed. The invariance of the free-energy surface in this temperature range is not surprising, as the protein unfolds at both lower (cold denaturation) and higher temperatures.

In the simplest case, when all of the friction (\(\Gamma\)) opposing the motion over the barrier top is due to the solvent viscosity, \(\eta^*/\omega^* \propto 1/D^\alpha^* \propto \eta^*\). However, we find a much smaller viscosity dependence, that is, 1/\(D^\alpha^* \propto \eta^*\), with an \(\alpha\) of only 0.3 (Fig. 4b). One might interpret this result as a breakdown of Kramers’ theory in which the Brownian assumption fails and causes a reduced viscosity dependence\(^2\). However, given the extremely weak viscosity dependence, the more likely possibility is that there is an additional source of friction from intramolecular interactions\(^20\). This so-called internal friction has been used previously to explain the decreased viscosity dependence observed for the relaxation rate of a protein conformational change\(^21\) and the

**Figure 2** | Determination of transition path times. a. A FRET efficiency (\(E = n_D/(n_D + n_{DF})\), where \(n_D\) is the number of acceptor photons and \(n_{DF}\) is the number of donor photons) trajectory (50-\(\mu s\) time bin) with photon trajectory of the yellow segment. b. Schematic of a FRET efficiency trajectory using a one-step model to describe the transition path from unfolded (U) to folded (F) states for a two-state protein. The average transition path time, \(t_{TP}\), is equal to the lifetime of a virtual intermediate state \(S\) (\(\tau_S = (2k_BT)^{-1}\), \(\epsilon\) is the difference of the log likelihood, \(\Delta L = \ln L(t_{TP}) – \ln L(0)\), plotted as a function of \(t_{TP}\) for folding and unfolding transitions at different temperatures (top row, 2.25 M guanidinium hydrochloride (GdmCl)) and at different solvent viscosities at 22 °C (bottom row). \(L(0)\) is the likelihood for a two-state model with instantaneous folding and unfolding transitions. Therefore, \(\Delta L\) quantifies how much better or worse the one-step model with a finite transition path time in b describes the photon trajectory compared to a two-state model with an instantaneous transition (Extended Data Fig. 2b).

The maximum values of \(\Delta L\) of all data are much greater than the 95% confidence limit (horizontal dashed line at \(\Delta L = 3\), which indicates that the transition path times determined by the maximum of \(\Delta L\) (Extended Data Tables 1 and 2) are highly statistically significant. The number of transitions analysed were 522 (22 °C), 355 (18 °C), 265 (14 °C), 284 (8 °C), 699 (\(\eta^*/\omega^* = 10\)), 541 (\(\eta^*/\omega^* = 38\)) and 423 (\(\eta^*/\omega^* = 53\)).
the lower denaturant concentrations and increased intra-molecular interactions slow the motion of the chain \(^{24}\).

Additional evidence for internal friction comes from the much greater temperature dependence of the transition path time than predicted by the temperature dependence of the solvent viscosity (in the absence of added viscojen) (Fig. 3b). Using the Arrhenius law, the activation energy for \(D^*\) is 11 \((\pm 4)\) kcal/mol. However, for protein folding Bryngelson and Wolynes \(^{22}\) showed that \(D^*\) should exhibit super-Arrhenius behaviour, that is, \(D^* \propto \exp[-(\Delta E/k_B T)]\), which fits the data equally well (Fig. 3b), in which \(\Delta E^2\) is the local mean-squared fluctuation in energy and is a measure of the underlying ‘landscape roughness’. With this temperature dependence \(\Delta E = 2.3 \pm 0.4\) kcal/mol. Interestingly, our predicted transition path time at 370 K, the temperature of the molecular dynamics simulations \(^{5}\), is 1.7 \(\mu\)s using the Arrhenius law and 2.3 \(\mu\)s using the super-Arrhenius equation. Both are in excellent agreement with the observed value of 0.9 \(\mu\)s in the molecular dynamics simulations. The agreement is even better if a viscosity correction were applied to the molecular dynamics value \(^{5}\), because the TIP3P water used in the simulations is threefold less viscous than real water.

Assuming that the total friction is a simple sum of the internal friction and the friction from the solvent (\(\zeta = \zeta_{\text{solvent}} + \zeta_{\text{internal}}\)), and that the internal friction is independent of solvent composition and viscosity\(^{20,23}\), one might have expected that the transition path time would be linear in viscosity with a non-zero intercept (Fig. 4b), as found for the myoglobin relaxation\(^{23}\) and atomic folding simulations where the friction of the implicit solvent was varied\(^{26}\). However, at the highest concentrations of viscojen (Extended Data Table 2), the solvent is largely non-aqueous (at \(\eta/\eta_0 = 53\) the solvent is only \(\sim 7\)% water by weight), and our assumptions of solvent-independent internal friction, two-stateness and unchanged curvatures of the free-energy surface may no longer hold.

An important remaining issue is the detailed structural origin of internal friction in protein folding. What are the dominant contributions of these microscopic dynamics that give rise to internal friction and thereby slow the diffusive motion along the reaction coordinate? For dynamics involving buried residues and therefore presumably less influenced by solvent viscosity, what is the relative importance, for example, of making and breaking inter-residue contacts compared to dihedral angle flips\(^{25}\)? How much of the internal friction results from the increased frustration caused by local non-native contacts that is more likely for a designed protein such as \(\alpha\)D than a naturally occurring protein\(^{27}\)? Do non-Markovian effects contribute to the reduced viscosity dependence\(^{20,21}\)? Why is much larger apparent internal friction observed in folding all \(\alpha\)-helical proteins\(^{15,28,29}\) compared to all-\(\beta\) or all-\(\beta\) proteins (see ref. 30)? The answers to these fundamental questions about protein folding dynamics will require transition path time measurements on proteins of different sequences and folds in combination with further investigations by theory and simulations.

**METHODS SUMMARY**

Detailed information on materials, single-molecule spectroscopy, temperature and viscosity measurements, calculation of likelihood function, and free-energy barrier height determination are described in Methods.

**Online Content**

Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Kramers, H. A. Brownian motion in a field of force and the diffusion model of chemical reactions. *Physica* 7, 284–304 (1940).
2. Hänggi, P., Talkner, P. & Borkovec, M. Reaction rate theory; fifty years after Kramers. *Rev. Mod. Phys.* 62, 251–341 (1990).
3. Oliveberg, M. & Wolynes, P. G. The experimental survey of protein-folding energy landscapes. *Q. Rev. Biophys.* 38, 245–288 (2005).

**References**

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**METHODS**

**Materials.** The preparation and purification of dye-labelled α3D has been described previously.

**Single-molecule spectroscopy.** Single-molecule FRET experiments were performed using a confocal microscope system (MicroTime200, Picoquant). The CW mode of a dual mode (CW/pulsed) 488 nm diode laser (LDH-D-C-488, PicoQuant) was used to excite donor dyes through an oil-immersion objective (PlanApo, numerical aperture 1.4, ×100, Olympus). Donor and acceptor fluorescence was collected by the same objective, split into two channels, and focused through optical filters (ET525/50m for the donor and E600LP for the acceptor, Chroma Technology) onto photon-counting avalanche photodiodes (SPCM-AQR-15, PerkinElmer Optoelectronics). Additional details for the optical setup and single-molecule experiments are found in refs 9 and 31.

Protein molecules were immobilized on a biotin–embedded, polyethylene glycol–coated glass coverslip (Bio-01, Microsurfaces Inc.) via a biotin (surface)–streptavidin–biotin (protein) linkage. To reduce dye bleaching and blinking, 1–2 mM L-ascorbic acid (ET525/50m for the donor and E600LP for the acceptor, Chroma Technology) was added to the 50 mM HEPES buffer (pH 7.6) solution12 for the temperature-dependence experiment. For the experiments in 50% and >50% glycerol, higher concentrations of 10 mM and 40 mM ascorbic acid/methyl viologen were used, respectively, to increase the bimolecular rates that reduce bleaching and blinking.

To collect a large number of trajectories, we used an automated data collection scheme as described in ref. 5.

**Temperature control and measurement.** Temperature was controlled by flowing dry nitrogen cooled by liquid nitrogen into a chamber surrounding the microscope objective and sample with a fixed flow rate. The temperature was varied by changing the flow rate and was measured at the confocal spot using the fluorescence lifetime of rhodamine B33, excited by a 485-nm laser in the pulsed mode, using the calibration equation (with T in °C):

$$\tau_m (ns) = 2.64 - 0.0572 T + 4.63 \times 10^{-4} T^2 - 1.34 \times 10^{-6} T^3$$

**Measurement of relative viscosities.** The relative viscosities (η/η0) of the solutions were obtained from the absolute temperature (T in K) and the translational diffusion times (τ) using the relationship η = τ T. The translational diffusion times were measured by fluorescence correlation spectroscopy experiments on an Alexa Fluor 488- and Alexa Fluor 594-labelled rigid rod molecule, 20-residue poly-proline, as described previously.

**Calculation of donor and acceptor cross-correlation.** To test further the assumption of a two-state model in our data analysis using the maximum likelihood method (see below), we calculated the donor–acceptor cross-correlation function, $$c(t) = A \exp(kt)$$, as shown in Extended Data Fig. 4 and compared its decay rate with the sum of the rate coefficients determined by the maximum likelihood method (Extended Data Table 1). To obtain the timescale of the unfolded state dynamics at high viscosity, the donor–acceptor cross-correlation function was calculated (Extended Data Fig. 6) for the photon trajectories in the unfolded segments that were extracted using the Viterbi algorithm34,35,36.

**Calculation of likelihood functions.** To determine parameters for the two-state model, we used the Gopich–Szaibő maximum likelihood method and its experimental application to a two-state protein system44 and for the transition path analysis45.

**Determination of the free energy barrier height.** The free-energy barrier height at 22 °C was determined (in Fig. 3d) using the ratio of the folding time to the transition path time tTP and the ratio of the curvatures at the barrier top and the bottom of the unfolded state (\(\alpha^\circ/\alpha_{\text{U}}\)). The value of \(\alpha^\circ/\alpha_{\text{U}}\) was evaluated for the measured tTP, 200 (continuous red curve), including its error, that is, 0.23 (top red dashed curve), and 167 (bottom red dashed curve). The most probable value of \(\alpha^\circ/\alpha_{\text{U}}\) obtained from the molecular dynamics simulations is 1.3, the average of 0.94 (using the fraction of native contacts as the reaction coordinate46) and 1.6 (using an optimized reaction coordinate), corresponding to \(\beta_{\text{G2}} = 4.2\). Assuming an uncertainty of a factor of 2 for \(\alpha^\circ/\alpha_{\text{U}}\), the possible range of \(\beta_{\text{G2}}\) becomes 3.2 to 5.1 for \(\alpha^\circ/\alpha_{\text{U}}\) between 0.65 and 2.6. Using the reported times from the molecular dynamics simulations of tTP = 27 ± 8 μs, and tFP = 0.9 ± 0.2 μs, and \(\alpha^\circ/\alpha_{\text{U}} = 1.6\) from the one-dimensional free-energy surface constructed with an optimized reaction coordinate, the ratio of equations (1) and (2) yields a barrier height of 1.2 ± 0.6 kBT, which is close to the 2.1 kBT (no error reported) barrier height for this surface. Using the fraction of native contacts as the reaction coordinate for all three quantities, the corresponding times are tTP = 264 ± 8.8 μs, tFP = 0.83 ± 0.17 μs (boundaries for the transition path are the two well minima), and \(\alpha^\circ/\alpha_{\text{U}} = 0.94 ± 0.18\), the calculated barrier height is 2.5 ± 0.6 kBT, compared to the barrier height of 2.4 ± 0.3 kBT (ref. 36).

31. Merchant, K.A., Best, R.B., Louis, J.M., Gopich, I.V. & Eaton, W.A. Characterizing the unfolded states of proteins using single-molecule FRET spectroscopy and molecular simulations. Proc. Natl Acad. Sci. USA 104, 1528–1533 (2007).

32. Vogelsang, J. et al. A reducing and oxidizing system minimizes photobleaching and blinking of fluorescent dyes. Angew. Chem. 47, 5465–5469 (2008).
33. Benninger, R. K. P. et al. Quantitative 3D mapping of fluidic temperatures within microchannel networks using fluorescence lifetime imaging. *Anal. Chem.* **78**, 2272–2278 (2006).

34. Viterbi, A. J. Error bounds for convolution codes and an asymptotically optimum decoding algorithm. *IEEE Trans. Inf. Theory* **13**, 260–269 (1967).

35. Rabiner, L. R. A tutorial on hidden Markov models and selected applications in speech. *Proc. IEEE* **77**, 257–286 (1989).

36. Best, R. B., Hummer, G. & Eaton, W. A. Native contacts determine protein folding mechanisms in atomistic simulations. *Proc. Natl Acad. Sci USA*; http://dx.doi.org/10.1073/pnas.1311599110 (in the press).

37. Zhu, Y. et al. Ultrafast folding of αD: a de novo designed three-helix bundle protein. *Proc. Natl Acad. Sci. USA* **100**, 15486–15491 (2003).

38. Liu, F. et al. A one-dimensional free energy surface does not account for two-probe folding kinetics of protein αD. *J. Chem. Phys.* **130**, 061101 (2009).
Extended Data Figure 1 | Amino acid sequences of polypeptides containing protein Α3D. Dyes were attached to the cysteine residues (red) and a biotin molecule was attached to the lysine residue (blue) in the AviTag sequence.

| AviTag | Linker |
|--------|--------|
| GMSGLNDIFE AQKIEWHESS GLVPRGSHM | |
| MGSWAEFKQR LAAIKTRLCA LGGSEAELAA FEKEIAAFES | |
| ELQAYKKGKN PEVEALRKEAAAIREDLQAY RHNC | Α3D |
Extended Data Figure 2 | Photon trajectory and kinetics models. a, The definition of photon indices and time interval of a photon trajectory with a folding transition. b, c, Photon trajectories were analysed using the two-state model to determine kinetics parameters (b) or the three-state model to determine the average transition path times \( t_{TP} = 1/2k_s \) (c).
Extended Data Figure 3 | FRET efficiency histograms of α₁D in 2.25 M GdmCl solution at different temperatures. The FRET efficiency histograms were constructed from 1-ms bins in the trajectories with the mean photon count rate > 40 ms⁻¹. Wide and narrow bars are the experimental histograms and the histograms constructed from re-coloured photon trajectories using the parameters obtained from the maximum likelihood method with the two-state model (Extended Data Table 1), respectively. The agreement between the two histograms validates the description of α₁D as a two-state folder. The similar ratio of the integral of the folded (high FRET) and the unfolded (low FRET) distributions indicates that the equilibrium constant is unchanged over the temperature range of the measurement, as shown more precisely in the maximum likelihood analysis. At high temperature and low pH, where the 11 glutamates and 1 aspartate are protonated, more than two states are observed.37,38
Extended Data Figure 4 | Donor–acceptor cross-correlation functions at different temperatures. Black solid lines are exponential functions that best fit the data. The fitting parameters are listed in Extended Data Table 1.
Extended Data Figure 5 | FRET efficiency histograms of α1D at various solvent viscosities. The FRET efficiency histograms were constructed from 1-ms bins in the trajectories with the mean photon count rate $>50 \text{ ms}^{-1}$ for 2.25 M and 3.2 M GdmCl and from 2-ms bins in the trajectories with the mean photon count rate $>30 \text{ ms}^{-1}$ for 4.6 M, 4.3 M and 3.8 M GdmCl concentrations. At the relative viscosity ($\eta/\eta_0$) 1, 10 and 38, the higher concentration of GdmCl was used to counteract the stabilization of proteins by glycerol to maintain the ratio of folded to unfolded molecules as close to unity as practically possible. The similar ratio of the integral of the folded (high FRET) and the unfolded (low FRET) distributions indicates that the equilibrium constant is unchanged at these conditions, as shown more precisely in the maximum likelihood analysis.
Extended Data Figure 6 | Donor–acceptor cross-correlation of the segments of the fluorescence trajectories corresponding to the unfolded state\textsuperscript{24}. a, Black solid lines are exponential functions that best fit the data. The fitting parameters are listed in Extended Data Table 2. b, The unfolded state dynamics are slowed approximately linearly by the solvent viscosity as previously observed at high denaturant concentrations\textsuperscript{24}. The relaxation time at $\eta/\eta_0 = 1$ (aqueous solution) is too fast to be measured by this method.
Extended Data Table 1 | Temperature dependence of the kinetic parameters obtained from the two-state maximum likelihood analysis, the relaxation rate obtained from the donor–acceptor cross-correlation analysis, and the transition path times.

| Temperature (°C) | 22.2 | 18.1 | 13.7 | 7.7 |
|------------------|------|------|------|-----|
| Viscosity (η/η_{0}) | 1.00 | 1.07 | 1.12 | 1.19 |
| $E_F$            | 0.91 (0.0005) | 0.90 (0.0006) | 0.89 (0.0006) | 0.89 (0.0006) |
| $E_J$            | 0.55 (0.0007) | 0.54 (0.0008) | 0.52 (0.0008) | 0.52 (0.0008) |
| $k$ (ms$^{-1}$)  | 0.85 (0.02) | 0.66 (0.02) | 0.52 (0.01) | 0.37 (0.01) |
| $\rho_F$        | 0.48 (0.008) | 0.49 (0.009) | 0.46 (0.010) | 0.47 (0.010) |
| Donor-acceptor cross correlation, $k$ (ms$^{-1}$) | 0.92 (0.04) | 0.64 (0.02) | 0.47 (0.02) | 0.34 (0.01) |
| Transition path time (μs) | 12.2 (2.0) | 14.7 (2.5) | 19.8 (3.3) | 19.9 (3.7) |

Errors are standard deviations obtained from the diagonal elements of the covariance matrix calculated from the likelihood function. $[\text{GdmCl}] = 2.25$ M.
Extended Data Table 2 | Viscosity dependence of the kinetic parameters obtained from the two-state maximum likelihood analysis, the transition path time and the correlation time of the unfolded state dynamics obtained from the donor–acceptor cross-correlation at 22 °C. Errors are standard deviations obtained from the diagonal elements of the covariance matrix calculated from the likelihood function.

| GdmCl (M) | 2.25 | 3.2  | 4.6  | 4.3  | 3.8  |
|-----------|------|------|------|------|------|
| Glycerol (%) | 0    | 50   | 55   | 58   | 61   |
| Viscosity (η/η₀) | 1    | 10   | 36   | 38   | 53   |
| $E_F$ (kcal/mol) | 0.91 (0.0005) | 0.84 (0.0004) | 0.79 (0.0007) | 0.79 (0.0004) | 0.81 (0.0005) |
| $E_J$ (kcal/mol) | 0.55 (0.0007) | 0.50 (0.0005) | 0.47 (0.0004) | 0.49 (0.0005) | 0.52 (0.0011) |
| $k$ (ms⁻¹) | 0.85 (0.02) | 0.48 (0.01) | 0.46 (0.01) | 0.40 (0.01) | 0.56 (0.01) |
| $\rho$ | 0.48 (0.008) | 0.50 (0.006) | 0.23 (0.004) | 0.50 (0.005) | 0.72 (0.005) |
| Transition path time (µs) | 12.2 (2.0) | 33.3 (2.7) | NA | 39.1 (4.0) | 49.0 (4.9) |

Donor-acceptor cross correlation time in the unfolded state, $1/k$ (µs) | NA | 0.80 (0.18) | 3.4 (0.6) | 3.8 (0.7) | 6.1 (1.6) |