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Mechanical unfolding of proteins: insights into biology, structure and folding

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Since user-friendly atomic force microscopes came onto the market a few years ago, scientists have explored the response of many proteins to applied force. This field has now matured beyond the phenomenological with exciting recent developments, particularly with regards to research into biological questions. For example, detailed mechanistic studies have suggested how mechanically active proteins perform their functions. Also, *in vitro* forced unfolding has been compared with *in vivo* protein import and degradation. Additionally, investigations have been carried out that probe the relationship between protein structure and response to applied force, an area that has benefited significantly from synergy between experiments and simulations. Finally, recent technological developments offer exciting new avenues for experimental studies.

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Introduction

The development of single-molecule atomic force microscopy (AFM) and other techniques, including molecular tweezers and the biomembrane force probe, enables scientists to measure the response of proteins to an externally applied force (Figure 1). In this fast-growing field, there are several problems being addressed. In this review, we concentrate on experimental insights into a few of these problems: how mechanical proteins perform their function; the molecular basis of how and why proteins respond to force differently; and technological developments that allow new problems to be investigated. The use of AFM techniques to investigate the structure, stability and activity of membrane proteins was reviewed recently in this journal [1] and will not be discussed here. AFM studies of protein–protein interactions are also increasing in number and are beyond the scope of this review (but see, for example, [2] and references therein). Computational groups using molecular dynamics and other simulation techniques have been very active in investigating forced unfolding of proteins. Again, the vast majority of these studies are beyond the scope of this review, but we comment on cases in which simulation has given particular insight into experimental results.

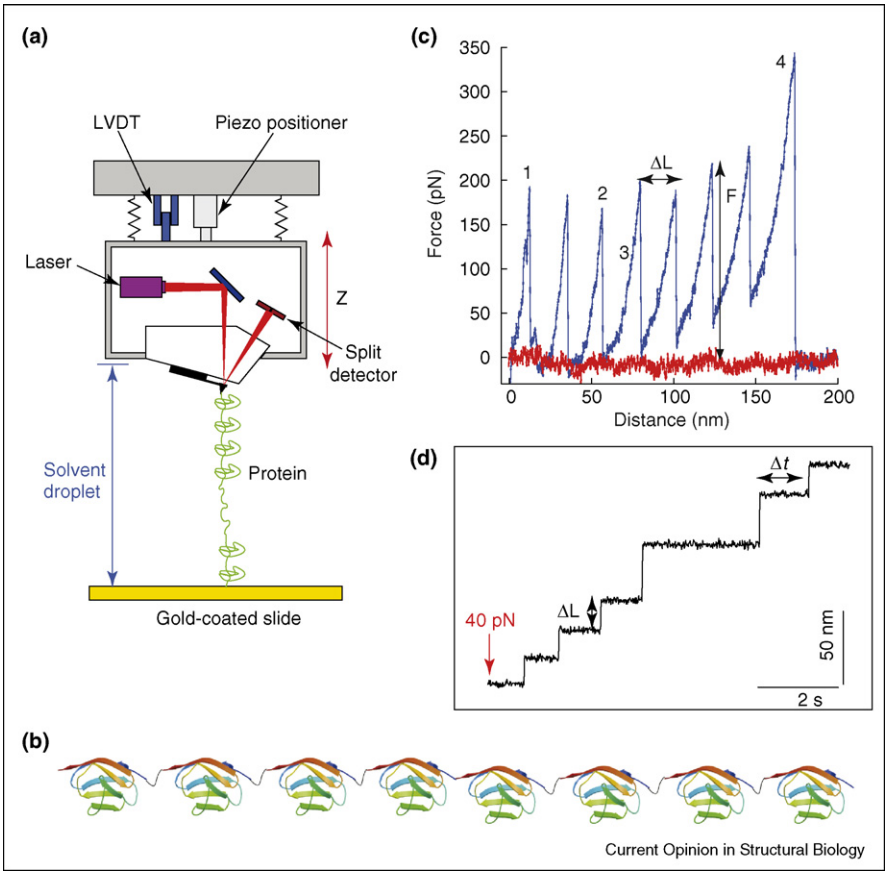
Mimicking *in vivo* forces: force and function

Since the development of instruments that unfold proteins mechanically, these tools have been used to mimic the mechanical stresses that proteins experience in the natural environment. One of the most important applications of AFM is the investigation of protein structure-function relationships: how so-called 'mechanical proteins' perform a variety of functions *in vivo*. Structural proteins, such as the giant muscle proteins titin and twitchin, and the intracellular matrix protein spectrin, are a subset of mechanical proteins responsible for maintaining structural integrity *in vivo*. A second subset, mechanosensory proteins, which includes several proteins of the extracellular matrix and cell adhesion molecules, participate in the transduction of mechanical signals to cell signalling pathways. Most proteins with mechanical functions are long proteins with a modular architecture (Figure 2), comprising repeating domains joined at the N and C termini.

Elastic protein architecture

Perhaps one of the most important questions to be considered is how 'mechanically active' proteins maintain their integrity when subject to forces that can be many tens of piconewtons. During normal activity, it is likely that the folded domains in these proteins remain folded. For instance, a simple study of the elasticity of intact fibrils of the extracellular matrix protein fibronectin with engineered GFP domains suggests that the effect of force on the fibril does not result in wholesale unfolding of molecules. Rather, there is extension of the entire protein from a compact to an extended conformation [3]. However, simulations suggest that cryptic binding sites in fibronectin might be exposed due to partial unfolding of the molecule under force [4]. The immunoglobulin (Ig) domains in the elastic portion of titin (the I-band) are highly resistant to force and are unlikely to unfold, unless the muscle is subject to extremely high loading rates [5]. In this case, one or two domains may unfold to prevent damage, and rapid refolding upon release of the force allows the protein to recover. Interestingly, the titin molecule seems to have 'strong' domains next to 'weak' ones. This lessens the likelihood of two adjacent domains

Figure 1



The use of AFM to study protein unfolding. **(a)** Schematic diagram showing the operation of the AFM. The protein is adsorbed onto a surface, often by covalent attachment to gold through cysteine residues engineered at the C terminus. It attaches to a microfabricated silicon nitride cantilever, most often by non-specific adsorption, when the cantilever is lowered onto the surface. As the cantilever is raised off the surface by a piezo positioner, a force is exerted on the protein. The position of the cantilever (and thus the force exerted) is determined using a laser and a split photodiode detector. The LVDT (linear voltage differential transformer) monitors displacement in the Z-direction. **(b)** Many AFM experiments make use of an engineered polyprotein, such as titin I27 (shown), with multiple copies of the same protein [62]. Several versatile cloning systems are available on request (e.g. [63]) that allow different domains to be cloned together to enhance expression, enable inclusion of an internal standard or allow interdomain interactions to be investigated (e.g. [18,64]). **(c)** A 'typical' AFM trace with the instrument used in constant velocity mode — the most common experimental setup. The unfolding force at a given pulling speed can be determined. The protein is a polyprotein containing multiple copies of titin I27 (as shown in b) and the trace was collected at a retraction speed of 600 nm/s. The red trace represents the cantilever deflection during the approach to the surface, the blue trace during the retraction. The first peak (1) is of variable height and reflects detachment of the tip and/or protein from the surface. Force is exerted on the protein until one domain unfolds (2). The unfolded polypeptide chain is then stretched (3) until another domain unfolds. The distance between unfolding events (ΔL) is characteristic of the unfolding of a domain of ~90 amino acids. The force (F) at which the domains unfold (~200 pN) is determined from the height of the unfolding peaks. Finally, all domains having unfolded, the protein detaches from the cantilever (4). **(d)** A 'typical' AFM trace showing the same protein being pulled while the AFM is used in constant force mode. At the time marked by the red arrow, the force was switched from −20 pN to 40 pN. At this force, the unfolding steps (ΔL) have a mean size of ~20 nm (the two larger steps correspond to two domains unfolding simultaneously). In this experiment, the time between unfolding events (Δ*t*) can be determined. Panel (a) reproduced with permission from [65].

unfolding and, consequently, upon release of force, reduces the chance of misfolding. Furthermore, domains that are most likely to unfold have the highest recovery (refolding) rates [6].

Recent studies have looked beyond single isolated domains to address large-scale architecture, for example, in muscle proteins. Commonly, domains in different parts of muscle proteins have different responses to force — mechanical hierarchies are observed. Titin and myomesin are two components of the muscle sarcomere. The molecular architecture of these proteins is apparently important in adapting the sarcomere to different elastic regimes; both proteins are expressed in different isoforms in different muscle types and in pathological conditions, such as cardiomyopathy. They are composed largely of all-β-sheet Ig-like domains, both Ig domains themselves and fibronectin type III (fnIII) domains.

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