Supporting Information

For

Interplay between viscoelasticity and force-rate affects sequential unfolding in polyproteins pulled at constant velocity

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1. Brownian dynamics (BD) simulations

Based on the procedure given in ref. [1], we performed BD simulations for a polyprotein comprised of a short linker and 8 folded domains in line with a cantilever. The evolution of the time dependent position for each one of the eight protein domains in the polyprotein is given by their length $x_{p,N}$, where the index, $N = 1 – 8$, denotes the domain number:

$$\dot{x}_{p,N} = \frac{D}{k_BT} \left[ F_{usc}(x_{p,N}) + F_p(x_{p,N}) + \Gamma(t) + K_S x_T \right],$$

where $D = 1500 \text{ nm/s}$ is the value of the diffusion coefficient taken here, representing the drag inflicted by the viscous hydrodynamic forces acting on the cantilever tip, $k_B = 1.38 \times 10^{-2} \text{ pN nm/K}$ is Boltzmann's constant, $T = 300 \text{ K}$ is the temperature, and $K_S = 25 \text{ pN/nm}$ taken as the spring constant of the cantilever.

The thermal random force resulting from the fluctuations of each element with its surroundings, $\Gamma(t)$, is described by the fluctuation-dissipation theorem, with $\langle \Gamma(t) \rangle = 0$, and $\langle \Gamma(t)\Gamma(t + \Delta t) \rangle = (2/D)(k_BT)^2 \delta(\Delta t)$, where $\delta(\Delta t)$ is a delta function. The contribution of the linker between the protein domains is accounted by the time evolution of its length, $x_l$:

$$\dot{x}_l = \frac{D}{k_BT} \left[ F_l(x_l) + \Gamma(t) + K_S x_T \right].$$

For a polyprotein of eight domains and a linker, the eight equations given by eq. (S1) and another by eq. (S2), are coupled by a constant velocity constraint:

$$x_T = Vt - x_l + x_{l,0} - \sum_{N=1}^{8} \left( x_{p,N} - x_{p,0} \right),$$

where $V [\text{ nm/s}]$ is the pulling velocity, $t [\text{ s}]$ is the time, $x_{l,0} = 0 \text{ [nm]}$ is the initial extension of the linker, and $x_{p,0} = R_c \text{ [nm]}$ is the size of a folded protein domain. The polymeric elastic extensibility forces experienced by the linker ($l$), and each one of the protein domains ($p$) (once unfolding occurred), are approximated by the worm like chain (WLC) interpolation formula,$^5, 6$

$$F_{usc}^k(x_k) = \frac{k_BT}{l_p} \left[ \frac{1}{4} \left( 1 - \frac{x_k}{L_{c,k}} \right)^2 - \frac{1}{4} + \frac{x_k}{L_{c,k}} \right].$$
Here $l_0 \sim 0.4$ nm is taken as the persistence length of the polypeptide polymeric linker, $L_{C,0} = 14$ nm is the contour length of the linker, and $L_{C,l} = 28$ nm is the contour length of the unfolded protein domain. Finally, the forces required to unfold each one of the protein domains are approximated with

$$F_p(x_{p,N}) = \nabla_x U_p = -4U_0e^{-\frac{b}{R_c}(x_{p,N}-R_c)} - e^{-\frac{b}{R_c}(x_{p,N}-R_c)} \left[ 1 - e^{-\frac{2b}{R_c}} \right]$$

where $U_p$ is the one-dimensional representation of a protein domain energy profile with $U_0 = 115$ pN nm $\sim 28$ kT is the free energy barrier height of a folded domain, $R_c = 4$ nm, is the size of a folded protein domain, and $b = 12$, is a curvature parameter in the folded domain potential. The total produces the recorded force restored by the polyprotein molecule:

$$F_T = K_S x_T$$

The time interval taken was $\Delta t = 2 \times 10^{-7}$ s. The solution of the expressions above guaranteed that there is no particular hierarchy in the unfolding events, such that when the domains are similar (as the case here), the unfolding sequence will be random.  

BD simulations were performed as specified above with pulling velocities varying between 40 – 4,000 nm/s, spanning over three velocity decades that are still within characteristic experimental range. $n = 100$ traces were collected for each pulling velocity. Figure S1 shows three exemplary BD simulation FX traces, each comprised of eight "unfolding" events, at three different velocities, $V = 40, 400,$ and $4,000$ nm/s colored with light blue, purple, and black, respectively. This color coding will remain with the all the relevant calculations and parameters concerning these velocities throughout the manuscript.
Figure S1. BD simulations of polyprotein unfolding. Three exemplary BD simulated FX traces of eight unfolding events at constant velocities, \( V = 40 \) (light blue), 400 (purple) and 4,000 (black) nm/s.

2. **Unfolding forces and stiffness from BD simulations**

We collected the maximal (unfolding) forces form all the traces for each event, and calculated their probability distribution functions (pdfs). Figure S2A shows the force pdfs at the pulling velocities for the 1\textsuperscript{st}, 5\textsuperscript{th} and 8\textsuperscript{th} events, from which the first and second moments are evaluated. Figure S2B shows similar distributions calculated from poly-\((I91)\) unfolding at \( V = 50, 100 \) and 1,000 nm/s for the 1\textsuperscript{st}, 3\textsuperscript{rd} and 7\textsuperscript{th} unfolding events.
Figure S2. Unfolding probabilities calculated from A. BD simulations, and B. Experimental measurements of poly-(I91). The pdfs are fitted with Gaussians.

The mean and standard deviations of the simulated unfolding forces are shown in Fig. S3A for the three representative pulling velocities. Consistent with the experimental data shown in Fig. 1B in the main text, the unfolding forces increase with event number (and with pulling velocity). Figure 3B shows the changes of stiffness with unfolding events. The stiffness values were estimated in as described in the main text, by taking the slope of the force with respect to the end-to-end extension in the linear regime before every unfolding event (as shown in red in Fig. S4A), and averaging these values with respect to the event number. Additionally, we estimated $K_{\text{eff}}$ using eq. (2) from the main text with respect to the parameters obtained from the simulations. Here, as with the experimental data shown in Fig. 1C in the
main text, the stiffness decrease with every consecutive unfolding event, where, in general, $K_{\text{eff}}$ underestimates $\langle dF/dx \rangle$ at high velocities and overestimate it at the low velocity, for $N > 4$. The stiffness obtained from the BD simulation is extremely high for relatively short linkers ($N < 4$), and particularly high before the 1st unfolding event. These values are very different from the ones observed experimentally, and result in part from the chosen functional form and parameters of the folded domains, and the short linker assumed in the BD simulations. Also, the experimental system experiences hydrodynamic drag effects that are highly prominent in the near vicinity of the surface to which the molecule is anchored 8-10 (and were not accounted for in the simulations). Comparing the behavior of $\langle dF/dx \rangle$ obtained from BD simulations to the one measured from poly-(I91) pulling experiments (Fig. 1C in the main text), a similar behavior is observed for $N > 3$, with the increase of the distance from the surface. Therefore, for the following, in our analysis of the BD simulations, we will disregard the 1st event.

![Figure S3](image)

**Figure S3.** Mean unfolding forces and stiffness with unfolding events from BD simulations. A. Maximal mean unfolding forces as a function of unfolding event number calculated at $V = 40$ (light blue blank circles), 400 (purple blank squares) and 4,000 (black blank triangles) nm/s. B. Mean stiffness of the linker as a function of chain length (given by the domain number), calculated by the slopes prior to unfolding events at $V = 40$ (light blue blank circles), 400 (purple blank squares) and 4,000 (black blank triangles) nm/s. $K_{\text{eff}}$, calculated with eqs. (2) and (3) in the main text using parameters obtained from the BD simulations at $V = 40$ (lined small light blue circles), 400 (lined small purple squares) and 4,000 (lined small black triangles) nm/s.
The observed reduction in chain stiffness of the linker with its increase in size (at constant \( \Delta L_C \) increments) can be intuitively illustrated. Fig. S4A shows an unfolding FX trace of poly-(I91) measured at a pulling velocity of 800 nm/s (blue). The linker extensions prior to every unfolding event are fitted with the WLM model given by eq. (S4) (black curves). These fits are obtained at a constant separation distance, \( \Delta L_C \), between each unfolding event. For clarity we also colored in red the segment used for the evaluation of the experimental stiffness \( dF/dx \) on one of the peaks. In Fig. S4B we plotted the numerical derivatives of the specific WLC curves from Fig. S4A, and plotted red triangles at the exact position of the end-to-end extension at which every unfolding event occurred. This illustrates how constant increment that forms a longer linker chain becomes "softer", although the mean force required to unfold a domain somewhere linearly connected with it increase.

![Diagram](image)

**Figure S4.** Illustration of stiffness reduction with progression of sequential unfolding events. A. Poly-(I91) unfolding trace measured at \( V = 800 \) nm/s (blue) fitted with the WLC model (black) showing constant \( \Delta L_C \) increments between unfolding events. The red line marked on a random peak demonstrate the region from which the experimental stiffness was evaluated (high stretch linear regime). B. Numerical derivatives of the WLC fits from (A), accounting for the stiffness of chains with increasing length (by \( \Delta L_C \)). The red triangles mark the specific unfolding positions at the FX trace in (A).
3. Variation of mean unfolding-forces stiffness and force-rates with pulling velocity at constant length

Maximal unfoldign forces were shown in the literature and in the main text to increase with the advancement of unfolding during the pulling experiments and simulatoins.\textsuperscript{7,11-13} In a typical analysis of FX experiments, these values are averaged per pulling velocity, and then plotted against the logarithm of the velocity. Here we are interested in the changes of the parameters monitored in this study (not only the mean unfolding forces) with the pulling velocity, with respect to their event number. While previously we dwelled on the effect of \( N \), where \( V = \text{const.} \), here we monitor their trends where \( V \) varies and \( N \) is considered constant. Figure S6 shows an increase of the unfolding forces, averaged with respect to their unfolding event, with the applied pulling velocities for poly-(I91) (Fig. S6A) at \( N = 1 \) (blank red four-pointed-star), 4 (blank blue hexagon), and 7 (blank green diamond), and for the BD simulations (Fig. S6B) at \( N = 2 \) (light blue four-pointed-star), 5 (purple hexagon), and 8 (black diamond).

![Figure S6](image)

**Figure S6.** Mean maximal unfolding forces as a function of the pulling velocity at similar number of unfolded domains (\( N = \text{const.} \)) for, A. Poly-(I91), and B. BD simulations.

The stiffness, evaluated at a given number of unfolded domains \( \langle dF/dx \rangle_N \), shows increase (although moderate) with the pulling velocity, which indicate the viscoelastic nature of the polyprotein as it unfolds for both poly-(I91) and the BD simulations in Figs. S7A and S7C respectively. Plotting \( K_{\text{eff}} \), as in Figs. S3B and 1B in the main text shows similar trends, as it underestimates \( \langle dF/dx \rangle \). As can be seen, the stiffness is affected by both the length of the unfolded domain and by the pulling velocity (conveyed \textit{via} the unfolding forces), where the effect of the latter is less pronounced.
Figure S7. Mean stiffness and force-rates as a function of the pulling velocity at similar number of unfolded domains ($N = \text{const.}$) for, A. & C. Poly-(I91), and B. & D. BD simulations.

Figs. S7 B and S7D show the increase of the force-rates with the applied velocity at a given linker chain length (constant $N$) for poly-(I91) and BD simulations respectively. The force-rates are given by the measured slopes of the maximal unfolding forces with respect to their occurrence time for a given $N$, $(dF/dt)_N$, and by the product of the mean stiffness with the pulling velocities at the same $N$, $\langle dF/dx \rangle \times V_N$, at different pulling velocities. Both experimental and simulated data shows an increase of the force-rates with the applied pulling velocity, and the difference between $(dF/dt)_N$ and $\langle dF/dx \rangle \times V_N$ that decreases as $N$ grows. The behavior of the mean stiffness and force-rates, averaged over all the $N$ events for both estimations is shown in Fig. 3 in the main text.
4. Zener (standard linear solid) model for the viscoelastic description of the linker

As mentioned above, and in the main text, the variation of the stiffness with the pulling velocity indicates the viscoelastic nature of the unfolding polyproteins. For this reason we chose to use the Zener model (illustrated in Fig. 4A in the main text) to describe the viscoelastic behavior of the extending linker as the polyprotein unfolds. The standard linear solid model, also known as the Zener model, is described in the Kelvin representation by two springs signifying the elastic moduli of the linker and probe ($K_l$ and $K_S$ respectively), with a viscous term given by $\mu$, the internal friction of the polymeric linker:

\[
(S7) \quad F + \left( \frac{\mu}{K_S + K_l} \right) \frac{dF}{dt} = \left( \frac{K_S K_l}{K_S + K_l} \right) x + \left( \frac{K_S \mu}{K_S + K_l} \right) V
\]

where $V = \frac{dx}{dt}$, is the velocity in which the linker is pulled. $K_l$ describes the stiffness of the linker with its extension, and it is expressed as a function of $L_C$, and $F$. We therefore take $L_C$ as the extension coordinate $x$, such that $K_l(x, F)$, and continue under the high force approximation:

\[
(S8) \quad F = \left( \frac{K_S \alpha x^{3/2}}{K_S + \alpha x^{3/2}} \right) x + \left( \frac{K_S}{K_S + \frac{\alpha}{x} F^{3/2}} \right) \mu V - \left( \frac{1}{K_S + \frac{\alpha}{x} F^{3/2}} \right) \mu \frac{\partial F}{\partial t}
\]

where $K_l = (\alpha x)^{3/2}$, $\alpha = 4(l_p/k_BT)^{1/2}$ according to eq. (2) in the main text. The overall stiffness of the system can be described by taking the derivative of the force with respect to the position:

\[
(S9) \quad \frac{\partial F}{\partial x} = \left[ \frac{K_S K_l}{(K_S + K_l)^2} \right] K_l + \left[ \frac{K_S K_l}{L_C (K_S + K_l)^2} \right] \mu V - \left[ \frac{K_l}{L_C (K_S + K_l)^2} \right] \mu \frac{\partial F}{\partial t}
\]

From eq.(S9) we can obtain an expression for $\mu$ at high extensions ($x/L_C \to 1$), which is described by the contour length of the chain, the stiffness of the probe and of the linker, but more importantly, with the overall stiffness of the system, $dF/dx$ (which is a combination of $K_S$ and $K_l$, and can be directly measured by the slope of the linear regime in the FX curve before unfolding event), of the applied pulling velocity $V$ and the force rate $dF/dt$:

\[
(S14) \quad \mu = \frac{\left[ \frac{L_C (K_S + K_l)^2}{K_l} \right] \frac{\partial F}{\partial x} - \frac{L_C K_S K_l}{K_S V - \frac{\partial F}{\partial t}}}{K_S V - \frac{\partial F}{\partial t}}
\]
Figure S8 presents the relation between $\mu$ and $\langle dF/dx \rangle$ as a function of $N$ and as a function of $V$. For constant $V$, $\mu$ shows an increase with $\langle dF/dx \rangle$ for poly-(I91) (Fig. S8A), and for the BD simulations (Fig. S8C), while for constant $N$ it exhibit the opposite trend (Figs. S8B and S8D). In both cases, the trend can be observed more explicitly in the BD simulations. When the polyprotein chain extends at constant pulling velocity, its stiffness reduces with every unfolding event, and with it the force-rate at which consecutive unfolding occurs. As the chain becomes more loose, it transfer momentum less efficiently, which is reflected through $\mu$ (viscosity is a measure for momentum transfer, the higher the viscosity, them better momentum transfer is). This can also explain why higher forces are required to unfold the preceding domains. We see this in Figs S8A and S8C, as the internal friction (viscosity) increases with the overall stiffness of the polyprotein (or decrease when the stiffness decreases). When $N$ is constant, i.e. a chain of fixed length is pulled at increasing velocities. The chain stiffness (mildly) grows with $V$, and with it the force rates (to larger extents) as shown in Fig. S7. The force-rates can be viewed as shear-rates, who reduces viscosity in polymeric liquids (shear thinning). Additionally, as discussed in the main text, the coupling with the term $\mu V$ predicts the decrease of $\mu$ as $V$ increases (and the stiffness and force-rates with it).
Figure S8. Variation in the internal friction, \( \mu \), with the mean stiffness of the polypeptide, calculated for poly-(I91) at constant \( V(A) \) and constant \( N(B) \), and for the BD simulations at constant \( V(C) \) and constant \( N(D) \).

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