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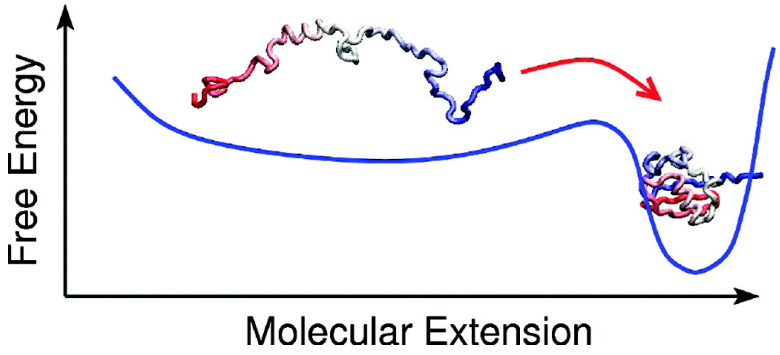

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Protein Folding Kinetics Under Force from Molecular Simulation

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Many proteins experience mechanical force *in vivo*: in structural roles in the cytoskeleton and muscle, as molecular anchors in cell adhesion and mechanosensing,[1] or during mitochondrial import[2] and protein degradation.[3] Nevertheless, even modest forces of 10−50 pN, common in biology, can effectively prevent refolding, as shown by manipulation of single protein molecules in atomic force microscopes and optical tweezers. Despite much work on protein unfolding[4] and folding/unfolding of RNA,[5] refolding of a protein against force has been achieved only recently[6] and so far only in a few cases.[6−10] An understanding of refolding under force is therefore relevant both biologically and for single-molecule experiments.

Here, we explore refolding kinetics in the presence of a pulling force using simulations of a coarse-grained model of ubiquitin. We show that the effects of force on the folding kinetics are captured by a microscopic Kramers-based theory of diffusive barrier crossing under force.[11] The fitted parameters are in almost quantitative agreement with free energy surfaces obtained from simulation, indicating that they are physically meaningful. By comparing parameters obtained from pulling in different directions (Figure 1), we gain insight into the role of the unfolded state in the refolding kinetics. The results explain why refolding becomes very slow at even moderate pulling forces[6] and suggest how it could be practically observed in experiments at higher forces.

Pulling experiments are usually performed on polyproteins. Ubiquitin forms polyprotein chains in the cell with several specific linkages[12] between residues 1−76, 11−76, 29−76, 48−76, and 63−76. The vectors connecting these residues (Figure 1A) thus define naturally occurring "pulling coordinates". We have investigated the refolding of ubiquitin when a constant stretching force F is applied to each pulling coordinate $\mathbf{r}_{ij}$ in Langevin simulations, by adding the term $-F|\mathbf{r}_{ij}|$ to the potential function (the term $-\mathbf{F}\cdot\mathbf{r}_{ij}$ may correspond more closely to experiment; however it is straightforward to correct for this difference). The protein is described by a coarse model in which residues are represented by α-carbon beads, with a Gō-like energy function (further details in the Supporting Information).[13,14] Gō-like models have successfully described protein folding rates[15,16] and other aspects of folding.[17] Our simulation procedure is intermediate between kinetic models that treat (un)folding as an escape from 1D potential wells (an assumption we will explicitly test) and all-atom molecular dynamics simulations (which, under force, would require ms simulations for a μs folder).

Starting from an equilibrium distribution of unfolded configurations at 100 pN, a large number of trials were performed where the force was instantaneously "quenched" to a much smaller value. Each trial was terminated when the fraction of native contacts,[18] Q, exceeded 0.9 or when the simulation time reached a maximum, τ_{sim} (a value of Q close to 1.0 identifies the folded state). From N trials, of which N_r refold with exponentially distributed first passage times τ_i, the maximum-likelihood estimate of the mean refolding time, is $\tau_{\mathrm{f}} = k_{\mathrm{f}}^{-1} = [\Sigma_i \tau_i + (N - N_r)\tau_{\mathrm{sim}}]/N_r$. The refolding times for all but one of the naturally occurring linkages, shown in Figure 2A, increase with force, with the sensitivity of the refolding rate

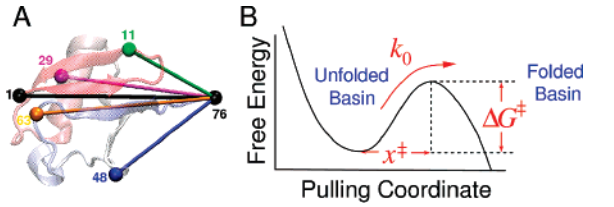

Figure 1. Structure of ubiquitin and a schematic 1D energy landscape for folding: (A) The five naturally occurring linkages are shown by lines connecting the attachment points. (B) Parameters of the 1D theory[11] in which the pulling coordinate is used as the reaction coordinate.

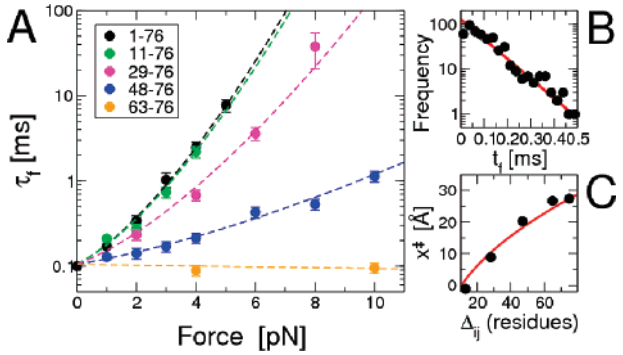

Figure 2. Effect of force on refolding kinetics. (A) Refolding times for different linkages (bold symbols) are distinguished by color and are fit globally using eq 1 with $\nu = {}^2/_3$ (broken lines). (B) Refolding time distribution at zero force. (C) $x^{\ddagger}$ as a function of sequence separation of attachment points Δ_{ij} from kinetics (symbols) and from WLC model (red line).

to force being dependent on the pulling coordinate. The resulting nonexponential dependence of folding times on force indicates that refolding rates do not follow the commonly used phenomenological Bell model for force-dependent rates, $k_{\mathrm{f}}(F) = k_0 \exp(Fx^{\ddagger})$, where k_0 is the "intrinsic" rate at zero force and $x^{\ddagger}$ is the distance from the unfolded state to the transition state (TS) at zero force.

We employ instead a recent microscopic formalism[11] for molecular transitions in the presence of a pulling force that characterizes the folding using a 1D energy landscape (Figure 1B). In the presence of a constant force F along the pulling coordinate, Kramers theory of diffusive barrier crossing predicts a folding rate[11]

$$k_{\mathrm{f}}(F) = \frac{1}{\tau_{\mathrm{f}}(F)} = k_0\left(1 - \frac{\nu F x^{\ddagger}}{\Delta G^{\ddagger}}\right)^{1/\nu - 1} \exp\left[\frac{\Delta G^{\ddagger}}{k_{\mathrm{B}}T}\left(1 - \left(1 - \frac{\nu F x^{\ddagger}}{\Delta G^{\ddagger}}\right)^{1/\nu}\right)\right] \quad (1)$$

where $\Delta G^{\ddagger}$ is the height of the barrier (Figure 1B). Although initially intended to describe molecular *rupture* (here: unfolding), the model is equally applicable to refolding, where $Fx^{\ddagger}$ is negative. The parameter ν is related to the shapes of the barrier and minimum: for a cusp-like barrier $\nu = {}^1/_2$, while for a smooth linear-cubic barrier $\nu = {}^2/_3$;[11] the Bell model is recovered with $\nu = 1$.

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Table 1. Parameters from Global Fits of Eq 1 to the Refolding Times in Figure 2, and Reference Values from 2D PMFs

fit parameter	$\nu = 1/2$	$\nu = 2/3$	$\nu = 1$	PMF
$x^{\ddagger}$ [Å] (1−76)	27.4	27.4	34.5	22
$x^{\ddagger}$ [Å] (11−76)	26.8	26.7	31.5	21
$x^{\ddagger}$ [Å] (29−76)	20.0	20.3	27.1	17
$x^{\ddagger}$ [Å] (48−76)	9.0	8.9	10.1	8
$x^{\ddagger}$ [Å] (63−76)	−0.9	−1.1	0.1	−4
$\Delta G^{\ddagger}$ [k_BT]	1.6	0.9	N/A	2
k_0 [ms^{-1}]	9.6	9.5	11.0	
χ^2	19.9	22.4	61.4	
$p(\chi^2)$	0.28	0.17	10^{-6}	

The refolding kinetics could be well fitted using eq 1 with a global k_0 and $\Delta G^{\ddagger}$ but separate $x^{\ddagger}$ for each linkage (Table 1). The parameters are very similar for fits to the cusp ($\nu = 1/2$) and linear-cubic barriers ($\nu = 2/3$), and a χ^2 test shows that both fits are significant. However, due to the curvature in the data, the fit of the Bell model ($\nu = 1$) is poor, with an insignificant χ^2 (p-value of 10^{-6}). For $\nu = 1/2$ or $\nu = 2/3$, the globally fitted intrinsic rate, 9.5 ms^{-1}, matches the direct calculation at zero force, 10.0 ms^{-1}. The global fit of k_0 and $\Delta G^{\ddagger}$ indicates that for all pulling directions folding occurs via the same, "intrinsic" barrier (i.e., that probed in the absence of force). In contrast, the unfolding mechanism at high forces has been found to differ from the intrinsic one for several proteins.[19−22]

The monotonic increase of the independently fitted $x^{\ddagger}$ with the sequence separation $\Delta_{ij} = |j - i|$ of the attachment points i and j (Figure 2C) suggests that the unfolded state is the main determinant of $x^{\ddagger}$. In fact, $x^{\ddagger}$ follows a worm-like chain (WLC) unfolded state, $x^{\ddagger} \approx (4 l_p l_c/3)^{1/2} - x_0$, with persistence and contour lengths of l_p = 5 Å and $l_c = \Delta_{ij} \times 3.81$ Å, respectively, for a transition state of fixed extension x_0. We note that for these parameters a harmonic potential is a good approximation for the free energy profile $G(r)$, justifying the use of the cusp model ($\nu = 1/2$) for the kinetics (Supporting Information).

Further insight into the refolding is obtained from projections of the folding free energy surface. Umbrella sampling and weighted histogram analysis were used to determine 2D potentials of mean force (PMFs) for ubiquitin, as functions of the pulling coordinates $|\mathbf{r}_{ij}|$ and Q. At zero force, the unfolded state is better separated from the TS and native state along $|\mathbf{r}_{1,76}|$ than $|\mathbf{r}_{48,76}|$ (Figure 3A and B). The distance to the TS on the PMF agrees well with $x^{\ddagger}$ fitted to the kinetic data (Table 1). Similarly, the fitted global barrier height $\Delta G^{\ddagger}$ is consistent with the low barriers in the PMFs. This suggests that physically meaningful parameters may be obtained from fitting eq 1 to experiment.

Force stabilizes the unfolded state in 1−76 more than in 48−76 because of the larger separation from the TS (Figure 3C and D), explaining both the different force sensitivity between the two linkages (Figure 2A) and the curvature in the folding times $\tau_f(F)$. Unlike a conventional "Hammond" picture,[23,24] where curvature is caused by a TS movement, here the unfolded ("reactant") state shifts to longer distances with force. The mechanically "soft" unfolded protein is most easily stretched by the pulling force.

In conclusion, our results indicate that, at the low forces where refolding is feasible, folding occurs over the intrinsic folding barrier. The relatively flat unfolded free energy profile causes a marked increase in distance to the TS with increasing force. The resulting superexponential force dependence explains why refolding is impractical above a few tens of pN.[6,9] The sensitivity of folding rates to force increases with the sequence separation of attachment points. Thus, from the point of view of single molecule experiments, pulling from the termini slows folding the most. We suggest that using linkages with smaller sequence separation may allow refolding to occur at higher forces and permit the observation of both folding and unfolding events at the same force. From a biological perspective, it seems imperative that proteins that experience force should evolve to resist unfolding since refolding becomes all but impossible unless the force is relaxed.

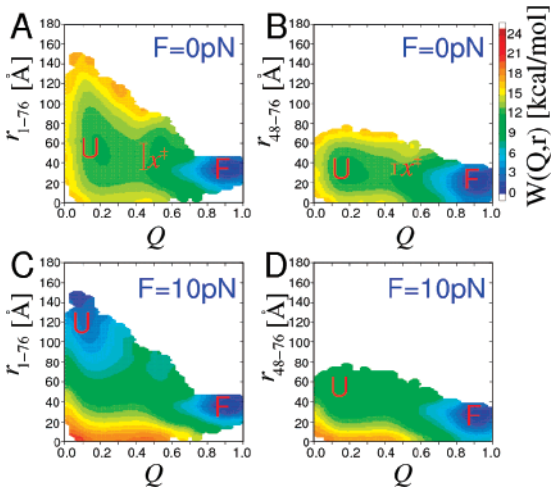

Figure 3. PMFs as a function of the fraction of native contacts Q and the pulling coordinate r for (A) linkages 1−76 and (B) 48−76 at zero force and (C) 1−76 and (D) 48−76 with a 10 pN pulling force. The folded and unfolded states are labeled "U" and "F", respectively. The $x^{\ddagger}$ from the kinetic fits are superimposed on the free energy surfaces in A and B.

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Supporting Information Available: Details of the simulation model and WLC approximation for the unfolded state. This material is available free of charge via the Internet at http://pubs.acs.org.

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