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Instrumental Effects in the Dynamics of an Ultrafast Folding Protein Under Mechanical Force

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Abstract

The analysis and interpretation of single molecule force spectroscopy (smFS) experiments is often complicated by the hidden effects from the measuring device. Here we investigate these effects in our recent smFS experiments on the ultrafast folding protein gpW, which has been previously shown to fold without crossing a free energy barrier in the absence of force (i.e. downhill folding). Using atomic force microscopy (AFM) smFS experiments, we found that a very small force of ~5 pN brings gpW near its unfolding midpoint and results in two-state (un)folding patterns that indicate the emergence of a force-induced free energy barrier. The change in the folding regime is concomitant with a 30,000-fold slowdown of the folding and unfolding times, from a few microseconds that it takes gpW to (un)fold at the midpoint temperature to seconds in the AFM. These results are puzzling because the barrier induced by force in the folding free energy landscape of gpW is way too small to account for such difference in time scales. Here we use recently developed theoretical methods to resolve the origin of the strikingly slow dynamics of gpW under mechanical force. We find that, while the AFM experiments correctly capture the equilibrium distance distribution, the measured dynamics are entirely controlled by the response of the cantilever and polypeptide linker, which is much slower than the protein conformational dynamics. This interpretation is likely applicable to the folding of other small biomolecules in

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smFS experiments, and becomes particularly important in the case of systems with fast folding dynamics and small free energy barriers.

Graphical abstract

INTRODUCTION

Single molecule force spectroscopy (smFS) experiments, either using the AFM or optical tweezers (OT), are now an essential part of the toolbox for the study of the conformational dynamics of biomolecules^{1–2}. While in conventional experiments one measures the behavior of an ensemble of molecules of the protein or nucleic acid of interest, smFS looks at a single copy of the molecule, which is manipulated by perturbing a well-defined progress variable for the biomolecular process of interest (e.g. the end-to-end distance). The perturbation is typically exerted on the molecule by the measuring device (a cantilever for AFM or two optically trapped beads for OT) through a long, flexible linker composed of a polyprotein construct in the case of AFM or two DNA handles in the case of OT. Both the measuring device and the flexible linkers should certainly have an effect on the observed phenomena. However, only recently these effects have been incorporated in the analysis of smFS experiments^{3–12}. Accounting for them is important to determine to what extent the information in the measured single molecule stochastic trajectories represents the underlying free energy landscape and the intrinsic dynamics of the molecule. Of particular note is the recent work by Cossio et al⁵, who interpret smFS experiments in terms of the three relevant time-scales involved: i) the usually slow conformational transition of the biomolecule (k_M^{-1}); ii) the fluctuations of the molecular extension (τ_M); and iii) the response of the instrument (τ_A) (see Fig. 1A). Depending on the relationship among these three time-scales, the relative contributions to the observed dynamics from the biomolecule of interest and from the instrument vary.

In this work we focus on smFS AFM experiments on a particularly challenging type of system, the ultrafast folding protein gpW¹³. In ensemble folding experiments this single domain protein, 62 amino acids in length and α + β secondary structure content (see Fig. 1A), was found to fold in a few microseconds and share all of the distinguishing features of ultrafast folding proteins^{14–15}. From the interpretation of the equilibrium and kinetic data, gpW was shown to have a small barrier ($\sim 1 k_B T$) at the thermal denaturation midpoint and to fold downhill in native conditions¹⁶. In our constant force experiments in the low force regime, we found that gpW behaved like a two-state folder, with a binary switching pattern indicative of a force-induced free energy barrier¹³ (Fig. 1B), as confirmed by molecular simulations using a coarse-grained model. This scenario may not be entirely surprising as it has been proposed by others before, based on simulation¹⁷ and theory¹⁸. However, the folding rate of gpW was $\sim 30,000$ -fold slower than the value measured in bulk at the thermal denaturation midpoint (343 K) from infrared relaxation decays after nanosecond laser-induced temperature jumps¹⁶. This result is difficult to reconcile with a modest folding free energy barrier, hinting that the instrument response may be playing a major role in the observed conformational dynamics.

Here we resolve these instrumental effects quantitatively, in order to understand the origin of the slow dynamics in our smFS experiments and the true molecular features of the ultrafast folder gpW under force. We start by presenting the theoretical methods we use in our analysis and discussing the simplest possible interpretation of the experimental results in terms of just a force-induced barrier. We then show that the observed dynamics are amenable to a description in terms of diffusion on the measured free energy landscape, which arises from the interplay between the true, molecular free energy landscape and the instrument response. Finally, we resolve the molecular free energy landscape using deconvolution, which together with a simple model of the measuring device allow us to explain the experimental slowdown.

METHODS

Lifetime and transition path analysis of experimental data.

We use the constant force (5 pN) segments of our AFM traces to extract folded and unfolded state lifetimes and transition path times. All the extension time-series data was shifted so that the folded state was centered consistently for all the traces. The characteristic extensions for the folded and unfolded states were obtained from Gaussian fits to the population distributions, resulting in values for the mean (q_F and q_U) and the standard deviation (σ_F and σ_U) of the extension for the folded and unfolded states, respectively. Following Woodside and his co-workers, we defined transition paths as those segments of the traces that reach between q_F and q_U and measured transition path times as the time spent between $q_1 = q_F + \sigma_F$ and $q_2 = q_U - \sigma_U$ ¹⁹.

Derivation of a diffusion model from the experimental traces.

We use Hummer's Bayesian method for deriving discretized diffusion models from time series data for an arbitrary coordinate Q ^{20–21}. Although the method was presented and has generally been applied to molecular simulation data^{20–22}, here we use the same procedure to

generate a diffusion model from the experimental time-series of measured extensions (q , see Fig. 1A). Briefly, in the method, the reaction coordinate is discretized into a number of intervals. The dynamics in the discretized space can be described using a rate matrix $\mathbf{R}$ for transitions between bins, whose elements are related to parameters representing the local free energies (G_i) and the position-dependent diffusion coefficients (D_i) for transitions between adjacent intervals along the coordinate Q . Free energies are related to the equilibrium probability of being in interval i , P_i , of width ΔQ via

$$G(Q_i) = -k_B T \ln \frac{P_i}{\Delta Q} \quad [1]$$

The position dependent diffusion coefficients are related to the elements of the rate matrix $R_{i+1,i}$ by

$$D_{i+1/2} \simeq \Delta Q^2 R_{i+1,i} \left(\frac{P_i}{P_{i+1}} \right)^{1/2} \quad [2]$$

From the rate matrix $\mathbf{R}$ it is possible to calculate the likelihood of an observed trajectory as

$$L = p(\text{data} | \{D_i\} \{G_i\}) = \prod_{i,j} \left[p(Q_j, t + \Delta t | Q_i, t) \right]^{N_{ji}}, \quad [3]$$

where the elements $p(Q_j, t + \Delta t | Q_i, t) = \exp[R \Delta t]_{ij}$ in the product are the that probability the propagators representing the dynamical instantaneous value of Q reaches bin Q_j at time $t + \Delta t$ provided that it was in bin Q_i at time t . Optimal values for $G(Q_i)$ and $D_{i+1/2}$ are chosen to maximize the likelihood L .

In practice, the optimization is done using a simple Metropolis Monte Carlo procedure²³ in which the iterative moves are made on the equilibrium populations and the elements of the rate matrix, and the effective energy function is the negative logarithm of the likelihood function, $-\ln L$ ^{20–21}. As it is a sensible assumption that at neighboring intervals the diffusion coefficients D_i should not differ much, we include a smoothness prior

$$p(\{D_i\} \{G_i\} | \text{data}) \propto L \prod_i \exp \left[- (D_{i+1} - D_i)^2 / 2\gamma^2 \right], \quad [4]$$

where the strength of the prior is determined by the parameter γ .

Deconvolution of the molecular potential of mean force.

In order to obtain the molecular free energy landscape, which is hidden in single molecule measurements, we have used a Monte Carlo deconvolution procedure. Following Woodside and Block^{8, 24} the “blurring” effect of the harmonic linker and tether are introduced by

convolving a trial molecular probability distribution $P(x)$ with a point spread function (PSF). We start from a discretized trial solution for $P(x)$ and iterate until the convolved potential of mean force closely matches the experimentally determined solution. As PSF we use a Gaussian distribution corresponding to a harmonic oscillator with a spring constant of 1.4 kJ/mol/nm². This value should reflect the combined effect of the linkers and cantilever tip, and has been obtained from the fluctuations in the native state before any transition occurs⁸.

Effects of the experimental apparatus in the dynamics.

To model the effects of an instrument in the measured dynamics we use the Cossio-Hummer-Szabo method⁵, where the measured potential of mean force (PMF) on the measured extension, $G(q)$, is given by

$$\exp(-\beta G(q)) = \int \exp(-\beta G(x, q)) dx = \int \exp[-\beta(G(x) + V(q - x))] dx, \quad [5]$$

Here, $G(x)$ is the PMF for the projection in the molecular extension (x , see Fig. 1A), while $G(x, q)$ is the two-dimensional PMF for both x and q . On the right hand side of Eq. 5 we find the free energy of the apparatus (linker and bead or cantilever tip), which is given by $V(q - x) = 0.5 \kappa (q - x)^2$, where κ is the spring constant of the apparatus. Using the deconvolved PMF and the value of κ from the PSF we generate the two-dimensional PMF, $G(x, q)$. Following Cossio et al, we run Brownian dynamics simulations on the resulting PMF with independent diffusion coefficients, D_x and D_q . The former was fixed in our simulations to $D_x = 1$ and different scaling factors were used in order to cover a range of 3 orders of magnitude in D_x/D_q . The time-step for the Brownian dynamics was $D_x \cdot t = 2e-5$.

RESULTS AND DISCUSSION

Reversible folding and unfolding of gpW at 5 pN.

Here we focus on the analysis of data from previously reported force-ramp experiments performed with an AFM on a chimeric polypeptide construct containing the protein gpW sandwiched between six copies of the I91 domain (formerly termed I27) from human cardiac Titin¹³ (Fig. 1A), which serve as a mechanical fingerprint. The mechanical midpoint, i.e. the force at which both states are equally populated, was determined to be ~5 pN from force ramp experiments¹³. A representative example of the constant force segment of the traces is shown in Fig. 1A. The data shows a distinct two-state like hopping pattern between the high (unfolded) and low extension (folded) states in the measured extension (q) vs time plots. In our previous work we described how this binary switching pattern, unexpected for an ultrafast downhill folder, is indicative of a force-induced barrier¹³. This was observed experimentally by us for the first time after predictions from simulations of nucleic acids by Hyeon and Thirumalai¹⁷ and, in the context of protein folding, by Fernández and his co-workers¹⁸.

In addition to the change in the folding regime from downhill to two-state, we found a surprisingly marked slowdown of the folding dynamics. Following Woodside and co-workers¹⁹, we estimate the kinetics of the interconversions between the folded and unfolded

states by simply defining dividing lines for the folded and unfolded states (Fig. 2A). We show that an exponential distribution is able to capture the histogram of the lifetimes for both the folded and unfolded states, with rate constants of $k_f = 1.8 \pm 0.1 \text{ s}^{-1}$ and $k_u = 1.7 \pm 0.2 \text{ s}^{-1}$ that are in quantitative agreement with our previous estimates from a hidden Markov model¹³. Relative to folding and unfolding rates of $\sim 60,000 \text{ s}^{-1}$ measured in bulk at the thermal denaturation midpoint (343 K)¹⁶, there is a 30,000-fold decrease.

Apparent transition paths determined under the AFM.

Another surprising result is that, in the midst of the sharp folding and unfolding transitions of gpW occurring in our AFM experiments, we could resolve transition paths. Transition paths are the reactive parts of the trajectories where folded and unfolded states interconvert. Experimentally, transition paths are defined as the trajectory fragments that connect the folded and unfolded states (reaching q_f from q_u or *vice versa*; see Fig. 2A) and their characteristic times are often estimated as the time spent in their central regions (i.e. between q_1 and q_2)¹⁹, which here we define from the averages and standard deviations of the distributions of measured extensions. Recently, there has been great interest in the experimental determination of transition path times (t_{TP}), which has first been possible with single molecule FRET²⁵ and more recently using smFS with optical tweezers^{19, 26–28}. However, recent theoretical work indicates that in the likely scenario of the instrument being slower than the molecule, the apparent transition path times measured may not be meaningful^{5–6}.

As illustration, in Fig. 2C we zoom in on several examples of transition paths in the folding and unfolding directions, and find that they extend over multiple experimental data points, which in these experiments were taken every 2 ms. The transition path times in the folding and unfolding directions are similarly distributed (Fig. 2D) and have almost identical average values (17 and 18 ms, respectively), as we expect from microscopic reversibility. Intriguingly, as it happens with the folding and unfolding rates, the average t_{TP} values are several orders of magnitude slower than previous estimates from single-molecule fluorescence experiments on several proteins (1.5–20 μs)^{29–30}. Even the transition path times for the formation of non-native structure in PrP dimers recently measured by optical tweezers (0.5 ms)¹⁹ are about 30-fold faster.

Barrier-centric interpretation of the smFS experiments.

The simplest possible way to interpret the experimental slowdown is to assume that an induced free energy barrier is the sole reason for the slow-down of the dynamics. To remove the effects of temperature, we can compare the folding rate in the AFM (1.8 s^{-1}) with that derived at 300 K from the two-state fit of the temperature jump data ($19,000 \text{ s}^{-1}$), resulting in a decrease of $\sim 10,000$ -fold. If this change were due exclusively to a free energy barrier induced upon application of a 5 pN force, it would need to be of $\sim 9 k_B T$, which is much higher than the temperature induced barrier ($\sim 1 k_B T$)¹⁶. The question here is whether the experimental data is consistent with such a high barrier.

We can obtain a lower bound of the free energy barrier from a direct Boltzmann inversion of the histogram of measured extensions (Fig. 3A), which results in a barrier of only $2.4 k_B T$

for folding and $2.7 k_B T$ for unfolding. These lower bounds are already sufficient to produce the two-state hopping patterns that we observe, but fall very short from the barrier required to explain the slow kinetics. A more realistic estimate of the barrier can be obtained by deconvolution of the effects of the linker on the observed landscape (discussed below).

Kinetics as diffusion on the measured landscape.

The discrepancy between the estimated barrier and observed slowdown strongly suggests that the response of the apparatus is contributing to the slow dynamics. Recently, Cossio et al have provided a prescription for understanding whether the rates measured in smFS experiments are a good approximation to the true molecular kinetics of the process (i.e. in case force could be applied directly on the molecule) or, alternatively, the kinetics are determined by the apparatus⁵. One of the key quantities that we must take into consideration here is the effective diffusion coefficient for the measured coordinate, D_q . The value of D_q can be derived from experiments using the expression $D_q = \langle \delta q^2 \rangle_{F(U)} \tau^{-1}$ ³¹, where the average runs over the segments in which gpW remains folded (unfolded), and τ is the autocorrelation time for q . From our gpW traces at 5 pN we obtain $D_{q,F} = 261 \pm 29 \text{ nm}^2 \text{ s}^{-1}$ and $D_{q,U} = 417 \pm 81 \text{ nm}^2 \text{ s}^{-1}$, which are comparable to the diffusion coefficient reported from AFM experiments by Brujic *et al.* on a poly-ubiquitin construct¹¹, and about 100-fold slower than the diffusion coefficients reported for DNA hairpins with optical tweezers²⁸. Notably, these values are much smaller than the intra-molecular diffusion coefficients of $\sim 1 \times 10^8 \text{ nm}^2 \text{ s}^{-1}$ measured on untethered molecules by single-molecule FRET spectroscopy³².

Taking these diffusion coefficients and the barrier estimates from above we can check whether our experimental data can be interpreted as diffusion on the measured landscape. For this to be true, the measured folding and unfolding rates should be close to the apparent rate k_A from the Kramers rate expression

$$k_A = \frac{\beta D (\kappa_{\ddagger} \kappa_{F(U)})^{1/2}}{2\pi} \exp(-\beta \Delta G_{\ddagger})$$

where $G_{\ddagger}$ is the barrier and $\kappa_{\ddagger}$ and $\kappa_{F(U)}$ denote the stiffness of the barrier and of the folded (unfolded) wells. Substituting the corresponding values into this expression we obtain rates of 2.5 and 1.6 s^{-1} for folding and unfolding. These values are surprisingly close to the actual rate constants determined directly from the dwell time analysis. We have used these parameters to check whether also the measured transition path times are consistent with this interpretation of the data. Using the expression³³

$$t_{TP} = \frac{1}{\beta D \kappa_{\ddagger}} \ln(2e^{\gamma} \beta \Delta G_{\ddagger})$$

where γ is the Euler-Mascheroni constant, we calculate a value for the transition path time of 9 ms, which is in only within a factor of 2 of the experimental value.

There are two important implications from these results. The first one is that the experimental kinetics seems to be well approximated by diffusion on the measured

landscape (with a $\sim 2.5 k_B T$ barrier). The second implication is that the low values of the diffusion coefficients confirm that the measured kinetics are unrelated to the true molecular kinetics⁵, but rather are due to the slow response of the instrument.

A diffusion model confirms the description of the measured kinetics.

In order to validate the description of the experimental data as diffusion on the measured free energy landscape, $G(q)$, we have inferred a diffusion model using the Bayesian method developed by Hummer²¹. The procedure allows to fit self-consistently the free energy surface and position dependent diffusion coefficients that best explain the observed time series data. The data could obviously come from simulation or, like in this case, from experiment. In order to avoid undesirable “spikes” in the resulting profiles, a “smoothing” prior can be introduced. In Fig. 3A we compare the estimated potential of mean force from a Boltzmann inversion with that resulting from the diffusion models, either using a uniform prior or with the smoothing prior. In both cases we obtain free energy profiles that are consistent with that derived from a Boltzmann inversion and which rendered a free energy barrier of $\sim 2.5 k_B T$. Moreover, the diffusion coefficients at the bottom of the folded and unfolded wells ($230\text{--}250 \text{ nm}^2 \text{ s}^{-1}$) are very close to those derived from the experimental data directly. It is important to bear in mind that these diffusion coefficients do not reflect the diffusivity of the molecule in the extension coordinate, but the combined effects of molecule and apparatus. We note that the diffusion coefficient as a function of the reaction coordinate is only slightly position dependent (Fig. 3B), with the value at the top of the folding barrier being ~ 2 times that in the folded or unfolded states, as it has been previously reported from a similar analysis of atomistic molecular dynamics (MD) simulations²².

Effects of the instrument response in the free energy surface.

Our AFM measurements appear to be in the undesirable regime where the dynamics are governed by the measuring instrument. Nevertheless, we can still resolve the true free energy landscape (i.e. removing the blurring caused by linkers and tether), using deconvolution²⁴. In this case we use an iterative Monte Carlo procedure with a weak smoothness prior and a point spread function of 1.4 kJ/mol/nm^2 (see Methods). This procedure results in a deconvolved free energy landscape, $G(x)$, which we show in red in Fig. 4A (see also the corresponding populations in Fig. 4B). The free energy barrier between the folded and unfolded states that is derived with this procedure is higher than from the Boltzmann inversion: $4.9 k_B T$ in the unfolding and $3.9 k_B T$ in the folding direction. However, these barriers are still far from the $9 k_B T$ needed to rationalize the full slowdown of the dynamics, as we have discussed above. We also note that the deconvolved PMF features a native state that is considerably narrower than the unfolded ensemble, as expected since the latter is much more flexible and likely to be stretched by pulling.

Incorporating the effects of the instrument response in the dynamics.

In order to account for the effects of the AFM in the experimental data we use the recent theory developed by Cossio, Hummer and Szabo⁵. We start from the deconvolved potential of mean force of gpW (Fig. 4A) and couple it to a modeled apparatus consisting of cantilever and polyprotein linker, which is simply described as a harmonic spring with the spring constant that was used for the deconvolution (see Methods). This coupled system

results in the 2D free energy surface, $G(x,q)$, represented in Fig. 5A, which we use here as proxy to model our single molecule AFM experiments⁵ because anisotropic diffusion in this landscape incorporates both the molecule and the apparatus response. Folding and unfolding take place when the saddle point of this free energy landscape is crossed, a process that involves fast diffusion on the molecular coordinate (x) and, typically, slow diffusion on the measuring coordinate (q)⁵.

The instrument response can be investigated via Brownian dynamics simulations on that 2D surface⁵. Simulations for the gpW-containing polyprotein linked to the AFM cantilever reveal that the dynamical effects of a “slow” versus a “fast” instrument (relative to the molecule’s dynamics) are in fact quite drastic (Fig. 5B) (bottom and center, respectively), relative to what one would find if force was applied on the molecule directly (top). For the fast instrument (i.e. low D_x/D_q), the measured dynamics are still similar to the molecular dynamics under force. Correspondingly, the measured rate would be comparable to the true, molecular (un)folding rate. However, an apparatus that is several orders of magnitude slower than the diffusion of the molecule (i.e. high D_x/D_q), as we have in the AFM experiments, results on a marked slowdown of the observed kinetics, which become decoupled from the true molecular kinetics. This effect is reflected in the relationship between the experimentally accessible rates for folding and unfolding (k_{MA}) and the true molecular rate (k_M) (Fig. 5C). The ratio k_{MA}/k_M approaches 1 at low D_x/D_q but it rapidly decreases, implying that in any experimentally accessible regime, the measured rates are very far from the true rates of the molecular process. The interval where one can obtain reliable measurements of the rates (i.e. the Langer plateau region^{5–6}) is particularly narrow due to the low barrier, as predicted by Cossio et al. A serendipitous but counterintuitive feature of our experiments is that the stiffness of the AFM allows it to track most of the folding and unfolding events, potentially in contrast to typical OT setups, a scenario previously discussed by Nam and Makarov³⁴.

Following Cossio and her collaborators⁷ we have also interrogated whether the surprisingly slow transition path times that we have been able to measure in our experiments represent true transition paths or are instead a reflection of the very slow drag of the AFM. In Figure 5C we show the ratio between average transition path times in the Brownian dynamics on the two-dimensional PMF for different values of D_x/D_q and that without an instrument, $\langle t_{TP} \rangle_{MA} / \langle t_{TP} \rangle_M$. Like in the case of the theoretical double-well potential used by Cossio et al, we find that the measured transition path times diverge rapidly from the true transition path times, even in the experimentally inaccessible range of values with $D_x/D_q < 1$.

CONCLUSIONS

In this work, we have analyzed the dynamics of recent smFS experiments on an ultrafast folding protein that lacks a free energy barrier in bulk, but exhibits a force-induced barrier in the presence of the very small forces required to bring it to its denaturation midpoint. We have combined a wide array of theoretical analysis tools to obtain reliable estimates of the size of the barrier induced by force and understand the puzzlingly slow dynamics that are observed in these experiments. From our analysis we conclude that the barrier induced by force is modest ($\sim 4\text{--}5\text{ }k_B T$), but considerably higher than the $\sim 1\text{ }k_B T$ barrier induced by

temperature for the untethered protein¹⁶. Thus, the effect that force has on the folding landscape of this protein is non-trivial and a consequence of the highly anisotropic nature of the perturbation produced on mechanical unfolding experiments.

We also show how the very slow hopping dynamics that we observe are in fact determined by diffusion of the AFM cantilever, which is orders of magnitude slower than the few μ s folding and unfolding times of gpW in the absence of force¹⁶. Using the Cossio-Hummer-Szabo method we are able to interpret the measured dynamics in terms of anisotropic diffusion on the fast molecular coordinate (x) and the slow measuring coordinate (q). For high barriers Cossio et al have shown that, for a sufficiently fast instrument, the folding dynamics can be recovered, whereas estimates of transition path times are extremely distorted⁵⁻⁶. Ultrafast folders lie at the other side of the spectrum, where neither folding rates nor transition path times can be reasonably recovered, explaining the 4 orders of magnitude slower conformational dynamics observed in the AFM relative to kinetic bulk measurements on the untethered protein and the extremely slow transition path times. A remarkable, and somewhat unexpected, result is that these AFM experiments still recapitulate the equilibrium mechanical unfolding landscape of the protein. This is so because the high stiffness of the cantilever and slow response of the AFM produces strong coupling to the molecular transitions, which permits to track most of the actual folding and unfolding events of the protein even though the overall dynamics are dictated by the instrument response. Our conclusions are likely general for the dynamics of other fast folding biomolecules when they are studied using smFS techniques.

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ABBREVIATIONS

smFS	single molecule force spectroscopy
AFM	atomic force microscope

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Figure 1.

(A) Cartoon of the AFM-based smFS experiment. A polypeptide construct consisting of six titin I91 domains (black) sandwiching a single copy of gpW (folded and shown in purple and yellow on the left, and stretched on the right panel) is anchored to a gold-coated substrate and picked up from the other end by the cantilever (not to scale). Dashed arrows mark the measured extension (q , black) and the hidden molecular extension (x , red). The folding/unfolding of the protein occurs in timescales of k_M^{-1} , while the conformational dynamics of the protein are much faster (τ_M). The measurement is affected with the timescales relevant to the instrument (τ_A). (B) Representative constant force trace of

measured extension (q) vs time at 5 pN with multiple unfolding and refolding events. Here an arbitrary offset is subtracted from q .

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Figure 2.

(A) Representative 5 pN constant force trace of measured extension (q) vs time (left) and detail corresponding to one of the observed transition paths (right). Dark red and green dashed lines indicate average values of the unfolded and folded states (q_U and q_F), and lighter red and green dashed lines indicate the limits for the assignment of transition paths (q_1 and q_2). The blue segment in the right panel corresponds to an unfolding transition path. (B) Multiple examples of folding (pink) and unfolding (cyan) transition paths. (C) Distributions of lifetimes of the folded (left) and unfolded (right) states fitted to single

exponential distributions. (D) Distribution of transition path times for transitions in the folding (left) and unfolding (right) directions.

Figure 3.

Results of the diffusion model derived using the Bayesian method by Hummer, including the potential of mean force (A) and position dependent diffusion coefficients (B). Results are shown for a uniform prior (red) and for a smoothness prior of $\gamma=0.1$ (blue). The potential of mean force from the Boltzmann inversion is shown as reference (gray) in (A).

Figure 4.

Results of the free energy landscape deconvolution. (A) Deconvolved potentials of mean force, $G(x)$, and (B) corresponding equilibrium distribution, $P(x)$, for the molecular coordinate x are shown in red. The potential of mean force $G(q)$ from the Boltzmann inversion and the equilibrium distribution $P(q)$ from the statistics on the experimental trajectory are shown in gray for reference. Individual solutions for $G(x)$ and $P(x)$ obtained in the Monte Carlo sampling are shown as thin red lines.

Figure 5:

(A) Two-dimensional potential of mean force on the molecular (x) and measured (q) coordinates. Units in $k_B T$. (B) Results of Brownian dynamics simulations in the absence of coupling to an instrument (top) and with coupling to a fast (center) and slow (bottom) instruments. Time units are relative to the simulated diffusion constant, $D_x=1$. (C) Left axis: Dependence of the rate for folding and unfolding ($k_{MA}=k_f+k_u$) relative to the molecular rate (k_M) with the ratio of instrumental and molecular diffusion coefficients (D_x/D_q). The green and blue dashed lines indicate the values corresponding to the traces in panel B. Right axis: Same for the ratio between average transition path times ($\langle t_{TP} \rangle_{MA}$) relative to the molecular transition path time ($\langle t_{TP} \rangle_M$).