Energy Landscape of Ubiquitin Is Weakly Multidimensional

Published as part of *The Journal of Physical Chemistry virtual special issue "D. D. Sarma Festschrift"*.

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Cite This: *J. Phys. Chem. B* 2021, 125, 8682–8689

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ABSTRACT: Single molecule pulling experiments report time-dependent changes in the extension (X) of a biomolecule as a function of the applied force (f). By fitting the data to one-dimensional analytical models of the energy landscape, we can extract the hopping rates between the folded and unfolded states in two-state folders as well as the height and the location of the transition state (TS). Although this approach is remarkably insightful, there are cases for which the energy landscape is multidimensional (catch bonds being the most prominent). To assess if the unfolding energy landscape in small single domain proteins could be one-dimensional, we simulated force-induced unfolding of ubiquitin (Ub) using the coarse-grained self-organized polymer–side chain (SOP-SC) model. Brownian dynamics simulations using the SOP-SC model reveal that the Ub energy landscape is weakly multidimensional (WMD), governed predominantly by a single barrier. The unfolding pathway is confined to a narrow reaction pathway that could be described as diffusion in a quasi-1D X -dependent free energy profile. However, a granular analysis using the P_{fold} analysis, which does not assume any form for the reaction coordinate, shows that X alone does not account for the height and, more importantly, the location of the TS. The f -dependent TS location moves toward the folded state as f increases, in accord with the Hammond postulate. Our study shows that, in addition to analyzing the f -dependent hopping rates, the transition state ensemble must also be determined without resorting to X as a reaction coordinate to describe the unfolding energy landscapes of single domain proteins, especially if they are only WMD.

INTRODUCTION

Many cellular functions are triggered by proteins that respond to mechanical stress.^{1,2} In cellular activities, such as muscle contraction and relaxation, cell adhesion, and transport of proteins through a membrane, proteins partially unfold when subject to an external force.^{3–5} These examples illustrate the need to quantitatively understand the molecular basis of the response of biomolecules to forces. Single molecule pulling experiments (optical tweezers, magnetic tweezers, or an atomic force microscope (AFM)), in the constant force mode, measure the extension of the molecule as a function of time, which could be used to sort out the structural transitions in a protein. In the force-ramp experiments,^{6–11} force is increased as a function of time by stretching the protein at a constant loading rate. Typically, but not always, force is applied to the ends of the protein of interest. Pulling at a constant force or constant loading rate unfolds the protein, leading to conformational transitions and eventually complete unfolding at sufficiently large forces. In the constant force mode (force clamp), structural transitions are detected by sudden changes in molecular extension (X) monitored in real time.¹² By performing the experiments at different values of force f , we

can extract the force-dependent unfolding rate constant $k_u(f)$. In the force-ramp experiments,¹³ the protein ends are pulled at a constant loading rate, and as f increases, the extension X can be measured. Abrupt changes in the force–extension plots (rips) signify the structural transitions in the protein. In each realization of the experiments, the unfolding force varies stochastically. By performing a large number of experiments, we measure the distribution of rupture forces $p_u(f)$. In principle, the data from the force clamp and constant loading rate modes produce the same information.

The measurements using single molecule pulling experiments have provided a quantitative estimate of the free energy profile (termed free energy surface (FES)) by constructing the histogram of X . Phenomenological and analytical models based on Kramer's theory¹⁴ are routinely used to analyze the data

Received: March 28, 2021

Revised: June 24, 2021

Published: July 28, 2021

assuming that the folding FES is one-dimensional with X as a reasonable reaction coordinate. The Bell model¹⁵ assumes that the force-dependent unfolding rate $k_u(f)$ decreases exponentially with the applied force f . The fit of the data to the Bell model may be used to extract the intrinsic unfolding rate $k_u(0)$ at $f = 0$ and the transition state (TS) location, $\Delta X^\ddagger$. A limitation of the Bell model is that it does not relate $k_u(f)$ to the barrier height $\Delta G^\ddagger$ or to the shape of the 1D FES. It has become clear that the Bell model is valid only for brittle proteins in which TS does not move with f .^{16–21} However, to account for ductile systems ($\Delta X^\ddagger$ changes with f), 1D FES (cubic FES or one with a cusp) for which $k_u(f)$ could be analytically calculated have proved to be particularly useful.^{7,23}

In an influential article, Dudko, Hummer, and Szabo modeled the FES as linear-cubic and harmonic cusp functions of X and derived analytical expressions^{24,25} for $k_u(f)$ by using a modified Kramer's theory.¹⁴ The unfolding rate $k_u(f)$ connects $k_u(0)$ to the free energy properties, $\Delta G^\ddagger$ and $\Delta X^\ddagger$. These quantities are extracted by fitting the data to the theoretical expression. The theory also assumes that FES is one-dimensional function of X , which might not always hold. Indeed, experiments and simulations provide evidence that the FES for some biomolecules could be multidimensional,^{26,27} involving multiple barriers.^{28–30}

If the energy landscape were multidimensional, then it could result in the nonlinearity in $\log k_u(f)$ vs f plot in force-clamp experiments^{19,31,32} or in the most probable rupture force f^* vs the logarithm of loading rate $\log r_f$.^{28,33–37} Is the multidimensionality of the unfolding energy landscape generic? There are only a few single domain proteins for which the presence of multiple unfolding pathways, which requires the use of multidimensionality to explain the experimental data, has been clearly documented.^{26,30,38–41} This suggests that at least strong multidimensionality in single domain proteins may not be the norm but cannot be ruled out unless scrutinized carefully. Ubiquitin (Ub) is an excellent model system to probe the validity of the assumption that the energy landscape is one-dimensional due to the availability of extensive single molecule pulling experimental data.^{42,43} To this end, we performed force-clamp and force-ramp pulling simulations of Ub using the coarse-grained self-organized polymer–side chain (SOP-SC)^{44,45} model, which allows us to use forces that are similar to those used in the experiments. We observed a downward curvature in the $\log k_u(f)$ vs f plots in force-clamp simulations, indicating that the FES is weakly multidimensional.²⁶ The nonlinearity observed in both the force-clamp and force-ramp simulation data due to the movement of the TS toward the folded state accords well with the Hammond postulate. The location of the TS extracted from the experiments does not satisfy the P_{fold} criterion.⁴⁶ Taken together, our work shows that, in addition to fitting experimentally measured rates to theories that assume that the free energy profile is one-dimensional, additional tests that do not rely on an assumed reaction coordinate or experimental data may be needed to understand the force-induced unfolding dynamics.

METHODS

SOP-SC Model and Simulation Method. Brownian dynamics simulations at temperatures $T = 300$ and 332 K are performed⁴⁷ using the SOP-SC model.^{44,45} The interactions between the side chains are given by the statistical potential.⁴⁸ The SOP-SC model and the simulation methodology^{49,50} are described in detail in the Supporting

Information. The SOP-SC energy function parameters are given in Tables S1–S3.

In the force-clamp simulations, we fixed the N -terminal end of Ub (backbone bead of residue 1) and applied a constant force f to the C -terminal end (backbone bead of residue 76). In the force-ramp simulations, the backbone bead of residue 1 is fixed, and the backbone bead of residue 76 is attached to a spring, with a spring constant $k_s = 35$ pN/nm. The other end of the spring is moved at a constant velocity v to ramp up the force to unfold the protein. The loading rate, $r_f = k_s v$, ranges from 8.75×10^4 to 8.75×10^7 pN/s. The end-to-end distance, X , of Ub is the distance between the backbone beads of residues 1 and 76. The structural overlap function,⁵¹ χ , is defined as $\chi = 1 - (1/N_{\text{tot}}) \sum_{i=1}^{N_{\text{tot}}} \Theta(\delta - |r_i - r_i^0|)$ to distinguish between different populated states of the protein. Here, $N_{\text{tot}} (= 11026)$ is the number of pairs of interaction centers in the SOP-SC model of Ub assuming that at least two bonds separate the interaction centers, r_i is the distance between the i th pair of beads, and r_i^0 being the corresponding distance in the folded state, Θ is the Heaviside step function, and $\delta = 2$ Å.

Transition State Ensemble. The numerically precise transition state ensemble (TSE) is identified from the unfolding trajectories obtained at different f values at $T = 300$ and 332 K by using the P_{fold} analysis.⁴⁶ Putative TSE structures are picked from the region in the unfolding trajectories where the folded state starts to unravel. This region satisfies the conditions $0.66 < \chi < 0.68$ and -50.0 kcal/mol $< E_{\text{CG}} < -40.0$ kcal/mol, where E_{CG} is the potential energy of a protein conformation in the SOP-SC representation. To compute the commitment probability P_{fold} for the probable TSEs, 500 short Brownian dynamics simulations are spawned with the friction value $\zeta = 1.0$ m/ τ_H , where m is the mass of the bead and τ_H is the time unit of the Brownian time step. If the P_{fold} of a structure is in the range $0.4 < P_{\text{fold}} < 0.6$, then the structure is classified as belonging to the TSE.⁴⁶

RESULTS AND DISCUSSION

Force-Clamp Pulling Simulations of Ub. We first performed force-clamp simulations by applying a constant f to the protein ends. At $T = 332$ K and $f = 0$ pN, the free energy difference (ΔG) between the Ub folded and unfolded states is ≈ 6.6 kcal/mol. The good agreement between the calculated and the experimental values⁵² for ΔG provides a measure of validation of the SOP-SC model. Upon application of f , the end-to-end distance of the protein, X increased by ≈ 22 nm, in agreement with experiments⁴³ and previous simulations^{53–60} (Figures S1 and S2).

Ub Unfolding Is Weakly Multidimensional. Force-induced unfolding of Ub occurs in a two-state manner without populating any intermediates (Figure S1). We observed a single dominant unfolding pathway of Ub at $T = 300$ and 332 K, at all forces (Figure S2). At each f , we performed 100 independent pulling simulations to compute the average unfolding time $\langle \tau(f) \rangle$. Assuming two-state kinetics, the force-dependent unfolding rate is taken to be $k_u(f) = \langle \tau(f) \rangle^{-1}$. The relative location of the TS, $\Delta X^\ddagger$, may be obtained by fitting the simulation data in the $[f, \log(k_u(f))]$ plot to the Bell model¹⁵

$$k_u(f) = k_u(0) \exp(f \Delta X^\ddagger / k_B T) \quad (1)$$

where k_B is the Boltzmann constant and $\Delta X^\ddagger = X^\ddagger - X^F$, where $X^\ddagger$ is the end-to-end distance of Ub in the TS and X^F is the end-to-end distance of Ub in the folded state at $f = 0$ pN. The

fit of $[f, \log(k_u(f))]$ to eq 1 at $T = 332$ K yields $\Delta X^\ddagger \approx 0.24 \pm 0.04$ nm, which agrees with the AFM pulling experiments at high forces,^{43,61–63} and $k_u(0) = 2.1 \pm 0.33$ s^{−1} (Figure 1). The fitting procedure is described in the Supporting Information.

Figure 1. Force-dependent unfolding rate, k_u , computed by using the force-clamp mode at (A) $T = 332$ K and (B) $T = 300$ K. The dashed line is a fit to the Bell model. At $T = 332$ K (300 K), the extracted $\Delta X^\ddagger = 0.24$ nm (0.19 nm) and $k_u(0) = 2.1 \pm 0.33$ s^{−1} (0.53 ± 0.12 s^{−1}). Deviation from linearity is observed, especially at higher forces leading to a downward curvature.

Although at lower forces ($\lesssim 170$ pN, $T = 332$ K) the plot is linear, deviation from linearity is observed for higher forces leading to a downward curvature. The Bell model predicts a constant slope ($\frac{\Delta X^\ddagger}{k_B T}$) for the $[f, \log(k_u(f))]$ plot based on the assumption that TS location is independent of the pulling force. However, curvatures are often observed at high forces either due to force-dependent switch in the unfolding pathways involving different TSs^{31,64} or due to the dependence of the TS on the applied force.²² A decrease in $\Delta X^\ddagger$ at higher values of force (in agreement with Hammond's postulate) can lead to a minor downward curvature (Figure 1). We computed residuals (= observed value − fitted value) to determine whether a linear or nonlinear model better fits the data set. Randomness in the residuals (errors) is a measure of how well a model fits the given data set. The residuals obtained from the Bell model fit to force-clamp data at $T = 300$ and 332 K formed a curved pattern, which indicates that linear models are not suitable to fit the data (Figure S3). Although randomness in the residuals is a criterion for selecting the suitable model, it does not necessarily ensure the adequacy of the model.

We believe that the downward curvature is due to the weak multidimensionality²⁶ (WMD) of the FES. We refer to this FES $F(\mathbf{r}, X)$ (where $\mathbf{r}$ represents conformational degrees of

freedom different from X) as weakly multidimensional²⁶ because the unfolding process is confined to a single, narrow reaction pathway in the multidimensional space and the absence of parallel unfolding pathways (Figure S2). A previous computational study⁶⁵ using an all-atom model also suggested that the Ub unfolding landscape could be multidimensional. In WMD landscapes, $k_u(f)$ satisfies the conditions $\frac{d \log k_u}{df} \geq 0$ and $\frac{d^2 \log k_u}{df^2} \leq 0$, which are the conditions for downward curvature, and Ub satisfies them at high f values (Figure 1). A minor downward curvature in a WMD energy landscape suggests a force-dependent movement of the TSE (see below).

Unfolding Involves Crossing a Single Major Barrier.

We also performed force-ramp simulations by pulling one end of Ub with a constant velocity at $T = 332$ K. The average force at which Ub unfolds, $\langle f_u \rangle$, depends linearly^{53,66} on the logarithm of the loading rate, $\log(r_f)$, for loading rates $r_f \leq 8.75 \times 10^7$ pN/s and deviates from linearity at larger r_f (Figure 2 and Figure S4). Assuming that the Bell model is valid, Evans

Figure 2. Average unfolding force $\langle f_u \rangle$ is plotted as a function of log-loading rate $\log(r_f)$. Solid lines are fit of the data to eq 3. At $T = 332$ K (300 K), the extracted parameters are $\nu = 0.58 \pm 0.0947$ (0.69 ± 0.29), $\Delta X^\ddagger = 0.66 \pm 0.19$ nm (0.54 ± 0.46 nm), $\Delta G^\ddagger = 26.07 \pm 4.49$ $k_B T$ (30.0 ± 15.36 $k_B T$), and $\kappa = 1.27 \times 10^7 \pm (3.95 \times 10^6)$ s^{−1} ($1.15 \times 10^7 \pm (1.02 \times 10^7)$ s^{−1}).

and Ritchie^{67,68} derived an equation for the most probable unfolding force f^* :

$$f^* = \frac{k_B T}{\Delta X^\ddagger} \log \left(\frac{r_f \Delta X^\ddagger}{k_u(0) k_B T} \right) \quad (2)$$

If $\langle f_u \rangle \approx f^*$, we can estimate $\Delta X^\ddagger$ from the slope of the linear region in the $[\log r_f, \langle f_u \rangle]$ plot. The values of $\Delta X^\ddagger$ extracted by fitting only the linear regime in the plots at $T = 332$ and 300 K are 0.34 and 0.38 nm, respectively (Figure S4). These values are in reasonable agreement with $\Delta X^\ddagger = 0.24$ nm (Figure 1). We also determined the TSE using the P_{fold} method for the lowest loading rate, $r_f = 8.75 \times 10^4$ pN/s, accessible in simulations at $T = 332$ K (Figure S5). The value of $\Delta X^\ddagger$ computed from the TSE is 1.43 nm, and it is in good agreement with the result from constant force simulations (Figure 3) but deviates from the values extracted from the Bell model analysis.

The deviation from linearity, with an upward curvature, in the $[\log r_f, \langle f_u \rangle]$ plots is evidence for the movement of the TS with force.⁶⁹ To interpret the curvature in $\log r_f$ vs $\langle f_u \rangle$ plot, a theoretical model¹⁷ could be used to assess if unfolding occurs by crossing a single barrier or multiple barriers in the one-

Figure 3. (A) Average end-to-end distance of the Ub conformations in the TSE, $\langle X^\ddagger \rangle$, as a function of the pulling force f . Data shown in circles and triangles are obtained at $T = 300$ and 332 K, respectively. (B) Relative location of the TS from the native state, $\Delta X^\ddagger (= \langle X^\ddagger \rangle - \langle X^F \rangle)$, as a function of force at $T = 300$ and 332 K in circles and triangles, respectively. The TS location shifts toward the native state as f increases.

dimensional free energy profile. In this model, unfolding transitions occur by thermal activation for $f \ll f_c$ and $\beta \Delta G^\ddagger \gg 1$, where f_c is the critical force and $\Delta G^\ddagger$ is the free energy of activation. The equation for most probable rupture force is given by

$$f^* \approx f_c \left[1 - \left(-\frac{k_B T}{\Delta G^\ddagger} \log \frac{r_f \Delta X^\ddagger}{\kappa k_B T} \right)^\nu \right] \quad (3)$$

where κ is the prefactor in Kramer's theory. The parameter κ contains information about the curvature of the FES near the folded basin and the barrier.⁷⁰ It estimates the mean first passage time for reaching the TSE from the folded basin under high-friction conditions. Sharp curvatures imply a smaller distance between the basin and the barrier for a fixed barrier height, leading to high transition rates. In Kramer's theory, random multiple-barrier recrossings are allowed, unlike transition state theory (TST), resulting in a lower estimation of transition rate compared to TST. Furthermore, TST provides an upper limit for Kramer's prefactor, which is $\approx \frac{k_B T}{h}$, where h is Planck's constant. It is known through a series of investigations dating back over two decades that the TST prefactor is simply wrong. For unfolding transitions involving a single barrier crossing, with f -dependent TS, the value of the exponent ν is bound by $\frac{1}{2} \leq \nu \leq 1$, while for transitions with multiple barriers $\nu < \frac{1}{2}$. Assuming that $\langle f_u \rangle \approx f^*$, we fit the data in $[\log r_f, \langle f_u \rangle]$ plot using eq 3 and obtained $\nu = 0.58$ (Figure 2) at $T = 332$ K. The relative errors from the fit at each data point are shown in Figure S6. The value $\nu = 0.58$ suggests

that a single barrier crossing explains the force ramp simulations, which also accords well with the force-clamp data. We conclude that the nonlinearity in the $[\log r_f, \langle f_u \rangle]$ plot is due to the force-dependent movement of the TS (see also Figure 3).

TS Location Depends on f . To check whether the TSE movement follows Hammond's postulate, we identified the TSE by using the P_{fold} method⁴⁶ (see the Methods section) at $T = 300$ and 332 K using the constant force simulation data. The average end-to-end distance of the structures in the TSE, $\langle X^\ddagger \rangle$, varied between ≈ 5.0 and 5.4 nm (Figure 3). The decrease in $\langle X^\ddagger \rangle$ at the higher temperature is reminiscent of temperature softening demonstrated long ago by using AFM experiments on ddFLN4.⁷¹ For a fixed T , the TS shifts to lower $\langle X^\ddagger \rangle$ values as f increases (Figure 3). The average end-to-end distances at $T = 300$ and 332 K in the absence of force ($f = 0$ pN) calculated from equilibrium simulations are nearly the same, $\langle X^F \rangle = 3.68$ and 3.70 nm, respectively.⁵⁰ The decrease in $\langle \Delta X^\ddagger \rangle (= \langle X^\ddagger \rangle - \langle X^F \rangle)$ as f increases at $T = 300$ K shows that the TSE moves closer to the folded state, in agreement with Hammond's postulate,^{16,69,72} which states that the TSE approaches the least stable species in a reaction (Figure 3). In force-induced unfolding, the stability of the native state decreases with an increase in pulling force, which results in the shift of the transition state toward the folded state. The TSE movement also explains the nonlinear behavior in the $[f, \log(k_u(f))]$ plot (Figure 1). We surmise that the gradual TS movement toward the native state with increasing f suggests that there is a single major free energy barrier separating the folded and the unfolded states.

The location of the TS ($\Delta X^\ddagger \approx 1.5$ nm) at $f = 195$ pN computed directly from the simulations using P_{fold} analysis, a method that does not rely on a reaction coordinate, differed significantly from the value ($\Delta X^\ddagger \approx 0.24$ nm) extracted by using the Bell fit to either the experimental^{42,43,62,63} or the simulation data. The discordance between the $\Delta X^\ddagger$ values indicates that a complete description of the forced-unfolding dynamics requires going beyond one-dimensional free energy profiles.

X Alone Is Not a Good Reaction Coordinate. The disagreement between the $\Delta X^\ddagger$ values extracted by using one-dimensional free energy profile, with X as the reaction coordinate, and computed directly by using the P_{fold} analysis raises the possibility that X alone may be an inadequate reaction coordinate for Ub unfolding. If X were a good reaction coordinate, then the P_{fold} for the ensemble of structures that are constrained to have $X \approx \langle X^\ddagger \rangle$ should be ≈ 0.5 . At $T = 332$ K and $f = 125$ pN, $\langle X^\ddagger \rangle$ is 5.1 ± 0.03 nm (Figure 3).

We picked 200 Ub conformations whose X values are within 5.1 ± 0.03 nm and performed P_{fold} analysis for these structures. The distribution, $P(P_{\text{fold}})$, of the P_{fold} values, shows that a significant fraction of these structures end up in the protein folded state with $P_{\text{fold}} = 1$. The rest of the structures lead to approximately a uniform distribution between $0 < P_{\text{fold}} < 0.9$ (Figure 4). This occurs because most of the Ub conformations with X in the range $\langle X^\ddagger \rangle \pm \sigma$ are closer to the folded basin than the TS. As a result, when short pulling simulations using these conformations are initiated, most of these structures reach the folded basin. We conclude that for Ub X alone is not a good reaction coordinate, although both the f -dependent unfolding rate and the dependence of the mean unfolding force as a function of r_f do not deviate significantly from the expectation based on an effective 1D energy landscape.

Figure 4. Probability distribution of P_{fold} values obtained from 200 Ub putative TS structures at $T = 332$ K and $f = 125$ pN. The 200 conformations have X within $\langle X^\ddagger \rangle \pm \sigma$ (5.1 ± 0.03 nm). Short pulling trajectories spawned by using these structures end up in the folded state basin. The rest of the structures lead to a broad distribution with P_{fold} between 0 and 0.9. Therefore, from the perspective of the TSE, X alone is not an adequate reaction coordinate.

The probability distribution, $P(X)$, in the absence of f , at close to the melting temperature,⁵⁰ $T = 353$ K, where both the folded and unfolded states are sampled, shows that X does not resolve the folded and unfolded basins of Ub. There is a cusp separating the two basins in the free energy (Figure 5). This finding is also in agreement with the previous simulation result,⁵⁴ which reported that X could be a poor reaction coordinate for Ub folding.

In single molecule pulling experiments of ductile systems with multidimensional FES, both $\Delta G^\ddagger$ and $\Delta X^\ddagger$ decrease with the increase in the magnitude of the applied force contrary to the assumptions in the theoretical models (Figure 3). In such FES, the molecular extension X alone cannot capture the details of the folding/unfolding process. The fact that we could not identify TS structures by using P_{fold} analysis when X was chosen to be the only order parameter reinforces the need for a multidimensional treatment of the FES in existing theoretical models. It is possible that a collective coordinate, such as the fraction of native contacts, may be a reasonable choice^{50,73} instead of X .

Structural Characterization of the TSE. The transition state structures (TSSs) of Ub in thermal⁵⁰ and force-induced unfolding are identified by using P_{fold} analysis.⁴⁶ The TSSs are globular and homogeneous, with most of the secondary structural elements and tertiary contacts intact as found in the folded state (Figure 6A), in agreement with Ψ -value analysis.^{74,75} Force-induced TSSs consist of a structured N-terminal region with α_1 helix and $\beta_1\beta_2$ sheet, in agreement with Φ -value analysis.⁷⁶ The average χ , which gives an estimate of the contact order in the thermal and force-induced TSSs, is ≈ 0.67 and 0.6 , respectively, in agreement with the estimated values for various proteins.⁷⁷ Although the structures are globular, there are some differences in the thermal and force-induced TSSs (Figure 6B). In the thermal TSSs compared to the folded structure, the contacts between the β -sheets $\beta_3\beta_5$ are ruptured completely. Although most of the significant tertiary contacts are present in the force-induced TSSs, the contacts between the sheets $\beta_1\beta_5$ and $\beta_3\beta_5$ are weakened considerably compared to the folded state (Figure 6B). In the initial stages of force-induced unfolding, we observe detachment of β_5

Figure 5. (A) Probability distribution of X , $P(X)$, and (B) FES of Ub projected onto X , $G(X)$, in the absence of external force ($f = 0$ pN) at $T = 332$ and 353 K. The melting temperature of Ub is ≈ 353 K.

strands disrupting contacts with β_1 and β_3 strands (Figure S2B,C). This is consistent with the fact that mechanical clamp in Ub is present between $\beta_1\beta_5$, the rupture of which constitutes the major energy barrier to unfolding.^{57,62,78} Furthermore, in line with our observation, simulations⁷⁹ also report force induced unfolding of Ub starting with the rupture of $\beta_3\beta_5$ contacts.

CONCLUSIONS

A major advantage of single-molecule pulling experiments is that the energy landscape of proteins can be measured without altering the intraprotein or protein solvent interactions. However, we need useful theoretical models to extract the relevant kinetic properties related to the protein's structural transitions from the experimental data. The common assumptions in the most fruitful models are (1) protein unfolding can be described as a barrier crossing in an effective one-dimensional energy landscape and (2) the location of the transition state is not very sensitive to the applied force. These assumptions need to be tested to ensure that extracted quantities are physically meaningful and consistent with independent experiments and/or reliable simulations.

Unfolding Ub by force, which has been extensively studied by experiments, is a curious case. The dependence of the unfolding rates on force and the mean rupture force as a function of loading rates only exhibit minor deviations from theoretical predictions based on 1D energy landscape. On the other hand, the structures that are generated by constraining

Figure 6. (A) Representative TSSs populated during thermal (left) and force-induced (right) unfolding. The secondary structural elements, five β strands (β_1 – β_5), and the two α helices (α_1 and α_2) are shown. (B) Contact map of the thermal TSE (left) shows that contacts between the sheets $\beta_3\beta_5$ are missing (shown in circle). In the force-induced TSE (right), contacts among the sheets $\beta_1\beta_5$ and $\beta_3\beta_5$ are weakened compared to the folded state. (C) Distribution of the final structural overlap parameter, χ , calculated from 500 simulation trajectories spawned by using the TSSs as the initial starting structure. Data are shown for five different structures. The distribution shows that roughly half of these trajectories go into the folded basin and the other half into the unfolded basin, demonstrating that these protein conformations are the TSSs.

the extension to the TS location extracted by fitting the simulation data to the one-dimensional analytical models do not satisfy the $P_{\text{fold}} \approx 0.5$ requirement. By combining both these findings, we demonstrate that the energy landscape of Ub is weakly multidimensional in which the location of the transition state moves in response to the applied force. Our study shows that only by combining analysis of experimental data using simple theories together with reliable simulations can one learn about the unfolding energy landscape, especially for systems which exhibit weak multidimensionality.

To test the validity of the theoretical models, we compared our simulation results to the AFM pulling experiments of Ub performed in the high-force regime.^{42,43,62,63} Using optical and magnetic tweezers, it is now possible to perform pulling experiments at low pulling forces (<1 pN) to gain insight into the mechanical properties and force regulation of proteins in the physiologically relevant low-force regime. However, thermodynamic parameters are still extracted by using simplistic theoretical models, which assume a one-dimensional FES with X as the order parameter. Thus, it is essential to

improve the existing theoretical models to incorporate features such as the multidimensionality of FES and force-dependent movement of the TS.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcb.1c02762>.

Description of the coarse-grained self-organized polymer–side chain protein model and simulation method; Table S1: SOP-SC energy parameter; Table S2: SOP-SC model parameters for ubiquitin; Table S3: side-chain radii of amino acids; Figure S1: multiple independent trajectories showing two-state unfolding of Ub; Figure S2: Ub dominant unfolding pathway; Figure S3: residual plot associated with a Bell fit on force-clamp simulation data of Ub; Figure S4: average unfolding force $\langle f_u \rangle$ as a function of the loading rate $\ln(r_f)$; Figure S5: P_{fold} analysis to identify the TSE from force ramp simulations; Figure S6: relative errors from the fit of eq 3 to the $[\log(r_f), \langle f_u \rangle]$ data (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

A part of this work is funded by the grant to G.R. by the National Supercomputing Mission (MeitY/R&D/HPC/2(1)/2014). D.T. acknowledges grants from the NIH (GM-107703) and the Welch Foundation (Grant F-0019) through the Collier-Welch chair. The computations are performed by using the TUE and Cray XC40 clusters at IISc.

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