



Mechanical unfolding studies of protein molecules

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Atomic force microscopy (AFM) enables the pick up of a single protein molecule to apply a mechanical force. This technique, called “force spectroscopy,” provides unique information about the intermediates and free energy landscape of the mechanical unfolding of proteins. In this review, we introduce the AFM-based single molecule force spectroscopy of proteins and describe recent studies that answer some fundamental questions such as “is the mechanical resistance of proteins isotropic?”, “what is the structure of the transition state in mechanical unfolding?”, and “is mechanical unfolding related to biological functions?”

Key words: protein folding, dynamic force spectroscopy, free energy landscape, ϕ -value analysis, single-molecule measurement

Viewing the three-dimensional structures of proteins displayed on a computer screen may be fascinating because of the beauty in their architecture. The interior is closely packed, and van der Waals interactions/hydrogen bonds are formed in reasonably effective ways, stabilizing the tertiary structures of proteins. Naive questions may arise in a viewer’s mind, such as “how soft is this protein?”, “how much is it fluctuating?”, or “how much force is required to break a bond here?”. Direct observation/measurement of such mechanical properties is apparently impossible because the dimensions of protein molecules are enormously small compared to those of our hands. Nevertheless, one

may desire to directly manipulate a protein using one’s own hands, introducing a deformation and measuring the response in order to investigate such mechanical properties.

Recent advances in the single-molecule manipulation technology including atomic force microscopy (AFM) and optical/magnetic tweezers have enabled us to perform direct mechanical measurements of single macromolecules. In 1996, Mitsui et al. observed the unfolding of a protein induced by mechanically applied force using AFM for the first time¹. In the following year, Rief et al. reported the mechanical unfolding of the muscle protein titin (also called connectin)². Since these pioneering works, the mechanical unfolding study of a protein has been performed experimentally and theoretically by protein researchers with great interest. Force spectroscopy is a powerful tool to probe subdomain structure and internal dynamics of proteins. In this review, we introduce the background of protein pulling experiments using AFM and discuss the recent progress and hot topics in this emerging field.

Subdomain structure(s) revealed by force–extension curve measurement

In a single-molecule pulling study using AFM, a protein of interest should be attached (“tethered”) between the AFM stage and an AFM cantilever. One of the most commonly used methods is that of gold–thiol bonding³; the sulfur atom in a cysteine residue (only at one end) is covalently linked to the gold-coated AFM stage by a gold–thiol bond. The other end of the protein is picked up by a cantilever with non-specific adhesion (called physisorption). Alternative tethering approaches include the use of non-specific interactions on both ends or the use of biological tags^{4,5}. Using molecular biological techniques, a designed fusion protein is often used, in which the domain of interest is

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flanked by a tandem of well-characterized domains (e.g., I27, ubiquitin, or GB1). These additional tandemly arranged domains serve as “handles” to be picked up by a cantilever; moreover, their well-characterized force–extension profiles can be used as fingerprints of the protein to distinguish data from contaminants.

As the AFM cantilever is continuously retracted from the surface at constant speed with a piezoelectric actuator, a protein tethered between the stage and the tip is stretched, and the deflection of the cantilever reports the mechanical tension applied to the molecule, which can be derived simply by Hooke’s law with the deflection and the pre-calibrated spring constant of the cantilever. Plotting applied force against the tip–surface separation, which can be obtained by subtracting the deflection of the cantilever from the AFM scanner movement trace, yields a force–extension spectrum that reveals a characteristic pattern for the molecule being studied.

The force–extension curve contains valuable information. For example, here we introduce the early studies on the titin I27 domain; these studies were mainly performed by the Fernandez group (experiment) and the Schulten group (simulation). Titin is a giant protein, connecting the Z-line and M-line in the sarcomere and functioning as a molecular spring, which relates to the passive elasticity of muscle⁶. In human cardiac titin, more than 300 immunoglobulin-like domains are arranged in tandem repeats. I27 (or I91 in the nomenclature of Bang et al.⁷) is one of such domain, and its tertiary structure has been determined by NMR spectroscopy⁸. Figure 1 shows a typical sawtooth pattern in the force–extension curve of a tandemly arranged recombinant I27 homo-polyprotein⁹. Upon stretching the protein molecule, the force gradually increases (Fig. 1(1–2)). This rising phase of the first peak fits well with the worm-like chain (WLC) model¹⁰, which reflects the entropic elasticity of the unstructured linker region. As the tensile force rises, one of the domains cannot resist the force and unfolds (Fig. 1(2)). Unfolding of a natively folded domain into an unstructured peptide chain results in an abrupt elongation of the molecule, by which the cantilever can snap back, exhibiting a sudden force drop (Fig. 1(2–3)). Upon further stretching, the next rising phase appears, reflecting a stretching process in both the linker and unfolded regions. Furthermore, this phase can fit well with the WLC model. The WLC model estimates molecule length known as the contour length. The increment in the contour length (ΔL) between the two rising phases must reflect the elongation length of a domain upon unfolding, which is predicted by the subtraction of the NC length of a folded domain from the unfolded polypeptide region (Fig. 1). In case of I27, the experimentally determined elongation length (ΔL) is 28 ± 1 nm, which is in good agreement with the value 28 nm, (32 nm (from the number of the amino acids (89 [AA]) and the length of single peptide bond (0.36 [nm/AA])) minus 4 nm (the NC length of the folded I27)).

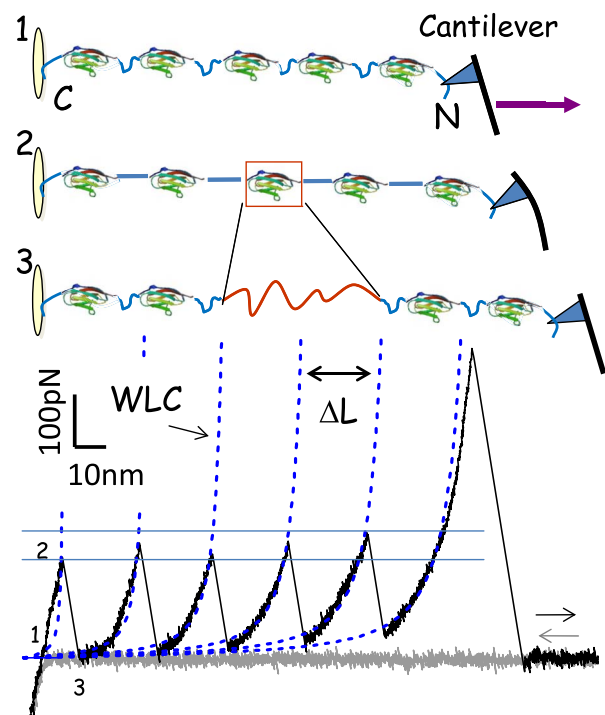


Figure 1 A sawtooth pattern found in the force–extension curve of the I27 polyprotein. At first, the flexible linker regions are extended (1→2). One of the I27 domains then unfolds, and the cantilever snaps back (2→3). These processes are repeated until all the domains have unfolded. The last high peak represents the detachment of the polyprotein from the cantilever.

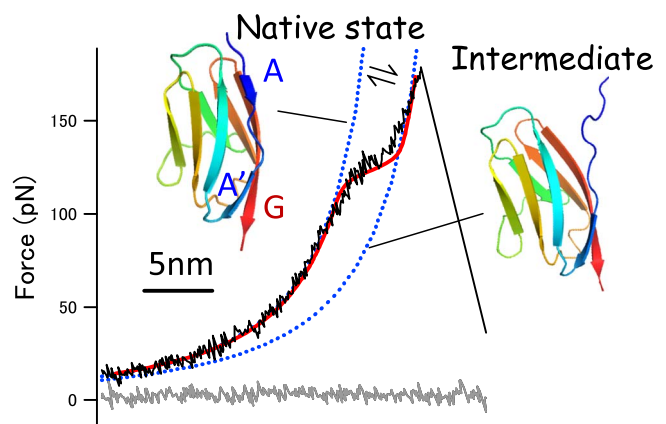


Figure 2 A “hump” structure found in the force–extension curve of the I27 polyprotein. The hump reflects the transition between the native and intermediate states. The red solid line is a fit with the two-state WLC model⁹.

On closer inspection of the rising phase, a “hump” is observed around 130 pN (Fig. 2). This hump is characteristic of the I27 force curve and reflects the partial unfolding event of I27. Molecular dynamics simulation and AFM experiments on the I27 mutant revealed that the hump is the result of the detachment of the β -strand A, which comprises

four amino acids, from the rest of the domain¹¹. This detachment process is in fast equilibrium with the reverse process compared with the time resolution of the AFM force–curve measurement (typically sub-milliseconds). Therefore, the detachment did not exhibit any distinct force peak, but a sigmoidal change was seen on the force–extension curve. While the β -strand A' is detached from the surface of the rest of the domain by mechanical force, the rest of the domain is mechanically stable and can withstand the tensile force. On further application of tensile force, the hydrogen bonds between the β -strand A and G are broken, and catastrophic unfolding of the entire domain occurs, giving a distinct force peak in the force–extension curve.

In addition, mechanical unfolding intermediates are observed in other domains. The FLN4 domain of the actin-binding protein filamin from *Dictyostelium discoideum* has an immunoglobulin-like fold, and both I27 and FLN4 have the same topology (geometric arrangement of β -strands). FLN4 shows an intermediate state in which roughly half of the domain unfolds¹². FLN4 and I27 unfold in different ways, reflecting the difference in interaction between β -strands. Maltose-binding protein (MBP) shows multiple unfolding force peaks, suggesting that MBP consists of four mechanically stable subdomains (called “unfoldons”)¹³. *Borrelia burgdorferi* outer surface protein A (OspA) has a non-globular single-layer β -sheet domain and unfolds by mechanical force through intermediates¹⁴. The majority of T4 lysozymes unfold in an all-or-none fashion, exhibiting a single distinct force peak; but a small fraction of T4 lysozymes unfold in a three-state fashion involving unfolding intermediate states, suggesting multiple distinct unfolding pathways¹⁵. Meanwhile, for the long coiled-coil region of myosin (known as the myosin rod or tail)^{16–18}, a different force–extension profile is found, wherein no distinct force peak is seen, but a plateau appears at around 50 pN (Fig. 3). At the beginning of the plateau, short coiled-coil segments begin to unfold. The segments undergo a rapid unfolding/refolding transi-

tion, similar to what is observed with the hump of I27. At the end of the plateau, the coiled-coil unfolds completely, and the force begins to rise again, reflecting the entropic elasticity of unfolded polypeptides. As mentioned above, by analyzing force–extension curves, we can acquire knowledge related to the intermediates, pathways, and dynamics of the mechanical unfolding of protein molecules.

Free energy landscape determines unfolding force

What does the unfolding force relate to? A mechanical unfolding of a single folded protein domain is a stochastic event (see unfolding forces in Fig. 1), and the unfolding force depends on the pulling speed (Fig. 4(A)). Therefore, a mechanical unfolding event is a kinetic process, not an equilibrium measurement; hence, the mechanical unfolding experiment will provide information about the folding free energy landscape including the height of the barrier.

To analyze the kinetics of mechanical unfolding, first let us assume native, unfolded, and transition states in the unfolding pathway, similar to other protein folding experiments (e.g., denaturation) (Fig. 4(B)). The mechanical unfolding coordinate is used along with the pulling direction, which is not necessarily the same as that of denaturant unfolding. A stretching force F is applied to a folded domain, and the Gibbs free energy at each state is lowered by Fx , the product of the tensile force (F) and distance from the native state (x); i.e., the elongated structure is energetically favored. Here, we assume that unfolding can be described as an escape from a free energy well around the native state, and relaxation time of the system is much faster than that of the escape over the barrier. As tensile force is increased, the height of the energy barrier to be overcome is decreased, and the escape rate becomes faster. Therefore, the unfolding rate at a given force, $k(F)$, correlates with the distance between the native state and the transition state (x_u), and the unfolding force depends on the loading rate. To analyze the unfolding force data measured at different loading rates, Bell's model, in which the x_u is assumed to be a constant, has been commonly used. However, recent studies showed that some experimental results cannot be rationalized (reproduced) with Bell's model^{19,20}. Recently, Dudko et al. have developed a new model, called the Dudko–Hummer–Szabo (DHS) model, which takes into account the force dependence of x_u ¹⁹. The DHS model has three parameters: the unfolding rate k_0 , the height of the barrier ΔG^* , and x_u at zero force. These parameters are derived by fitting to the histograms of the unfolding forces measured at various pulling speeds. (For details of the DHS model and data analysis, refer to the original papers^{19,21}). Once the parameters are determined, the prefactor (A) can be expressed by $A = k_0 \exp(\Delta G^*/k_B T)$. Moreover, the spring constant of the domain (D) and the magnitude of thermal fluctuation along with the pulling direction ($\langle x \rangle$) can be estimated by the equipartition theorem as $D = 2\Delta G^*/x_u^2$ and $\langle x \rangle = (k_B T/D)^{1/2}$,

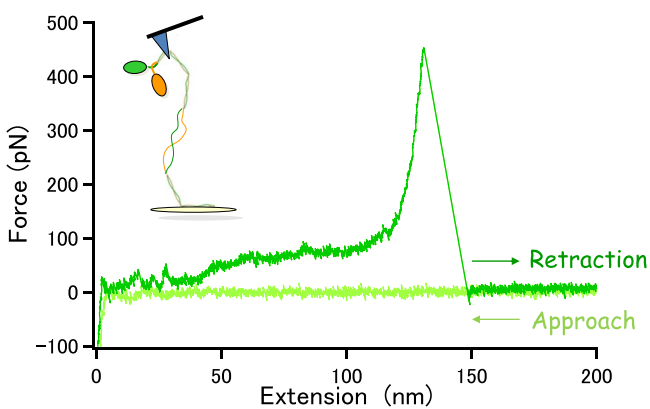


Figure 3 The force–extension curve of the myosin rod. The unfolding–refolding transition of the coiled-coil segments results in a plateau around 50 pN.

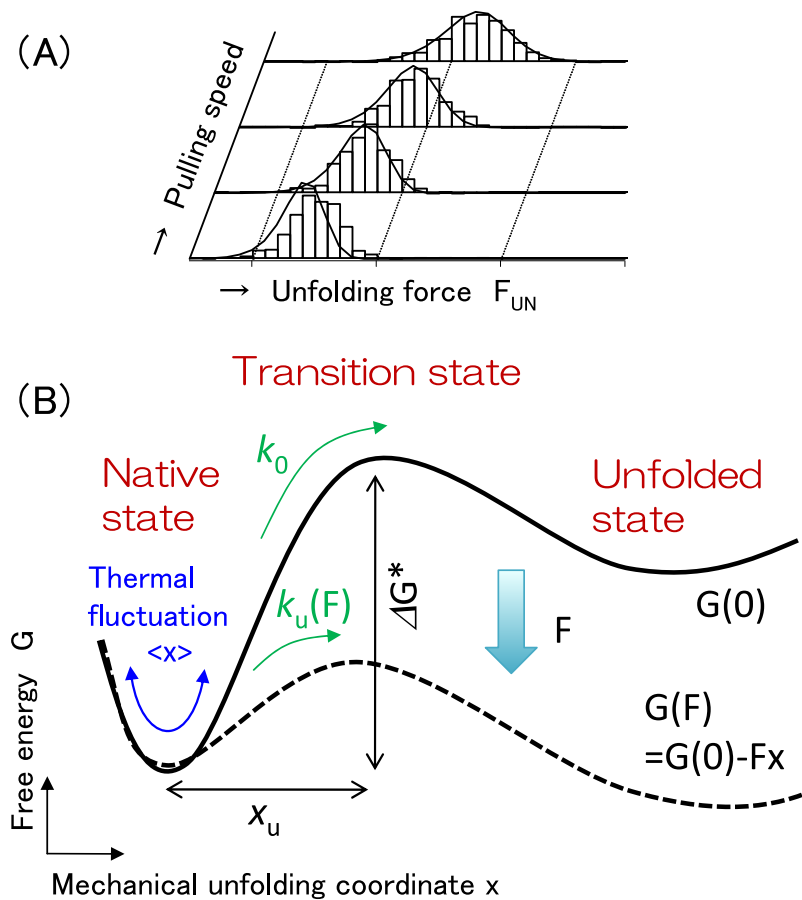


Figure 4 (A) Unfolding force histograms at various pulling speeds. The pulling speed dependence of the unfolding force distribution contains information about the free energy landscape. (B) The free energy landscape along the mechanical unfolding reaction coordinate. The external force F reduces the free energy by Fx . The thermally-activated molecule escapes the potential well in a stochastic manner.

respectively, assuming the parabolic potential well²². The spring constant D provides a measure of the softness of protein domains. For example, x_u of I27 at 30°C is estimated to be $5.1 \pm 0.2 \text{ \AA}$ by our analysis using the DHS model, where ΔG^* is $22 \pm 0.4 k_B T$, D is $0.7 \pm 0.06 \text{ N/m}$, and A is $2.0 \pm 1.3 \times 10^5 \text{ s}^{-1}$. (Note that these values characterize the unfolding from the intermediate state of I27 shown in Fig. 2). These parameters show temperature dependence, indicating that the I27 domain softens^{9,22} and the prefactor A increases significantly upon heating (Taniguchi et al., unpublished data). The increase of the prefactor is a remarkable finding, suggesting a contribution of the viscosity and/or roughness in the free energy landscape to the unfolding rate. Thus, the mechanical unfolding experiments at different temperatures may lead to a detailed description of the free energy landscape.

Another important factor that determines the unfolding force of a protein molecule is the pulling geometry. The mechanical resistance of a domain is anisotropic, correlated to the topological and tertiary structure. Brockwell et al. have demonstrated the anisotropy in the dihydrolipoyl acetyltransferase subunit of the pyruvate dehydrogenase (E2lip3)

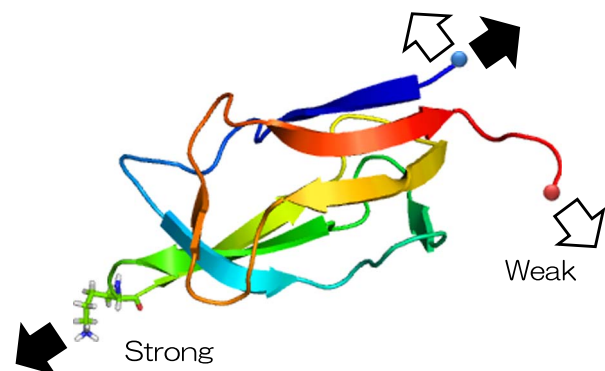


Figure 5 A drawing showing the relation between the mechanical resistance and the pulling direction in E2lip3. E2lip3 can resist relatively higher forces ($177 \pm 3 \text{ pN}$ at the pulling speed of 700 nm/s), which is applied in the direction indicated by the black arrows. On the other hand, E2lip3 unfolds at below 15 pN when it is pulled through its N and C termini (white arrows)²³.

from *Escherichia coli*²³. E2lip3 can resist high forces ($>100 \text{ pN}$) when the tensile force is applied to shear between β -strands, as indicated by black arrows in Figure 5;

whereas it is unfolded at a very weak force (<15 pN) when pulled by the N and C termini to “unzip” the β -strands. Similar results have been reported with ubiquitin²⁴ and GFP²⁵, and these results imply that hydrogen bonds between anti-parallel β -strands act as mechanical clamps when the load is applied along the shearing direction. Meanwhile, for globular α -helical proteins, mechanical stability is relatively low, and they unfold at forces lower than 100 pN.

Pathway of mechanical unfolding

One of the fundamental questions in protein folding is the structure of the transition state. The widely used approach to probing the structure of the transition state is the “ ϕ -value analysis,” which is an experimental method based on protein engineering. This method has also proved to be useful in mechanical unfolding studies²⁶. The ϕ -value of the folding reaction is defined as $\Delta\Delta G_{U-TS}/\Delta\Delta G_{U-N}$, where $\Delta\Delta G_{U-TS}$ is the change in ΔG between the transition state and the unfolded state caused by the substitution of a single amino acid residue, and $\Delta\Delta G_{U-N}$ is the change in ΔG between the native and unfolded states. Thus, the ϕ -value indicates the extent of native (-like) structure preservation around the mutated residue at the transition state.

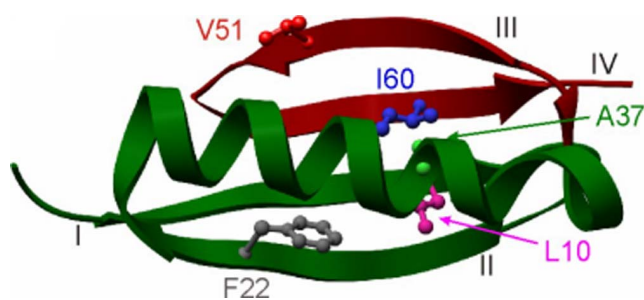
The B1 domain of protein L shows all-or-none-type unfolding upon pulling from its N and C termini. Sadler et al. investigated the mechanical stability of protein L variants (Fig. 6)²⁷. The four mutants, except I60V showed ϕ^F -values close to 1, which suggests that the structure in the vicinity of these residues is preserved in the transition state of mechanical unfolding. On the other hand, the ϕ^F -value of I60V was significantly low, indicating that the structure near I60 is disrupted in the transition state. By contrast, in case of denaturant-induced unfolding, all these mutants showed ϕ^D -

values of less than 0.5. These inconsistent results between mechanical and chemical unfolding experiments may reflect the difference in the mechanisms of unfolding; mechanical force causes local perturbation in the interaction between the terminal β -strands, whereas denaturation affects an entire domain. Another possible explanation may be given by the heterogeneity of unfolding pathways. Mechanical unfolding pathways are restricted compared with those of denaturant-induced unfolding, where the transition state is an ensemble average of multiple pathways.

Such information would be useful for the rational design of mechanically stable proteins. For example, the unfolding force of I60V, in missing a single methylene group of the isoleucine side chain, was decreased by 25% compared with that of the wild type. On the other hand, for I60F, which has a larger hydrophobic volume at position 60, the unfolding force was increased by 50%. These results suggest that I60 is a special amino acid that functions as a mechanical rheostat in the hydrophobic core.

Direct AFM observation of refolding

Is it possible to observe protein refolding by AFM? Rief et al. performed the following experiment in 1997². First, they stretched a native titin molecule to observe a sawtooth pattern in the force–extension curve, and the cantilever was then approached close to the AFM stage to relax the molecule. After a delay, they retracted the cantilever again. In the approaching phase, there was no evident force peak observed, but some force peaks appeared in the second retraction force curve, depending on the length of the delay time³. This observation indicates that titin immunoglobulin-like domains are required to be fully relaxed for their refolding. Although this was the first experiment to follow a refolding reaction at the level of a single molecule by AFM, direct detection of the refolding reaction was not performed. For a direct observation of the refolding reaction, a real-time observation of the contraction of the unfolded polypeptide must be performed; this has been extremely difficult because it requires piconewton-level force resolution and a sub-nanometer scale-stabilized AFM setup against optical and mechanical drift. Recently, the Rief group developed a low drift AFM, which enabled them to stretch a protein at a very slow speed, and a long time-averaging window was taken in the force–extension curve measurement, which provided a high S/N ratio of the force curve. With the low drift AFM setup, they successfully showed a stepwise fluctuation of the molecular length of calmodulin at a tensile force of approximately 10 pN, which reflects individual unfolding/refolding events²⁸. The Fernandez group developed a force-clamp AFM system with which they performed refolding of ubiquitin²⁹, kinetic studies of the mechanical unfolding³⁰, and enzymatic reactions³¹ at single-molecule resolution.



	L10A	F22A	A37G	V51A	I60V
(a) ϕ^D	0.43	0.41	0.11	0.19	0.22
(b) ϕ^F	0.95	1.00	0.99	0.78	0.33
	± 0.04	± 0.03	± 0.04	± 0.11	± 0.08

Figure 6 A comparison of the ϕ -value between denaturant unfolding (a) and mechanical unfolding (b) in case of protein L (modified from Ref. 27 with permission).

Mechanical unfolding *in vivo*

As described above, the mechanical stability of a protein domain is not necessarily identical to its chemical stability or thermal stability. In addition to conventional folding studies, mechanical unfolding studies can provide information about protein architecture in a different way. This fact gives protein folding researchers a strong motivation to investigate the mechanical unfolding of proteins. For many proteins, mechanical unfolding relates directly to their biological functions, which is also of interest to many biologists. Recently, novel strain sensor proteins have been discovered. In addition to immunoglobulin-like domains, titin has a kinase domain. In the absence of external load, the ATP-binding site of the kinase domain is covered by another intramolecular region for auto-inhibitory regulation. The mechanical stretching of titin detaches the inhibiting region; hence kinase function is activated³². Thus, titin kinase controls muscle gene expression and protein turnover in a load-dependent manner. Another example is talin, a key player in cell adhesion, signaling, and migration. The external tensile load causes a stretching of the talin molecule and exposes a cryptic binding site for vinculin³³, which enhances subsequent vinculin recruitment. The partial unfolding by external stretching force is the mechanism for the strain sensing function of these proteins.

Mechanical unfolding is an important process even for proteins that are not directly related to a mechanical func-

tion. Many proteins need to be unfolded for their translocation across membranes (i.e., import into mitochondria or the endoplasmic reticulum for secretory proteins) and for their degradation by some proteases including proteasomes. To mechanically unfold a protein molecule for the aforementioned purposes, cells have special ATP-fueled machineries. These machineries are believed to tug a part of a protein and destabilize it. The import rates of I27 variants to mitochondria are correlated with their mechanical stabilities³⁴. Recently, tugging and unfolding of a substrate protein by the ClpXP protease system was observed directly at a single-molecule level using optical tweezers^{35,36}. The ClpX is an AAA+ ATPase that generates mechanical force, unfolds protein molecules, and translocates the unfolded polypeptides into the ClpP domain for degradation.

Mechanical resistance is the crucial function of scaffold proteins. A tandem repeat structure is frequently found in the extracellular region of integral membrane proteins involving cellular adhesion (e.g., protein L or cadherins). Such tandem repeat regions show a sawtooth pattern in their force–extension profiles (as in Fig. 1), where the entropic elasticity of the unstructured linker region and the unfolding of individual domains are involved. On the other hand, the force–extension profile of an ankyrin repeat, which mediates the attachment of the intracellular region of integral membrane proteins to the membrane skeleton, shows a different pattern. Figure 7 shows the retract/approach cycle of ankyrin repeats of ankyrin-R, armadillo repeats of β -

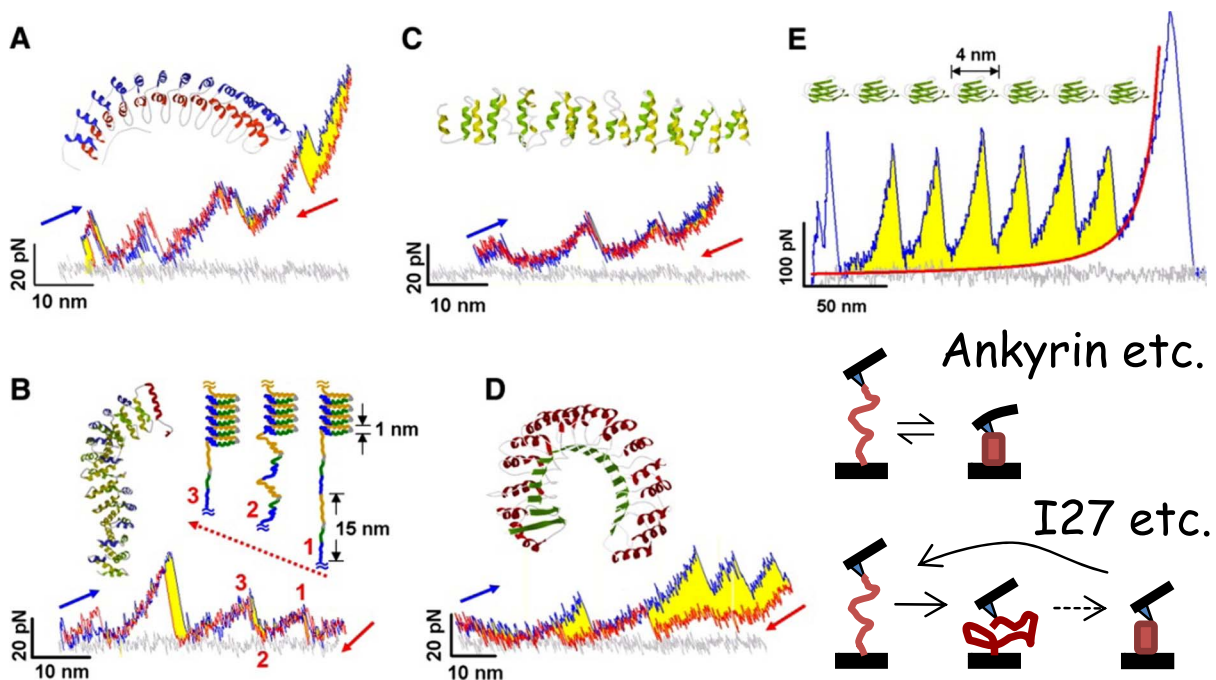


Figure 7 Force–extension curves of some repeat proteins: (A) ankyrin repeats of ankyrin-R, (B) armadillo repeats of β -catenin, (C) HEAT repeats of clathrin, (D) leucine-rich repeats of ribonuclease inhibitor, and (E) I27. The degree of hysteresis (yellow areas) is relatively small in the domains consisting of mainly α -helices (A–C) (modified from Ref. 37 with permission).

catenin, HEAT repeats of clathrin, leucine-rich repeats of ribonuclease inhibitor, and I27³⁷. These domains consist of repeating units of 30–50 amino acids that primarily form α -helical structures. The mechanical unfolding/refolding of these structural units defines their mechanical property. An interesting feature in the force–extension curves of these repeat proteins is a small hysteresis, which is also seen with the coiled-coil structure of the myosin rod¹⁶ but is in contrast with the huge hysteresis of I27. In these repeat proteins, they can refold rapidly even against high loads (approximately 30 pN), suggesting that the mechanical unfolding occurs as a quasi-equilibrium process with minimal energy dissipation³⁷. Cells may properly use different molecular springs to tune their mechanical properties.

Future prospects

Until date, single-molecule force spectroscopy has been performed with only a small number of proteins. Many proteins are related to cellular mechanical function, and they remain to be elucidated. However, most proteins are not mechanically stable like immunoglobulin-like domains, and in order to investigate such mechanically weak proteins or intermediates, force measurement with piconewton resolution is required. However, the thermal fluctuation of an AFM soft cantilever typically has a noise level of approximately 10 pN, which limits the resolution in force measurement. Noise may be eliminated by time averaging²⁸, but the fluctuation itself is not suppressed. The same can be said for optical tweezers, which can measure forces at piconewton or sub-piconewton resolution because of the low stiffness of the probe. We should not overlook the fact that the amplitude of the fluctuation is sometimes comparable to the dimension of a molecule of interest, which may greatly affect the dynamics of the molecule. The thermal fluctuation can be used as a perturbation probe for measuring single molecule viscoelasticity³⁸.

The mechanical unfolding of proteins has been studied by computer simulation, commonly with a Go-like model^{39,40}. Molecular dynamics (MD) simulations have successfully described the unfolding scenarios of many protein molecules^{11,17,21,23–34,32,41,42}. However, there are some exceptions. For example, in case of protein L, MD simulation failed to reconstruct the experimental result of the single point mutation at I60, where amino acid substitution caused a drastic effect on the mechanical stability. In addition, we should note that there is still a huge gap between the timescales of computer simulations and AFM experiments. Moreover, the pulling speed in simulations is typically chosen to be several orders of magnitude faster than that in experiments. To fully understand mechanical unfolding in detail, a close collaborative study between computer simulations and AFM experiments with mutant proteins would be necessary.

Mechanical unfolding studies can provide unique information on protein architecture, and they promise to reveal

the mechanisms underlying their mechanobiological functions. For more details, see original papers and reviews in the references^{43–45}.

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