RELEVANCE OF THE SPEED AND DIRECTION OF PULLING IN SIMPLE MODULAR PROTEINS

Running title: PULLING IN SIMPLE MODULAR PROTEINS

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ABSTRACT

A theoretical analysis of the unfolding pathway of simple modular proteins in length-controlled pulling experiments is put forward. Within this framework, we predict the first module to unfold in a chain of identical units, emphasizing the ranges of pulling speeds in which we expect our theory to hold. These theoretical predictions are checked by means of steered molecular dynamics of a simple construct, specifically a chain composed of two coiled-coils motives, where anisotropic features are revealed. These simulations also allow us to give an estimate for the range of pulling velocities in which our theoretical approach is valid.

INTRODUCTION

Protein folding and unfolding plays a central role in many biological processes. The emergence of single molecule manipulation techniques, mainly Laser Optical Tweezers and Atomic Force Microscopy (AFM), has made it possible to improve our understanding of elastic properties of protein systems and other biomolecules (1–4). Specifically, a large effort has been devoted to the analysis of the so-called force-extension curves, obtained when pulling the biomolecule by either controlling its end-to-end distance or the applied force (5–8).

From a theoretical standpoint, a typical approach is to consider that each of the biomolecule’s structural units can be modelled by a particle moving in an effective potential or free energy landscape (6–11). In a certain range of forces, the free energy has two minima corresponding to different system lengths $I(F)$ and $I(U)$ ($I(F) < I(U)$), see Fig. 1. Thus, as shown in Fig. 2, a multistable region appears in the equilibrium branches of the force-extension curve (7). Within this range, each unit unfolds by jumping from the minimum corresponding to $I(F)$ to the minimum corresponding to $I(U)$ at a value of the force that depends on the pulling velocity (8, 12).

FIGURE 1 Schematic free energy landscape of a single repeat for three different forces $F_1 < F_2 < F_3$. The system starts in the F state, which is the absolute minimum for $F_1$. If thermal noise is neglected, the repeat remains in the folded state for $F_2$ even when the unfolded state is the most stable state. For $F_3$, the F state disappears and the repeat finally unfolds.
FIGURE 2 Qualitative picture of the stability branches in a modular system with two units. The blue line stands for the unfolding pathway followed in the limit of maximum hysteresis, when the pulling speed is high enough to make the system sweep the whole branches and the jumps between consecutive branches takes place by the mechanism shown in Fig. 1. For low enough pulling speed (quasistatic limit), jumps occur at the lengths (dashed red lines) for which the branch with one more unfolded unit becomes more stable, that is, when its free energy is lower.

Here, we use this theoretical treatment to investigate the unfolding pathway, that is, the order and the way in which the elementary constituents of the biomolecule unravel. Specifically, some recent work has put forward the significance of both the pulling direction and speed in the unfolding pathway of proteins (13–17). In this respect, there are still a lot of unanswered questions, particularly in modular systems, which we attempt to clarify.

Let us focus on the first unfolding event for pulling processes in which the end-to-end length of the biomolecule is the controlled quantity. If pulling is very slow and quasi-static, then, the first unfolding event occurs at the length value for which the free energy minima over the branch with all the units folded and the branch with only one unit unfolded are equally deep (7). Then, the completely folded branch is swept only partially and the jump between branches occurs by thermal activation over the free energy barrier separating them. In contrast, there is a range of fast pulling velocities that do not give the system enough time to be thermally activated over the barrier, but are slow enough to allow it to sweep completely the metastable part of the branches. In this case, the jump between branches comes about at the limit of metastability, only when the folded minimum disappears (8), see Fig. 2. This range of velocities has been called “adiabatic” (8) or said to lead to the “maximum hysteresis path” (10).
Here, we introduce a theory for predicting the unfolding pathway of homopolyproteins (tandem repeats of a single protein separated by a non-interacting protein linker), as a specific example of the general model in (17). To do so, we make use of a Langevin description within the so-called macroscopic approximation (18). Moreover, we test predictions of our model by steered molecular dynamic (SMD) simulations in a particularly simple system composed of coiled coils. These kind of structures are common in nature, which makes it extremely useful as a model system (19–21).

MATERIALS AND METHODS

Simple Model

We consider a homopolyprotein composed of \( N \) non-interacting identical repeats. When the molecule is submitted to a pulling force along a defined axis, the simplest description is one-dimensional. As depicted in Fig. 3, we define the coordinates \( q_i, \ (i = 0,1,\ldots,N) \), in such a way that the \( i \)-th repeat extends from \( q_{i-1} \) to \( q_i \). Thus, the extension of the \( i \)-th repeat can be found as a simple subtraction \( x_i = q_i - q_{i-1} \).

\begin{equation}
\gamma q_i = -\frac{\partial}{\partial q_i} A(q_1,\ldots,q_N) + \xi_i, \quad i > 0, \quad (q_0 = 0), \quad (1)
\end{equation}

where \( \xi_i \) represent Gaussian white noise forces that verify

\begin{equation}
\langle \xi_i(t) \rangle = 0, \quad \langle \xi_i(t) \xi_j(t') \rangle = 2\gamma k_B T \delta_{i,j} \delta(t-t'), \quad (2)
\end{equation}

Above, \( k_B \) is the Boltzmann constant, and \( \gamma \) and \( T \) are the friction coefficient and the temperature of the fluid in which the molecule is immersed, respectively. As usual, the symbol \( \langle \cdots \rangle \) in Eq. (2) stands for the average over the noise. We assume the global free energy of the system to be

\begin{equation}
A(q_1,\ldots,q_N) = \sum_{i=1}^{N} a(q_i - q_{i-1}) + \frac{1}{2} k_e (L - q_N)^2. \quad (3)
\end{equation}
The previous equation assigns to each repeat contribution $a$, which only depends on the extension, and an elastic term that tries to keep the total length of the molecule $q_N$ equal to the desired program $L(t)$. In this work, we focus on length-controlled experiments at constant rate $v_p$, therefore $dL/dt = v_p$.

Note that when $k_c \to \infty$ the control over the length is perfect and $q_N \to L$ for all times in such a way that $k_c(L - q_N) \to F$, becoming a Lagrange multiplier that assures that the constraint over the total length holds. In order to keep the model as simple as possible, we consider (i) perfect control over the length and (ii) the so-called macroscopic approximation (18), that is, we neglect the noise terms in the Langevin approach. Note that the system evolution still depends on the temperature, because the single-repeat free energy $a$ contains the temperature. Taking into account these approximations, we reach the following evolution equations for the extensions

$$\gamma \ddot{x}_i = -a'(x_i) + a'(x_{i+1}), \quad (4a)$$
$$\gamma \ddot{x}_i = -2a'(x_i) + a'(x_{i+1}) + a'(x_{i-1}), \quad (4b)$$
$$\gamma l_N = -2a'(x_N) + a'(x_{N-1}) + F, \quad (4c)$$
$$F = \gamma v_p + a'(x_N). \quad (4d)$$

The reader familiarized with AFM experiments may note that our model does not completely match the usual AFM setup, in which the elastic force stemming from the bending of the cantilever is located on the opposite end (see Fig. 1 of (2)). Nevertheless, in the limit of perfect length control considered here, it can be proven that the location of the elastic reaction does not change the evolution equations.

Up to this moment, we have made no assumptions about the particular shape of the contribution of a single repeat to the free energy $a$. In a simplifying view, each repeat has two states, folded (F) and unfolded (U). Therefore, they can be represented by a potential $a(x)$ if the function $a - Fx$ has a double-well shape for some interval of forces, see Fig. 1. In this interval, the chain presents a metastable behaviour (7). For low values of the force the F state has a lower energy than the U state, making the first one more stable. Increasing the force, we start making the U state more and more stable until the F state ceases to exist. The macroscopic approximation, that is, neglecting the thermal noise terms in the Langevin equations, means that the only way to surpass from F to U state is to reach this limit of stability, as qualitatively shown in Fig. 1.

The dynamical Eqs. (4a) can be solved by means of a perturbative expansion in the pulling speed $v_p$. The perturbative solution, up to a linear order, starting from an initial condition where all the repeats are folded, is (17, 22)
\[ x_i = \ell + \nu_p \frac{3i(i-1)-(N^2-1)}{6Na''(\ell)} , \]  

where \( \ell = L/N \) is the specific length per repeat. According to Eq. (5), the closer a repeat is to the pulled end (greater \( i \)), the faster its length is increased. Note that there exists a value of \( \ell \) for which \( a''(\ell) = 0 \) and therefore the approximate solution given by Eq. (5) diverges.

However, we expect our solution to be useful along the initial evolution of the system and, specifically, to predict which repeat is the first to unfold. In a homopolyprotein, the answer seems to be simple within this approach: the fastest repeat is the pulled one (the closest to the moving end of the pulling device) and thus this will be the first to unfold. The idea is that the first unit that unfolds is the first that reaches the stability threshold \( \ell \), which marks the end of the metastability region.

It must be emphasized that this picture remains valid as long as the velocity is slow enough to allow the system sweep the metastable region of the equilibrium branches but not so slow to allow the system jump over the barrier separating the folded and unfolded states by thermal activation. Therefore, the system completely sweeps the metastable region of the equilibrium branches and the “adiabatic or deterministic limit”, using the terminology of (8), or the “maximum hysteresis path”, using the terminology of (9, 10), is attained. In other words, the biomolecule only unfolds at the value of the force that makes the folded minimum disappear, as already said before and qualitatively shown in Figs. 1-2.

In summary, our theory predicts that the first repeat to unfold in a homopolyprotein would be the pulled one, if thermal activation can be neglected and the unfolding pathway is thus mainly deterministic, as described above.

**Candidate to test**

Our theoretical approach is clearly a drastic simplification of reality. In fact, our theory is deterministic, in the sense that the unfolding pathway is a definite one, the randomness coming from thermal fluctuations being effectively “suppressed” by the fast enough pulling velocity. In reality, the unfolding pathway will have some stochasticity, stemming from the interactions between the molecule under study and the fluid where it is immersed, which are encoded in the Gaussian white noises of the Langevin description. Therefore, one expects our theoretical approach to hold for some molecules within a specific range of pulling velocities. One of the obvious requirements our protein candidate must meet is a negligible interaction between repeats, since we have assumed no nearest-neighbour interaction terms in the global free energy. Regarding the range of velocities, we need to be in the regime of the maximum hysteresis path, in which the unfolding of a repeat comes about because its extension has reached its limit of stability.

We have designed a homopolyprotein that fits these parameters which we can use to test whether it falls into the regime our model predicts. We have extracted the structure of an antiparallel coiled-coil motif (CC) from the archreal box C/D sRNP core protein (Protein Data Bank entry 1NT2), which comprises 67 residues and whose N-terminus and C-terminus are, respectively, arginine and isoleucine (23). This structure has been proven to be useful as a mechanical folding probe (24). We use this CC as the building blocks of the
molecule: our system is simply a concatenation of two CC motives connected by a linker, which is composed of two consecutive pairs of alternated residues of glycine and serine. We expect this linker not to introduce any significant interaction between the two domains. The initial conformation of the constructed model structure and orientation of the two CC repeats is shown in Fig. 4. The end-to-end vector points from the N-terminus to the C-terminus, aligned with the $x$-axis, whereas both axial directions of the two CC structures are located as parallel as possible to the $z$-axis.

FIGURE 4 Initial conformation of the homopolyprotein composed of two CCs in the SMD simulations. The pulling direction is aligned with the $x$-axis, whereas the axial directions of the CCs are aligned with the $z$-axis.

All-atom Molecular Dynamics Simulation

In order to minimize any technical difficulty stemming from experimental setup, we restrain the analysis in this work to all-atom molecular dynamics simulations. Moreover, the typical pulling speeds in molecular dynamics simulations are higher than the experimental ones, which make them especially suitable to explore the range in which the maximum hysteresis path is expected to be relevant. Such simulations start from the initial conformation shown in Fig. 4. First, we add hydrogen atoms using VMD Automatic PSF Builder (25). Then, a water box is created with a size large enough to unfold the protein in the direction of pulling, the $x$-axis. Also, NaCl is introduced in the system replacing water molecules, until the concentration reaches 150 mM/L and the charge is neutralized. Finally, simulations are performed using NAMD2 2.10 (26): first in the equilibration phase at 310 K and then in the pulling stage at $1.4\cdot10^{10}$ nm/s with a force constant 4860 pN/nm. Note that this value for the stiffness of the elastic reaction is also two orders or magnitude higher.
than the typical one in AFM experiments, and thus closer to the perfect length control situation assumed in our theory.

A notable number of trajectories are needed in order to obtain a meaningful statistical analysis of the unfolding pathway. Thus, we generate different trajectories varying the time in the first stage of equilibration. The duration of the pulling stage, 1.6 ns, is chosen in order to let the molecule unravel. We have considered also pulling velocities that are 2 and 5 times faster, in order to investigate whether or not increasing the pulling speed makes the unfolding pathway more deterministic in the NAMD simulations.

RESULTS AND DISCUSSIONS

According to the theoretical framework we have developed, we expect that if we pull from one end of the designed molecule, the first repeat to unfold will be precisely the closest to the moving end. To test this theory we perform SMD simulations to analyse the degree of agreement between theory and simulation.

For the sake of accuracy, we define a criterion for distinguishing between different kinds of trajectories; this is done by giving a quantitative measurement of the degree of unfolding for each repeat. Since the size of the CC motif in its axial direction is around 5 nm (4.82 nm between the two CAs most separated in the axial direction), we say that a motif is completely unfolded when its end-to-end distance (measured between their CAs in the terminal residues ARG and ILE) exceeds 10 nm. In our theory, the unfolding is sequential: when the first unit unfolds, the second one remains in the folded state. Therefore, we have to define a criterion to check this fact in our simulations, by introducing an “unfolding threshold”, i.e. a length below which we consider the unit to be still folded. Specifically, we consider this unfolding threshold to be 7 nm, which corresponds to an opening angle of 90° in a rigid rods picture, see Fig. 5.

FIGURE 5 Simple unfolding criterion for the CC under study based on a rigid-rod picture.
The choice of the unfolding criterion has some degree of arbitrariness. In order to give a physical basis for this choice, we study the fraction of native contacts in a CC motif versus its total length in Fig. 6. To do this, we have performed a SMD simulation in which the CC motif is pulled from its folded state at a speed of $3.75 \times 10^7$ nm/s. It can be observed how the number of native contacts decreases along the trajectory. The above defined thresholds for considering the molecule folded/unfolded fit quite well to the borders of the plateau observed in the figure. Therefore, this plateau can be understood as the region where the main bonds that keep the double-stranded CC folded are broken.

![Figure 6](image)

**FIGURE 6** Percent of native contacts as a function of the total length. The borders of the plateau agree quite well with the thresholds up to (from where) the molecule is considered to be folded (unfolded), marked with vertical dashed lines.

**C-pulling**

Consistently with the above-described criteria, let us index the different types of trajectories by (I, II, III, IV), attending to the degree of agreement with the theoretical prediction. The distinction between the different cases, in a C-pulling simulation, is shown in Table 1.

**TABLE 1** Definition of the different type of trajectories in a SMD C-pulling simulation. Types I and IV are the closest trajectories to a deterministic pathway, agreeing and disagreeing, respectively, with the prediction of our model.

| Type | First repeat to unfold | Length of the “folded” repeat |
|------|------------------------|-------------------------------|
| I    | C-terminus             | < 7 nm                        |
| II   | C-terminus             | > 7 nm                        |
| III  | N-terminus             | > 7 nm                        |
| IV   | N-terminus             | < 7 nm                        |
In Fig. 7, we plot the evolution of the distance between the end terminals of each repeat. The red line stands for the pulled repeat (C-terminus) whereas we plot in blue the length of the other repeat (N-terminus). It can be seen how in type I the pulled repeat clearly unfolds first. Although from different categories, types II, III and IV seem to share a common feature. In the initial part of the trajectory, it is the pulled repeat the fastest to lengthen but its unfolding comes to a standstill before being completed, and the second repeat takes advantage of this impasse to increase its extension.

Due to thermal fluctuations, we do not expect to obtain a perfect agreement with our theory, but a preponderance of the deterministic (type I) trajectories. In fact, the statistical information of 31 simulations, with a pulling speed of $1.4 \cdot 10^{10}$ nm/s, collected in Table 2 is completely compatible with our expectation from theory. Furthermore, as discussed above, a detailed analysis of the rest of type trajectories highlights a branching from type I due to an impasse of the length of pulled repeat. Therefore, our theory seems to predict the unfolding mechanism displayed in the SMD of this CC homopolyprotein.

**TABLE 2** Statistical analysis of the output of 31 runs SMD C-pulling simulations of the two CCs construct. A clear preponderance of type I trajectories is observed, in agreement with our theoretical prediction.

| Type | Occurrence (%) |
|------|----------------|
| I    | 45.2           |
| II   | 16.1           |
| III  | 22.6           |
| IV   | 16.1           |

According to our theory, we expect the pathway to become more deterministic as the pulling speed is increased. Indeed, this is what simulations show. Specifically, for ten trajectories with a pulling speed twice as fast, the unfolding statistics is almost deterministic (80% type I and 20% type III). The increase in the pulling speed effectively diminishes the relevance of thermal activation effects, as we thoroughly discuss below.
FIGURE 7 Representative plots of the different types of trajectories for SMD C-pulling simulations of the two CC construct. Each panel corresponds to a given type, as labelled. The extensions of both repeats are plotted: N-repeat (blue) and C-repeat (red). Our model predicts that the pulling repeat (C-terminus) is the first that unfolds.

N-pulling

Of course, our one-dimensional theory is completely left-right symmetric, since the free energy only depends on the extensions. Therefore, if we perform the same kind of SMD simulations, but pulling from the N-terminus, we expect the unfolding to start, preponderantly, from the unit closer to the N-terminus. Nevertheless, for a pulling speed of $1.4 \cdot 10^4 \text{nm/s}$, the observed unfolding pathway is basically random; none of the trajectory types prevail. We understand this disagreement with the theoretical prediction as a signature of the molecule being anisotropic.

Anisotropy of biological systems has been extensively studied (27). Indeed, in (28), a system very similar to ours presents different unfolding kinetics depending on the direction of the pulling: N- or C-pulling. Therein, the observed N-pulling transition rates between folded and unfolded states were much higher than the C-pulling rates. This property is
compatible with our observation: if transition rates are higher in N-pulling, thermally activated jumps from the folded to the unfolded state could be relevant for the considered pulling velocity, although they were not for C-pulling. This would lead to the failure of our theory, since the “deterministic” or “maximum hysteresis” path would lose its preponderance.

In order to check the above hypothesis, we have repeated the NAMD simulations with a faster pulling speed. Specifically, when the construct is pulled from its N-terminus with a velocity $v = 7 \cdot 10^{10}$ nm/s, that is, five times faster, the unfolding pathway is purely deterministic: for ten trajectories, all of them show that the first unit to unfold is the pulled one.

**CONCLUSIONS**

We put forward a model that predicts the unfolding pathway of homopolyproteins that are pulled from one of their endpoints. The predictions of the model have been verified by means of all-atom steered molecular dynamics simulations in a very simple system composed of two repeats of an antiparallel CC motif.

The aforementioned model gives a mathematical foundation to the physical intuition about how a pulling force acts on a macromolecule that is being stretched. According to our theory and simulation results for high enough pulling speeds, the pulled repeat in a homopolyprotein is the fastest to increase its length and thus the first to reach the unfolded state. This new insight improves our understanding about the dependence on the pulling direction and speed of the unfolding pathway of polyproteins.

The chosen molecule and the range of velocities play a central role for testing the theory. For instance, for the first considered velocity, N-pulling in simulations leads in our simple modular protein to a random unfolding pathway, in contrast with the mostly deterministic pathway found in C-pulling. This randomness in the unfolding pathway can be understood as stemming from the molecule being anisotropic, analogously to the reported behaviour of a similar structure in Ref. (28). Consistently with our theoretical approach, increasing the speed with which the system is pulled increases the preponderance of the deterministic path, both in C- and N-pulling.

One drawback of our results is the range of pulling speeds used in molecular dynamics simulations, which are several orders of magnitude above those typical of atomic force microscopy experiments. In this sense, an interesting prospect for future work is choosing a polyprotein for which the “maximum hysteresis” or “deterministic” path is found in the range of experimentally accessible pulling speeds (or, at least, closer to it).

Finally, a more general theory predicts the unfolding order of polyproteins comprising repeats with different stability properties (17). Therein, a set of critical velocities emerged: when the pulling speed crosses these critical velocities, the first repeat to unfold changes. There are particularly simple cases in which this theory should be testable, as a macromolecule composed by identical repeats except for a mutated one. Then, another
possible course of action for future investigations would be checking our theoretical approach in these, more complex, systems.

AUTHOR CONTRIBUTIONS

A. P. and P. E. M. designed the research project, A. P. and C. A. P. developed the theoretical model with feedback from P. E. M. and Z. N. S., C. A. P. performed and analysed the computer simulations with help from Z. N. S. and all the authors equally contributed to the writing of the manuscript.

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