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Understanding biology by stretching proteins: recent progress

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Abstract

Single molecule manipulation techniques combined with molecular dynamics simulations and protein engineering have enabled, during the last decade, the mechanical properties of proteins to be studied directly, thereby giving birth to the field of protein nanomechanics. Recent data obtained from such techniques have helped gain insight into the structural bases of protein resistance against forced unfolding, as well as revealing structural motifs involved in mechanical stability. Also, important technical developments have provided new perspectives into protein folding. Eventually, new and exciting data has shown that mechanical properties are key factors in cell signaling and pathologies, and has been used to rationally tune these properties in a variety of proteins.

Keywords

Protein nanomechanics; Mechanical Stability; Single-Molecule Force Spectroscopy; Molecular Dynamics; Protein folding

1. Introduction

Modern biology compares the cell to a factory: a place crowded with machine-like molecular devices that work together in a highly organized fashion to sustain life. Classical biochemical techniques extract information regarding the function of these molecules from large ensembles; however, they do not directly address the details of their inner workings. Recent technological advances have enabled single molecules to be studied, avoiding ensemble averaging. For instance, these techniques can capture transient intermediates and alternative conformers. In

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particular, the techniques used to manipulate molecules individually include atomic force microscopy (AFM), laser optical tweezers, magnetic tweezers [1].

An important feature of single molecule experiments is that they are closely comparable to molecular dynamics (MD) simulations of individual molecules. MD simulations provide an atomic description of the system, not accessible experimentally. They have been proved to be very accurate, and even predictive, such that they offer an unprecedented and fruitful interplay between the theory and the experiments [2].

Here, we review the most recent advances in mechanical unfolding/folding of proteins achieved using AFM-based single molecule force spectroscopy (SMFS), often associated to computer simulations. More comprehensive descriptions of the field are provided elsewhere [3–8].

2. Local structure as the origin of mechanical stability

In 1997, a pioneering AFM study (see fig. 1a) of stretching a single molecule of the muscle protein titin was performed [9]. The observed pseudo-periodic (sawtooth) pattern in the force extension plot has been associated with the unfolding of separate immunoglobulin (Ig) modules of the proteins. The obvious question that aroused was where does such high forces (150–350 pN) come from? Mechanical stability, which is measured as the average rupture force upon stretching, enables a protein to remain folded under certain mechanical stress. By using MD simulations, it was found to be a local property, associated to specific patches of secondary structure of each Ig module [10]. Interestingly, these simulations could even explain subtle details observed experimentally, such as deviations from the expected saw-tooth pattern [3], even at pulling speeds close to those used in experiments [11]. Each force peak was associated with the breakage of a patch of backbone hydrogen bonds due to a shearing force applied to the N and C termini.

This so-called mechanical clamp involves two neighboring β-strands usually located near the termini (see fig. 1b). Subsequently, such mechanical clamps have been discovered in many other modules and proteins from metazoa, such as fibronectin type III and even in proteins with no known mechanical function, like ubiquitin [4]. Recently, they have also been observed in putatively mechanical proteins from evolutionarily distant organisms such as the cohesin I domains in scaffoldins from bacteria [12]. These domains are the most mechanostable proteins found experimentally to date (see table 1c) and they were even predicted, using a coarse grained model, to be very resistant to stretching [12]. Since they have also been recently discovered in archaea [13], the mechanical clamp motif seems to be ubiquitous and present in all domains of life. However, it should be noted that mechanical stability is not only determined by mechanical clamps, but also by other geometries. Moreover, critical contacts in the mechanical transition state (TS: see below) further modulate this property [14,15].

In addition to mechanical clamps, other highly stable geometries (up to 1000 pN) have been predicted using MD simulations in the most recent PDB-wide survey (for ungapped proteins shorter than 250 amino acids) [16]. These results include a wide range of mechanical stabilities and are available at info.ifpan.edu.pl/BSDB/. The top 13 strongest proteins turned out to have a different geometry: the cysteine slipknot (CSK: see fig. 2a). Upon stretching, a segment of protein backbone is dragged through a cysteine “knot” (not a knot in the topological sense, but a ring) by a disulfide bond. This cysteine knot is formed by two other segments of the backbone, which are linked into a closed loop by two additional disulfide bonds. Interestingly, this geometry relies on overcoming steric constraints rather than breaking bonds through shearing. These simulations were done with a coarse grained model, which only takes into account contacts present in the deposited structure. It is interesting to note that there is evidence for other contacts stabilizing some proteins mechanically [13,17].

Other protein structures respond differently to the application of force. Gankyrin, an ankyrin-repeat protein, follows alternative mechanical unfolding pathways, showing non-cooperative behaviour with no local breakpoint[17].

Apart from the aforementioned intrinsic factors, mechanical stability can also be affected by the proteins environment. For instance, molecular crowding (using dextran) induces a slight increase in mechanical stability and changes in the unfolding rate of ubiquitin [18]. On the other hand, the presence of the denaturing agent guanidinium chloride decreases the unfolding free energy barrier of GB1, leaving the unfolding pathway unaffected [19]. Furthermore, binding of different antibody fragments was found to enhance the mechanical stability of GB1, even when the binding site was distant from the TS [20,21]. Moreover, smaller ligands like peptides or even ions affect calmodulin mechanostability [22].

3. Recent advances in mechanical folding

Until recently, the only way to study unfolding/folding was through bulk chemical or thermal experiments. Such experiments are usually carried out at high temperatures or extreme denaturant concentrations, conditions not found *in vivo* [23]. By contrast, SMFS enables the use of mechanical force, a parameter involved in numerous cellular processes [5]. Furthermore, this method provides a well defined reaction coordinate: typically the end-to-end distance of the protein (with forces projected on this direction). An additional difference is that force acts locally on the protein instead of globally [7].

3.1. Investigating the transition state

In the classical two-state model of protein unfolding, a protein passes from a native state to an unfolded state through an energy barrier. The peak of this barrier, known as the TS, is the maximum free energy conformation.

Since the TS of a protein cannot be isolated, information on this state can only be extracted from indirect studies such as φ-value analysis [24], SMFS experiments [25] or a combination of both [26,27]. With SMFS, the location and height of this state can be determined, although the structure cannot be obtained. Hence, combining MD simulations with this technique provides more detailed information, such as the putative structure or the native contacts already present in the TS [26].

Changes in the nature of the solvent can give us important information on the TS. Pulling experiments in the presence of glycerol [28] or heavy water [29] have been shown to change the kinetics of the unfolding process, implying a modification of the position and height of this barrier. These experiments suggest the involvement of the solvent within the TS structure [30], whereby both the size and chemical nature of the solvent influence its properties, as predicted by simulations. These experiments provide important hints on the unfolding mechanism.

3.2. Pushing the frontiers of protein folding

When a protein is fully stretched, it acquires a relatively well defined state where inter-residue contacts are limited due to breakage of secondary and tertiary structures. SMFS refolding experiments use this state as a starting point to follow the folding reaction. Due to technical limitations, only few studies have reported direct measurements of the refolding forces [31].

In 2004, the folding of an ubiquitin polyprotein (a tandem repeat of the protein of interest, typically used as a single molecule marker [25]) was reported [32]. In this study, a quenching force was applied to a previously stretched protein using the force-clamp mode of SMFS (fig. 3a) allowing it to fold while monitoring its end-to-end distance. The different stages observed

(see fig. 3a) were initially interpreted as follows: a fast entropic recoil of the extended protein followed by a fluctuating step, in which the collapsed protein explores the energy landscape, until it further collapses to its native state. These observations were controversial [6,33,34], as they supported consecutive stages as the folding mechanism, against the classical view. Moreover, refolding using force-clamp does not, in general, proceed along the thermal folding pathway [35].

These studies were recently extended through experiments that further support the original observations, providing further insight into the properties of each stage. First, the possibility of artifacts derived from aggregation [36] or the use of polyproteins [37,38] was addressed, concluding that the mechanical unfolding of polyproteins is not affected by these factors. These results further validate the use of polyproteins, as they represent the properties of the monomer while providing a series of additional advantages [25].

The nature of the first stage in folding [32] has been further investigated using different force-clamp protocols [39] and cosolvents [39,40], concluding that hydrophobic forces seem to direct this first collapse. A detailed study of the compact structures formed after the hydrophobic collapse suggests that these belong to an ensemble of minimum energy compact structures (MECS), a population that is on-pathway in the folding process [41] (see fig. 3b). Remarkably, the existence of MECS was predicted by lattice theories of folding [42]. MECS were also found to be unaffected by cosolvents [40], suggesting that they are structures that are preferentially solvated by water molecules that must be released in the last folding step.

The development of so-called lock-in SMFS has increased the force sensitivity and range of the technique into the sub-pN regime [31]. The method involves a low-frequency oscillation of the sample combined with a low pulling velocity. This has been applied to the study of the three-state folding process of the 4th Ig domain from filamin. A hopping pattern was obtained (see fig. 4), reporting an apparent equilibrium between states: unfolded and intermediate, as well as intermediate and native.

The two aforementioned approaches [31,32] suggest different views of the folding process: stages *versus* transitions. In the absence of further experimental data, MD simulations are being used to gain more insight into such processes (see for example ref. [43]).

4. Other mechanical studies

Mechanical unfolding experiments have also been used to study a wide range of biological processes. As a result, titin has been suggested to also function as a mechanical signal transducer [44]. Moreover, mechanically induced exposure of cryptic (hidden) sites have been described as a transduction mechanism in talin [45]. Also, the ability of ankyrin to generate force upon refolding has been proposed to be relevant for biological functions that require specific elastic responses, such as the generation of tension in biological membranes [46].

Furthermore, disease associated mutations in polycystic kidney disease (PKD) domains of polycystin-1 (a kidney mechanosensor) display distinct mechanical phenotypes [47]. Considering that neurotoxic proteins may jam the unfoldase motors [7], the recent attempts to measure their mechanical properties [48–50] constitute a particularly interesting biologically relevant line of research.

Several other applications for this methodology have recently been published. For instance, detailed information has been obtained on the single disulphide-bond breakage under force [51,52]. Moreover, rational modification of the mechanical properties of proteins is an interesting emerging field. Different approaches have been followed, such as fragment shuffling between structurally related proteins [14], changing the unfolding pathway by using

disulphide bonds [53], adding metal chelation sites to the mechanical clamp [54], adding amino acids that extend a loop near the break point of the protein [55], or modulating hydrophobic contacts by complementary changes on related residues [27].

5. Conclusions and future directions

SMFS studies have provided exciting and promising results in studying large conformational changes, including protein folding. However, further developments are required in order to gain a better insight in the mechanics of protein folding.

For instance, these studies have been performed on a limited number of proteins, and the general rules that govern protein mechanical stability and folding need to be worked out in more detail. Also, some technological developments are needed, such as single-molecule markers that allow proteins with low mechanical stability to be studied (*e.g.* natively unstructured proteins), or single-event markers to unequivocally analize the nanomechanics of protein interactions. Furthermore, better functionalizing protocols that permit greater control of the protein sample are needed.

Finally, further development in computer science and modeling is also required in order to be able to use all-atom simulations in biologically relevant timescales, and in conditions comparable to experiments.

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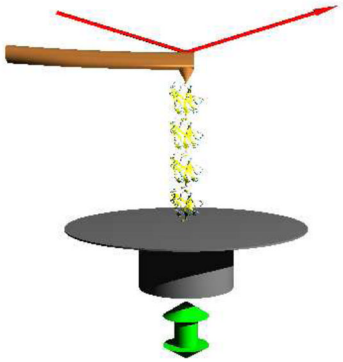
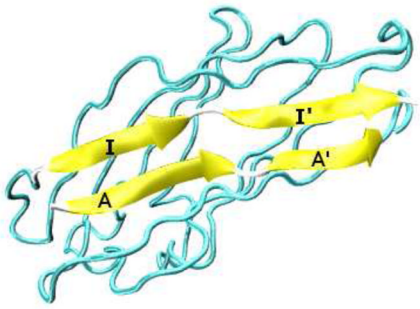


(a) Atomic Force Microscope



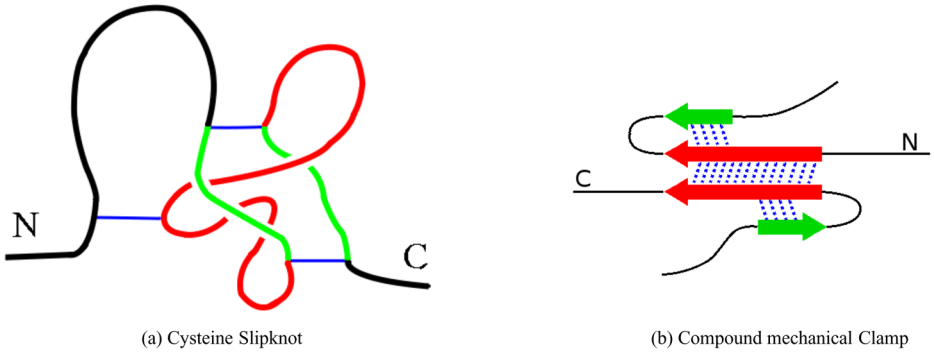
(b) Tertiary structure of a cohesin module

	$F^{exp}(pN)$	$k_U^{exp}(s^{-1})$	$F_{max}^{CG}(pN)$	AI+A'I'	$T_m(°C)$
c7A	480 ± 14	$(6 \pm 5)10^{-6}$	473	5+5	84.6 ± 0.6
c1C	425 ± 9	$(1.1 \pm 0.5)10^{-4}$	429	5+5	74.3 ± 0.2
c2A	214 ± 8	$(2.1 \pm 0.3)10^{-2}$	296	4+3	87.5 ± 0.6

(c) Stability of cohesins

Figure 1.

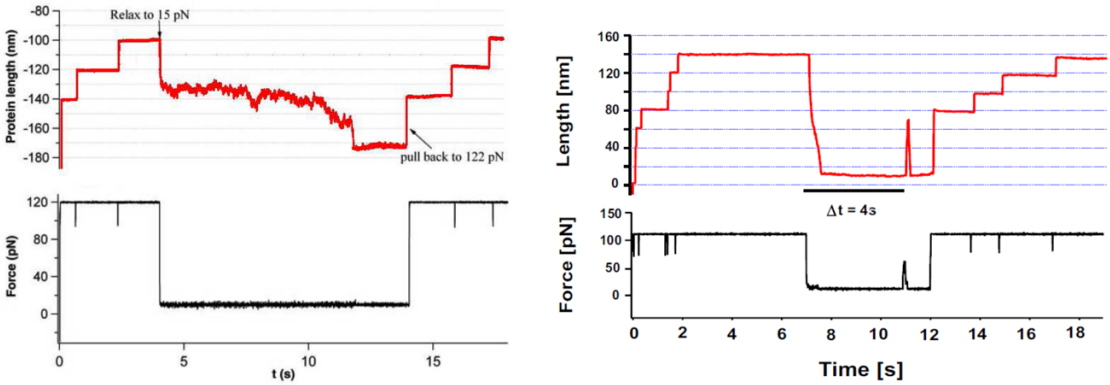
AFM-based SMFS can explore the high range of protein mechanical stability. (a) **Atomic Force Microscopy (AFM)** is based on stretching proteins deposited on a substrate using a cantilever to exert a pulling force. Typical AFM has a better spatial resolution than other techniques, although forces resolved are comparatively higher (in the order of tenths of pN). (b) **Tertiary structure of a cohesin**, in which the shear mechanical clamp motif is highlighted. Figure done with VMD [56]. (c) **Comparative analysis of cohesin modules from scaffoldin**. The properties shown include the unfolding force (F^{exp}) and spontaneous unfolding rate (k_U), measured experimentally by AFM; the maximum unfolding force from coarse-grained MD simulations (F_{max}^{CG}), the number of hydrogen bond present between the different chains (AI+A'I') and the melting temperature (T_m). We can observe that the mechanical stability correlates with the unfolding constant, and that simulations are also in close agreement with experiments, while thermal stability does not correlate with the other measurements. Data obtained from ref. [12] and [16].



(a) Cysteine Slipknot (b) Compound mechanical Clamp

Figure 2.

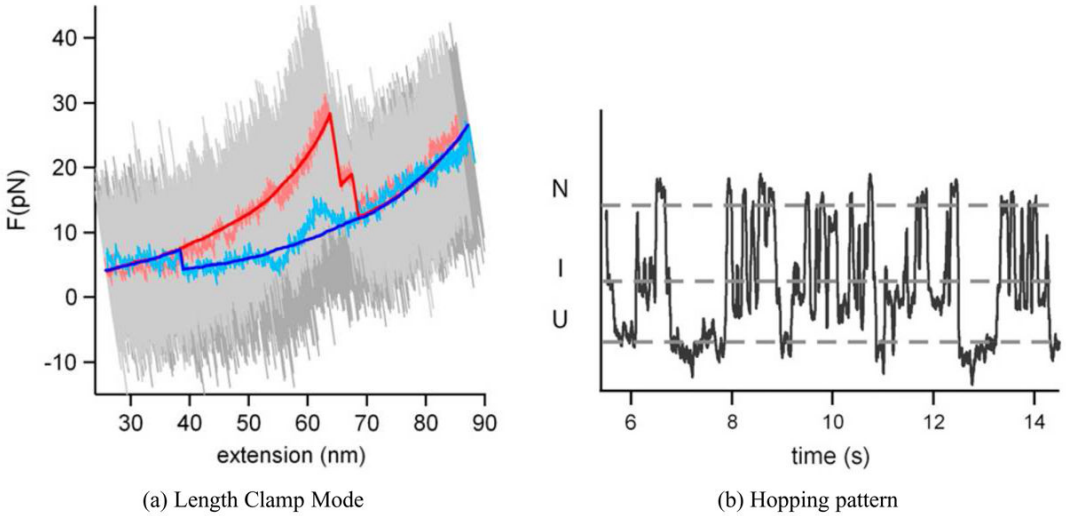
Protein breakpoints with the highest predicted mechanical stability. (a) The recently reported **Cysteine Slipknot (CSK)** is formed by a disulfide bond between two cysteine residues that constitute the knot, together with two additional disulfide bonds closing a ring (shown in green). In the absence of the disulfide bonds, this geometry would be trivial to undo by pulling, although it is the most stable structure predicted up to date using simulations. The cartoon shows the knot from the human vascular endothelial growth factor (PDB code 1vpf). (b) **Compound mechanical clamp:** This cartoon shows the compound shear mechanical clamp of the β domain of streptokinase (PDB code 1c4p), formed by three hydrogen-bonded regions. Both geometries are predicted to have higher mechanical stability than cohesin modules (fig. 1b, table 1c). Figures modified from ref. [16].



(a) Force Clamp Mode (b) Proving Minimum Energy Collapsed State (MECS)

Figure 3.

Exploring protein folding with force clamp spectroscopy. (a) In the **force clamp** mode, a feedback mechanism corrects the substrate-tip distance, which is measured as a function of time, in order to control the force. In the typical protocol, the force is initially set to a high value to trigger the unfolding of a polyprotein. Each step reports the unfolding of each module, and kinetic data can be directly obtained from this kind of traces. The first arrow marks the force reduction that allows folding stages to be observed. Eventually, to check that the protein has folded successfully, the high force is restored, expecting to reproduce the unfolding pattern. (b) **MECS:** Using a lower quenching force protocol, the fluctuating stage (see panel 3a) is probed by applying a force pulse. Comparing the effect of this perturbation to unperturbed controls, it can be concluded that these structures are true precursors of the protein's native state. Figures obtained from ref. [32] and [41] with permission.



(a) Length Clamp Mode (b) Hopping pattern

Figure 4.

Lock-in SMFS. (a) In the **length clamp** mode, the sample is withdrawn at a constant velocity, while measuring the bending force of the cantilever. In this mode, each unfolding event is represented by a force peak. The figure shows a comparison between the results obtained with the lock-in mechanism (dark red and blue) [31] and the usual length clamp protocol, showing raw data (dark and light gray) as well as low-pass filtered (light red and blue) curves. This set up not only reduces noise of the curve, but also increases the sensitivity of the technique. (b) A **hopping pattern** is observed in the 4th Ig module of filamin by keeping a constant average length and amplifying the oscillation: the molecule jumps between the native, intermediate and unfolded states in apparent equilibrium. Figures obtained from ref. [31] with permission.