SUPPLEMENTARY DATA for the manuscript: "Changes of protein stiffness........." by K. Małek and R. Szoszkiewicz

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DETAILED ANALYSIS OF AN ELASTIC SPRING CONSTANT FOR FOLDED PROTEINS

Elastic spring constants of a folded protein can be directly obtained from the slopes of the FX-AFM data plotting a stretched length of a folded protein as a function of an applied stretching force. Those measurements yield values of the elastic spring constants for Ig domains between 20 to 60 pN/nm for poly-I27s [Carrion-Vazquez M, Oberhauser AF, Fisher TE, Marszalek PE, Li HB, Fernandez JM. Mechanical design of proteins-studied by single-molecule force spectroscopy and protein engineering. Progress in Biophysics and Molecular Biology. 2000;74(1-2):63-91]. However, the FX-AFM curves are noisy and contain calibration errors as well as errors coming from subtraction of the AFM cantilevers displacement. Thus, several other reports have appeared, like the developments of Dietz et al. [Dietz H, Berkemeier F, Bertz M, Rief M. Anisotropic deformation response of single protein molecules. Proceedings of the National Academy of Sciences of the United States of America, 103, 12724-12728 (2006)], where an elastic spring constant for a protein has been calculated as a spring constant of an unfolding potential.

The method proposed by Dietz et al. relies on a substantial amount of data, which needs to be collected, and thus, by a virtue of statistics is expected to provide more precise estimates than direct AFM measurements. This method involves three steps. First, an experimental measure of the mean unfolding force, \( \langle f \rangle \), as a function of the loading rate \( (df/dt) \) is obtained in a series of many FX-AFM experiments. A distribution of \( \langle f \rangle \) vs. \( (df/dt) \) is fitted with appropriate models, like the Bell-Evans-Ritchie model to provide the values of an apparent (at zero force) unfolding rate constant \( k_u^0 \) and an averaged distance to the unfolding transition state along a pulling coordinate \( \Delta x_u \) [Evans E, and Ritchie K. Dynamic strength of molecular adhesion bonds. Biophys. J. 72:15411555 (1997)]. Noteworthy, the values of \( \Delta x_u \) for proteins are typically of the order of the length of a typical covalent bond, i.e., several angstroms, but an actual extension of a folded protein to an unfolding transition are up to several nm to tens of nanometers even for simple proteins [Borgia A, Williams PM, Clarke J. Single-molecule studies of protein folding. Annual Review of Biochemistry. 2008;77:101-125, Carrion-Vazquez M, Oberhauser AF, Fisher TE, Marszalek PE, Li HB, Fernandez JM. Mechanical design of proteins-studied by single-molecule force spectroscopy and protein engineering. Progress in Biophysics and Molecular Biology. 2000;74(1-2):63-91].
Furthermore, the loading rates are obtained as tangents to the force-extension plots (obtained by AFM) in the vicinity of the unfolding transition for each protein. In the second step, an apparent activation barrier height $\Delta G^*$ is calculated using the Arrhenius equation $\Delta G^* = k_B T \ln(k_o^u/k_A)$, where $k_B$ is the Boltzmann's constant, $T$ is an absolute temperature, and $k_A$ is the Arrhenius frequency factor, a.k.a. the attempt frequency. The value of $k_A$ is typically taken as 1 GHz. However, despite some reports [O. Bieri et al., The speed limit for protein folding measured by triplet-triplet energy transfer, Proc. Natl. Acad. Sci. USA 96, 9597-9601, 1999] it is not clear why such a value of an attempt frequency should reflect molecular vibrations at which the protein is most likely to jump over an energy barrier. Finally, in a third step, the unfolding potential is assumed to be harmonic and its spring constant $k_s$ is calculated mimicking equation for a potential energy of a harmonic oscillator, i.e., $k_s = 2\Delta G^*/(\Delta x_u)^2$. As such the method of Dietz et al. represents an insightful and extremely valuable approach, but we would argue that it provides an elastic spring constant for just-about-to-unfold proteins and using several approximations, which can be questioned.

Polymer physics models, like the WLC model, predict that an elastic constant of a polymer is supposed to increase during its stretching. Thus, an elastic spring constant of a folded protein might be much smaller than an elastic spring constant of a protein near an unfolding transition. And indeed, recently, several direct measurements of elastic spring constants of several folded proteins yielded values of several to only a few tens of pN/nm. For example, Wang and Zocchi stretched 24 kDa protein bound to a nanoparticle in an alternating electric field and reported the elastic spring constant of this protein as 5 pN/nm [Ref. 18 in the paper]. Similarly, Taniguchi et al., vibrated NP-attached myosin rods and reported their elastic spring constant of several pN/nm [Ref. 14 in the paper]. Those results are much closer the results obtained by AFM alone and the results in our current paper.

Overall, our results are about two orders of magnitude smaller than the typical spring constants obtained using a method of Dietz et al. However, our results correlate very well with several other existing measurements of the elastic spring constants of the folded proteins. Since the issue of elastic spring constants of folded proteins is still in its infancy, we expect many upcoming developments in this arena.
FIG. S1. An examples of the FQ-AFM trace for a non-refolder with 1 domain of I274 unfolded. Note initial entropic protein stretch due to sudden force increase as well as the first unfolding step of 24 nm.

FIG. S2. Another FQ-AFM trace for a non-refolder with 1 domain of I274 unfolded.
FIG. S3. An FQ-AFM trace for a non-refolder with 2 domains of I27\textsubscript{4} unfolded.

FIG. S4. Another FQ-AFM trace for a non-refolder with 2 domains of I27\textsubscript{4} unfolded.
FIG. S5. An FQ-AFM trace for a non-refolder with 3 domains of I274 unfolded.

FIG. S6. Another FQ-AFM trace for a non-refolder with 3 domains of I274 unfolded.
FIG. S7. An FQ-AFM trace for a non-refolder with 4 domains of I274 unfolded.

FIG. S8. Another FQ-AFM trace for a non-refolder with 4 domains of I274 unfolded.
FIG. S9. An FQ-AFM trace for a refolder with 1 domain of I274 unfolded and then refolded.

FIG. S10. An FQ-AFM trace for a refolder with 2 domains of I274 unfolded and then refolded.
FIG. S11. An FQ-AFM trace for a refolder with 3 domains of I274 unfolded and then at least two of those refolded.

FIG. S12. An FQ-AFM trace for a refolder with 4 domains of I274 unfolded and then at least two of those refolded.
FIG. S13. Normalized autocorrelation plot of fluctuations in the end-to-end length vs molecular compliance for two traces of I274 molecules, which initially unfolded one domain and that domain managed to refold during the force quench period. This is similar to Fig. 3 in the paper, but now obtained for a completely refolded one domain of I274. First three (highest) molecular stiffness values are marked on the graph.

FIG. S14. Normalized autocorrelation plot of fluctuations in the end-to-end length vs molecular compliance for four traces of I274 molecules, which initially unfolded two domains and those domains managed to refold during a force quench period.
FIG. S15. Normalized autocorrelation plot of fluctuations in the end-to-end length vs molecular compliance for four traces of I274 molecules, which initially unfolded three domains and at least two of those domains managed to refold during a force quench period.
FIG. S16. Normalized autocorrelation plot of fluctuations in the end-to-end length vs molecular compliance for three traces of I274 molecules, which initially unfolded four domains and at least two of those domains managed to refold during a force quench period. One of the values of a transient molecular stiffness (1.1 pN/nm) is marked to guide the eye.
FIG. S17. Fast Fourier Transform of the end-to-end protein length within a region of interest in Fig. 1 in the main paper for 10 non-refolders. Not much is seen beyond electronic noise of 60 Hz and its multiplicities.