Comment to the Editor

Response to the Comment by Ainavarapu et al.

In their comment, Ainavarapu et al. argue the discrepancy in ligand-free dihydrofolate reductase (DHFR) stability between their and our study could be due to a low force cutoff of 30 pN used in our study for unfolding force histograms. The simplicity of this argument may be tempting at first sight, but does not withstand closer examination.

In almost all our curves, both in the absence and in the presence of ligands, we observe unfolding of DHFR after one or two Ddfilamin domains have unfolded. Such a curve for ligand-free DHFR is shown in Fig. 1 *a*. Since this class of curves constitutes the vast majority of events we observe, a cutoff of 30 pN introduced to ensure reliable force and distance measurements will not influence the measured distribution. This cutoff must not be confused with the minimum force detectable in our experiments, which is determined by thermal noise. Our experimental assay allows detection of DHFR events reliably at forces above 20 pN (see the 20 pN line in Fig. 1). We can therefore readily check the potential presence of a dominant population of low-force DHFR unfolding events postulated by Ainavarapu et al. The fraction of such events where ligand-free DHFR unfolds before the filamin domains at forces <30 pN is almost negligible ($<5\%$). One of the rare examples we find in our data is shown in Fig. 1 *b*, where we observe DHFR unfolding at a force of 23 pN followed by unfolding of the DHFR intermediate at 17 pN. This sample curve shows that we do have the resolution to detect such low-force events. Extremely rare observation of low-force events preceding filamin unfolding is hence in perfect agreement with a mechanically stable conformation of DHFR but not with the force distribution reported by Ainavarapu et al. Moreover, the design of our modular protein is such that in all events where at least three Ddfilamin domains, including ddFLN4, unfold we can be sure that also DHFR must have unfolded (see *inset* in Fig. 1). In contrast to Ainavarapu et al., we do not observe a significant fraction of events with featureless spacers preceding Ddfilamin unfolding. Taken this evidence together, we can exclude a low-force population of ligand-free mouse DHFR in our experiments.

Our data hence show that in the absence of ligands, mouse DHFR can exist in a conformation mechanically equally stable than the ligand-bound forms. Interestingly, in a recent study, Wilcox et al. investigated mechanical stability of DHFR from *Escherichia coli* and also reported high unfolding forces for the wild-type protein in the absence of ligands (1) and no change in unfolding force upon methotrexate (MTX) addition (A. Matouschek, Northwestern University, personal communication, 2006). This is in perfect agreement with our results. Even though the sequences of DHFR from *E. coli* and mouse differ, this comparison is relevant since in mitochondrial import experiments, addition of MTX blocks import for both DHFR variants (1,2).

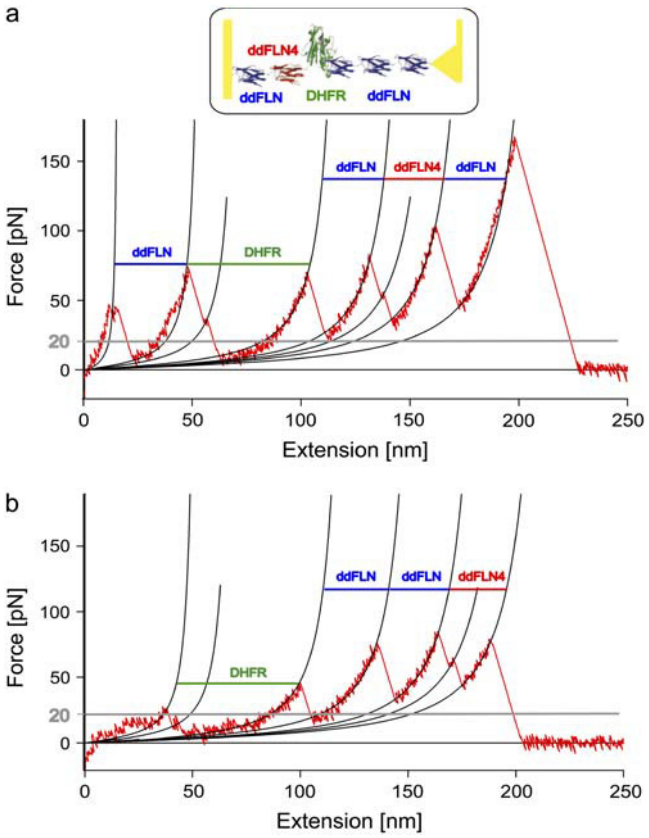

FIGURE 1 Force curves for the DHFR-Ddfilamin-protein construct. Our detection limit of 20 pN is indicated by the gray line. (*a*) In the vast majority of cases, DHFR unfolds at 50–80 pN after one or more Ddfilamin domains. (*b*) Only in very rare cases, DHFR unfolds at forces below 30 pN. Although these events are extremely rare, they are easily detectable with our assay.

What could be a possible explanation for the apparent discrepancy between the two studies? It is important to note that the DHFR variants (mouse and Chinese hamster ovary) used in the two studies are homologous but not identical (sequence identity of 96%). We propose that differences in the proteins as well as in the experimental conditions (e.g., temperature) are likely explanations for the apparently different stabilities. It has been reported that DHFR, in the absence of ligands, can exist in at least two different conformations that are populated to varying degrees at different temperatures (3). We hence propose that the different results reflect a complex conformational behavior of DHFR enzymes rather than

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potential shortcomings of the experimental design. Further support for this interpretation comes from the consistent observation of an unfolding intermediate in mouse DHFR, which Ainavarapu et al. did not report. Exploring these differences will be an important task for the future.

Mechanical stability measurements are an important contribution to understanding protein import. However, relating unfolding force measurements to import efficiencies is not simple and will require a bigger picture. Otherwise, it will be difficult to explain that the mechanically very stable domain I27 from titin (200 pN unfolding force) is readily imported into mitochondria, whereas DHFR complexed with MTX (60 pN unfolding force) blocks import (4).

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