Comment to the Editor

Fingerprinting DHFR in Single-Molecule AFM Studies

In a recent issue of the *Biophysical Journal*, we reported that ligand binding modulated the mechanical stability of Chinese hamster ovary dihydrofolate reductase (DHFR), an enzyme essential for cell survival. In our work, we found that, in the absence of ligands, DHFR displayed low mechanical stability, with an average unfolding force of only 27 pN (1). By contrast, we found that in the presence of micromolar concentrations of ligands (e.g., methotrexate (MTX), nicotinamide adenine dihydrogen phosphate, or 7, 8-dihydrofolate), DHFR was far more stable with an average unfolding force of 83 pN (1). In the same issue, Junker et al. (2) reported a contradictory finding claiming that the unfolding force of mouse DHFR does not depend on ligand binding (2). In this Comment to the Editor, we explain this discrepancy by showing how the experimental design and analysis method used by Junker et al. (2) could have prevented them from detecting the effects of a ligand on the mechanical stability of this important enzyme.

In our experiments (1), we used a polyprotein chimera that combined the I27 titin module together with the DHFR protein, (I27-DHFR)$_4$. The I27 protein is mechanically stable and its properties are well understood (3,4) providing an unmistakable mechanical fingerprint. More importantly, the chimera approach (5–7) allows the observer to be certain that DHFR proteins are being extended by force, regardless of whether DHFR is mechanically stable or not. Fig. 1 A shows

that stretching the (I27-DHFR)$_4$ chimera in the absence of ligands gives an initial spacer region (corresponding to the elongation of the mechanically weak DHFR molecules), followed by a regularly spaced saw-tooth pattern (representing the unfolding of the I27 markers). For example, in Fig. 1 A, due to the alternating arrangement of I27 and DHFR in the polyprotein, observation of four I27 domains unfolding is unequivocal indication that mechanical force has been applied to at least three DHFR molecules. We were therefore able to identify the DHFR unfolding events by constructing the appropriate number of worm-like chain fits backward from the first I27 unfolding signature (Fig. 1 A). We then estimated the unfolding forces of DHFR by recording the intercept values between the worm-like chain fits and the experimental force-extension curve, and obtained an average unfolding force of 27 pN (Fig. 1 B). Therefore, the chimera approach has enabled us to identify DHFR unfolding events, even though most of the time DHFR did not give a discernible saw-tooth pattern fingerprint. By contrast, we found that in the presence of a ligand like MTX, DHFR always gave a clear unfolding saw-tooth pattern preceding the I27 fingerprint (Fig. 1 C). In this case, the average unfolding force was 82 pN (Fig. 1 D).

Junker et al. (2) did not use the polyprotein chimera approach. Instead, they engineered a polyprotein that consisted of a single DHFR protein flanked on either side by
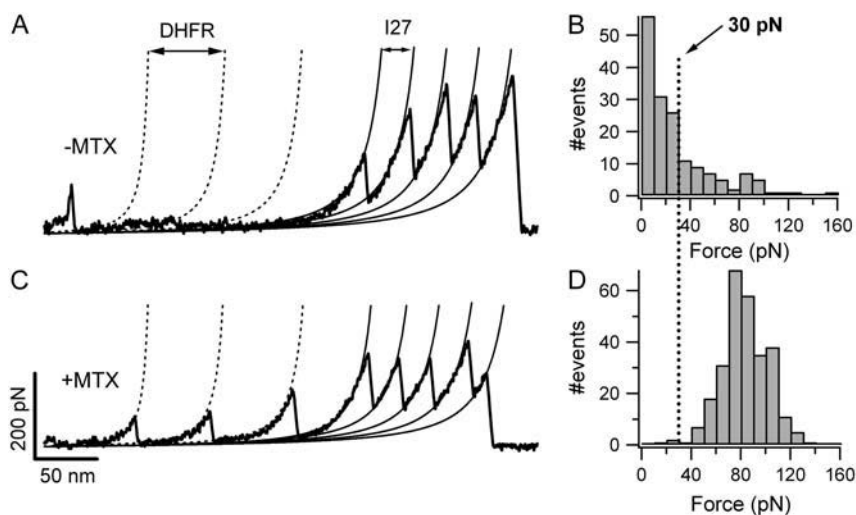

FIGURE 1   Force-extension curves obtained using the chimeric polyprotein (I27-DHFR)$_4$ in the absence (*A*) and in the presence (*C*) of 190 μM MTX. The corresponding histograms of unfolding forces of DHFR are shown in *B* and *D*, respectively.

Ddfilamin domains. Using this construct, they reported observing clear DHFR unfolding events both in the presence and in the absence of ligands. However, Junker et al. imposed a minimum force threshold of 30 pN in the detection of force

peaks (Supplementary Material of Junker et al. (2)), missing the majority of the weak, ligand-free, DHFR unfolding events. The choice of a 30 pN threshold was necessary because in contrast to our chimera polyprotein approach (see above), in the absence of a clear force-peak, Junker et al. could not be sure that a DHFR protein had been unfolded. As it is plain from our data (Fig. 1 *B*), a cutoff of 30 pN would have shifted the average unfolding force of ligand-free DHFR up to a value comparable to that of MTX-bound DHFR (Fig. 1D).

Finally, it is well known that DHFR unfolds and traverses the protein translocation channels in mitochondria (8,9) and the degradation channel in the proteasome (10–12). Binding to MTX significantly reduces the rate of both mitochondrial import (8,9) and the proteasomal degradation of DHFR (10–12). Our finding that ligands increase the mechanical stability of DHFR directly explains these observations.

REFERENCES

1. Ainavarapu, S. R., L. Li, C. L. Badilla, and J. M. Fernandez. 2005. Ligand binding modulates the mechanical stability of dihydrofolate reductase. *Biophys. J.* 89:3337–3344.

2. Junker, J. P., K. Hell, M. Schlierf, W. Neupert, and M. Rief. 2005. Influence of substrate binding on the mechanical stability of mouse dihydrofolate reductase. *Biophys. J.* 89:L46–L48.

3. Carrion-Vazquez, M., P. E. Marszalek, A. F. Oberhauser, and J. M. Fernandez. 1999. Atomic force microscopy captures length phenotypes in single proteins. *Proc. Natl. Acad. Sci. USA.* 96:11288–11292.

4. Carrion-Vazquez, M., A. F. Oberhauser, S. B. Fowler, P. E. Marszalek, S. E. Broedel, J. Clarke, and J. M. Fernandez. 1999. Mechanical and chemical unfolding of a single protein: a comparison. *Proc. Natl. Acad. Sci. USA.* 96:3694–3699.

5. Li, H., A. F. Oberhauser, S. B. Fowler, J. Clarke, and J. M. Fernandez. 2000. Atomic force microscopy reveals the mechanical design of a modular protein. *Proc. Natl. Acad. Sci. USA.* 97:6527–6531.

6. Best, R. B., B. Li, A. Steward, V. Daggett, and J. Clarke. 2001. Can non-mechanical proteins withstand force? Stretching barnase by atomic force microscopy and molecular dynamics simulation. *Biophys. J.* 81:2344–2356.

7. Li, L., H. H. Huang, C. L. Badilla, and J. M. Fernandez. 2005. Mechanical unfolding intermediates observed by single-molecule force spectroscopy in a fibronectin type III module. *J. Mol. Biol.* 345:817–826.

8. Eilers, M., and G. Schatz. 1986. Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature.* 322:228–232.

9. Matouschek, A., N. Pfanner, and W. Voos. 2000. Protein unfolding by mitochondria. The Hsp70 import motor. *EMBO J.* 1:404–410.

10. Johnston, J. A., E. S. Johnson, P. R. Waller, and A. Varshavsky. 1995. Methotrexate inhibits proteolysis of dihydrofolate reductase by the N-end rule pathway. *J. Biol. Chem.* 270:8172–8178.

11. Thrower, J. S., L. Hoffman, M. Rechsteiner, and C. M. Pickart. 2000. Recognition of the polyubiquitin proteolytic signal. *EMBO J.* 19: 94–102.

12. Lee, C., M. P. Schwartz, S. Prakash, M. Iwakura, and A. Matouschek. 2001. ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. *Mol. Cell.* 7:627–637.

Sri Rama Koti Ainavarapu, Lewyn Li, and Julio M. Fernandez

Biological Sciences
Columbia University
New York, New York 10027