Published in final edited form as:

J Phys Chem Lett. 2016 October 06; 7(19): 3798–3803. doi:10.1021/acs.jpclett.6b01722.

Reconciling Intermediates in Mechanical Unfolding Experiments With Two-State Protein Folding in Bulk

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Abstract

Most experimentally well-characterized single domain proteins of less than 100 residues have been found to be two-state folders. That is, only two distinct populations can explain both equilibrium and kinetic measurements. Results from single molecule force spectroscopy, where a protein is unfolded by applying a mechanical pulling force to its ends, have largely confirmed this description for proteins found to be two state in ensemble experiments. Recently, however, stable intermediates have been reported in mechanical unfolding experiments on a cold-shock protein previously found to be a prototypical two-state folder. Here, we tackle this discrepancy using free energy landscapes and Markov state models derived from coarse-grained molecular simulations. We show that protein folding intermediates can be selectively stabilized by the pulling force and that the populations of these intermediates vary in a force-dependent manner. Our model qualitatively captures the experimental results and suggests a possible origin of the apparent discrepancy.

TOC image

The development of single molecule technologies over the past two decades has allowed the observation of details of protein free energy landscapes that had been inaccessible to ensemble experiments.¹ In single molecule force spectroscopy experiments a pulling force is applied directly to the termini of the protein, resulting in unfolding. Evidence from both

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experiment and computer simulations indicates that the folding mechanism may change as a function of force, including the population of intermediates^{2–6} as well as changes in folding transition state (via ϕ -value analysis in experiment).^{6–12} However, for most proteins which have been shown to be two-state¹³ from bulk experiments in the absence of force, only a folded and an extended unfolded state become observable in the pulling experiments, confirming the existing description. In other cases, in which more than two stable states were identified in force experiments, the unfolding mechanism was generally consistent with previous equilibrium and kinetic measurements.¹⁴ The recent increase in stability of the instruments and the use of different measurement modes such as force clamp^{15,16} and force ramp¹⁷ have further helped to reveal discrepancies between mechanical and ensemble unfolding mechanisms.^{18,19}

Here we focus in the thermostable protein CspB from *Thermotoga maritima*, Csp Tm (see Fig. 1a). For this system, equilibrium fluorescence measurements and stopped flow kinetic experiments have universally indicated two-state folding,²⁰ a finding later confirmed at the single molecule level using FRET.^{21,22} AFM experiments in the constant speed mode also fit with a two-state description.²³ However, recent work has clearly identified multiple metastable states which are populated when pulling the protein from its ends in the constant force mode.²⁴ Intermediates in single molecule force experiments had previously been identified, perhaps the best known example being Titin I27.^{2–5} What is remarkable in this case is the longevity (milliseconds in the presence of a pulling force) and heterogeneity of the intermediates, considering the small size of Csp Tm (66 residues), and the fact that no intermediates were resolved in ensemble experiments. To tackle this problem we run molecular simulations of a coarse-grained topology based model of Csp Tm and derive a force-dependent Markov state model (MSM) that captures the kinetics of the system. In accord with experiment, we find that while in the absence of force the system is a two-state folder by thermodynamic and kinetic criteria, in the presence of a constant biasing force, certain intermediate species are stabilized. The kinetic model we present recapitulates the shift from two-state to multi-state folding upon application of force recently discovered experimentally.

Using the experimental NMR structure of the protein²⁵ as a starting point (see Fig. 1a) we have used well established methods to build a topology-based coarse-grained (i.e. G) model²⁶ (see SI for details). Such models, in which only native contacts are considered attractive, are justified in the context of a funneled folding energy landscape.²⁷ First we check whether this simple model is able to recapitulate the two-state description in the absence of force. We have run simulations without a pulling force at multiple temperatures and combined them using WHAM.²⁸ The projection on the folding coordinate Q (fraction of native contacts)²⁹ of one of these trajectories, corresponding to a temperature where we observe reversible folding ($T=310$ K) shows how the protein inter-converts in sharp, cooperative, transitions between the folded and unfolded states (Fig. 1b). The calculated heat capacity thermogram and potential of mean force (PMF) on Q both show unequivocal two-state features (see Fig. 1c–d). The former presents a single, well defined, narrow peak as a function of the temperature, with the simulation midpoint temperature located around $T_m \approx 315$ K (Fig. 1c). This is low compared to the experimental value (*Thermotoga maritima* grows up to 90°C²⁰) but enough to yield a folding barrier in native conditions. In the same

way, from the PMF we can also diagnose two-stateness for the Csp *Tm* model: there are two well defined wells, albeit with a small shoulder at an intermediate value of the fraction of native contacts ($Q = 0.6$). The two-state description is consistent with the simulation work carried out with a similar native-centric coarse-grained model where the denaturation effects from chemicals, instead of temperature, are explicitly accounted for.³⁰

To analyze the effects of an external force in the system we have run multiple simulations pulling from the protein termini at different forces (see Table S1). The situation that we encounter is quite different from that at zero force. In Fig. 2a we show time series data for the projection on the pulling coordinate (d , the end to end distance) for simulations carried out at different pulling forces and 300 K. While at zero force the protein remains in the folded state but for transient excursions to higher distance conformations (i.e. the shoulder in the PMF from Fig. 1d), at non-zero forces intermediate species are clearly stabilized in the 2–6 nm range. At forces between 10 and 15 pN we see an additional shorter-lived intermediate at ~ 7 –8 nm (see traces in Fig. S1). Our projected data resembles some of the experimental traces obtained with the AFM at constant force.²⁴ In the experiments, up to 5 different intermediates could be observed, although in the majority of individual pulls the number of intermediates was either zero or just one. Also, considering an approximate value of $L_0 \approx 1.3$ Å for the native state, three of the experimental intermediates (C1 at $L = 2.8 \pm 0.7$ nm, C2 at $L = 5.3 \pm 0.7$ nm and C3 at $L = 8.6 \pm 0.9$ nm²⁴) have total extensions within the range of d identified for metastable species in our pulling simulations (Fig. 2a).

The emergence of intermediates can be rationalized from the changes in the folding free energy landscape upon the application of force. Using the umbrella sampling technique we have run simulations at different pulling forces and calculated 2D PMFs for the folding and extension order parameters (Q and d , see Fig. 2b and SI for details). At low forces the dominant free energy barrier separates the unfolded well from a broad native state, with no barrier between regions with intermediate values of the order parameters and the folded state. However at 5–10 pN a free energy barrier emerges between intermediate states that now separate from the native well. At the highest forces used in our simulations (15–20 pN), the free energy well with $Q \approx 0.6$ and $d \approx 5$ nm becomes even more stable than the native state. Hence, an external force can tilt the free energy landscape, stabilizing more extended conformations, and modulating the population of the stable forms of the protein and the barriers between them. Extended intermediates which are only transiently visited at zero force become significantly populated as the force is increased.

In order to better understand the information present in the pulling simulations and define the dominant intermediate species more systematically we integrate the data at different forces in a minimal Markovian kinetic model (i.e. an MSM^{31–33}). In this type of model the dynamics of the system are encapsulated in a master equation, $d\mathbf{P}(t)/dt = \mathbf{K}\mathbf{P}(t)$, where $\mathbf{P}(t)$ is a vector with the populations of the states in the model and $\mathbf{K}$ is the rate matrix, containing the rates k_{ji} for all the microscopic transitions $i \rightarrow j$. To derive this model from the simulations we have built on established procedures,^{34,35} with the additional development that in this case the MSM was estimated at the different pulling forces (see full details in SI Methods). We have used a contact map-based clustering^{34,36} to identify a small number of states ($N = 4$), two of which are intermediates (I_1 and I_2 , see Fig. 2c) plus the

unfolded (U) and native (N) states. On the PMFs in Fig. 2b we show the mean values of Q and d of the four states in the MSM. The two intermediates I_1 and I_2 differ in the fraction of native contacts for β -strand 1 that are broken, with only the N-terminal residues being unfolded in I_2 , and the whole of strand 1 being unfolded in I_1 (see contact maps in Fig. S5). The intermediate states have extensions of 2.8 and 4.8 nm, respectively, which are close to the extensions of the experimental intermediates C1 and C2,²⁴ and agree well with the locations of the free energy minima in the PMF, which were not used in the clustering process. We recolour the time series in Fig. 2a based on the MSM state to show how the assignment procedure captures the important slow dynamics (see also Fig. S4). After assigning the trajectory frames to the MSM states, we calculate the microscopic rate coefficients, k_{ji} (from state i to state j), at the different pulling forces using a lifetime based method.³³

The force dependence of the rate coefficients shows the expected behaviour (see Fig. 3a–b), with refolding processes being more sensitive to the pulling force than unfolding processes, due to the larger changes in molecular extension associated with refolding, and the extensible nature of the unfolded state.³⁷ Naturally, in some cases transitions are not observed due to the finite time of the simulations. This is the case, for example, of refolding events at high pulling forces (e.g. $U \rightarrow I_1$ or $I_1 \rightarrow F$), where the bias is too strong for the refolding process to be observed. To overcome this difficulty, we fit the calculated rates to the Bell or Dudko-Hummer-Szabo models for barrier crossing processes induced by force^{38,39} (in the case of k_{UI_1} and k_{UI_2} an additional intrinsic rate was also included to model the variation of folding pathway with pulling force,¹⁰ see Supplementary Methods). We find that in all cases the microscopic rate coefficients can be well captured by the functional form of the model. From the fits we can recover a rate matrix at any desired pulling force, $\mathbf{K}_{\text{fit}}(F)$, even at forces where some transitions are not observed directly. Although in some cases this requires an extrapolation, it is based on the fit to a physical model.

Having assembled this model, we can attempt to recover the phenomenology observed in different conditions. We use the solution to the master equation, $\mathbf{P}(t) = \exp(\mathbf{K}_{\text{fit}} t) \mathbf{P}(0)$ to propagate the dynamics and relax from a given initial condition, $\mathbf{P}(0)$. In native conditions we relax from the unfolded state, mimicking a stopped flow refolding experiment or the average behaviour of many refolding trajectories by quenching the force in the AFM. We find that the system relaxes to the equilibrium distribution with most of the amplitude (99%) corresponding to the slowest exponential phase (see Fig. 3b, top). This slowest mode corresponds to the first eigenvector of the rate matrix (ψ^1), which indicates exchange of density between the unfolded state and the folded and intermediate states (i.e. the folding process, see Fig. 3c). This observation is the kinetic counterpart of the dominant barrier separating the unfolded and folded wells, the latter of which here incorporates the MSM states F, I_1 and I_2 (Fig. 1b). The intermediates equilibrate rapidly with the folded state as dictated by fast processes corresponding to eigenvectors ψ^2 and ψ^3 (see Fig. 3c, bottom). In ensemble experiments, this situation, involving a single exponential phase, would unequivocally correspond to two-state folding.²⁰

As we increase the force the model yields a substantially different scenario (see Fig. 3b). Propagating the rate matrix from the folded state we find that, particularly for forces > 10 pN an intermediate state accumulates as the protein unfolds, reaching up to $\sim 80\%$ of the population. When we propagate the rate matrix from the folded configuration, we find that, in a first kinetic phase, the population of the intermediates increases substantially (mostly for I_2 , as dictated by the eigenvector corresponding to the slowest process), and only later do they interconvert with the unfolded conformation. Again, the eigenvectors help us understand how the slowest process at higher forces involves the interconversion between I_1 and U (see ψ^1 in Fig. 3) while equilibration between the folded state and the intermediates is faster (ψ^2 and ψ^3). This explains the long-lived formation of intermediates when unfolding upon application of a pulling force, as seen experimentally.²⁴

A natural question is why the application of force is able to expose intermediates hidden at equilibrium, and why other perturbations such as chemical denaturants do not have a similar effect: Csp *Tm* has always appeared two-state in ensemble kinetics experiments using chemical denaturants to destabilize the protein.²⁰ A possible explanation lies in the relative sensitivity of the intermediates to force or denaturant. Since the effect of denaturant is, to a first approximation, proportional to the solvent-accessible surface area (*SASA*) of a given state,⁴⁰ its effect can be estimated from a free energy landscape projected onto (Q , *SASA*), analogous to the (Q , d) projections for force (Fig. S6). The obvious difference between the two PMFs is the good correlation between *SASA* and Q for the metastable free energy basins, compared with the situation for d and Q . The reason for that difference is most likely that *SASA* is more closely related to the global degree of folding, captured by the fraction of native contacts. On the other hand, larger extensions can be sampled by intermediates via only local unfolding (as indeed evident from the structures in Fig. 2). This poor correlation between extension d and Q means that force can selectively stabilize intermediates which have a larger extension. For example intermediate I_1 has a similar extension to the unfolded state, and so at low forces is similarly stabilized; as force increases, the softer unfolded state shifts to larger extensions and becomes more stable.

Recent efforts using highly stable atomic force microscopes are starting to resolve interesting features of energy landscapes that were not observable before, as in the case of the protein Csp *Tm*.²⁴ This work has resulted in an apparent discrepancy between conventional (i.e. two-state) descriptions of protein folding and more intricate descriptions where alternative paths^{10,11,19} and novel intermediate states²⁴ need to be accounted for. Here we have shown how a simple coarse-grained simulation model can explain many of the experimental observations. Using a force-dependent MSM derived from the simulation data in combination with models for force-induced transitions^{38,39} we find that low pulling forces can stabilize intermediates. A molecular explanation for the stability of the I_1 and I_2 intermediates may lie in the orientation of the force-bearing beta-sheet relative to the applied force. An analysis of each of the stable states shows that while the hydrogen bonds in the force-bearing sheet are aligned with the force in the case of the native state (corresponding to an unzipping mechanism), for I_1 and I_2 , the corresponding hydrogen bonds are closer to perpendicular to the applied force (a shearing mechanism, see Fig. S7). Since proteins whose force-bearing beta sheets are oriented perpendicular to the force (such as titin I27 or the B1 domain of protein G) are known to be mechanically strong, this may help to explain

the relative stability of intermediates I_1 and I_2 . Consistent with this, the force-dependent unfolding rates for the two intermediates $I_1 \rightarrow U$ and $I_2 \rightarrow U$ show an initial insensitivity to the pulling force, one of the signatures of a shearing mechanism,^{10,11} in which the mechanism only shifts at sufficiently high force from the intrinsic unfolding pathway to that which is accelerated by pulling. As a result, describing the force dependence of these unfolding rates required the sum of a force independent intrinsic rate and a force-dependent rate from a one-dimensional (1D) rate theory to fit the simulation data, while all of the other unfolding rates (including $N \rightarrow I_1$ and $N \rightarrow I_2$) could be described by 1D rate theory alone. At zero force these intermediate states may be only marginally populated and remain on the folded side of the dominant barrier, hence becoming invisible in the bulk.

We anticipate that, with further improvements in instruments, intermediates may in future be observed in force experiments on other proteins in future. Evidence of intermediates with populations that can be tuned by the combination of temperature and force has also been reported in simulations using a similar structure-based model for immunoglobulin-like ddFLN.^{41,42} We have shown that apparent discrepancies between the results of bulk and single-molecule experiments on mechanical unfolding of a small single-domain protein can be reconciled within the context of a coarse-grained folding model. Application of an alternative bias (force, rather than denaturant) helps to reveal more details of the energy landscape by exposing previously hidden intermediate states which are more easily perturbed by the application of a biasing force than by chemical denaturant or other perturbations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

D.D.S. acknowledges support from the Spanish Ministry of Economy and Competitiveness (MINECO, grant CTQ2015-65320-R) and an Ikerbasque Research Fellowship. R.B.B. was supported by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health. DDS acknowledges Jörg Schönfelder, Raul Pérez-Jiménez, Victor Munoz and Frank Noé for helpful discussions.

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Figure 1.

(a) Cartoon representation of the cold shock protein from *Thermotoga maritima* (PDB: 1g6p). The C α trace of the protein is shown to emphasize the resolution of the simulation model. The C and N termini of the protein from which we pull are shown as spheres. (b) Time series data corresponding to the projection on the fraction of native contacts (Q) at zero force and 310 K. (c) Heat capacity thermogram obtained from equilibrium runs at multiple temperatures. (d) Potential of mean force at the midpoint temperature.

Figure 2.

(a) Projection of simulation trajectories at different pulling forces on the end to end distance, d . Data points are coloured based on the MSM state they are assigned to (U: dark blue; I_1 : cyan; I_2 : yellow; F: brown). (b) Potentials of mean force from umbrella sampling calculated at forces between 0 pN and 20 pN using the fraction of native contacts Q and the end to end distance d as order parameters. Free energies are in kcal/mol. Note that due to the changes in stability of the states induced by force different free energy scales are used for 0, 5 and 10 pN (top) and 15 to 20 pN (bottom). We show the mean values of Q and d for MSM states calculated from snapshots assigned at the corresponding pulling forces. (c) Representative snapshots of the MSM intermediates I_1 and I_2 .

Figure 3.

(a) Microscopic rate coefficients for transitions in the refolding (top) and unfolding (bottom) directions. Lines are fits to the one dimensional barrier model (see text). (b) Relaxation to equilibrium of the population of different states $P(t)$ at different forces. (c, d) Eigenvectors of the fitted rate matrix at 0 and 15 pN, respectively.