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Protein mechanics probed using simple molecular models

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Structured Abstract

Background

Single molecule experimental techniques such as optical tweezers or atomic force microscopy can be used as a direct probe of the mechanical unfolding/folding of individual proteins. They are also a means to investigate free energy landscapes. Single-protein force spectroscopy alone provides limited information; theoretical models relate measurements to thermodynamic and kinetic properties of the protein, but they do not reveal atomic level information. By building a molecular model of the protein and probing its properties through numerical simulation, one can gauge the response to an external force for individual interatomic interactions and determine structures along the unfolding pathway. When used in combination, single molecule force probes and molecular simulations have contributed to uncover the rich behavior of proteins when subjected to mechanical force.

Scope of Review

We focus on how simplified protein models have been instrumental in showing how the general properties of the free energy landscape of a protein relate to its response to mechanical perturbations. We discuss the role of simple or toy protein models to explore the complexity of free energy landscapes and highlight important conceptual issues that more chemically accurate models with all-atom representations of proteins and solvent cannot easily address.

Major Conclusions

Native-centric, coarse-grained models, despite their simplifications in chemical detail compared to all-atom models, can be used to reproduce and interpret experimental

results. They have also highlighted instances where the theoretical framework used to interpret single molecule data is too simple. However, in some cases where non-native contacts are important on unfolding, these simple models are not able to reproduce the experimental findings.

General Significance

Mechanical forces are ubiquitous in the cell and it is increasingly clear that the way a protein responds to mechanical perturbation is important.

Introduction

Single molecule force probes and molecular simulations, in particular simulations with simplified molecular models, have together contributed to uncover the rich and sometimes unexpected behavior of proteins when subjected to mechanical force. Single molecule experimental techniques such as atomic force microscopy and laser optical tweezers have played a major role in defining the field of "protein mechanics" and opening a new 'window' onto protein free energy landscapes. Force measurements through single molecule force spectroscopy experiments are a direct probe of the resistance to mechanical unfolding of individual proteins. They are also a means to investigate folding and collapse, elasticity and plasticity. In this short and partial review we focus on how the response of a protein to a mechanical force is dictated by its free energy landscape, and simplified protein models have been instrumental in showing how general properties of the free energy landscape of a protein are related to its response to mechanical perturbations. Below we discuss the role of simple or toy protein models to explore the complexity of free energy landscapes and highlight important conceptual issues that more chemically accurate models (*i.e*., all-atom representations of proteins and solvent) cannot easily address. We also focus on showing how simple native-centric, coarse-grained models can qualitatively reproduce experimental results and highlight where the theoretical models used to interpret experimental data are too simple to reproduce the experimental phenomenology.

Proteins and forces

The relationship between the sequence, structure and biological function of proteins has been the centerpiece of biomolecular research over the past few decades. Advances in structural determination techniques have driven the field and been of considerable importance in the quest for unveiling the molecular basis for protein organization, function and regulation. However, the static picture that emerges from structural studies has obscured to some extent the fact that proteins are dynamic systems. Dynamics is key if proteins are to fulfill their biological role as components of a complex system adapting to external signals or environmental changes, including temperature, osmolytes, pH and mechanical forces.

Mechanical forces are ubiquitous in the cell and it is increasingly clear that the way a protein responds to mechanical perturbation is important, at some stage in their lifetime,

to most, if not all, proteins and not only those with a clear mechanical function such as actin, myosin and titin.

There are numerous processes *in vivo* that depend on the sensing and subsequent transduction of mechanical signals into appropriate biochemical or physical responses [1], such as protein degradation [2-4] and import [5-7], cell adhesion [8, 9] and motility [10-12]. Similarly, mechanical forces are clearly related to the function of protein nanomachines, especially those operating as nanomotors. An example would be F_1F_O-ATP synthase, a two-motor complex involved in the synthesis of ATP from ADP during the respiratory chain. ATP synthesis is accomplished through the conversion of electrochemical energy (transmembrane electrochemical gradient) to chemical energy through mechanical torque produced by the membrane electromotor. The mechanical coupling has been proposed [13] to involve elastic protein components acting as energy buffer.

Polypeptide-based matter can display extremely variable mechanical properties. There are some extremely force-resilient proteins, such as the much-studied immunoglobulin domains from titin [14], and protein complexes like titin–telethonin [15] that require hundreds of pN of force to rupture/unfold (1 pN = 10^{-12} N = 0.0144 kcal/mol/Å). In contrast, forces of just a few pN can destabilize other more labile disordered [16] or even folded [17] proteins. The response of individual proteins to force also depends on the direction and point of application of the force [18]. This directional dependence of a protein's mechanical properties is not just of academic interest but has implications for the rate of protein degradation or import *in vivo* [3, 19-21] and exposure of cryptic binding sites upon force activation [22]. Adding to this complexity is the fact that the mechanical properties of a protein are not straightforwardly related to its properties in the absence of an external force. That is, the unfolding pathway in the presence or absence of a force may differ [22-24]: a thermally stable protein may be mechanically labile and *vice versa*.

The complexity of a protein's response to mechanical, directional forces emerges particularly at relatively small forces (of the order of 10 pN). Forces of such magnitude are most likely experienced by many proteins, in many different situations, *in vivo*. Meanwhile mechanical forces are found to play a role in an increasing number of biological processes, and the complex response of proteins to such forces may indeed be crucial to their function. From a conceptual point of view, the complexity of a protein's response to a constant applied force can be understood in terms of free energy

landscapes and how the force deforms such landscapes. The response of a protein to a variable force is instead a non-equilibrium property that depends also on the history of how the force has been applied; if the force is applied with a constant loading rate the most likely force at which a protein unfolds can be exactly related to the average unfolding time under constant force conditions [25].

Single protein force spectroscopy

In the past two decades, single molecule force spectroscopy (SMFS) employing the atomic force microscope (AFM) or optical/magnetic tweezers has been increasingly used to explore the physical properties of proteins [14, 26-28]. With ever improving instrumentation, force spectroscopy is fast becoming one of the most valuable tools to study the finest details of the kinetics and underlying free energy landscape of proteins. The single molecule nature of these techniques means that information on individual unfolding/folding trajectories can be obtained, in contrast to the 'averaged' behavior measured by conventional ensemble techniques. By moving the probe (be it the AFM cantilever or a bead held in an optical trap) at constant velocity, unfolding/folding forces as a function of loading rate are acquired, or alternatively unfolding/folding times are given by adjusting the position of the probe to maintain a constant force.

Typical loading rates employed in AFM-based force spectroscopy experiments range from 10–10,000 nm s^{-1} with force constants in the range 1–100 pN nm^{-1}. This range is to a large extent determined by the piezoelectric manipulation of the cantilever and/or surface to which the protein is attached, and any associated drift in the measurement of cantilever deflection. If unfolding corresponds to elongating a protein by 1 nm, the unfolding times induced and monitored in AFM experiments are in the range of 0.1–100 ms, which exceed the timescales that can be simulated using atomistic, chemically accurate models. Recent high-speed studies of protein unfolding [29] and unbinding [30] pushed the pulling speed to ~30 mm s^{-1} using small cantilevers with force constants of 100–600 pN nm^{-1}. These speeds begin to overlap with those used in all-atom molecular dynamics (MD) simulations and are easily compatible with coarse-grained models.

Interpretation of experimental data

The simplest kinetic models used to extract parameters for the free energy landscape of a protein from raw experimental data treat the problem as one of diffusion along a one-dimensional (1D) free energy profile. The first kinetic model of mechanical strength was

formulated by Zhurkov [31] for solids subjected to stretching forces. He observed that the lifetimes of solids under mechanical stress could be described by a van 't Hoff–Arrhenius like expression

$$\tau(F) = \gamma \exp\left[\beta[\Delta G^{\ddagger}(0) - Fx^{\ddagger}]\right] = \tau(0)\exp\left[-Fx^{\ddagger}\right]$$

where β is the inverse temperature, γ is a kinetic prefactor, which gives the time scale of mechanical rupture in the absence of a barrier; $\Delta G^{\ddagger}(0)$ is the height of the unperturbed free energy barrier opposing the fracture reaction; $x^{\ddagger}$ is a coefficient describing the force dependence of $\tau(F)$. The equation above is referred to as "Bell model" after George Bell who was the first to apply it in a biological context, modeling cell adhesion rates in the presence of hydrodynamic stress [32].

Although the Zhurkov–Bell (ZB) model is widely used in the analysis of single molecule force spectroscopy experiments, the modern framework in which it is understood was provided by Evans and Ritchie [33] who rigorously derived it using Kramers' reaction rate theory [34]. In this context, the process of mechanical fracture can be thought as diffusion over a free energy barrier on a 1D energy profile defined by the extension (*i.e.*, the distance between the points where force is applied) of the protein (see Figure 1).

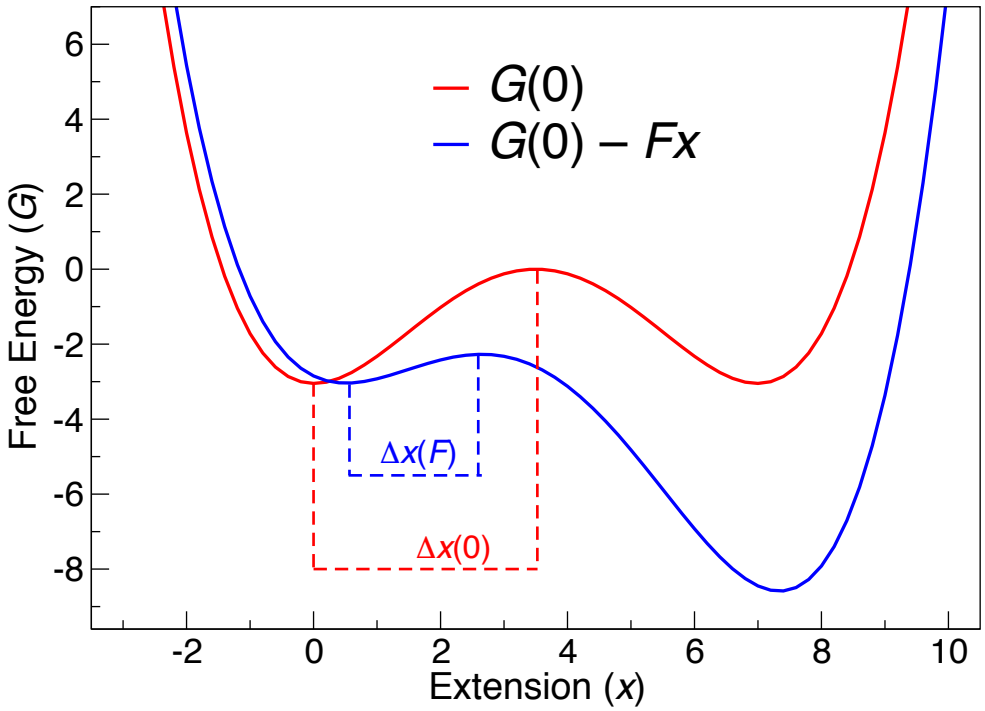

Figure 1. One-dimensional free energy profile before (red) and after the application of a force (blue). The distance between the native and transition state, Δx, depends in general on the force applied.

If it is assumed that both the free energy barrier and friction are high, a van 't Hoff–Arrhenius like expression can be obtained from Kramers' theory. The coefficients $x^{\ddagger}$ can then be interpreted as the position of the barrier on the 1D free energy profile assumed to describe the reaction. The ZB model, despite its simplicity, has been, and still is the model most often used to fit single molecule force spectroscopy measurements [35].

If the force applied to the two ends of a protein is not constant, but increases linearly, as in experiments where an atomic force cantilever with elastic constant κ is retracted at constant speed v, the most likely unfolding force is (with the caveats discussed in [35])

$$F = \frac{1}{\beta x^{\ddagger}} \ln\left[\kappa v \beta x^{\ddagger} \tau(0)\right]$$

Regardless of the protein under investigation, experimental results have generally shown good compliance with the ZB model; straight-line plots of unfolding force against the logarithm of loading rate pervade the literature. Despite being calculated only for the limited range of forces or pulling rates dictated by the technique, the values of $x^{\ddagger}$ and $\tau(0)$ (or its inverse the unfolding rate at zero force $k(0)$) are then assumed to apply for all forces or pulling velocities by linear extrapolation.

If this were true at all forces (or pulling speeds), then one could assume that the same process and parameters $x^{\ddagger}$ and $\tau(0)$ characterize the unfolding observed in simulations performed at the pulling speeds several orders of magnitude higher, as generally used in molecular dynamic simulations of detailed, atomistic models.

Below we will show how simulations using coarse-grained models have demonstrated how the ZB model provides parameters that cannot always be related to physical quantities, and that in some specific cases, fails to reproduce even qualitatively the force dependence of unfolding rates at low forces. The failure of such widely used 1D models does not in itself undermine single molecule force spectroscopy techniques. On the contrary, it shows the potential of single-molecule force probes to reveal the unique features of the free energy landscape of proteins at conditions normally experienced *in vivo*.

The main limitation of single-protein force spectroscopy, whatever the model used to interpret the results, is the limited information that can be gained. All that can be measured are forces and extensions and their dependence on pulling speeds or loading rates. Models relate such measurements to thermodynamic and kinetic properties of the protein, but they do not reveal atomic level information. For instance, it would be useful to know which bonds or other interactions are important for protein mechanical stability; what is the order of rupture of these bonds and what other physical changes does the protein undergo during unfolding? By building a molecular model of the protein and probing its properties through numerical simulation, one can in principle gauge the response to an external force for individual interatomic interactions and determine the structure in the rate limiting state.

Simulation offers atomic-resolution movies of mechanical unfolding

Molecular dynamics simulation of proteins has become a tool of choice to study the dynamics of proteins and provide a bridge between theory and experimental phenomenology. Atomistic, classical, empirical but transferable models, are now considered reliable enough to rank the energy of folded and unfolded conformations and even to reversibly fold small, fast folding proteins *ab initio* [36]. The single force spectroscopy experiments described above have introduced a novel way to induce and follow mechanical folding and unfolding reactions. The perturbation to which proteins are subject in SMFS experiments is microscopically well defined and can be straightforwardly implemented in a simulation [37, 38]. Whether one wants to introduce the variable force exerted by a AFM cantilever retracted at constant speed or the

constant force applied by a force-clamp apparatus, this requires just a few lines of code in a MD program and virtually no additional computational time. Atomistic simulation, where an external force is added to two atoms so as to mimic and thereby interpret structurally raw single molecule experimental data, has become a computational approach that has highlighted and rationalized the importance of mechanical force to perturb and probe the free energy landscapes of biological macromolecules.

One of the first simulations of mechanical unfolding was used to interpret AFM force spectroscopy results for the I27 domain from titin; probably the most well-studied 'mechanical' protein [37-39]. Despite the pulling rate being a factor of ~10^7 higher, and the cantilever force constant significantly higher than used experimentally—due to computational time constraints associated with the fully atomistic model—these first simulations were able to identify key interactions associated with the main rupture event, and were a great stimulus to the field. However, some researchers were concerned about the short time scales covered by all-atom simulations and have questioned whether there is actual predictive power in molecular dynamics methodology. Here we will focus on results obtained by simulation of simple molecular models and highlight how they can provide a high degree of overlap with experimental findings and, perhaps more importantly, deep theoretical insight into protein unfolding. Recent advances focusing on all-atom simulation of protein unfolding have been reviewed elsewhere [40].

Model simplification allows for larger scale, longer timescale, lower force simulations and rigorous statistics.

Ideally, atomistic protein models with explicit solvent should be employed to provide a fully accurate picture of the molecular mechanism of the process being probed experimentally. Yet, folding and unfolding of most proteins, occurs on timescales longer than those that can be realistically and routinely simulated; this is even more the case if statistically meaningful results are needed.

Typically, forces (or pulling speeds) much larger than those used in experiments have been applied to accelerate computations, although the gap is narrowing steadily [40]. Analogously, forces much larger than those experienced by proteins *in vivo* are typically imposed in mechanical unfolding AFM experiments. While it is now generally accepted that forces of different orders of magnitude have qualitatively different consequences on the unfolding pathway of proteins, and thus different free energy barriers may be probed at different forces, this fact was largely ignored when the first mechanical unfolding

experiments and simulations were reported. It can be tempting with very detailed all-atom models, and therefore very detailed findings, to make predictions that go beyond the scope of the short time durations to which they are related. Ng and Clarke [41] for instance provide an example of experimental evidence for the failure (or perhaps over-interpretation) of predictions from all-atom simulations.

One way to tackle the problem of computational efficiency, as well as to permit computations for large protein complexes, is to reduce the complexity of the model, *i.e.*, decrease the number of degrees of freedom and the range of the interactions. Simpler models are not only computationally more manageable, but also an effective tool to identify and focus on hypothetical factors that are relevant to the mechanics of proteins. The first step towards model simplification is the replacement of the solvent environment—water molecules, co-solvents and salts—with a continuum and/or effective energy potentials, taking into account the solvation effects on intra-protein interactions. Implicit solvation reduces drastically the degrees of freedom of a system.

Since the largest part of a solvated protein system is composed of solvent molecules, replacing the solvent models by an implicit solvent model accounting for the solvent environment, can reduce the computational time by orders of magnitude, permitting the application of smaller forces and/or allowing collection of multiple simulation runs to provide statistically relevant results. The water molecules are expected to participate in hydrogen bonding with the protein backbone, and neglecting them may alter the actual mechanism compared to the explicit solvent system. However, one argument for the use of implicit solvent, besides computational efficiency, is that it is also physically more appropriate when mechanical unfolding simulations are performed many orders of magnitude faster than in the experiments. This is because water relaxes fast relative to the conformational changes induced in the protein by mechanical pulling. In unfolding simulations where pulling occurs at a much faster rate than in experiment, the solvent may not have the time to fully equilibrate. In contrast, an implicit solvent relaxes instantaneously, and while the hydrogen bonding properties of water are only included on average, this prevents the attribution of specific importance to potentially arbitrary configurations of the solvent. Paci and Karplus [38] using a Gaussian-type effective energy to account for solvent effects and a polar-hydrogen atomistic model to describe proteins [42] investigated the mechanical unfolding mechanism of two β-sandwich proteins and two α-helical proteins revealing significant differences in mechanical response both within the same class and between different classes related to differences in topology and energetics. In the case of the titin immunoglobulin domain I27,

simulations pointed to the early detachment of the A-strand during unfolding resulting in a metastable intermediate, observed earlier experimentally with AFM and studied with explicit solvent simulations [43]. The intermediate was characterized in terms of mechanical behavior using an I27 mutant lacking the A-strand, which implicit solvent simulations indicated as a good representative of the observed intermediate of wild type I27 [39]. In subsequent work [44], implicit solvent simulations combined with experimental data from mutation and AFM provided a microscopic picture of the transition state during mechanical unfolding. Comparison with results observed for denaturant-induced unfolding, revealed differences in the native-like features of the transition state. The same methodology was also applied with some success to a mechanical study of the fibronectin type III domain (TnFn3) [45].

Despite their simplification relative to explicit solvation models, timescales accessible with implicit solvent models are only one or two orders of magnitude longer, which is not a great help when comparing with unfolding times at realistically high forces. Also, implicit-solvent simulations of large protein assemblies and nanomachines with atomistic detail still demand significant computational resources when large conformational changes occur, even if mediated by the application of external forces, unless forces greatly exceeding those used experimentally are employed. To reduce computational cost further, more drastic approximations of protein chemistry are employed resulting in simplified, coarse-grained (CG) protein models, with or without explicit treatment of solvation. Apart from being computationally efficient, CG models are well suited to understand general, fundamental physical and chemical properties of biomolecules whether external mechanical force is applied or not [46].

Structure-based coarse-grained models

The development and use of simplified protein models can be traced back to the first simulations of protein folding and dynamics with the seminal work of Levitt and Warshel [47, 48], and Gō and co-workers [49]. Since then, considerable advances have been made in CG modeling and its application in molecular biosciences. CG models, despite the limitations due to their underlying assumptions, permit computer simulation of biomolecular systems at timescales where direct comparison between computer simulations and experimental data is possible.

Simulations of chemically accurate models, when practically feasible, can provide essential insight into the microscopic determinants of macroscopic experimental observations. Assessment of the quality of models relies on the availability of relevant

experimental information as well as on the possibility of accurately sampling their conformation space to calculate macroscopically observable properties as averages of microscopic properties. This scenario complicates the definition of an "accurate model" and by extension also that of a "simplified model".

When defining a molecular model with the purpose of performing biologically relevant dynamics simulations, it is not a matter of whether to simplify or not, but how to do it and what for. Given a system and the duration of a particular process, a model should cover the whole process at reasonable computational cost. If the aim is the reproduction of a complete single molecule experiment (~milliseconds) for a protein (~1000 atoms for a small protein not considering the solvent) a simplified description involves replacing groups of atoms with one interaction site, which is the usual method for coarse-graining (Figure 2A–B). Coarse-grained descriptions need carefully designed energetic definitions to compensate for this simplicity. Obviously, there are countless ways in which these force fields can be designed, but they usually lie within two main categories: mean-field potentials or topology-based ones. The former describe the protein Hamiltonian by means of what has been observed in a large number of proteins. In this way, the interaction between, for instance, arginine and phenylalanine always obeys the same rules no matter which specific protein is involved, as the applied force field reproduces the average behavior of these amino acids. This approach has been widely used in protein folding studies. However, the mean-field strategy often fails when studying the whole folding process or protein mechanics.

The lack of an accurate native state within this approach constitutes the main weakness of mean-field potentials, but is also the strength of topology-based potentials, generally referred to as native-centric or Gō-like models. Since mechanical unfolding is mainly, but not always (see later), driven by native interactions, the importance of these specific interactions is utilized as the predominant feature of these models.

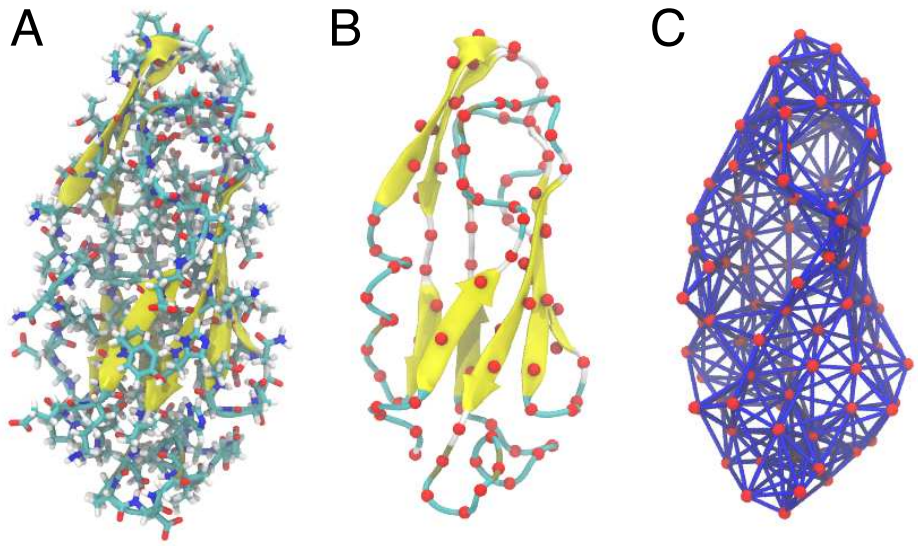

Figure 2. Three different models of the 100 amino acid protein filamin. A) All-atom representation; not including the solvent, and not assuming a cut-off, the number of possible interacting pairs are ~1,000,000. B) Cα representation, *i.e.*, each residue is represented by a single interaction site; the number of possible interacting pairs is ~5000. C) Elastic network model based on Cα atoms only; the chain connectivity is not explicitly represented and the number of interactions, represented by lines, is here ~500 (assuming that only atoms within 8 Å interact).

'Gō-like' is an umbrella term encompassing explicit-chain polymer models for proteins where a reference experimental structure (the so-called native structure) serves as a bias towards the native configuration of the protein. The details of the actual model, *i.e*. the degree of coarse-graining and the potential-energy functional form, may vary from model to model, all of them having the common characteristic feature that only native residue contacts as identified in the reference structure contribute favorably to the system's Hamiltonian, while non-native contacts are less favorable, usually modeled as repulsive forces between coarse-grained particles. Despite their simplicity and the apparent artificial character of the configurational bias towards a particular structure, Gō-like models partly stem their legitimacy from the fact that evolutionary pressure has led proteins to the adaption of a minimally frustrated funnel-like energy landscape, in contrast to random heteropolymers. Native protein structures lie in the lowest basin of such an energy landscape, so that structure-based coarse-grained models in the sense of Gō-like models constitute a parameterization of that specific part of the energy landscape. The original Gō model [49] was developed for simulating protein folding dynamics adapting a native structure-centered potential for a lattice-based one-bead polymer, while the configuration space was sampled using a standard Metropolis Monte Carlo algorithm. Many extensions of the original Gō model have been developed, which attempt to capture the variety of interactions possible in a real protein. The problem of insufficient cooperativity of Gō-like models has been partly addressed by the inclusion of extra energetic terms in model Hamiltonians, accounting for the effective desolvation

barrier [50, 51] and sequence effects on protein folding [52]. An example of a versatile Gō-like model with finite extensible nonlinear elastic energetics terms for backbone chain connectivity is the self-organized polymer (SOP) model introduced by Thirumalai and coworkers [53]. A recent example used this model to probe the forced-rupture of ligands from cell adhesion molecules showing good agreement with experiment [54]. This model was also successfully applied to the mechanical folding/unfolding of proteins and the dynamical simulation of allosteric transitions in ATP-dependent nanomachines, like chaperonin GroEL–GroES [55] and myosin [56]. Another nanomachine, namely hexameric helicase interacting with DNA was studied [57] by means of Gō-like models for both the protein and nucleic acid components following Hoang and Cieplak [58]. More recently all-atom Gō-like models have been introduced and used in simulations of folding, aiming to bridge the gap between CG native-centric models and all-atom empirical force fields [59-61].

Elastic network models (ENMs), a popular class of coarse-grained models for proteins, consider the folded state of proteins as an elastic body, where a set of interconnected nodes interact via simplified potentials, giving rise to an elastic network [62] following the ideas of Tirion [63]. In ENMs, the coarse-graining stage consists of mapping the nodes onto a protein's structural elements. On the basis of residue-based coarse-graining, there is one bead/node corresponding to each Cα atom (Figure 2C). A cut-off distance is used to assign interacting nodes and a simple harmonic potential is applied between interacting nodes (Gaussian network model, GNM). In an important extension of the simple GNM, anisotropic residual fluctuations are taken into account resulting in an anisotropic network model [64]. A handful of papers extend the use of ENMs to estimate weak deformation modes [65, 66] which in general correlate with those directions where the protein unfolds faster upon application of a mechanical force.

An alternative approach to studying the mechanical properties of proteins has been proposed by Thorpe and co-workers on the basis of graph-theoretic rigidity theory [67, 68]. They proposed an algorithm, FIRST, that analyzes the local flexibility/rigidity of a protein based on single structures as input. Protein stereochemistry and intermolecular forces, such as hydrogen bonds, are taken into account as restraints. A single-structure rigidity analysis can provide a natural way of coarse-graining the protein, reducing the total number of degrees of freedom. Combining the results from FIRST, Monte Carlo simulations can then be employed to explore the coarse-grained conformation space [69, 70] to find alternative basins on the energy surface. The method has been extended

to produce low-energy transition pathways between states [71] and simulate the force-induced unfolding of proteins. The method is computationally efficient and could find applications in protein modeling and computational structural biology. However, as it is based on a Monte Carlo simulation protocol it lacks a realistic description of protein dynamics.

While native-centric models such as Gō-like or ENMs have provided considerable insight into the fundamental effect of forces on protein free energy landscapes and in several cases predicted the mechanical features of specific proteins, there are cases where they are doomed to fail. One notable example is that of the mechanosensing PKD domain; its remarkable mechanical properties could not be predicted based on structure, or from simulation using native-centric models, because they are due to the formation of mechanically strong non-native interactions; their formation is triggered by application of the force itself [72]. Those findings, obtained using an all-atom model with implicit solvent could be confirmed directly by experiment: by performing mutations to prevent the formation of non-native hydrogen bonds the PKD domain became mechanically weak, while its folding behavior in the absence of force was unchanged [73].

The following three sections describe a number of unprecedented findings obtained using native-centric, CG Gō-like models.

Extension as reaction coordinate for mechanical unfolding

By describing the process of mechanical unfolding through the molecule's extension alone one assumes that the extension discriminates all states along the unfolding pathway. For a two-state system under high mechanical stress, this will be the case for the native and unfolded (highly extended at high forces) states. But how do smaller, physiologically relevant forces affect the unfolding of a protein, and under what forces can a protein refold?

A structure-based, minimally frustrated Gō-like model is often the only viable option to investigate equilibrium sampling of protein folding or unfolding in the absence of force or at small forces. With such a model [52] and by analyzing the trajectory without projecting it onto the protein extension or other arbitrary geometric variable [74] the equilibrium free energy landscape of the protein filamin in a small range of forces (≤6 pN) could be determined [75]. The resulting free energy landscape can be represented as a network of interconnected states each with a well-defined probability and transition. The crucial finding was that while in the absence of force the protein can be described as a two-state

system, by applying a 3 pN force between the two ends an obligatory intermediate appears. At 6 pN a second intermediate is populated and transitions between native and denatured state may occur that pass either through both intermediates or only one (Figure 3A).

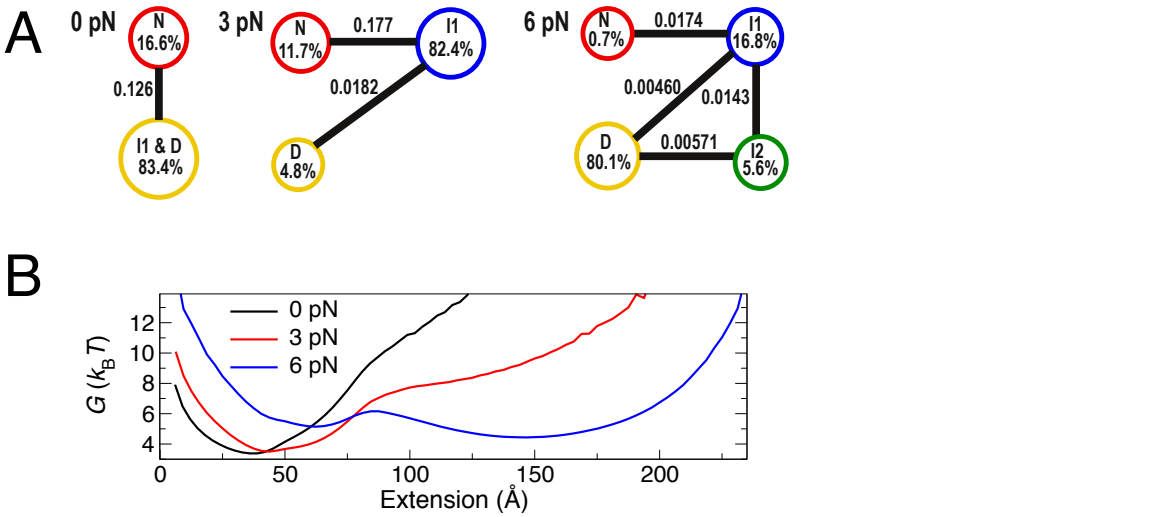

Figure 3. A) Equilibrium network of states for filamin in the presence of forces applied to the N- and C-termini; the population fraction of each state is given within each circle and the transition rate (in ns^{-1}) between the states is given on the connecting lines. B) Potential of mean force as a function of the extension (figure modified from Ref. [75]).

The potential of mean force along the extension (Figure 3B) shows a single shallow barrier between two broad local minima, which in fact do not correspond to any of the "true states" (for the model). Even defining a coordinate dependent diffusion coefficient, the force dependence of the kinetics of folding or unfolding cannot be obtained from diffusion on such a 1D free energy profile.

Free energy of protein extension

Single molecule manipulation of proteins with force spectroscopy provides, when data are fitted with a kinetic model as discussed above, a rate and the position of a barrier on the free energy projection over the extension (and, with an improved model, also the barrier height). But is the full free energy profile along the extension measurable? The Jarzynski relation [76, 77] provides one way (and not the only one) to estimate the free energy along the extension coordinate from single molecule force spectroscopy experiments. In this method, the free energy, $\Delta G(z)$ is given by

$$e^{-\beta \Delta G(z)} = \left\langle e^{-\beta W(z)} \right\rangle_{\lambda(t)}$$

where $W(z)$ is the nonequilibrium work when modifying a parameter z and the average taken over multiples trajectories $\lambda(t)$. Hummer and Szabo [76] have shown how to use this to extract $\Delta G(d)$, where d is the distance between two atoms being pulled apart using a single molecule force spectroscopy method, the two atoms usually being at the N- and C- termini. The average $\langle \quad \rangle$ is meant to be performed over all initial conditions consistent with the Boltzmann distribution. Hence, non-equilibrium experiments can be used to obtain equilibrium free energies, and thus the resulting free energy should be independent of history, and thus of the pulling speed. A problem with single molecule force probes is that only events in which folded structures are unfolded are selected for analysis. Other, alternative initial structures, including those that may be unfolded but have a native-like extension, do not leave a signature. This fact has been clearly illustrated using a Gō-like model of E2Lip3 protein [78], for which $\Delta G(d)$ could be estimated by simply performing a long equilibrium simulation close to the melting temperature. By selecting native initial structures it could be shown that the Jarzynski equality above only holds in the limit of zero pulling speed (Figure 4A); on the other hand, if applied on a sample population where folded and unfolded compact conformations are represented with their Boltzmann weight, the Jarzynski equality holds at all pulling speeds (Figure 4B).

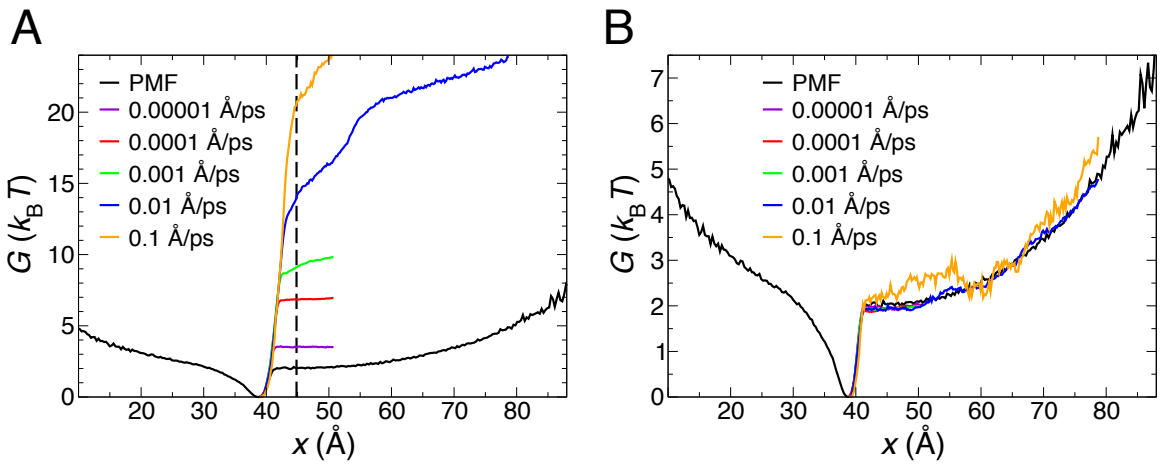

Figure 4. Free energy as a function of the distance between two specific residues of the protein E2Lip3. In black the true potential of mean force obtained from a long equilibrium simulation. A) Using the Jarzynski relation but averaging over an ensemble of native conformations. B) Using the Jarzynski relation and averaging over a Boltzmann population of structures with extension equal to the inter-residue distance in the native state (figure modified from Ref. [78]).

Such a result demonstrates the utility of the Jarzynski equality, how it works in practice and provides an important caveat when trying to use single molecule force spectroscopy methods (or steered molecular dynamic simulations) to determine the free energy by pulling proteins irreversibly.

Breakdown of the Zhurkov–Bell postulate

In the treatment of mechanical unfolding kinetics, it is generally assumed, as seen above, that there exist well-defined transition and native states. The example of filamin above shows that the transition rate between states can also be determined by the depletion or gain in pathways at different forces, which implies the existence of multiple distinct transition states. In fact, the presence of alternative folding routes had already been shown from discrepancies observed between the unfolding rates measured by chemical denaturants and those inferred from mechanical unfolding rates measured at high force [79]. The difference between the two rates has been attributed to differences in the energy landscapes for denaturant and mechanical unfolding [39, 50]. What simulations of Gō-like models have highlighted is that parallel pathways are likely to coexist at small forces: a corollary of the assertion is that the unfolding rate is a continuous function of the applied force. If there are parallel pathways, a plot of the logarithm of the unfolding rate against the applied force will reveal curvatures (Figure 5) instead of being linear as predicted by the ZB model [80, 81]. The type of curvature and range of forces for which these curvatures are observed will depend on the characteristics of the various pathways (*i.e.*, barrier heights and force dependence).

Thus, "anomalous" kinetic signatures provide a glimpse into the underlying free energy landscape, revealing its multidimensionality, as well as its architecture (*i.e.*, the number of pathways and the relationship between pathways). The "catch bond", a bond that appears stronger under load, experimentally observed for the unbinding of protein–protein/protein–ligand complexes, is a prime example of anomalous kinetics.

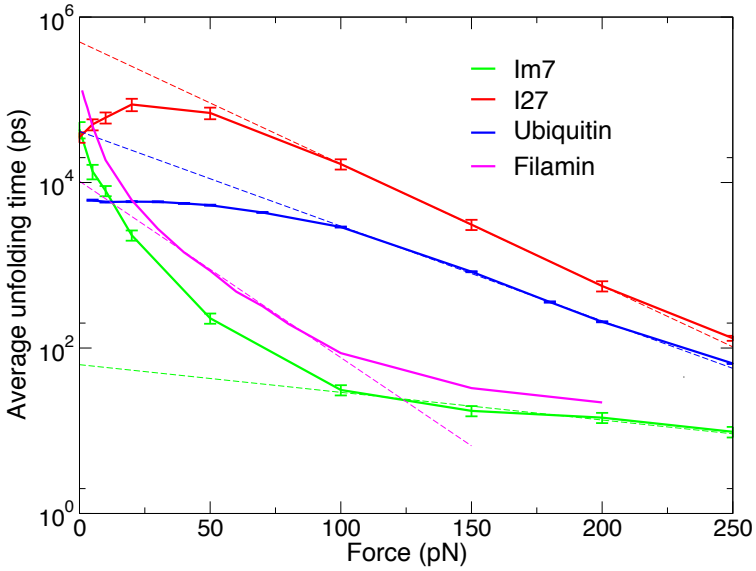

Figure 5. Plot of the unfolding time of Gō models of four different proteins as a function of the applied force. At low forces, there are significant deviations from 1D models, such as the ZB model, which predict logarithmic

dependence of the unfolding rate. Dashed lines show that such dependence of the unfolding rate on the force only holds for a limited range of forces (figure modified from Ref. [82]).

The unfolding kinetics of filamin is another example of anomalous kinetics: filamin is stronger at low force (*i.e.*, unfolds slower) than any 1D kinetic model can predict from its behavior at higher force [83, 84]. Molecular dynamics simulations of a coarse-grain model of filamin reproduce the same phenomenon and indicate that the anomalous deviation at low force can be explained by a switch between two distinct pathways. A two-path model fits the experimentally determined distributions of unfolding forces very well [83, 84]. These results demonstrate a kinetic complexity not previously described in single-domain proteins, which are predominantly thought of as simple two-state systems. Furthermore, the curvature of the type described for filamin had not been previously observed for any other single-domain protein, although it has been observed for protein–ligand unbinding [85]. More recently, analogous deviation from exponential dependence of the unfolding rate on force has been experimentally shown for spectrin [86], where, as for filamin, at higher forces the increase in unfolding rate with force becomes faster than that predicted by fitting the lower force response with a single exponential. For filamin [83, 84] the low-force behavior was highlighted as anomalous relative to the high-force one. This highlights how, for a small range of forces, the dependence of unfolding rate on force can always be fitted with a single exponential; a fact that has contributed to misleadingly validate the hypothesis that a 1D approximation of the free energy surface, such as the ZB model, is generally acceptable.

The complexity of the free energy landscape has implications for the function of proteins that have a mechanical role *in vivo*. The force-dependent stabilization of different states with distinct structural properties not only allows the protein to tune its response based on the magnitude of the external force, but also enables it to respond rapidly to changes in the external force. Although deviations from 1D models can be rationalized by the existence of multiple pathways, a structural explanation is less forthcoming. To this end, atomistic resolution of simulations and subsequent validation of the simulations using site directed mutagenesis is very important.

Discussion

A large variety of atomistic molecular models have been used in conjunction with the application of an external force to mimic single molecule force experiments. These models range from all-atom, fully solvated systems to greatly simplified models where

clusters of atoms are combined to form interacting coarse beads, or models in which atomic detail is lost and the protein is represented as an elastic body. While coarse-grained models are an approximation of all-atom models, there is little consensus on how accurate models are in general. The accuracy of models can only be assessed by comparing predictions from simulation with appropriate experiments, and experiments provide only a limited amount of information for comparison. One general advantage of coarse-grained, computationally efficient models is that statistically rigorous results can be more easily obtained. Rigorous estimations of unfolding rates, only possible with models that do not treat individual interactions between all atoms of the protein and its environment explicitly, have been instrumental in showing that the response of proteins to forces is considerably more complex (and interesting) than initially suggested by experiments.

Atomistic simulation has been broadly used to pull proteins apart, mainly due its simplicity and its direct relation with equivalent single molecule experiments. The most accurate protein models certainly provide the closest representation of real processes, and have sometimes precisely identified "load-bearing" elements or interactions responsible for the force peaks observed, *e.g.*, in atomic force measurements. However, as in other areas of protein biophysics, conceptual understanding of protein mechanics has benefited significantly from simpler, highly approximate and certainly "chemically less accurate" models. In particular, simpler models have anticipated the experimental observation of multiple mechanical unfolding pathways, the non-exponential dependence of the unfolding rate on applied force, and predicted the existence of proteins whose unfolding rate decreases upon application of a force.

The spatial and temporal resolution of single molecule force spectroscopy techniques is still limited, and is not sufficient for a complete understanding of the processes being investigated. Therefore, simulations are needed not only to provide an atomistic picture but also to aid in the evaluation of the theoretical framework used to analyze mechanical unfolding experiments.

Simplified models demonstrated the inability of the ZB model to account for the effect of mechanical forces on unfolding rate. For this Gō models have been very good; neglecting non-native interactions is less dramatic than assuming 1D landscapes. Gō models have been useful to rank the mechanical strength of proteins [87]. But examples of proteins have been found in which non-native interactions are important during unfolding, and therefore, by their very nature Gō-models would not be appropriate and

will give spurious results (*e.g*. ref. [88, 89]). If by "good" we mean universal reproduction of experimental results then maybe Gō models, and coarse-grained models in general, are not as good as we and others previously claimed. If by "good" we mean the ability to learn, assess the validity of theories and current experimental interpretations, and propose novel ideas about the mechanical response of proteins and their broad importance, then they are good and possibly very good.

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