PROTEIN NANOMECHANICS: THE POWER OF STRETCHING

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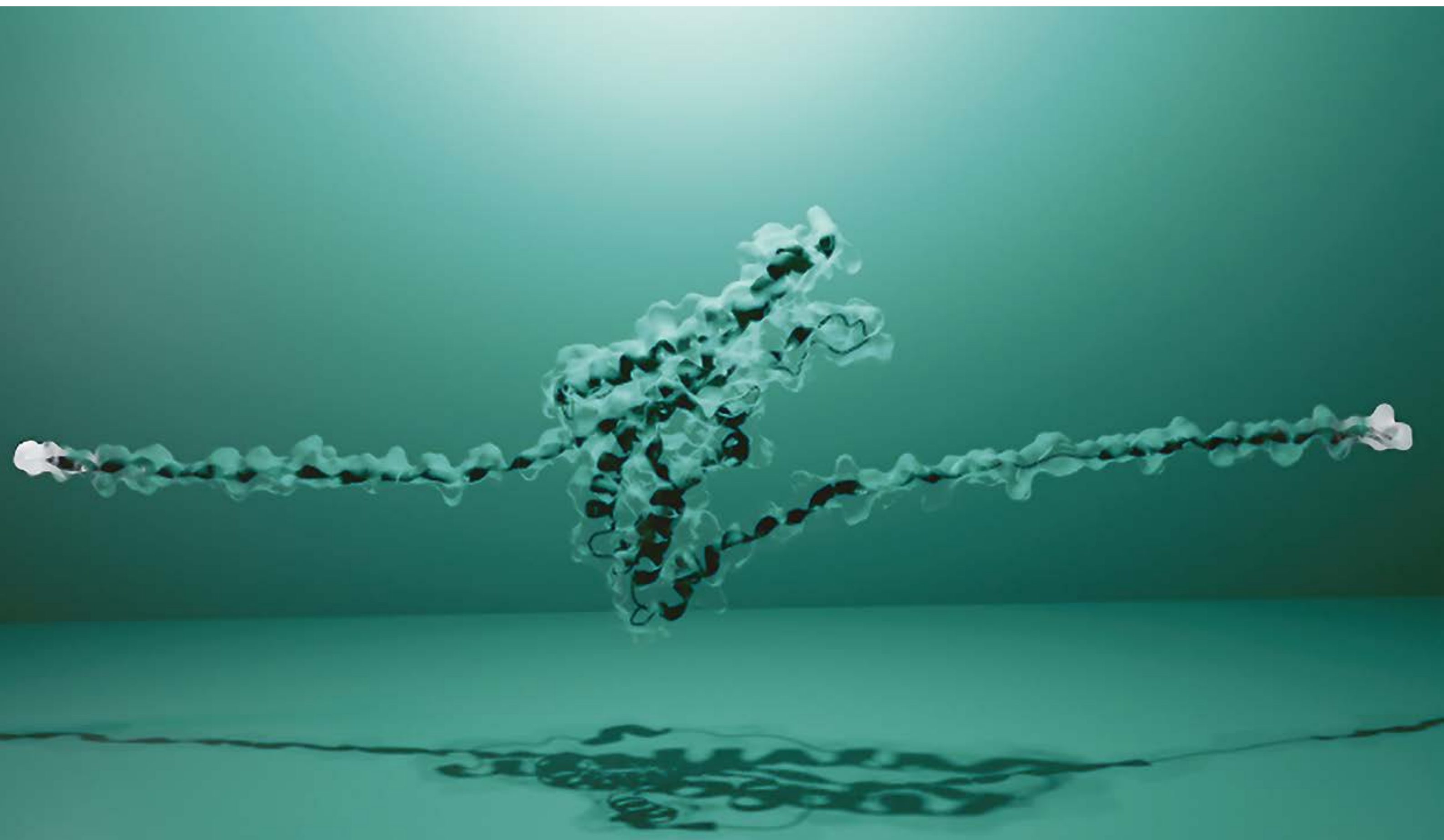
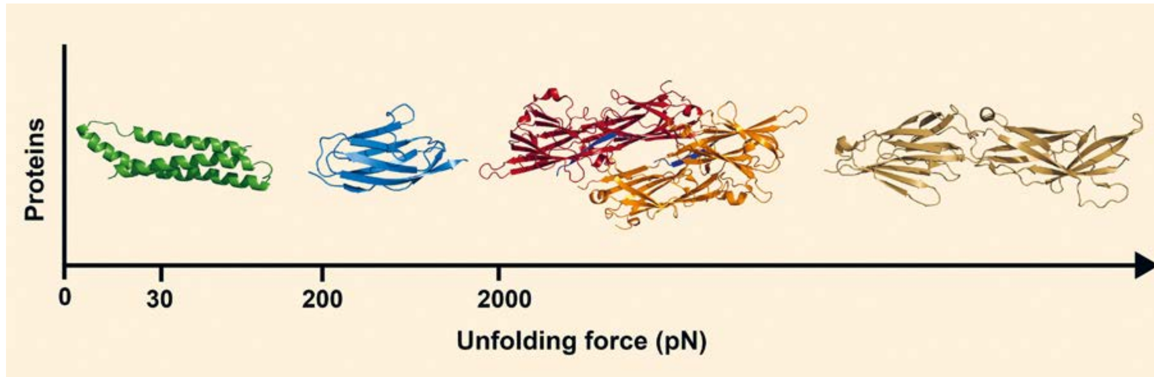
Protein nanomechanics is a rapidly evolving field at the intersection of physics, chemistry and biology focused on the characterisation of the conformational dynamics of proteins under force, of common occurrence *in vivo*.

Mechanical forces regulate important biological functions, including muscle contraction, differentiation, migration, and even cancer metastasis. Yet, in most cases the underpinning molecular mechanisms remain largely elusive. Those mechanically-regulated cellular processes are often ultimately controlled by the elastic properties of the individual constituent proteins — which need to constantly stretch and recoil under mechanical load under physiological conditions. Capturing the individual unfolding and refolding trajectories of relevant proteins under force is challenging. Progress in that direction has been mainly halted by the lack of techniques able to probe the dynamics of individual proteins under force. Thanks to the advent of single-molecule technology, we are now in the position of investigating the molecular mechanisms that confer mechanical resistance to proteins. Borrowing concepts from polymer physics, protein chemistry and molecular biology, complemented with innovative technical developments, we are now beginning to understand the molecular basis of protein elasticity, with single-molecule resolution.





◂ **FIG. 1: Proteins display different mechanical stabilities.** From left to right: Spectrin repeat (~30 pN), Titin Ig27 domain (~200 pN), adhesin SdrG:Fgβ complex (~2300 pN) and adhesive pilin Spy 0128 (which is inextensible due to its internal covalent isopeptide bond).

Force as a denaturing agent

Proteins are essential biological polymers consisting of the covalent linkage of individual amino acids. They are involved in nearly all cellular processes and most of their changes in functionality rely on structural changes, mainly involving the rupture and reformation of their constitutive molecular interactions. In order to be fully functional, the majority of the proteins need to fold into a well-defined three-dimensional structure that defines the natively folded conformation.

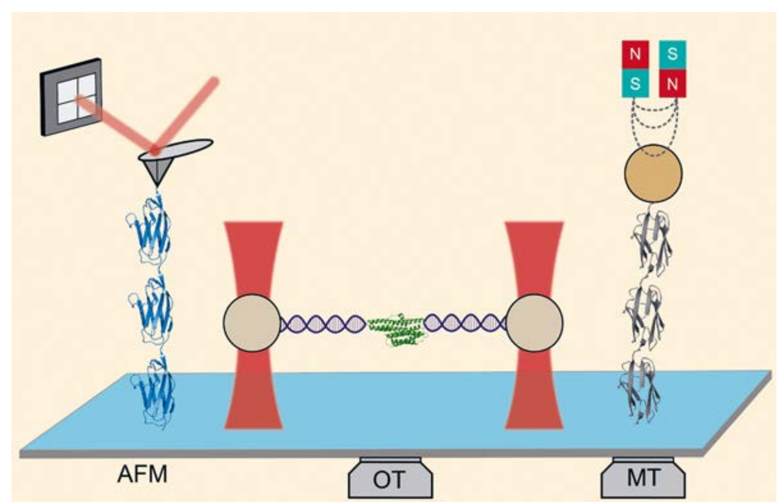
Traditionally, characterising protein (un)folding consisted in exposing proteins to harsh environmental changes by using strong chemical denaturants, abrupt changes in pH or temperature. These experiments led to the important observation that protein folding was a reversible process, and overall provided with a thorough description of the folding energy landscape for a large number of studied proteins. However, these experiments cannot probe the effect of mechanical force on the folding energy landscape of elastic proteins. The development of single-molecule force-spectroscopy techniques in the late 90s complemented the classical protein folding field by experimentally testing the implications of using force as a denaturing agent.

To date, this approach has been used to monitor the force-induced conformational changes of a wide range of proteins. When a stretching force is applied to the protein by a force probe, the protein's restoring force is measured as a function of its end-to-end length. Alternatively, the extension of the protein can be monitored over time. These experiments use the mechanical stability of proteins as their structural fingerprints. Given that proteins exhibit a wide range of mechanical stabilities according to their different structures and topologies (Fig. 1), three complementary main single-molecule techniques (namely optical tweezers, magnetic tweezers and atomic force microscopy) have been developed [1], each of them excelling at measuring forces with unprecedented resolution within a defined force regime (Box). Combined, the enabling experiments have provided us with a general framework to understand the molecular mechanisms that regulate protein nanomechanics.

The fundamentals of protein nanomechanics: the mechanical clamp

During the past two decades, an impressive body of literature has unravelled the basic rules governing protein (un)folding under force [2]. The first implication of using force (a vectorial quantity) as a denaturant is that it distributes non-isotropically throughout the protein backbone. Consequently, not all the amino acids forming the polypeptide chain are equally exposed to the stretching force. Accordingly, and in sharp contrast with the isotropic nature of the biochemistry unfolding experiments, mechanical unfolding is a highly localised process, whereby the main barrier to unfold typically lies in the rupture of a few non-covalent bonds between key amino

▾ **FIG. 2: The three main single-molecule protein nanomechanics techniques.** From left to right: single molecule atomic force-microscope, optical tweezers and the magnetic tweezers.



Mechanical unfolding is a highly localised process, whereby the main barrier to unfold typically lies in the rupture of a few non-covalent bonds between key amino acids that act as the structural force-bearing motif – the so-called mechanical clamp ❞

BOX: SINGLE-PROTEIN FORCE SPECTROSCOPY TECHNIQUES

Atomic force microscopy

Developed in 1986 by Binning and co-workers, the atomic force microscopy (AFM) was initially conceived as an imaging technique to elucidate the topography of both conducting and insulating surfaces with sub-nanometre resolution. By using the sharp cantilever tip as a force probe, it soon became an ideal tool to manipulate individual biomolecules. In a typical experiment, a polyprotein is usually tethered between the substate (commonly a gold coated cover slide) and the cantilever tip. Protein stretching and unfolding is directly monitored by the deflection of the cantilever tip (Fig. 2 left). The AFM is ideally suited to work with proteins with intermediate to high mechanical stabilities, ranging from ~20 pN up to ~1 nN. It can work under force-extension and force-clamp operational modes.

Optical tweezers

Based on the operational principles of the single-beam gradient force trap, optical tweezers (OT) can be used as a force-spectroscopy technique able to apply pN forces to individual biomolecules while recording their position with sub-nanometre resolution. In the particular case of proteins, the experimental strategy consists of tethering a single monomer of interest between two long DNA tethers which are, in turn, attached to two optically trapped dielectric beads (Fig. 2 middle). By controlling the bead displacement and the laser intensity, optical tweezers can apply forces from ~0.1 pN up to 100 pN. OT can work both under the force-extension and force-clamp modes, and is typically used to study the unfolding and refolding trajectories of single proteins with low mechanical stability.

Magnetic tweezers

The magnetic tweezers (MT) technique uses a magnetic field, generated with a pair of permanent magnets placed on top of the sample, to apply calibrated forces to individual biomolecules. In a typical protein-based experiment, individual polyproteins are tethered from both ends. One end is tethered to the cover slide (usually using HaloTag chemistry) and the other terminus is linked to the paramagnetic bead using Streptavidin-Biotin technology (Fig. 2 right). By precisely controlling the position of the permanent magnets — and hence the magnetic field strength — MT can apply forces spanning ~1- 200 pN under passive mode conditions. The development of new covalent strategies has enabled the study of protein (un)folding with exceptional stability over long periods of time (of even week-long continuous stretching cycles).

acids that act as the structural force-bearing motif – the so-called mechanical clamp (Fig. 3a). Once the mechanical clamp is disrupted, mechanical unfolding typically proceeds in a downhill manner, without any additional resistance to the applied force (unless further mechanical intermediates are found during the unfolding pathway). A second immediate consequence of the directionality imposed by the applied force is that the mechanical stability of proteins depends on the pulling direction.

Reconstructing protein energy landscapes with force

Two main operational modes are typically used to study protein unfolding under force: (i) by stretching proteins under constant velocity [3] and, (ii) by applying a constant force [4] – the force-clamp mode. (i) Stretching a polyprotein under constant velocity – as first reported in the seminal paper form Gaub's lab [5] – results in the characteristic saw-tooth unfolding trajectories, where each force peak in the force versus protein extension trajectory corresponds to the independent unfolding of an individual monomer within the polypeptide chain (Fig. 3b). (ii) Alternatively, unfolding a polyprotein under force-clamp conditions results in a staircase-like unfolding trajectory, where each step-wise length increase corresponds to the unfolding of an individual protein monomer (Fig. 3c). The force-clamp approach monitors the time-dependency of protein (un)folding under a constant stretching force, enabling direct extraction of the kinetic parameters defining the unfolding energy landscape.

A qualitative representation of the effect of force on the protein conformation (ultimately dictated by the underpinning (un)folding energy landscape) can be rationalised using simple energetic considerations. In the absence of force, the protein lies in its energy minimum, which typically corresponds to the folded state. The application of a constant force introduces a mechanical work term ($-Fx$, where F is the applied force and x is the protein's extension), which tilts the energy landscape by reducing the activation energy barrier ΔE and introduces a new free energy minimum towards extended conformations that is populated once the protein crosses the ΔE barrier upon unfolding (Fig. 3d). The position of this new equilibrium minimum is force-dependent, as predicted by the worm-like chain (WLC) model of polymer elasticity.

A quantitative reconstruction of the protein's energy landscape is accurately obtained by measuring the force-dependent unfolding rates $\alpha(F)$ at different stretching constant forces. While different models relating $\alpha(F)$ with the pulling force have been developed, they are ultimately all refinements of the simple phenomenological Bell-Evans-Ritchie model, which describes the exponential relationship between the life-time of individual bonds and the externally applied force:

$$\alpha(F)=\alpha_0 \exp - \frac{F\Delta x}{k_B T} \quad (1)$$

where α_0 is the unfolding rate in the absence of force and Δx is the distance to the transition state. Recent efforts in the field have focused on understanding how well-defined chemical perturbations (such as single-point mutations, protein-ligand interactions and post-translational modifications) affect the (un)folding energy landscapes of proteins under force.

Conclusions

Force has emerged as a key physical perturbation that precisely controls the conformation of individual proteins, with important implications for the elastic regulation of those proteins that are physiologically exposed to mechanical forces. The advent of single-molecule force-spectroscopy techniques (AFM, OT and MT) has enabled mapping of the conformational dynamics of individual proteins under force with unprecedented force-, length- and time-resolution. While the single protein nanomechanics field is reaching maturity, arguably the main challenge in the field is the translation of these *in vitro* nanomechanical experiments into the cellular context (with potential knock-on effects at the functional level). Despite recent promising achievements, we lack global understanding of whether individual molecules working inside the cell respond to force according to the same fundamental physical laws established by the single-molecule experiments. Closing such a scale gap has become a timely, necessary and unavoidable requirement to enable the natural advancement of the mechanobiology field.

About the authors

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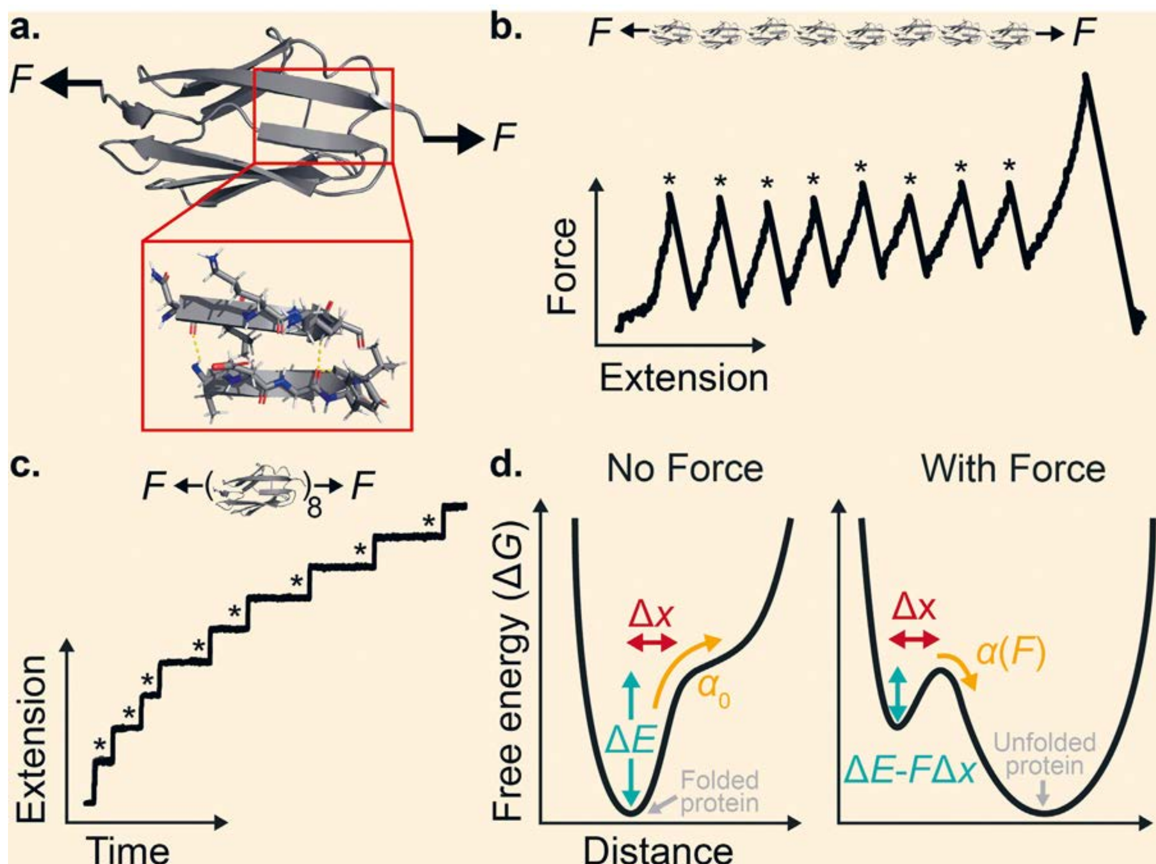

◂ **FIG. 3: The nanomechanical characterization of protein unfolding. a. The folded structure of the titin Ig27 domain, with a zoom on its mechanical clamp. b. Representative force versus extension unfolding trajectory of a polyprotein made of eight identical repeats of the Ig27 protein when stretched at constant velocity. c. Representative force-clamp unfolding trajectory of the same polyprotein stretched under a constant force, where each step length increase corresponds to the unfolding of a single protein domain. d. Schematic representation of the 1-D energy landscape representation of a protein in the absence and presence of an externally applied constant force.**