

Magnetic tweezers to capture the fast-folding λ_{6-85} in slow motion

Corresponding Author: Professor Michael Schlierf

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript, Quintana-Cataño et al use magnetic tweezers (MT) to measure the reversible folding dynamics of the fast-folding λ_{6-85} protein. The authors previously measured the dynamics of this protein using optical tweezers (OT) and, intriguingly, they report that the folding dynamics measured with MT are substantially slower. To rationalize this discrepancy, they develop an analysis framework to account for the contribution of the molecular construct (and optical trap) which allows them to interpret the energetics of these transitions when captured through different experimental techniques. They reason that MT alters the transition kinetics by increasing the entropic barrier, owing to the stiff protein linker and the intrinsic force clamp conditions, resulting in slowed down kinetics. Overall, I find their study sound from a technical viewpoint and their interpretation of the results plausible. However, I believe that, while they offer a possible explanation for the observed discrepancy, further controls should be done to convincingly demonstrate that such different folding dynamics arise indeed from the effect of the molecular spacer. More specifically:

1) The model the authors propose accounts for the elastic nature of their protein construct in the MT experiments, consisting of two repeats of the inextensible Spy0128 protein, plus the HaloTag, which are treated as rigid bodies, using the FJC to model the elasticity of the full protein. Presumably, the specifics of this construct result in the increased entropic barrier that slows down the folding kinetics. Therefore, their model could be used to predict how different molecular constructs impact the folding kinetics of the same protein. Ideally, using a DNA linker akin to that employed in the OT experiments should accelerate these kinetics, approaching the MT measurements to the OT ones—although it remains unclear to me what is the relative effect of the optical trap versus the spacer. I understand that this suggestion is technically challenging, as MT experiments require short tethers. However, the authors could change the molecular construct to some other protein linker that can be unfolded, therefore dynamically reducing the stiffness of the spacer protein. For example, using eight repeats of protein L or Ig27 as spacer, which are commonly used proteins in MT experiments, the authors could start measuring the folding dynamics of their λ_{6-85} protein (which should resemble those measured with the Spy0128 linker), and then unfold all eight protein spacers, to measure again the dynamics of the λ_{6-85} protein. Extracting the (un)folding rates before and after unfolding the spacers and fitting the data to their theory would convincingly demonstrate that the energy landscape of the folding protein is indeed altered by the entropic elasticity of the linker proteins.

2) Have the authors considered that the different folding dynamics might simply arise from a different diffusion coefficient in each technique? This effective diffusion coefficient indeed owes to the combined linker+protein and the tethering system (the magnetic beads in MT and the polystyrene beads in the OT experiments have likely very different diffusing properties), so it is also probably dependent on the elasticity of the spacers. The authors assume that the prefactor in the Kramers rates is the same across all techniques, citing reference 28, where this quantity is measured using bulk experiments. However, I'm not fully convinced about this; this prefactor includes the diffusion coefficient and that can be very different depending on the way the folding pathway is probed. The authors should also consider this alternative explanation, and attempt to estimate the effective diffusion coefficient in their MT and OT experiments. For example, for AFM, it was demonstrated that the cantilever dominates the diffusive dynamics of proteins (Berkovich et al PNAS 2012), while in MT it seems that the diffusion coefficient of the bead is orders of magnitude larger than that of the measured folding dynamics (Tapia-Rojo et al Biophys J 2024). Optical tweezers experiments in proteins have generally reported higher diffusion coefficients than those in AFM and MT, which would naturally result in faster folding dynamics—see, for example, Yu et al PNAS 2012. This simpler, alternative explanation could also account for the different folding rates they report.

3) I'm not fully convinced by the use of the FJC to model the elasticity of their spacer construct. They assume that the construct consists of rigid body-like structures, which corresponds to the Kuhn elements of the FJC, but they neglect the

contribution of linkers connecting the proteins, which should be quite soft, and which therefore would dominate an arrangement of elastic elements in series. The effective persistence length (or Kuhn length) of the folded protein elements should be directly measurable with MT by stretching the construct over a range of forces that keeps the λ 6-85 protein folded. From the unfolding force ramps in Fig. 1C, it seems to me that the folded construct can be stretched and behaves as a polymer of low persistence length—the polymer behaves pretty similarly before and after the λ 6-85 protein unfolds in Fig. 1C. The authors could correct their model using parameters directly extracted from their experimental data by stretching the folded and unfolded construct.

4) How well does their model predicts/explains non-equilibrium unfolding forces? The authors could do additional force-ramp experiments in MT (it seems they have these measurements for OT in their reference 27) and compare their unfolding force distributions. I would expect higher unfolding forces for MT—given the slower folding kinetics—which should be also explained by their model.

5) For clarity, I believe the manuscript would benefit from including some OT data to allow for direct comparison with the MT trajectories—for example in Fig. 3.

6) Is there any predicted effect on the value of the coexistence force? I assume that, since it is an equilibrium parameter, it should be the same regardless of the measuring technique, but the authors could report and discuss this quantity as well in light of their model.

Reviewer #2

(Remarks to the Author)

The work by Quintana-Castaño et al investigates new methods for studying fast-folding proteins under mechanical force. In particular, the authors work with magnetic tweezers and λ repressor (6-85) to develop a model that considers the constant-force nature of the magnetic tweezers technique to make suitable for studying such fast-folding proteins akin optical tweezers. In this model the authors consider the contribution of the spacers and introduce an constant force field. In general, the authors obtain results comparable to that of OT upon extrapolation and can estimate the spacer and device contributions to the energy barrier. Overall, this is a good work and that unifies criteria of single-molecule techniques. The results are justified, and conclusions are appropriate. I recommend publication after addressing some concerns.

1. I would advise the authors against direct comparison with thermal denaturation experiments, even upon extrapolation. Thermal stability operates considering molecular interactions that are different from those conferring mechanical stability. Also, in thermal denaturation the proteins are never fully extended rendering molecular species different than those observed in mechanical experiments. At least the authors should comment on these molecular differences to advice the reader.

2. In figure 1c the authors show a slow-drop in force, but I wonder why they have not done a slow-increasing force to observe unfolding transitions. In fact, they observe repetitive steps in the slow-drop that I would assume may also happen in the slow-increase. Have the authors considered such scenario?

3. In figure 1b, between 320 and 360 s approx., the protein seems to be trapped in the unfolded state. Is this an instrumental artifact or a real phenomenon to be observed?

4. Would it be possible to use the same exact molecular construct (e.g., DNA handles) for both MT and OT for a more direct comparison? I am not suggesting that the authors should do it but discussing such possibility.

Reviewer #3

(Remarks to the Author)

The paper by Quintana-Catano et al. investigates the unexpected ability of a magnetic tweezers instrument to capture the folding behavior of a protein, despite a small bandwidth relative to the intrinsic timescales of folding (as estimated using other techniques). The authors carefully document the folding behavior of the protein, demonstrating it behaves as expected based on established thermodynamics of mechanical folding, and show extensive data supporting the measured kinetics. They then present an expanded Bell model (adapted from similar models applied to optical tweezer data) to account for the slowing, which includes terms associated with the free energy of stretching the spacer and protein, and the work associated with moving the probe. The result is that the effective activation barrier for folding is raised dramatically relative to the zero force case, apparently (per the model) due to the extra work involved in moving the probe and stretching the spacers. Generally, the article was a pleasure to read. The results are carefully documented and the argument was clear throughout. The results should be of interest to the community. A few comments:

1) I felt the discussion of the results of the model was a bit lacking. Specifically, the most interesting part of the paper for me was the discussion on page 13 presenting how the activation barrier changed for the MT versus OT geometries. These differences were presented very minimally, with just the numbers for each contribution listed. I would love to see an expansion of this discussion—particularly, one key difference appears to be regarding the type of spacer used, where the OT requires long dsDNA tethers that do not contribute heavily to the raised activation barrier, whereas the MT uses small folded proteins that do contribute heavily to the activation barrier. This is interesting, and of course not really a function of the type of instrument but more to the biochemistry of tethering. This implies that, to achieve the desired kinetic slowing, it is best to use short folded protein spacers. More discussion of this would be interesting, e.g. about what would happen if a more flexible spacer was used in the MT, or whether OT can use short folded proteins. A second key MT/OT difference

contributing to the extra activation barrier for the MT is the work done in moving the probe (apparently much larger for the MT than OT, 9.1 vs 5.7 kT). This is more directly tied to the instrument than the choice of spacer, but I don't quite know why it happens; comments about this would be helpful.

2) As with many recent protein-manipulation papers using voice-coil MTs, the authors calibrate the instrument w.r.t. the folding of a model protein. I have reviewed multiple such papers over the years, and I still am confused as to why these papers never benchmark this calibration scheme in comparison to analysis of thermal fluctuations of the probe particle—the advantage of analysis of thermal fluctuations is that it is a 'pure physics' approach, completely independent of the nature of the biomolecular tether, and so a truly precise benchmark. One worries that any given prep of a protein can have chemical modifications that alter the folding behavior, so protein-folding benchmarking seems less precise. Further it depends on various parameter choices (persistence length and so on).

3) I am not convinced of the utility of presenting the 'incorrect' model, Eq. 5. My understanding is that it is well-established in the literature that one needs to account for spacer and probe behavior—indeed, the authors' model is based on a prior OT version (Eq. 7). Presenting the results from the Eq. 5 analysis adds clutter to the plots that would seem to detract from the paper, and I don't see the upside. I wonder if they would consider removing this part of the analysis.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have convincingly addressed my concerns. Still, I believe that repeating some of their experiments with other extensible protein spacers to compare the folding/unfolding rates in magnetic tweezers would have strengthened their case (which at the moment it's a theoretical prediction), but I understand that it can be considered to be out of scope. The manuscript is therefore suitable for publication.

Reviewer #2

(Remarks to the Author)

Reviewer #3

(Remarks to the Author)

The author's responses to my comments about the main topic of the paper are extremely convincing; I appreciate the additional detail on page 13, and the new Figure 5.

I could quibble a bit about their comments about thermal calibration-- in a properly done experiment, one does not need to know the dissipation ('frictional forces') a priori; instead the dissipation (along with the spring constant) can be found from the analysis of thermal fluctuations. Thus their comments about needing to, e.g., apply the Faxen correction are not really relevant.

But this is really just a minor quibble; I appreciate their response, and this issue does not affect the experiment in a significant way.

I am in favor of publication of the revised manuscript.

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Response to reviewers

We thank the editor and the reviewers for their work, taking the time to read our manuscript and for their constructive feedback. Below we address point-by-point the reviewers' comments.

Reviewer 1

Reviewer 1: In this manuscript, Quintana-Cataño et al use magnetic tweezers (MT) to measure the reversible folding dynamics of the fast-folding $\lambda 6-85$ protein. The authors previously measured the dynamics of this protein using optical tweezers (OT) and, intriguingly, they report that the folding dynamics measured with MT are substantially slower. To rationalize this discrepancy, they develop an analysis framework to account for the contribution of the molecular construct (and optical trap) which allows them to interpret the energetics of these transitions when captured through different experimental techniques. They reason that MT alters the transition kinetics by increasing the entropic barrier, owing to the stiff protein linker and the intrinsic force clamp conditions, resulting in slowed down kinetics. Overall, I find their study sound from a technical viewpoint and their interpretation of the results plausible. However, I believe that, while they offer a possible explanation for the observed discrepancy, further controls should be done to convincingly demonstrate that such different folding dynamics arise indeed from the effect of the molecular spacer. More specifically:

Response: We thank Reviewer 1 for the positive view on our manuscript. We are commenting below in detail on the helpful suggestions.

Reviewer 1: 1) The model the authors propose accounts for the elastic nature of their protein construct in the MT experiments, consisting of two repeats of the inextensible Spy0128 protein, plus the HaloTag, which are treated as rigid bodies, using the FJC to model the elasticity of the full protein. Presumably, the specifics of this construct result in the increased entropic barrier that slows down the folding kinetics. Therefore, their model could be used to predict how different molecular constructs impact the folding kinetics of the same protein. Ideally, using a DNA linker akin to that employed in the OT experiments should accelerate these kinetics, approaching the MT measurements to the OT ones—although it remains unclear to me what is the relative effect of the optical trap versus the spacer. I understand that this suggestion is technically challenging, as MT experiments require short tethers. However, the authors could change the molecular construct to some other protein linker that can be unfolded, therefore dynamically reducing the stiffness of the spacer protein. For example, using eight repeats of protein L or Ig27 as spacer, which are commonly used proteins in MT experiments, the authors could start measuring the folding dynamics of their $\lambda 6-85$ protein (which should resemble those measured with the Spy0128 linker), and then unfold all eight protein spacers, to measure again the dynamics of the $\lambda 6-85$ protein. Extracting the (un)folding rates before and after unfolding the spacers and fitting the data to their theory would convincingly demonstrate that the energy landscape of the folding protein is indeed altered by the entropic elasticity of the linker proteins.

Response: We appreciate the interest in the details of our underlying model to extrapolate the measured kinetics to different potential forces. We would like to address the three points: (i) prediction of the behavior with other linkers, (ii) clarification of OT contribution to the energetic barrier, and (iii) experimental verification of linker effects. To address point (i) and (ii), we have thoroughly revised our manuscript and supporting information. In particular, we introduce in the main text Figure 5, which illustrates energetic contributions for magnetic and optical tweezers. In brief, assuming the typical experimental conditions of spacers as in our experiments for $\lambda 6-85$, the energetic contributions arising from the spacers dominate the

overall energetic barrier formation. We discuss the new figure in detail in the manuscript and in the supplementary information (see Figure S7 and S9). In the supplementary section 9, we present the effects of different types of spacers, e.g. DNA handles or long unstructured polypeptide, as suggested by the reviewer. Overall, our predictions agree well with data and previous literature, see (Forns, N. et. al., Biophysical Journal 2011) or (Chang et. al. Phys. Rev. 2013). With this in mind, while Reviewer 1 suggested also (iii) to test different linkers experimentally is interesting, though we think that there is plenty of literature already showing that linker stretching corrections are necessary and such an extension goes beyond the scope of this manuscript. Additionally, we predict for the suggested experiments its own layers of complexity. For instance, Protein L and Ig27 refold at higher forces than our observed λ_{wt} equilibrium (un)folding, hence λ_{wt} equilibrium kinetics would only be measured with folded Protein L / Ig27, yielding limited new insights. Yet, to gather new insights on the folding, we discuss now more detailed the analysis of Langevin dynamics including the linker effects. We added this as well to the main text.

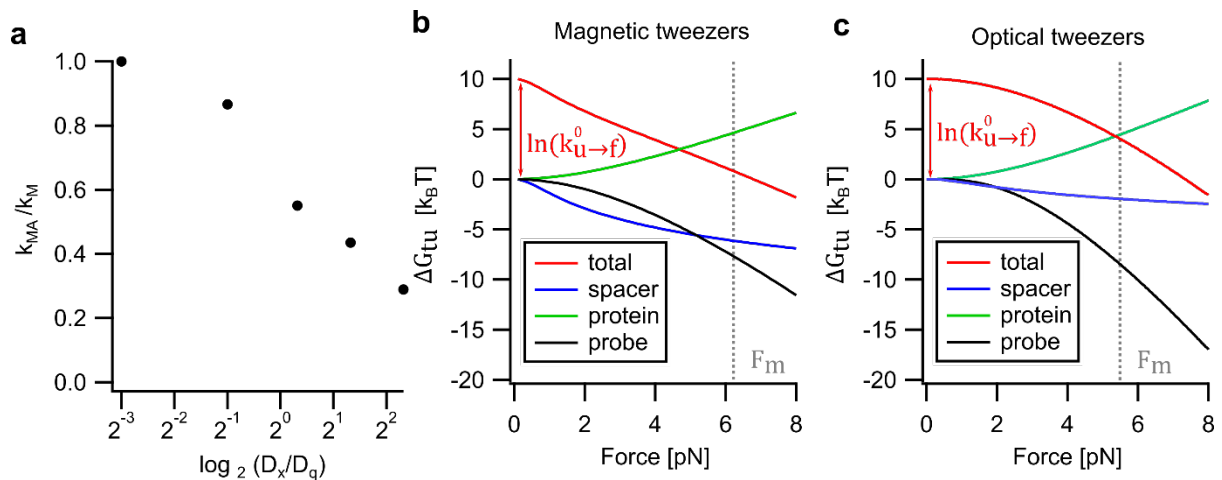


Figure 5. Contributions of each element in the kinetics of folding. To estimate the difference in kinetics between optical tweezers and magnetic tweezers we start with a Langevin dynamics simulation (a) for different apparatus diffusion coefficients (see Supplementary section 11). k_M represents the molecular rate and k_{MA} the observed rate using the same nomenclature as in ²⁰. On the other side, (b) and (c) shows entropic barrier contributions of each mechanical elements in our models for both devices (with $x_t^s = 0$), which modulates the force response of each instrument. We offset the sum of every contribution (labelled as total) by $\ln(k_{uf}(F=0))=10$ to resemble our data. In gray is labelled as well the mechanical midpoint (F_m) of each instrument which has a small change from 5.5 pN (in OT) to 6.2 pN (in MT).

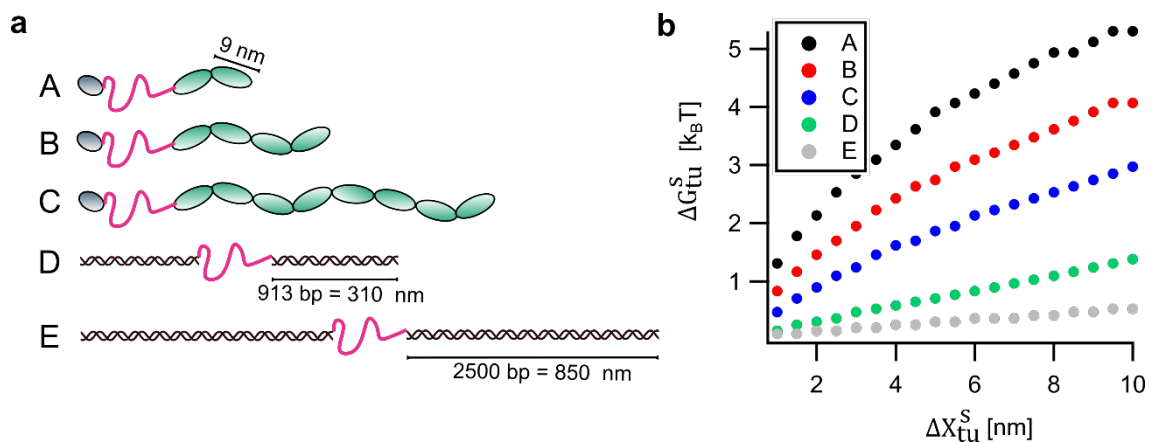


Figure S7. Entropic barrier for different spacers. (a) Cartoon models of different spacers configurations starting from the one we used in our magnetic tweezers setup (A), then with additional spy0128 domains (B and C), the DNA tether used in our optical tweezers setup (D), and a longer DNA tether (E). (b) Entropic barrier contributions of each model in (a) as a function of Δx_{tu}^S the distance in stretches when the protein is in the transition state.

Reviewer 1: 2) Have the authors considered that the different folding dynamics might simply arise from a different diffusion coefficient in each technique? This effective diffusion coefficient indeed owes to the combined linker+protein and the tethering system (the magnetic beads in MT and the polystyrene beads in the OT experiments have likely very different diffusing properties), so it is also probably dependent on the elasticity of the spacers. The authors assume that the prefactor in the Kramers rates is the same across all techniques, citing reference 28, where this quantity is measured using bulk experiments. However, I'm not fully convinced about this; this prefactor includes the diffusion coefficient and that can be very different depending on the way the folding pathway is probed. The authors should also consider this alternative explanation, and attempt to estimate the effective diffusion coefficient in their MT and OT experiments. For example, for AFM, it was demonstrated that the cantilever dominates the diffusive dynamics of proteins (Berkovich et al PNAS 2012), while in MT it seems that the diffusion coefficient of the bead is orders of magnitude larger than that of the measured folding dynamics (Tapia-Rojo et al Biophys J 2024). Optical tweezers experiments in proteins have generally reported higher diffusion coefficients than those in AFM and MT, which would naturally result in faster folding dynamics—see, for example, Yu et al PNAS 2012. This simpler, alternative explanation could also account for the different folding rates they report.

Response: Thank you for the very insightful suggestion. Indeed, we have taken the diffusion coefficients into consideration with Langevin dynamics simulations. We regret that this important point has not become clear in our manuscript and have emphasized this in the revision. In brief, to take diffusion into consideration we used Langevin dynamics simulations including the corresponding reported diffusion coefficients showing that the expected effect on kinetics (2-fold change) is minor compared to the experimentally observed change in kinetics (16-fold). We now discuss this discrepancy in the revised manuscript and have added Figure 5a. In particular we take into consideration earlier work by Cossio, Hummer & Szabo, PNAS 2015 and Sancho et. al., JPC B 2018. Overall, both entropic barrier change due to different energetic contributions as well as diffusion times contribute to changed kinetics. This might depend on each individual instrumentation and experimental design, though we offer in this manuscript a general approach to include specifics of the experimental design in a model and obtain an extrapolation going beyond the Bell-Evans model, allowing for comparison between different experimental approaches.

Reviewer 1: 3) I'm not fully convinced by the use of the FJC to model the elasticity of their spacer construct. They assume that the construct consists of rigid body-like structures, which corresponds to the Kuhn elements of the FJC, but they neglect the contribution of linkers connecting the proteins, which should be quite soft, and which therefore would dominate an arrangement of elastic elements in series. The effective persistence length (or Kuhn length) of the folded protein elements should be directly measurable with MT by stretching the construct over a range of forces that keeps the $\lambda 6$ -85 protein folded. From the unfolding force ramps in Fig. 1C, it seems to me that the folded construct can be stretched and behaves as a polymer of low persistence length—the polymer behaves pretty similarly before and after the $\lambda 6$ -85 protein unfolds in Fig. 1C. The authors could correct their model using parameters directly extracted from their experimental data by stretching the folded and unfolded construct.

Response: Thank you. We have now added supplementary section 10 which address the possibility of fine-tuning the spacer model considering short unstructured amino acids sequences in energetics (see Fig. S8). As the reviewer mentions, adding to the FJC model of the folded domains a few unstructured amino acids indeed increases the resemblance between model and data and we show that this addition changes energetics by $\sim 1 k_B T$. Albeit being a change in energy, we would like to point out that such a correction might be a small contribution compared to other sources of unknown deviation and error. We would like to point out that such an additional correction is prone to error by the lack of an absolute extension

measurements. With magnetic tweezers we are measuring relative extensions between the reference bead and the tracked bead rather than absolute extensions and a small deviation in the y-offset has a large impact in the fitting parameters. Hence, we think it is better to keep theoretical predictions given that they agree well with our constructs.

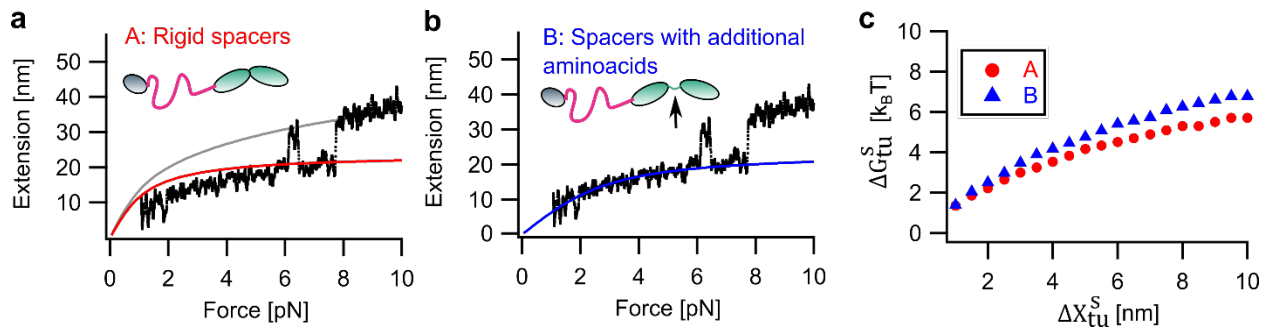


Figure S8. Effects of unstructured amino acids in the folding barrier. a) and b) show an exemplary force extension curve extracted from a force ramp. In grey (a) is depicted the hybrid polymer model used to describe the tether with unfolded λ_{wt} as described in supplementary section 3, which we used as a reference to offset in y the extension data. In red, is the function x_s without unstructured aminoacids and in blue, x_s accounting for 20 unstructured amino acids modeled with a WLC of contour length 7 nm. C) shows the contribution in the entropic barrier of each model as described in supplementary section 7.

Reviewer 1: 4) How well does their model predicts/explains non-equilibrium unfolding forces? The authors could do additional force-ramp experiments in MT (it seems they have these measurements for OT in their reference 27) and compare their unfolding force distributions. I would expect higher unfolding forces for MT—given the slower folding kinetics—which should be also explained by their model.

Response: We thank the reviewer for this new suggestion which we agree is very interesting for the field, but out of the scope of this paper. We are indeed now working on the interface between equilibrium and out-of-equilibrium force protocols which reveal further interesting possibilities of extracting transition rates.

Reviewer 1: 5) For clarity, I believe the manuscript would benefit from including some OT data to allow for direct comparison with the MT trajectories—for example in Fig. 3.

Response: Thank you for the great suggestion. We have included now comparative time traces as supplementary figure 5.

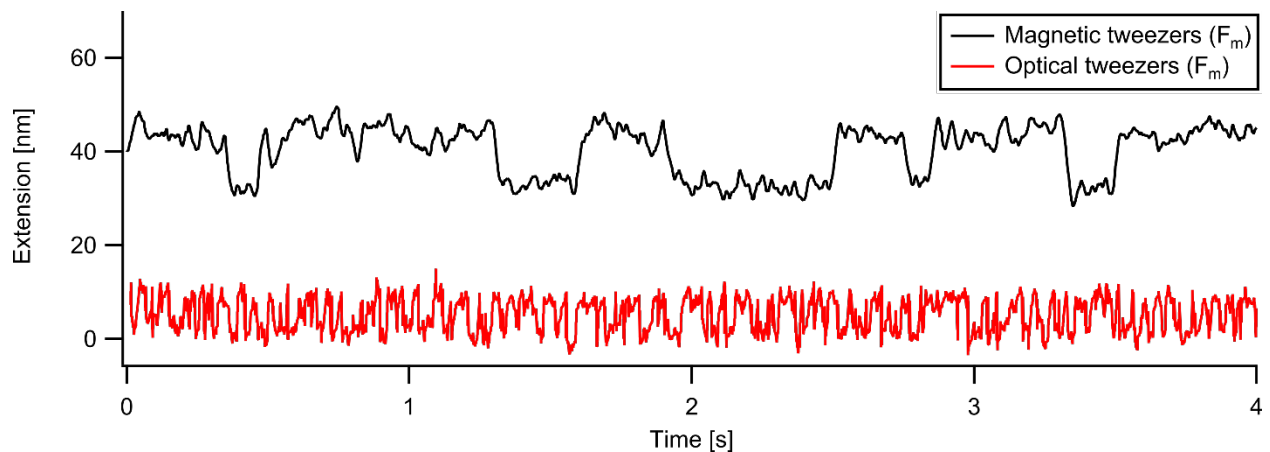


Figure S5. Side by side comparison of magnetic tweezers and optical tweezers data. Both datasets are taken from their respective mechanical midpoint, i.e. 6.2 pN for magnetic tweezers and 5.5 pN for optical tweezers. Both traces are excerpts from tens to hundreds of seconds long equilibrium transitions.

Reviewer 1: 6) Is there any predicted effect on the value of the coexistence force? I assume that, since it is an equilibrium parameter, it should be the same regardless of the measuring technique, but the authors could report and discuss this quantity as well in light of their model.

Response: Yes, we saw a small effect on the mechanical midpoint which we discuss now in the main text (pages 7 and 14). We saw as well this effect in Langevin dynamics simulations. Such an effect becomes intuitive if one considers Eqs. 4.1 and 4.2 from the main text.

Reviewer 2

Reviewer 2: The work by Quintana-Castaño et al investigates new methods for studying fast-folding proteins under mechanical force. In particular, the authors work with magnetic tweezers and λ repressor (6-85) to develop a model that considers the constant-force nature of the magnetic tweezers technique to make suitable for studying such fast-folding proteins akin optical tweezers. In this model the authors consider the contribution of the spacers and introduce an constant force field. In general, the authors obtain results comparable to that of OT upon extrapolation and can estimate the spacer and device contributions to the energy barrier. Overall, this is a good work and that unifies criteria of single-molecule techniques. The results are justified, and conclusions are appropriate. I recommend publication after addressing some concerns.

Response: We thank Reviewer 2 for the detailed comments, the insightful recommendations and positive feedback.

Reviewer 2: 1. I would advise the authors against direct comparison with thermal denaturation experiments, even upon extrapolation. Thermal stability operates considering molecular interactions that are different from those conferring mechanical stability. Also, in thermal denaturation the proteins are never fully extended rendering molecular species different than those observed in mechanical experiments. At least the authors should comment on these molecular differences to advice the reader.

Response: We agree with the reviewer that such a comparison is not straight forward and contains lots of ambiguities. We have added a note of caution to the main text (page 9).

Reviewer 2: 2. In figure 1c the authors show a slow-drop in force, but I wonder why they have not done a slow-increasing force to observe unfolding transitions. In fact, they observe repetitive steps in the slow-drop that I would assume may also happen in the slow-increase. Have the authors considered such scenario?

Response: We thank the reviewer for the close inspection of our data. When we reduce the loading rate for unfolding we also observe reversible kinetics. Yet, our typical experimental protocol reaches high forces rapidly, to allow for a quick estimation of extensions and a slow reduction of forces to quickly estimate the mechanical midpoint force. As pointed out for reviewer 1, in the future, we would like to explore a wide range of loading rates and compare out-of-equilibrium with equilibrium data.

Reviewer 2: 3. In figure 1b, between 320 and 360 s approx., the protein seems to be trapped in the unfolded state. Is this and instrumental artifact or a real phenomenon to be observed?

Response: We thank again the reviewer again for the insightful and close inspection of the data. This is indeed not an experimental artifact, but a kinetic trap that we managed to capture with the ultrastable magnetic tweezers device. Due to the limited scope of the manuscript, we have not extensively discussed this previously. We show now it is a kinetic trap in a new supplementary section 6. For this purpose, we reanalyzed all our data and discovered more of these rare events (5 out of ~3600 transitions) from which after a pause of several tens of seconds the molecule resumes its normal folding transitions. Most likely, these kinetics traps originate from proline isomerization as reported earlier for a different protein in (Rognoni et. al., PNAS 2014), which can prevent λ_{wt} from folding. We hypothesize about this observation in the revised manuscript, but do not explicitly test it due to the limited scope of this article.

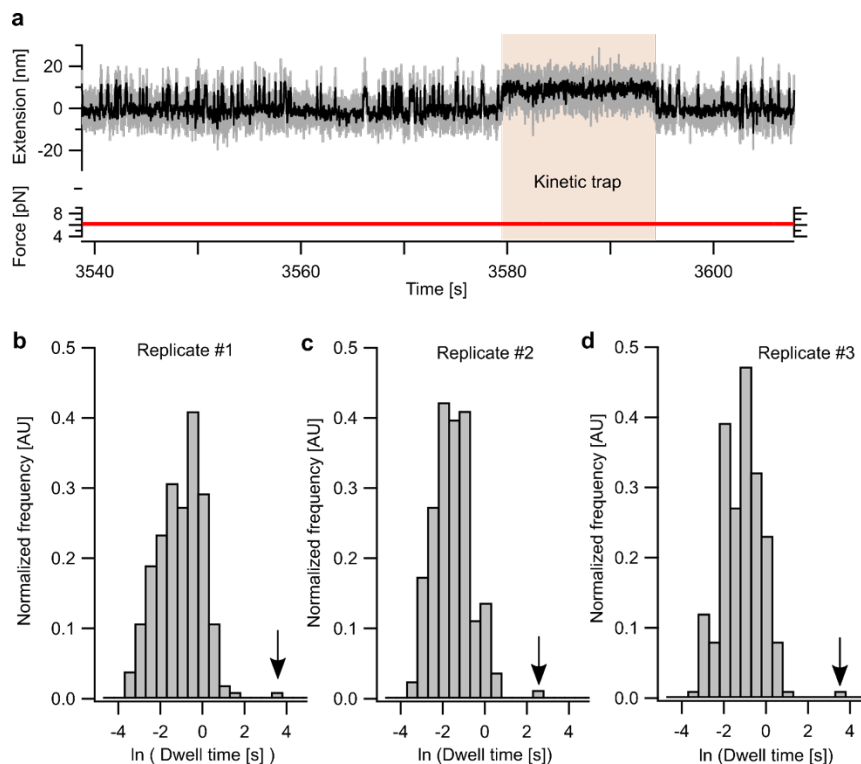


Figure S6. Long λ_{wt} recordings show rare kinetic traps. Kinetic traps could be identified as long dwell times of λ_{wt} unfolded state that do not resemble the ongoing kinetics. **a)** Exemplary kinetic trap where λ_{wt} and in the orange shaded area takes a long time to refold.

Long dwell times can be identified as isolated points far from our logarithmic dwell times distribution, as shown in **b**), **c**) and **d**) (black arrows) for three independent tethers.

Reviewer 2: 4. Would it be possible to use the same exact molecular construct (e.g., DNA handles) for both MT and OT for a more direct comparison? I am not suggesting that the authors should do it but discussing such possibility.

Response: Indeed, in principle one could use a very similar construct in MT as used in OT, albeit new surface chemistry approaches need to be developed. The effects of such a construct are now discussed in supplementary section 9. In brief, Figure S7a contains in model D the same molecular tether as that of our optical tweezers construct. Such a long, soft tether would indeed accelerate folding kinetics again, although it might increase also thermal noise and reduce potentially the resolution (Forns, N. et. al., Biophysical Journal 2011). This might limit its applicability for MT experiments.

Reviewer 3

Reviewer 3: The paper by Quntana-Catano et al. investigates the unexpected ability of a magnetic tweezers instrument to capture the folding behavior of a protein, despite a small bandwidth relative to the intrinsic timescales of folding (as estimated using other techniques). The authors carefully document the folding behavior of the protein, demonstrating it behaves as expected based on established thermodynamics of mechanical folding, and show extensive data supporting the measured kinetics. They then present an expanded Bell model (adapted from similar models applied to optical tweezer data) to account for the slowing, which includes terms associated with the free energy of stretching the spacer and protein, and the work associated with moving the probe. The result is that the effective activation barrier for folding is raised dramatically relative to the zero force case, apparently (per the model) due to the extra work involved in moving the probe and stretching the spacers. Generally, the article was a pleasure to read. The results are carefully documented and the argument was clear throughout. The results should be of interest to the community. A few comments:

Response: We thank Reviewer 3 for the positive comments and the insightful recommendations.

Reviewer 3: 1) I felt the discussion of the results of the model was a bit lacking. Specifically, the most interesting part of the paper for me was the discussion on page 13 presenting how the activation barrier changed for the MT versus OT geometries. These differences were presented very minimally, with just the numbers for each contribution listed. I would love to see an expansion of this discussion—particularly, one key difference appears to be regarding the type of spacer used, where the OT requires long dsDNA tethers that do not contribute heavily to the raised activation barrier, whereas the MT uses small folded proteins that do contribute heavily to the activation barrier. This is interesting, and of course not really a function of the type of instrument but more to the biochemistry of tethering. This implies that, to achieve the desired kinetic slowing, it is best to use short folded protein spacers. More discussion of this would be interesting, e.g. about what would happen if a more flexible spacer was used in the MT, or whether OT can use short folded proteins. A second key MT/OT difference contributing to the extra activation barrier for the MT is the work done in moving the probe (apparently much larger for the MT than OT, 9.1 vs 5.7 kT). This is more directly tied to the instrument than the choice of spacer, but I don't quite know why it happens; comments about this would be helpful.

Response: Thank you for the enthusiastic comment. We have added a more detailed discussion and a new figure to the revised manuscript (Fig. 5). There, we discuss indeed different “biochemical” tether forms and their respective effects on imposed additional barriers during protein (un)folding experiments. We also discuss your second observation in great detail in the revised manuscript.

Reviewer 3: 2) As with many recent protein-manipulation papers using voice-coil MTs, the authors calibrate the instrument w.r.t. the folding of a model protein. I have reviewed multiple such papers over the years, and I still am confused as to why these papers never benchmark this calibration scheme in comparison to analysis of thermal fluctuations of the probe particle—the advantage of analysis of thermal fluctuations is that it is a ‘pure physics’ approach, completely independent of the nature of the biomolecular tether, and so a truly precise benchmark. One worries that any given prep of a protein can have chemical modifications that alter the folding behavior, so protein-folding benchmarking seems less precise. Further it depends on various parameter choices (persistence length and so on).

Response: We thank the reviewer for the appreciation of our calibration in magnetic tweezers. The reason for us to not think of such a comparison was that with time and experience handling the instrument it became quickly evident for us that a thermal calibration of our short tethers (<50 nm tethers) was far from accurate. Stokes description of frictional forces assumed that the microsphere is infinitively deep in solution, which would not be the case for our experimental design (microsphere diameter 1-2 μm ; distance from surface 10s of nanometers). Thermal fluctuation induced motion of a microsphere close to the surface are affected by hydrodynamic effects. Such effects can be partially corrected using Faxén’s law (as discussed for instance for optical tweezers close to the surface here <https://doi.org/10.1063/1.1645654>). Yet, such corrections depend on the absolute distance to the surface. To avoid too many uncertainties in corrections, we decided to follow the established procedure to use a known protein as reference. Notably, the biochemical production of the protein is independent in different laboratories, yet the calibration parameters agree very well between the laboratories, suggesting within error a reliable method of force calibration for short tethers. We have added this explanation to the Supplementary document.

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Response: We thank the reviewer for the helpful feedback. There are some advantages on keeping the linker free model in the main text: 1) It gives a lower bound estimation of estimated parameters. 2) It promotes researchers in the field to test for different possible models and gives a sense of the striking differences that arise from changing the model. We indeed learned a lot from testing different models. 3) It is illustrative to show the 4-orders of magnitude deviation in extrapolation. Given these reasons, we would prefer to keep the “incorrect” model in the main text also as a resource for future researchers.

This document contains:

- 1. Rebuttal letter.** Response to reviewers submitted on the 31st of October of 2024.
- 2. Response of the referees to rebuttal letter.** Received on the 2nd of December, 2024.

1. Rebuttal letter.

We thank the editor and the reviewers for their work, taking the time to read our manuscript and for their constructive feedback. Below we address point-by-point the reviewers' comments.

Reviewer 1

Reviewer 1: In this manuscript, Quintana-Cataño et al use magnetic tweezers (MT) to measure the reversible folding dynamics of the fast-folding λ 6-85 protein. The authors previously measured the dynamics of this protein using optical tweezers (OT) and, intriguingly, they report that the folding dynamics measured with MT are substantially slower. To rationalize this discrepancy, they develop an analysis framework to account for the contribution of the molecular construct (and optical trap) which allows them to interpret the energetics of these transitions when captured through different experimental techniques. They reason that MT alters the transition kinetics by increasing the entropic barrier, owing to the stiff protein linker and the intrinsic force clamp conditions, resulting in slowed down kinetics. Overall, I find their study sound from a technical viewpoint and their interpretation of the results plausible. However, I believe that, while they offer a possible explanation for the observed discrepancy, further controls should be done to convincingly demonstrate that such different folding dynamics arise indeed from the effect of the molecular spacer. More specifically:

Response: We thank Reviewer 1 for the positive view on our manuscript. We are commenting below in detail on the helpful suggestions.

Reviewer 1: 1) The model the authors propose accounts for the elastic nature of their protein construct in the MT experiments, consisting of two repeats of the inextensible Spy0128 protein, plus the HaloTag, which are treated as rigid bodies, using the FJC to model the elasticity of the full protein. Presumably, the specifics of this construct result in the increased entropic barrier that slows down the folding kinetics. Therefore, their model could be used to predict how different molecular constructs impact the folding kinetics of the same protein. Ideally, using a DNA linker akin to that employed in the OT experiments should accelerate these kinetics, approaching the MT measurements to the OT ones—although it remains unclear to me what is the relative effect of the optical trap versus the spacer. I understand that this suggestion is technically challenging, as MT experiments require short tethers. However, the authors could change the molecular construct to some other protein linker that can be unfolded, therefore dynamically reducing the stiffness of the spacer protein. For example, using eight repeats of protein L or Ig27 as spacer, which are commonly used proteins in MT experiments, the authors could start measuring the folding dynamics of their λ 6-85 protein (which should resemble those measured with the Spy0128 linker), and then unfold all eight protein spacers, to measure again the dynamics of the λ 6-85 protein. Extracting the (un)folding rates before and after unfolding the spacers and fitting the data to their theory would convincingly demonstrate that the energy landscape of the folding protein is indeed altered by the entropic elasticity of the linker proteins.

Response: We appreciate the interest in the details of our underlying model to extrapolate the measured kinetics to different potential forces. We would like to address the three points:

(i) prediction of the behavior with other linkers, (ii) clarification of OT contribution to the energetic barrier, and (iii) experimental verification of linker effects. To address point (i) and (ii), we have thoroughly revised our manuscript and supporting information. In particular, we introduce in the main text Figure 5, which illustrates energetic contributions for magnetic and optical tweezers. In brief, assuming the typical experimental conditions of spacers as in our experiments for $\lambda 6-85$, the energetic contributions arising from the spacers dominate the overall energetic barrier formation. We discuss the new figure in detail in the manuscript and in the supplementary information (see Figure S7 and S9). In the supplementary section 9, we present the effects of different types of spacers, e.g. DNA handles or long unstructured polypeptide, as suggested by the reviewer. Overall, our predictions agree well with data and previous literature, see (Forns, N. et. al., Biophysical Journal 2011) or (Chang et. al. Phys. Rev. 2013). With this in mind, while Reviewer 1 suggested also (iii) to test different linkers experimentally is interesting, though we think that there is plenty of literature already showing that linker stretching corrections are necessary and such an extension goes beyond the scope of this manuscript. Additionally, we predict for the suggested experiments its own layers of complexity. For instance, Protein L and Ig27 refold at higher forces than our observed λ_{wt} equilibrium (un)folding, hence λ_{wt} equilibrium kinetics would only be measured with folded Protein L / Ig27, yielding limited new insights. Yet, to gather new insights on the folding, we discuss now more detailed the analysis of Langevin dynamics including the linker effects. We added this as well to the main text.

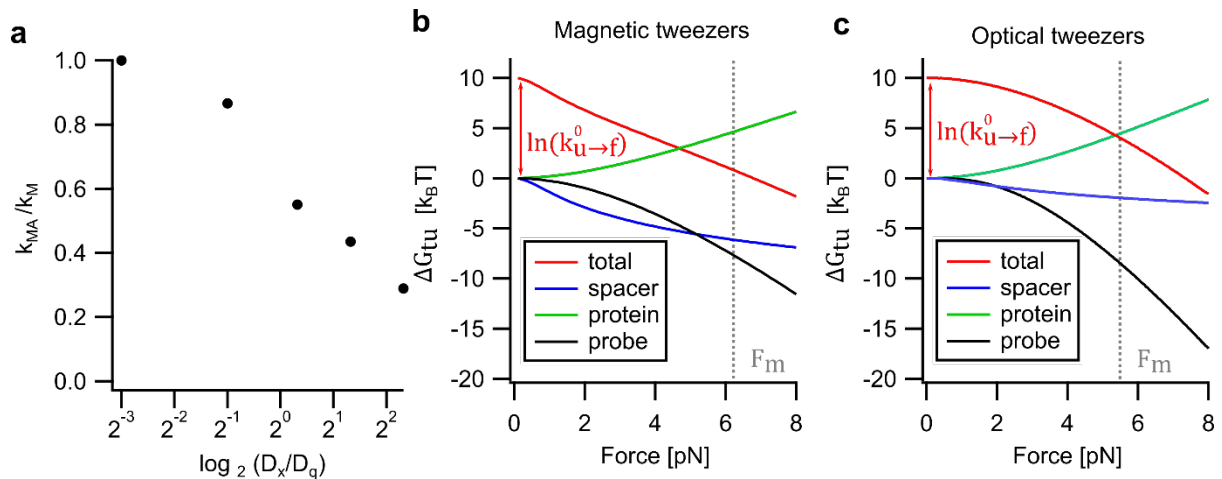


Figure 5. Contributions of each element in the kinetics of folding. To estimate the difference in kinetics between optical tweezers and magnetic tweezers we start with a Langevin dynamics simulation (a) for different apparatus diffusion coefficients (see Supplementary section 11). k_M represents the molecular rate and k_{MA} the observed rate using the same nomenclature as in ²⁰. On the other side, (b) and (c) shows entropic barrier contributions of each mechanical elements in our models for both devices (with $x_i^s = 0$), which modulates the force response of each instrument. We offset the sum of every contribution (labelled as total) by $\ln(k_{uf}(F=0))=10$ to resemble our data. In gray is labelled as well the mechanical midpoint (F_m) of each instrument which has a small change from 5.5 pN (in OT) to 6.2 pN (in MT).

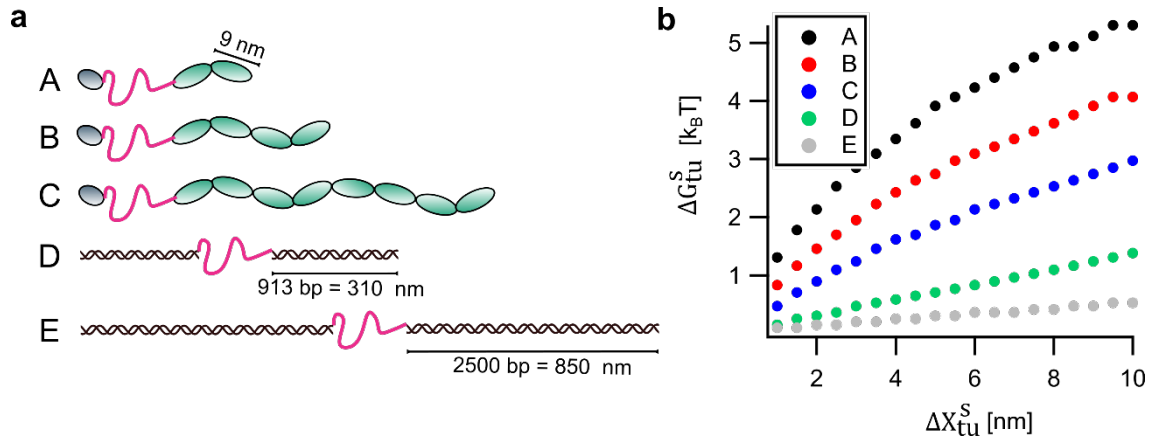


Figure S7. Entropic barrier for different spacers. **a)** Cartoon models of different spacers configurations starting from the one we used in our magnetic tweezers setup (A), then with additional spy0128 domains (B and C), the DNA tether used in our optical tweezers setup (D), and a longer DNA tether (E). **b)** Entropic barrier contributions of each model in **a)** as a function of Δx_{ttu}^S , the distance in stretches when the protein is in the transition state.

Reviewer 1: 2) Have the authors considered that the different folding dynamics might simply arise from a different diffusion coefficient in each technique? This effective diffusion coefficient indeed owes to the combined linker+protein and the tethering system (the magnetic beads in MT and the polystyrene beads in the OT experiments have likely very different diffusing properties), so it is also probably dependent on the elasticity of the spacers. The authors assume that the prefactor in the Kramers rates is the same across all techniques, citing reference 28, where this quantity is measured using bulk experiments. However, I'm not fully convinced about this; this prefactor includes the diffusion coefficient and that can be very different depending on the way the folding pathway is probed. The authors should also consider this alternative explanation, and attempt to estimate the effective diffusion coefficient in their MT and OT experiments. For example, for AFM, it was demonstrated that the cantilever dominates the diffusive dynamics of proteins (Berkovich et al PNAS 2012), while in MT it seems that the diffusion coefficient of the bead is orders of magnitude larger than that of the measured folding dynamics (Tapia-Rojas et al Biophys J 2024). Optical tweezers experiments in proteins have generally reported higher diffusion coefficients than those in AFM and MT, which would naturally result in faster folding dynamics—see, for example, Yu et al PNAS 2012. This simpler, alternative explanation could also account for the different folding rates they report.

Response: Thank you for the very insightful suggestion. Indeed, we have taken the diffusion coefficients into consideration with Langevin dynamics simulations. We regret that this important point has not become clear in our manuscript and have emphasized this in the revision. In brief, to take diffusion into consideration we used Langevin dynamics simulations including the corresponding reported diffusion coefficients showing that the expected effect on kinetics (2-fold change) is minor compared to the experimentally observed change in kinetics (16-fold). We now discuss this discrepancy in the revised manuscript and have added Figure 5a. In particular we take into consideration earlier work by Cossio, Hummer & Szabo, PNAS 2015 and Sancho et. al., JPC B 2018. Overall, both entropic barrier change due to different energetic contributions as well as diffusion times contribute to changed kinetics. This might depend on each individual instrumentation and experimental design, though we offer in this manuscript a general approach to include specifics of the experimental design in a model and obtain an extrapolation going beyond the Bell-Evans model, allowing for comparison between different experimental approaches.

Reviewer 1: 3) I'm not fully convinced by the use of the FJC to model the elasticity of their spacer construct. They assume that the construct consists of rigid body-like structures, which corresponds to the Kuhn elements of the FJC, but they neglect the contribution of linkers

connecting the proteins, which should be quite soft, and which therefore would dominate an arrangement of elastic elements in series. The effective persistence length (or Kuhn length) of the folded protein elements should be directly measurable with MT by stretching the construct over a range of forces that keeps the $\lambda 6$ -85 protein folded. From the unfolding force ramps in Fig. 1C, it seems to me that the folded construct can be stretched and behaves as a polymer of low persistence length—the polymer behaves pretty similarly before and after the $\lambda 6$ -85 protein unfolds in Fig. 1C. The authors could correct their model using parameters directly extracted from their experimental data by stretching the folded and unfolded construct.

Response: Thank you. We have now added supplementary section 10 which address the possibility of fine-tuning the spacer model considering short unstructured amino acids sequences in energetics (see Fig. S8). As the reviewer mentions, adding to the FJC model of the folded domains a few unstructured amino acids indeed increases the resemblance between model and data and we show that this addition changes energetics by $\sim 1 k_B T$. Albeit being a change in energy, we would like to point out that such a correction might be a small contribution compared to other sources of unknown deviation and error. We would like to point out that such an additional correction is prone to error by the lack of an absolute extension measurements. With magnetic tweezers we are measuring relative extensions between the reference bead and the tracked bead rather than absolute extensions and a small deviation in the y-offset has a large impact in the fitting parameters. Hence, we think it is better to keep theoretical predictions given that they agree well with our constructs.

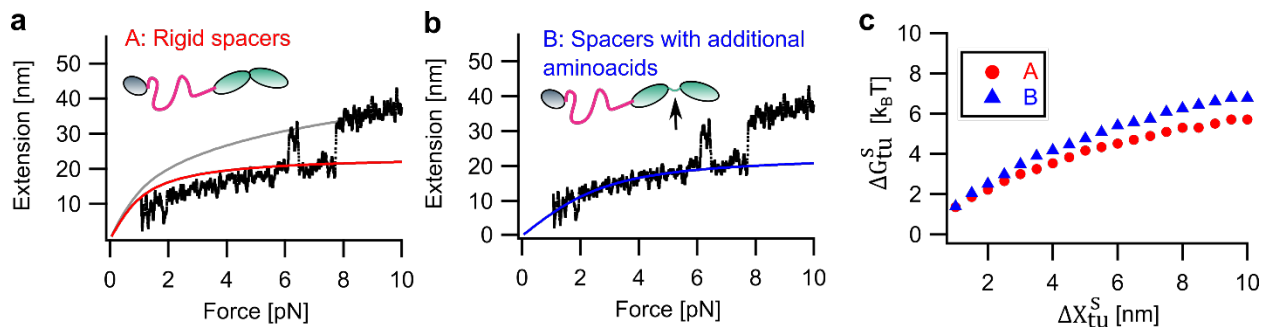


Figure S8. Effects of unstructured amino acids in the folding barrier. a) and b) show an exemplary force extension curve extracted from a force ramp. In grey (a) is depicted the hybrid polymer model used to describe the tether with unfolded λ_{wt} as described in supplementary section 3, which we used as a reference to offset in y the extension data. In red, is the function x_s without unstructured aminoacids and in blue, x_s accounting for 20 unstructured amino acids modeled with a WLC of contour length 7 nm. C) shows the contribution in the entropic barrier of each model as described in supplementary section 7.

Reviewer 1: 4) How well does their model predicts/explains non-equilibrium unfolding forces? The authors could do additional force-ramp experiments in MT (it seems they have these measurements for OT in their reference 27) and compare their unfolding force distributions. I would expect higher unfolding forces for MT—given the slower folding kinetics—which should be also explained by their model.

Response: We thank the reviewer for this new suggestion which we agree is very interesting for the field, but out of the scope of this paper. We are indeed now working on the interface between equilibrium and out-of-equilibrium force protocols which reveal further interesting possibilities of extracting transition rates.

Reviewer 1: 5) For clarity, I believe the manuscript would benefit from including some OT data to allow for direct comparison with the MT trajectories—for example in Fig. 3.

Response: Thank you for the great suggestion. We have included now comparative time traces as supplementary figure 5.

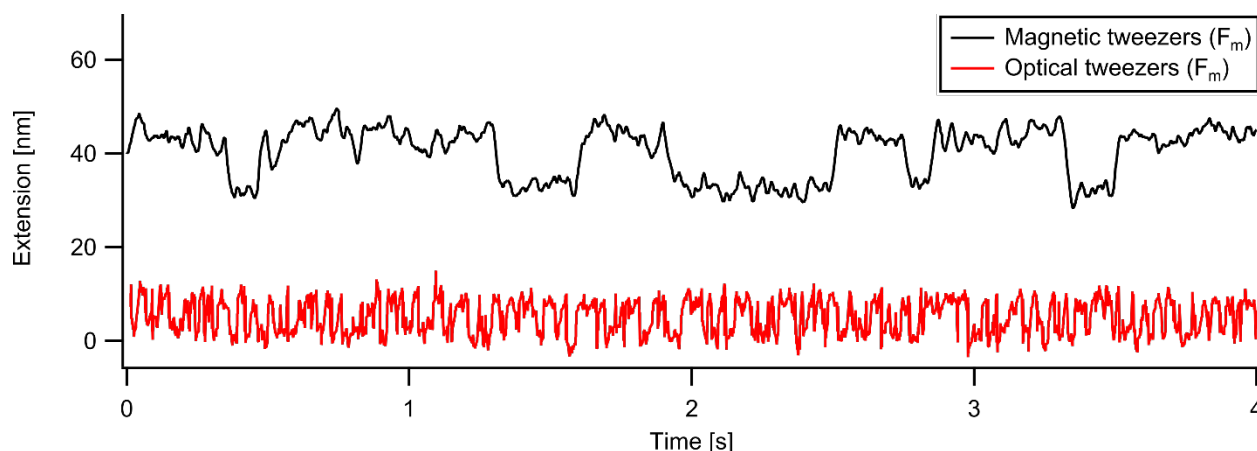


Figure S5. Side by side comparison of magnetic tweezers and optical tweezers data. Both datasets are taken from their respective mechanical midpoint, i.e. 6.2 pN for magnetic tweezers and 5.5 pN for optical tweezers. Both traces are excerpts from tens to hundreds of seconds long equilibrium transitions.

Reviewer 1: 6) Is there any predicted effect on the value of the coexistence force? I assume that, since it is an equilibrium parameter, it should be the same regardless of the measuring technique, but the authors could report and discuss this quantity as well in light of their model.

Response: Yes, we saw a small effect on the mechanical midpoint which we discuss now in the main text (pages 7 and 14). We saw as well this effect in Langevin dynamics simulations. Such an effect becomes intuitive if one considers Eqs. 4.1 and 4.2 from the main text.

Reviewer 2

Reviewer 2: The work by Quintana-Castaño et al investigates new methods for studying fast-folding proteins under mechanical force. In particular, the authors work with magnetic tweezers and λ repressor (6-85) to develop a model that considers the constant-force nature of the magnetic tweezers technique to make suitable for studying such fast-folding proteins akin optical tweezers. In this model the authors consider the contribution of the spacers and introduce an constant force field. In general, the authors obtain results comparable to that of OT upon extrapolation and can estimate the spacer and device contributions to the energy barrier. Overall, this is a good work and that unifies criteria of single-molecule techniques. The results are justified, and conclusions are appropriate. I recommend publication after addressing some concerns.

Response: We thank Reviewer 2 for the detailed comments, the insightful recommendations and positive feedback.

Reviewer 2: 1. I would advise the authors against direct comparison with thermal denaturation experiments, even upon extrapolation. Thermal stability operates considering molecular interactions that are different from those conferring mechanical stability. Also, in thermal denaturation the proteins are never fully extended rendering molecular species different than

those observed in mechanical experiments. At least the authors should comment on these molecular differences to advice the reader.

Response: We agree with the reviewer that such a comparison is not straight forward and contains lots of ambiguities. We have added a note of caution to the main text (page 9).

Reviewer 2: 2. In figure 1c the authors show a slow-drop in force, but I wonder why they have not done a slow-increasing force to observe unfolding transitions. In fact, they observe repetitive steps in the slow-drop that I would assume may also happen in the slow-increase. Have the authors considered such scenario?

Response: We thank the reviewer for the close inspection of our data. When we reduce the loading rate for unfolding we also observe reversible kinetics. Yet, our typical experimental protocol reaches high forces rapidly, to allow for a quick estimation of extensions and a slow reduction of forces to quickly estimate the mechanical midpoint force. As pointed out for reviewer 1, in the future, we would like to explore a wide range of loading rates and compare out-of-equilibrium with equilibrium data.

Reviewer 2: 3. In figure 1b, between 320 and 360 s approx., the protein seems to be trapped in the unfolded state. Is this an instrumental artifact or a real phenomenon to be observed?

Response: We thank again the reviewer again for the insightful and close inspection of the data. This is indeed not an experimental artifact, but a kinetic trap that we managed to capture with the ultrastable magnetic tweezers device. Due to the limited scope of the manuscript, we have not extensively discussed this previously. We show now it is a kinetic trap in a new supplementary section 6. For this purpose, we reanalyzed all our data and discovered more of these rare events (5 out of ~3600 transitions) from which after a pause of several tens of seconds the molecule resumes its normal folding transitions. Most likely, these kinetics traps originate from proline isomerization as reported earlier for a different protein in (Rognoni et. al., PNAS 2014), which can prevent λ_{wt} from folding. We hypothesize about this observation in the revised manuscript, but do not explicitly test it due to the limited scope of this article.

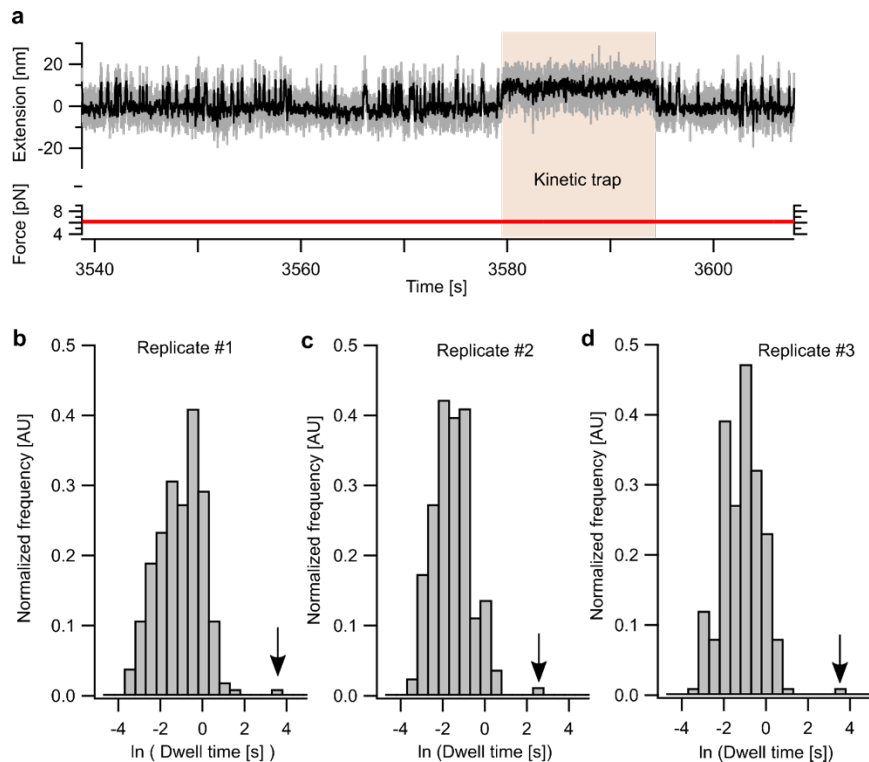


Figure S6. Long λ_{wt} recordings show rare kinetic traps. Kinetic traps could be identified as long dwell times of λ_{wt} unfolded state that do not resemble the ongoing kinetics. **a)** Exemplary kinetic trap where λ_{wt} and in the orange shaded area takes a long time to refold. Long dwell times can be identified as isolated points far from our logarithmic dwell times distribution, as shown in **b)**, **c)** and **d)** (black arrows) for three independent tethers.

Reviewer 2: 4. Would it be possible to use the same exact molecular construct (e.g., DNA handles) for both MT and OT for a more direct comparison? I am not suggesting that the authors should do it but discussing such possibility.

Response: Indeed, in principle one could use a very similar construct in MT as used in OT, albeit new surface chemistry approaches need to be developed. The effects of such a construct are now discussed in supplementary section 9. In brief, Figure S7a contains in model D the same molecular tether as that of our optical tweezers construct. Such a long, soft tether would indeed accelerate folding kinetics again, although it might increase also thermal noise and reduce potentially the resolution (Forns, N. et. al., Biophysical Journal 2011). This might limit its applicability for MT experiments.

Reviewer 3

Reviewer 3: The paper by Quntana-Catano et al. investigates the unexpected ability of a magnetic tweezers instrument to capture the folding behavior of a protein, despite a small bandwidth relative to the intrinsic timescales of folding (as estimated using other techniques). The authors carefully document the folding behavior of the protein, demonstrating it behaves as expected based on established thermodynamics of mechanical folding, and show extensive data supporting the measured kinetics. They then present an expanded Bell model (adapted from similar models applied to optical tweezer data) to account for the slowing, which includes terms associated with the free energy of stretching the spacer and protein, and the work associated with moving the probe. The result is that the effective activation barrier for folding is raised dramatically relative to the zero force case, apparently (per the model) due to the

extra work involved in moving the probe and stretching the spacers. Generally, the article was a pleasure to read. The results are carefully documented and the argument was clear throughout. The results should be of interest to the community. A few comments:

Response: We thank Reviewer 3 for the positive comments and the insightful recommendations.

Reviewer 3: 1) I felt the discussion of the results of the model was a bit lacking. Specifically, the most interesting part of the paper for me was the discussion on page 13 presenting how the activation barrier changed for the MT versus OT geometries. These differences were presented very minimally, with just the numbers for each contribution listed. I would love to see an expansion of this discussion—particularly, one key difference appears to be regarding the type of spacer used, where the OT requires long dsDNA tethers that do not contribute heavily to the raised activation barrier, whereas the MT uses small folded proteins that do contribute heavily to the activation barrier. This is interesting, and of course not really a function of the type of instrument but more to the biochemistry of tethering. This implies that, to achieve the desired kinetic slowing, it is best to use short folded protein spacers. More discussion of this would be interesting, e.g. about what would happen if a more flexible spacer was used in the MT, or whether OT can use short folded proteins. A second key MT/OT difference contributing to the extra activation barrier for the MT is the work done in moving the probe (apparently much larger for the MT than OT, 9.1 vs 5.7 kT). This is more directly tied to the instrument than the choice of spacer, but I don't quite know why it happens; comments about this would be helpful.

Response: Thank you for the enthusiastic comment. We have added a more detailed discussion and a new figure to the revised manuscript (Fig. 5). There, we discuss indeed different “biochemical” tether forms and their respective effects on imposed additional barriers during protein (un)folding experiments. We also discuss your second observation in great detail in the revised manuscript.

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2. Response of the referees to rebuttal letter.

Reviewer #1 (Remarks to the Author):

The authors have convincingly addressed my concerns. Still, I believe that repeating some of their experiments with other extensible protein spacers to compare the folding/unfolding rates in magnetic tweezers would have strengthened their case (which at the moment it's a theoretical prediction), but I understand that it can be considered to be out of scope. The manuscript is therefore suitable for publication.

Response: We thank the reviewer for the positive evaluation of our revised manuscript. We take up the reviewer's suggestion of a future study with variable spacers.

Reviewer #3 (Remarks to the Author):

The author's responses to my comments about the main topic of the paper are extremely convincing; I appreciate the additional detail on page 13, and the new Figure 5.

I could quibble a bit about their comments about thermal calibration-- in a properly done experiment, one does not need to know the dissipation ('frictional forces') a priori; instead the dissipation (along with the spring constant) can be found from the analysis of thermal fluctuations. Thus their comments about needing to, e.g., apply the Faxen correction are not really relevant.

But this is really just a minor quibble; I appreciate their response, and this issue does not affect the experiment in a significant way.

I am in favor of publication of the revised manuscript.

Response: We thank the reviewer for the positive evaluation of our revised manuscript. We take up the reviewer's suggestion to look into thermal calibration for the very short tethers in future studies.