Supporting Information

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SI Text

Description of the Protocol Used to Estimate the Parameters Defining the Energy Landscape of the Native Conformations. In our experiments, we probe the mechanical properties of the native state of mechanically stable proteins by studying their unfolding kinetics under the effect of a constant stretching force. To get insight into the parameters defining the energy landscape of the unfolding reaction, we measure the force dependency of the unfolding rate of single polyproteins. Under the presence of a constant pulling force, the rate of unfolding can be approximated by the simple Bell model (1–4), which describes the unfolding reaction as a 2-state process limited by a well-defined energy barrier. The height of this energy barrier is modulated by the stretching force according to the relationship $k(F) = k_0\exp(F\Delta x/kT)$, where F is the pulling force, k_0 is the rate constant in the absence of force, and Δx is the distance to the transition state. Furthermore, given that $k_0 = A\exp(-\Delta G/kT)$ and assuming a prefactor $A = 10^6\ s^{-1}$ (5), we can readily estimate the height of the activation energy barrier of unfolding, ΔG.

The normalized average of an ensemble of a few trajectories such as that shown in Fig. 1 in the manuscript (F_1 regime) gives the probability of unfolding as a function of time, $P(t)$, for each particular force (Fig. 2*A*). As a first approximation, we use single exponential fits to the average time-course of unfolding at each particular force to describe the average rate of unfolding of ubiquitin as $k_2(F) = 1/\tau(F)$, where $\tau(F)$ is the time constant of the exponential fits to the averaged unfolding traces, shown in Fig. 2*A*. Furthermore, we obtain an estimate of the standard error of $k_2(F)$, using the bootstrapping technique. As we have previously demonstrated, a single exponential fit captures about 81% of the unfolding events and thus represents a reasonable measure of the unfolding rate (6). The remainder of the data demonstrates that the native state of ubiquitin is not unique; instead, it is composed of an ensemble of similar structures with slightly different energies (2, 6–8). By repeating these measurements over the force range between 90 pN and 190 pN we obtained the force-dependency of the unfolding rate in standard PBS-buffered aqueous solution (Fig. 2*C*, gray triangles) (9). Fitting the Arrhenius rate equation to the data Fig. 2*D* (gray dashed line) resulted in $k_{0,2} = 8.8 \times 10^{-3} \pm 5.0 \times 10^{-4}\ s^{-1}$ and $\Delta x_2 = 1.6 \pm 0.1$ Å.

Description of the Protocol Used to Estimate the Parameters Defining the Energy Landscape of the Ensemble of Collapsed Conformations. We aim to investigate the response of the recently identified ensemble of collapsed states upon the application of a stretching force under different solvent environments. In particular, we aim to gain insight into the features of the energy landscape associated with unraveling such conformations; such as the distance to the transition state, $<\Delta x_1>$, and the height of the energy barrier, $<\Delta G_1>$. By using a force-quench protocol, we identified the ensemble of collapsed states as a necessary conformation visited by the protein during its time-course evolution to the native state (9).

To study the force-dependent unraveling rate of this ensemble of conformations in standard PBS solution, we devised a force quench protocol (Fig. 4*A*) that specifically probes the mechanical resistance of these structures under force. In this protocol, we first unfold ubiquitin at a high pulling force of 110 pN, resulting in a series of steps of 20 nm. The force is subsequently quenched to a low value of 10 pN (F_2) for a period $\Delta t = 2$ s to trigger the extended polypeptide to collapse into its folded length. Allowing the protein to refold during longer Δt values results in the successful refolding of ubiquitin, and the absence of the collapsed conformations (9). A test pulse to a force of 50 pN for 7 s (F_3 regime in Fig. 4*A*) tests the time-course of unraveling of the ensemble of collapsed states without unfolding the ubiquitin modules that have already regained mechanical stability. By changing the pulling force of the test pulse (F_3) within a range spanning from 30 pN to 70 pN, we measured the force-dependent time-course of unraveling them in standard PBS aqueous solution (9). Fig. 4*E* shows a logarithmic plot of the rate of unraveling of the collapsed conformations, k_1, as a function of the pulling force (gray triangles). We fitted the force-dependency of k_1 with a simple Arrhenius term, obtaining $k_{0,1} = 0.7\ s^{-1}$ and $\Delta x_1 = 2.0$ Å. Assuming the same prefactor value used to describe the unfolding energy barrier of the native conformations (5, 10) of $A = 10^6\ s^{-1}$ we calculate an average activation energy of $<\Delta G_1>$ approximately 8.4 kcal/mol (9).

1. Schlierf M, Li H, Fernandez JM (2004) The unfolding kinetics of ubiquitin captured with single-molecule force-clamp techniques. *Proc Natl Acad Sci USA* 101:7299–7304.
2. Garcia-Manyes S, Brujic J, Badilla CL, Fernandez JM (2007) Force-clamp spectroscopy of single-protein monomers reveals the individual unfolding and folding pathways of I27 and ubiquitin. *Biophys J* 93:2436–2446.
3. Dougan L, Feng G, Lu H, Fernandez JM (2008) Solvent molecules bridge the mechanical unfolding transition state of a protein. *Proc Natl Acad Sci USA* 105:3185–31890.
4. Bell GI (1978) Models for the specific adhesion of cells to cells. *Science* 200:618–627.
5. Li MS, Klimov DK, Thirumalai D (2004) Thermal denaturation and folding rates of single domain proteins: Size matters. *Polymer* 45:573–579.
6. Brujic J, Hermans RI, Garcia-Manyes S, Walther KA, Fernandez JM (2007) Dwell-time distribution analysis of polyprotein unfolding using force-clamp spectroscopy. *Biophys J* 92:2896–2903.
7. Lindorff-Larsen K, Best RB, Depristo MA, Dobson CM, Vendruscolo M (2005) Simultaneous determination of protein structure and dynamics. *Nature* 433:128–132.
8. Brujic J, Hermans RI, Walther KA, Fernandez JM (2006) Single-molecule force spectroscopy reveals signatures of glassy dynamics in the energy landscape of ubiquitin. *Nat Phys* 2:282–286.
9. Garcia-Manyes S, Dougan L, Badilla CL, Brujic J, Fernandez JM (2009) Direct observation of an ensemble of stable collapsed states in the mechanical folding of ubiquitin. *Proc Natl Acad Sci USA* doi/10.1073/pnas.0901213106.
10. Schuler B, Lipman EA, Eaton WA (2002) Probing the free-energy surface for protein folding with single-molecule fluorescence spectroscopy. *Nature* 419:743–747.

Table S1. Energy parameters defining the distance to the transition state, Δx, the unfolding rate in the absence of force, $k_{0,1}$ and $k_{0,2}$ and the height of the energy barrier, ΔG, obtained for unfolding the native state (*Upper*) and unraveling the ensemble of collapsed states (*Lower*) under different solvent environments (Figs. 2 and 4)

Native state	Δx_2 (Å)	$k_{0,2}(s^{-1})$	ΔG_2 (kcal/mol)
Glycerol 30%	2.3 ± 0.1	$4.2 \times 10^{-5} \pm 2.4 \times 10^{-5}$	14.2
Ethanol 30%	1.7 ± 0.1	$8.5 \times 10^{-3} \pm 2.5 \times 10^{-3}$	11.0
PBS solution	1.6 ± 0.1	$8.8 \times 10^{-3} \pm 5.0 \times 10^{-4}$	11.0
Ensemble of collapsed conformations	$<\Delta x_1>$ (Å)	$k_{0,1}$ (s^{-1})	$<\Delta G_1>$ (kcal/mol)
Glycerol 30%	1.4 ± 0.2	0.5 ± 0.2	8.6
Ethanol 30%	1.4 ± 0.6	0.7 ± 0.6	8.4
PBS solution	2.0 ± 0.4	0.7 ± 0.4	8.4