Supporting Results and Discussion

Worm-Like Chain Fits to Force-Extension Data Predict Step Size in Force-Clamp

The data presented in Fig. 6*A* demonstrate the unfolding of a single $(I27_{G32C\text{-}A75C})_8$ molecule when stretched at a constant velocity (400 nm/s). This results in a sawtooth pattern in force-extension spectroscopy, the most common type of protein unfolding experiment conducted using the atomic force microscope (1). Each peak in the sawtooth results as either a protein module unfolds or a single disulfide bond is reduced (followed by trapped residue unfolding). The extension of the unfolded polypeptide after each peak will correlate with the number of amino acids being stretched and can be fit with the Worm-Like Chain model of polymer elasticity (WLC),

$$F = \frac{k_B T}{p}\left(\frac{1}{4(1-x/L_C)^2} + \frac{x}{L_C} - \frac{1}{4}\right), \qquad [1]$$

where p is the persistence length, x is the end-to-end length, and L_C is the contour length of the molecule. When fitting multiple peaks, we can measure ΔL_C, the contour length increase per unfolding event. The contour length of a polymer is its extrapolated length under an infinite pulling force, when the conformational entropy of the polymer is reduced to zero (2). However, we do not approach this limit in our experimentation; the contour length for an unfolded polypeptide is only approached at forces near 1 μN, whereas all of the forces we examine are at well under 1 nN. Thus, at a given force the actual elongation of the molecule after an unfolding event will be shorter than ΔL_C.

As described in the main text, force-clamp spectroscopy involves the application of a constant force to a single protein, resulting in a stepwise elongation of the molecule as a function of time. Each of these steps results from the extension of a polypeptide in

the molecule. These steps can be a result of unsequestered unfolding, full-length unfolding, or thiol/disulfide exchange (Fig. 1*C*). In the sawtooth pattern in Fig. 6*A*, we see a similar series of events: unsequestered unfolding (red fits), thiol/disulfide exchange events (blue fits), and full length unfolding of modules with reduced disulfides (green fit). WLC fits to the sawtooth pattern in Fig. 6*A* resulted in: ΔL_C = 12.6 nm for unsequestered unfolding, ΔL_C = 16.5 nm for thiol/disulfide exchange, and ΔL_C = 28.6 nm for full-length unfolding. By using these WLC fits, we also can predict the amplitude of the stepwise elongation of the polyprotein under force-clamp for unsequestered unfolding (Un), thiol/disulfide exchange (T/D), and full length unfolding (FL) (dashed arrows in Fig. 6*A*).

Fig. 6*B* demonstrates a force-extension trace of wild-type $(I27)_8$. Here all peaks are equally spaced, which is not the case in $(I27_{G32C\text{-}A75C})_8$. Note that the step size predicted at 130 pN for wild-type $(I27)_8$ is much longer than that predicted for unsequestered unfolding, but is approximately equal to that predicted for full-length unfolding in $(I27_{G32C\text{-}A75C})_8$. Importantly, at 130 pN the predicted step size for unsequestered unfolding (10.4 nm) and that for thiol/disulfide exchange (13.5 nm) can be added to approximately equal that for full-length unfolding (24.5 nm). From the above calculation it is clear that unsequestered unfolding extends part of total $I27_{G32C\text{-}A75C}$ polypeptide, while thiol/disulfide exchange and trapped unfolding extends the remainder. Thus, we can ensure that we are monitoring two distinct processes within the same protein module.

As demonstrated in Fig. 6 *C* and *D*, the experimentally determined step sizes for unsequestered unfolding and thiol/disulfide exchange recover the predicted step sizes.

The histogram in Fig. 6*C* is a compilation of unsequestered unfolding step sizes from multiple single-molecule extensions at F = 130 pN. The data was fit with a Gaussian distribution, with an average step size of 10.6 ± 0.7 nm (n = 254), which is very close to the predicted value for unsequestered unfolding at F = 130 pN (10.4 nm in Fig. 6*A*). In Fig. 6*D*, step sizes for single thiol/disulfide exchange reactions were compiled from double pulse force-clamp experiments at 12.5 mM DTT (data in Fig. 4*A*). Again, each data set was fit with a Gaussian distribution. The average step size was very similar to the predicted values obtained from Fig. 6*A* (see Table 1). Note that the step size distributions for unsequestered unfolding and thiol/disulfide exchange events do not overlap at any force. Thus, we could consistently and easily distinguish amongst these steps using our force-clamp instrumentation.

In our force-extension experiments examining disulfide reduction (Fig. 6), disulfide reduction occurs at higher forces than protein unfolding. In the force-extension trace, the disulfide bond is exposed to the solvent for only a brief period of time; the entire recording only lasts ≈1 s. Thus, as expected from the force-dependent reduction kinetics presented in this work, a large force is necessary to catalyze disulfide reduction within this brief time frame. This is why the reduction peaks in the sawtooth pattern are much higher than those for protein unfolding.

Unsequestered Unfolding as a Function of the Pulling Force

Fig. 7 shows the result of multiple force-clamp experiments in the absence of DTT. Similar to the analysis conducted for ubiquitin (3), we averaged multiple traces (5 to 14) obtained at different forces. As expected, we only observed unsequestered

unfolding events in $(I27_{G32C-A75C})_8$, and no disulfide reduction events are noted. A single exponential was fit to each averaged trace, resulting in a time constant for unfolding τ_u at each force. The rate of unsequestered unfolding, $\alpha_u = 1/\tau_u$, was plotted in Fig. 5*A* as a function of force. By fitting this data to the equation $\alpha_u(F) = \alpha_u(0)e^{\frac{(F\Delta x_u)}{k_B T}}$, we obtained a rate of spontaneous (zero-force) unsequestered unfolding $\alpha_u(0) = 6.22 \times 10^{-3}$ s^{-1}. This value is larger than that measured for the spontaneous unfolding of wild-type I27, 3.3×10^{-4} s^{-1} (4). Furthermore, the value of $\Delta x_u = 1.75$ Å, compared with the reported value of $\Delta x_u = 2.5$ Å for wild-type I27 unfolding reported by Carrion-Vazquez *et al.* (4). Note that in Fig. 6, it is apparent the peak unfolding force of $I27_{G32C-A75C}$ (Fig. 6*A*) is somewhat lower than for wild-type I27 (Fig. 6*B*) at the same pulling speed (400 nm/s) in force-extension. Interestingly, this is true even in the case of full-length unfolding, where the disulfide bond is reduced before unfolding. Thus, it appears that these cysteine mutations, in an oxidized or reduced state, slightly affect the mechanical properties of I27. These changes result in a decrease of peak unfolding force in force-extension mode, a higher value of spontaneous unfolding, and a different value of Δx_u.

Unsequestered Unfolding Events During the Second Pulse

In Fig. 7, note that at a constant pulling force of 200 pN, ≈70% of unsequestered unfolding events occur after only 40 ms. Our instrumentation has a time resolution of ≈ 5 ms per unfolding event. Thus, when we unfold the unsequestered region of $(I27_{G32C-A75C})_8$ at 200 pN, we are approaching our resolution limit. Because this resolution is necessary to identify the fingerprint of a single molecule in our experiments, we first stretch at 130 pN, the standard force for the first pulse of double-pulse experiments. At

this force, ≈70% of unfolding events occur after 1 s. Yet that means that up to 30% of events may not yet have occurred at the time when the force is changed to the second pulse. In double-pulse experiments with increases to 200-400 pN, these remaining folded domains would rapidly unfold (within 50 ms) and are easily separated kinetically from thiol/disulfide exchange events, which are much slower. This rapid unfolding cannot be distinguished from the entropic extension of the polypeptide that occurs during the step increase to a higher force (e.g., Fig. 3*B*). To obtain second pulse data at 100 pN, however, step size was the sole factor in discriminating unsequestered unfolding events from thiol/disulfide exchange reduction events, because both types of events could occur over a time course of seconds at this lower force.

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2. Marko, J. F. & Siggia, E. D. (1995) *Macromolecules* **28,** 8759-8770.
3. Schlierf, M., Li, H. & Fernandez, J. M. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 7299-7304.
4. Carrion-Vazquez, M., Oberhauser, A. F., Fowler, S. B., Marszalek, P. E., Broedel, S. E., Clarke, J. & Fernandez, J. M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 3694-3699.