Electronic Supplementary Material (ESI) for Nanoscale.
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Electronic Supplementary Information

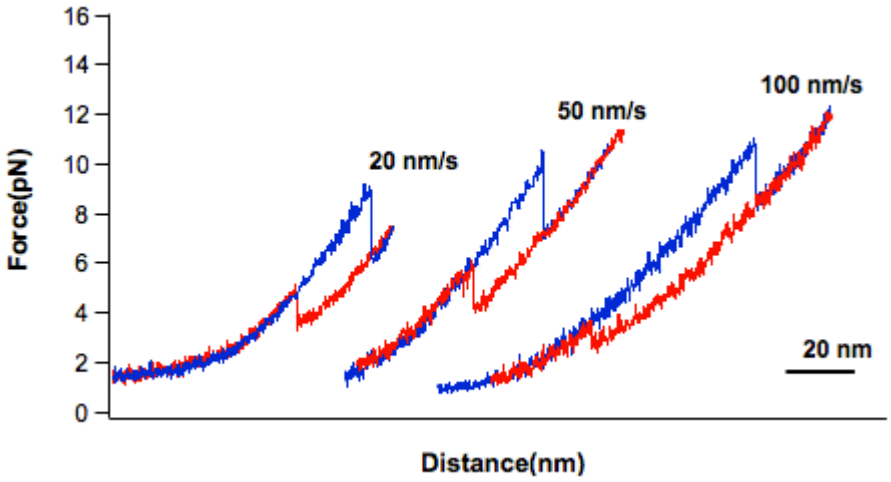

Figure S1. Force-induced unfolding-refolding of AFV3-109 occurs under non-equilibrium in constant velocity experiments. The hysteresis between the unfolding (blue) and refolding cycle (red) is evident, and depends on the pulling speed. The higher the pulling speed, the larger the hysteresis.

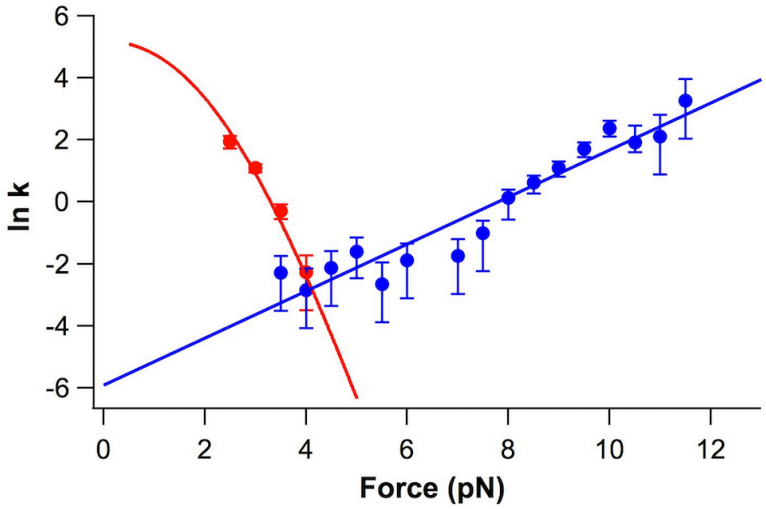

Figure S2. Force dependency of the unfolding rate (blue) and folding rate (red) of AFV3-109. Filled circles are experimental results and solid lines are fittings using Bell-Evans model for unfolding and a modified model for folding, which takes into account of polymer elasticity. It is important to note that the force dependency of the unfolding and folding rate constants obtained this way is an indirect measurement, which could lead to potentially large uncertainty in determining $F_{1/2}$, at which the unfolding and refolding rates are equal to each other. To directly and more precisely measure this dependency and determine $F_{1/2}$, constant force experiments will be required. Moreover, $F_{1/2}$ is only meaningful in constant force experiments, as in constant velocity experiments, due to the length difference of the folded and unfolded states, the unfolding will lead to the relaxation of the force and the refolding will lead to the increase of the force.

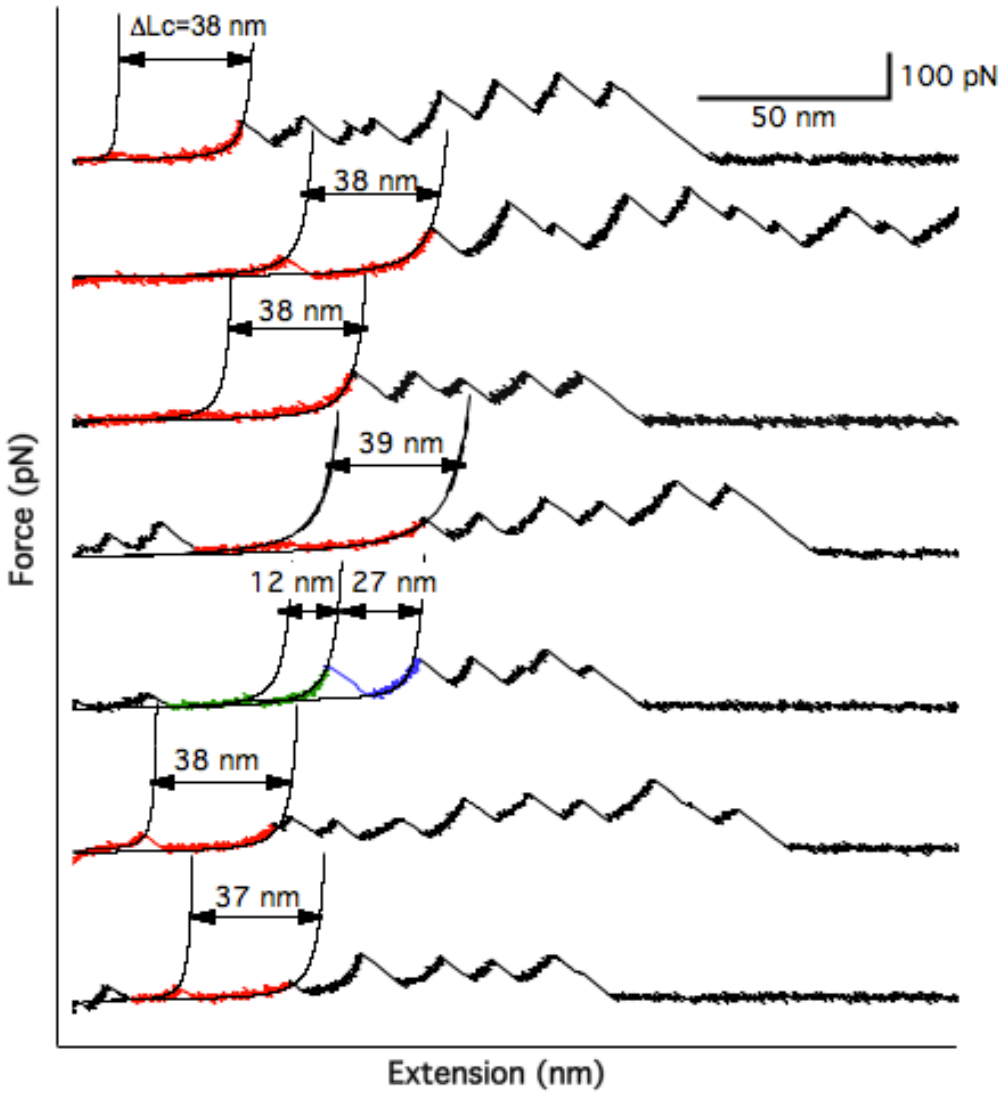

Figure S3. Representative force-extension curves of $(GB1)_4$-AFV3-109-$(GB1)_4$ measured using softer AFM cantilevers (with a spring constant of 6 pN/nm). Unfolding events of GB1 are colored in black, two-state unfolding of AFV3-109 are colored in red and the three-state unfolding event is colored in green and blue. In these experiments, 70% AFV3-109 unfolded via the two-state pathway. This is in sharp contrast to the observation in AFM experiments using stiffer AFM cantilevers, where only 39% AFV3-109 unfolded in a two-state fashion.

Table S1. Fitting parameters from chemical and mechanical denaturation experiments.

	$D_{1/2}$ (M)	m (kCal/M)	ΔG_0 (k_BT)
Equilibrium Chemical Denaturation	3.97	1.85	12.2 ± 0.7
Mechanical Denaturation (CFT)			13.5±2.0
Mechanical Denaturation (β_0/α_0)			11.0 ± 1.0

D1/2: denaturant concentration at which 50% of the protein is unfolded
m: equilibrium m value
ΔG_0: equilibrium free energy difference between the folded and unfolded states.

Table S2. Kinetic parameters from optical trapping and AFM experiments.

	OT (two-state, N-U)	AFM (three-state, N-I)	AFM (three-state, I-U)	AFM (two-state, N-U)
α_0 (s^{-1})	0.003	0.011	0.72	1.8
Δx_u (nm)	3.5	0.59	0.27	0.24
β_0 (s^{-1})	180	NA	NA	NA

Table S3. Bifurcation of the unfolding pathways of AFV3-109 in force spectroscopy experiments with different probe stiffness (kc).

	OT (kc: 0.25 pN/nm)	AFM (kc: 6 pN/nm)	AFM (kc: 40 pN/nm)
Two-state	100%	70%	39%
Three-state	0	30%	61%