# Engineering proteins with tailored nanomechanical properties: a single molecule approach

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Elastomeric proteins underlie the elasticity of natural adhesives, cell adhesion and muscle proteins. They also serve as structural materials with superb mechanical properties. Single molecule force spectroscopy has made it possible to directly probe the mechanical properties of elastomeric proteins at the single molecule level and revealed insights into the molecular design principles of elastomeric proteins. Combining single molecule atomic force microscopy and protein engineering techniques, it has become possible to engineer proteins with tailored nanomechanical properties. These efforts are paving the way to design artificial elastomeric proteins with well-defined nanomechanical properties for application in nanomechanics and materials sciences.

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## Introduction

Cells are miniature factories consisting of a wide spectrum of molecular machinery of the dimensions of nanometres to micrometres,[1] which represent perfect examples that scientists and engineers are attempting to mimic and re-engineer. Amongst them, mechano-machines are those directly involving mechanical force. They constantly sense, generate and bear mechanical force under their biological settings and play indispensable roles in achieving their optimum functions across different length scales, from a single molecule to whole tissue.[1–3] Protein-based mechano-machines consist of diverse members, ranging from "active" elements, such as molecular motors[3,4] that convert chemical energy into mechanical work, to passive elastic elements, such as elastomeric proteins that function as molecular springs.[5–8] Such mechano-machines have aroused tremendous interest not only from a biological perspective,[2,9] but also from a nanoscience and nanotechnology point of view.[10,11] These mechano-machines are ideal building blocks for the bottom-up construction of nanomechanical devices and thus will have great potential in nanotechnology. Molecular motors have been successfully incorporated into nanomechanical devices,[10,11] demonstrating the great potential of using protein-based mechanical elements in nano-devices. With the development of nanotechnology, it is conceivable that nanomechanical devices will soon incorporate more diverse protein-based components to function as molecular springs, switches, sensors and motors, including both naturally occurring ones as well as rationally designed and synthesized ones. Investigating the mechanics of these protein-based mechanical elements, as well as their molecular design principles, has become increasingly important. Single molecule force spectroscopy techniques,[12] such as optical tweezers, biomembrane force probe[13] and atomic force microscopy (AFM), are playing particularly important roles for fulfilling such important tasks.

Among the mechanical machinery and components, elastomeric proteins are passive elastic elements and serve as molecular springs inside cells as well as in extracellular matrix space to establish elastic connections and provide mechanical strength and extensibility.[7,8,14] Elastomeric proteins can also function as

structural materials of superb mechanical properties, such as spider silk protein.[8,15] Elastomeric proteins can withstand significant deformation without rupture, and can return back to their original state when the stretching force is removed. One of the common features of the elastomeric proteins is their tandem modular construction.[7,14] For example, the giant muscle protein titin is composed of hundreds of individually folded immunoglobulin-like (Ig) domains and fibronectin type III (FnIII) domains, interspersed by random coil-like unique sequences.[5] It was not until very recently that the mechanical properties of individual proteins could be directly measured. In 1997, three landmark papers demonstrated for the first time that it is possible to mechanically manipulate individual proteins and measure their force-extension relationships at the single molecule level and with pico-Newton resolution.[16–18] Subsequently, protein engineering techniques were employed to construct polyproteins made of identical tandem repeats, enabling the studies of molecular determinants of protein's mechanical stability.[19] These pioneer works opened up a new field of inquiry: single protein mechanics. Due to its high force sensitivity, superb spatial resolution and no need of specific chemical immobilization, single molecule AFM has evolved into the power house in the field of single protein mechanics. Fig. 1 shows the schematics of a single molecule AFM experiment in which a tandem modular protein is being stretched to measure its elastic behaviors.

Over the last ten years, there has been tremendous progress in single protein mechanics. Extensive single molecule AFM studies and molecular dynamics simulations have been carried out to determine the mechanical properties of a wide range of elastomeric proteins and to illustrate their underlying molecular design principles.[19–34] It was discovered that stretching force can trigger sequential mechanical unfolding of individually folded domains. Such a "modular" unfolding mechanism conveys high toughness to elastomeric proteins and makes them perfect shock-absorbers.[6,16,35] This mechanism has been extensively exploited in nature and can be found in a wide variety of materials, ranging from muscle fibres,[5] spider silk[15] to biological adhesives.[36] The elastic properties of individual protein domains are combined collectively to determine the overall mechanical properties of the elastomeric proteins. For example, single molecule AFM studies on titin have provided deep insights into the molecular mechanism of how the passive elasticity of muscle is finely regulated by the collective mechanical properties of its constituting folded Ig-like domains as well as random coil-like sequences.[35,37–39] In parallel to these single molecule AFM efforts to uncover biophysical principles underlying elastomeric proteins and their associated biological significance, efforts to engineer/design proteins of well-defined mechanical properties are also under way. Inspired by naturally occurring elastomeric proteins, researchers have started to explore and develop new methodologies to tailor the mechanical properties of proteins in a rational way with the aim to exploit the engineered artificial elastomeric proteins for specific nanomechanical applications.[40–46] For example, the mechanical unfolding of green fluorescent protein (GFP) was investigated in detail by single molecule AFM,[42,47] with the ultimate goal of correlating the mechanical unfolding with the fluorescence change of GFP and employing GFP as molecular force sensors. Such efforts represent a burgeoning new field in single protein mechanics, and hold the promise to significantly expand the toolbox of elastomeric proteins and to develop artificial protein-based nanomechanical elements for material science and nanomechanical applications. Here I provide a personal account of this emerging area.

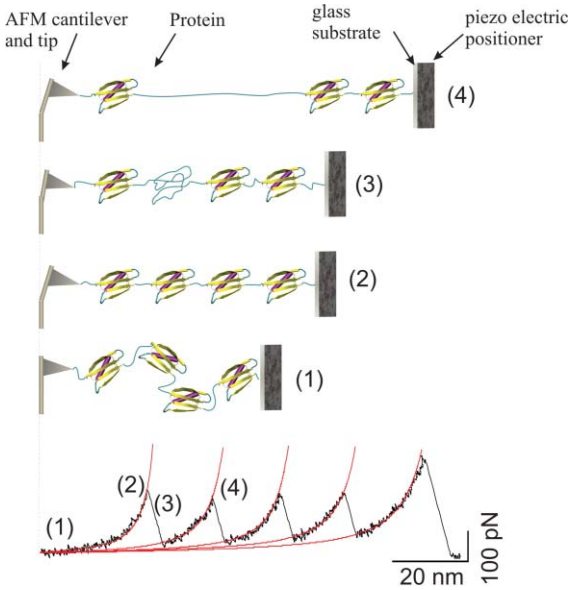


**Fig. 1 Using single molecule atomic force microscopy to probe the mechanical properties of single proteins**. In a typical single molecule AFM experiment, a tandem modular protein molecule, which is deposited onto a glass cover slip, is picked up by the AFM tip and stretched between the AFM tip and the solid substrate, which is mounted onto a high precision piezoelectric positioner. Stretching a tandem modular protein results in force-extension curves of the characteristic saw-tooth pattern appearance of force peaks. The force can be measured from the deflection of the AFM cantilever. The individual saw-tooth peak corresponds to the sequential unravelling of individual domains in the tandem modular protein. The unfolding force is a measure of the mechanical stability of the protein domains. As the piezoelectric positioner moves away to increase the end-to-end distance of the molecule (from state 1 to state 2), the protein generates a restoring force following the worm-like-chain model of polymer elasticity. Upon domain unfolding, the contour length of the protein increases and the force acting on the cantilever is relaxed. Further extension again results in an increase of force (state 4). The last peak in the force-extension curve represents the extension of the fully unfolded tandem modular protein prior to its detachment from the AFM tip or substrate.

## Non-mechanical proteins can exhibit significant mechanical stability

The significant mechanical stability shared by elastomeric proteins is perfectly suited to their biological functions under stressful physiological conditions. In contrast, many other proteins are not subject to stretching forces under their normal working conditions; we refer to these proteins as non-mechanical proteins. It was unknown whether mechanical stability is a property that is unique to mechanical proteins and whether non-mechanical proteins can be used for nanomechanical purposes. Although an early single molecule AFM study on a non-mechanical protein, barnase,[48] suggested that proteins that are not selected for mechanical functions may not resist force in the same way as mechanical proteins, recent single molecule AFM studies proved the opposite. Inspecting the structures of mechanical proteins revealed that

 

mechanical proteins do not contain unique structural elements and characteristics that provide mechanical strength, suggesting that the mechanical stability is determined by the same set of non-covalent interactions that determine the overall three dimensional structures and thermodynamic stability of proteins. This is very similar to the scenario of hyperthermophilic proteins, which possess extreme thermostability yet do not have unique structural characteristics. Therefore, the mechanical stability of a protein must depend on its optimal use and arrangement of known interactions, such as hydrogen bonds, hydrophobic interactions *etc.* Hence, if non-mechanical proteins are structurally similar to natural mechanical proteins, it is likely that non-mechanical proteins will have significant mechanical stability.

Steered molecular dynamics simulations (SMD) revealed that protein topology plays critical roles in determining the mechanical stability of proteins.[25,26,49,50] Shear topology of the two terminal force-bearing β strands appears to be a common feature amongst most of the mechanically stable proteins. In these proteins, the two terminal force-bearing β strands are arranged in parallel and are pointing towards opposite directions. The arrangement of A′ and G β strands in I27 is a typical example of the shear topology (Fig. 2A). Shearing the backbone hydrogen bonds and hydrophobic interactions between the two force-bearing strands results in mechanical resistance to unfolding and constitutes the molecular basis for the mechanical stability of the protein.[49,50] If this view is correct, non-mechanical proteins in principle can display or be engineered to display significant mechanical stability just like their mechanical counterparts, provided that these non-mechanical proteins possess, either by nature or by engineering, desired structure and topology.

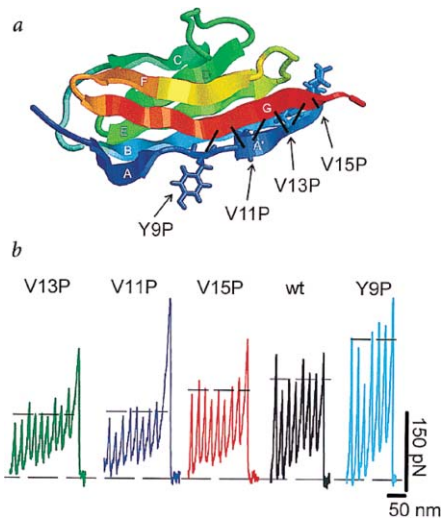


**Fig. 2 Point mutations in the mechano-active site of I27 alter its mechanical stability**. (A) Cartoon diagram showing the β-sandwich structure of the I27 module and the amino acids that were substituted by proline residues. Black bars indicate the six backbone hydrogen bonds linking the A′ and G β-strands that are predicted to be the mechano-active site and hold the key to the mechanical stability of I27. (B) The force extension relationships for the I27 polyproteins: wild type (black), Y9P (cyan), V11P (blue), V13P (green), and V15P (red). The mutations V11P, V13P and V15P decrease the force required to unfold the I27 module. By contrast, the mutation Y9P increases this force. Adapted by permission from Macmillan Publishers Ltd: Nature Structural Biology. H. Li, M. Carrion-Vazquez, A. F. Oberhauser, P. E. Marszalek and J. M. Fernandez, *Nat. Struct. Biol.*, 2000, **7**, 1117, copyright (2000).

Experimental efforts proved it is indeed the case. Based on the search criterion of shear topology, non-mechanical proteins of significant mechanical stability have been successfully identified and characterized. Non-mechanical proteins B1 IgG binding domain of protein L,[41] B1 IgG binding domain of protein G[45,46] and Top7[43] are three representative examples. All three proteins share the feature of shear topology arrangement of the terminal force-bearing β strands. Single molecule AFM experiments showed that protein L[41] and protein G,[45,46] both belonging to the β-grasp fold, exhibit significant mechanical stability and unfold at forces of ∼130 pN and ∼180 pN, respectively, which are comparable to that of elastomeric proteins, such as the I27 domain from titin.[51] In comparison, a *de novo* designed protein, Top7,[52] was also demonstrated to exhibit significant mechanical stability and yet the Top7 fold is distinct from the Ig-like fold and β-grasp fold, representing a novel mechanically stable protein fold.[53] Non-mechanical proteins do not just exhibit mechanical properties that are similar to those of natural elastomeric proteins; instead, artificial polyproteins can show mechanical features that surpass the natural ones. For example, artificial polyprotein made of protein G was shown to fold much faster than any elastomeric protein that has been studied to date.[46]

These studies demonstrate the great potential of non-mechanical proteins to achieve desirable mechanical properties, and will greatly expand the toolbox of mechanically stable proteins for nanomechanical applications. Moreover, the finding that Top7 carries significant mechanical stability is of particular significance. Top7 was *de novo* designed by Baker and coworkers[52] a few years ago and was shown to have a novel fold that has not been sampled by nature. The finding that Top7 is mechanically stable demonstrates that it is possible to use computational methods to *de novo* design proteins of novel topology to possess tailored nanomechanical properties, although Top7 was not designed for mechanical purposes *per se*.

Along the same line, other non-mechanical proteins have also been identified with significant mechanical stability. Green fluorescent protein is one typical example.[42,47] With the further development of this field, I anticipate that in the near future a much expanded toolbox of artificial elastomeric proteins will be built and proteins with well-defined biological functions and mechanical stability will be engineered and used in well-defined nanomechanical applications.

## Tuning the mechanical stability of proteins by tuning the mechano-active site of the mechanical protein

Single molecule AFM and SMD simulations showed that the mechanical stability of proteins is largely a local property. Local topology and interactions play critical roles in defining the overall mechanical stability of a given protein. Taking the 27$^{th}$ Ig domain of titin as an example, the AB and A′G regions are believed to be key to the mechanical stability of I27 (Fig. 2A).[49,50] Such critical region(s) of a mechanical protein can be considered as the mechano-active site of a mechanical protein, analogous to the active site for an enzyme. Therefore, tuning the mechanical stability of a protein is similar in many ways to the tuning of the enzymatic activity of a given enzyme.

The initial efforts[54–57] of tuning mechanical stability of proteins started with I27, a paradigm for single protein mechanics. The mechano-active sites of I27 are believed to be the AB and A′G regions, where backbone hydrogen bonds connecting the two β strands are believed to be the key for mechanical stability of I27[49,50] (Fig. 2). Using site-directed mutagenesis, residues in the A′ strand that are involved in the formation of key backbone hydrogen bonds were mutated to proline to prevent the formation of backbone hydrogen bonds and to disrupt the local β sheet structure.[55] As anticipated, proline mutations at positions V11, V13 and V15 significantly reduced the mechanical unfolding force of I27. In addition, these mutations also increased the distance from the native state to the transition state. However, proline mutation at position Y9 increased the mechanical stability by almost 50 pN. Due to the lack of detailed structural information on the Y9P mutant, it remains a mystery how a supposedly disruptive mutation Y9P makes the protein mechanically more resistant. Nonetheless, this work demonstrated the possibility that one can systematically tune the mechanical stability of protein by fine tuning the non-covalent interactions in the mechano-active site, if the molecular determinants of protein's mechanical stability are fully understood. Since then, extensive studies have been carried out to investigate the phenotypical effects of point mutations on the mechanical stability of proteins, and tuning the key interactions in the mechano-active site has become a widely used approach.[37,58–62]

Despite extensive efforts in this direction, the molecular determinants of mechanical stability of proteins are still not fully understood. As such, decreasing the mechanical stability of a given protein is readily achievable, however, rationally increasing the mechanical stability of the given protein proves challenging. The coupling of the mechano-active site with the rest of the protein structure may be a contributing factor. For example, recent studies showed that, despite the local attributes of mechano-active sites, mutations outside the mechano-active site may also affect the mechanical stability.[53,56]

## Ligand binding provides an effective approach to modulate the mechanical stability of proteins

Ligand binding is ubiquitous in biological processes. It is well known that ligand binding can affect the thermodynamic stability of proteins by affecting the equilibrium between the folded and unfolded states of proteins.[63,64] Ligand binding has been widely exploited to stabilize proteins of interest. However, since the mechanical stability does not correlate with the thermodynamic stability of proteins, it was unknown whether ligand-binding affects the mechanical stability of proteins in the same way it affects thermodynamic stability. Using dihydrofolate reductase (DHFR) from a Chinese hamster as a model system, Fernandez and coworkers[65] elegantly demonstrated that ligand binding may serve as a valuable tool to modulate the mechanical stability of DHFR. As shown in Fig. 3, in the absence of ligands, DHFR unfolds at very low forces (below ~50 pN) and does not produce typical unfolding force peaks. Instead, the stretching of DHFR results in a featureless mechanical response that is typical of random coil-like polymers. Upon adding its ligand or inhibitor, the mechanical stability of DHFR was significantly enhanced, resulting in unfolding events of DHFR at ~80 pN. In addition, they also found that the binding of multiple ligands to DHFR simultaneously, such as MTX and NADPH, does not result in additive stabilizing effects. These findings not only provide effective ways to enhance protein's mechanical stability, but also carry important biological significance as they provide a possible explanation for the observed slowing down effect for protein translocation across membranes upon binding of a ligand or inhibitor.[65]

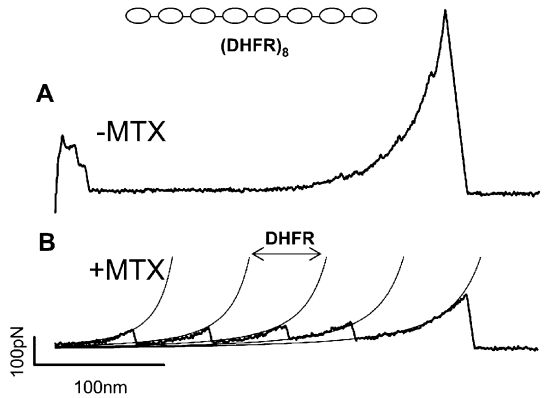


**Fig. 3 Ligand methotrexate (MTX) modulates the mechanical stability of DHFR**. (A) Force-extension curve of the polyprotein $(DHFR)_8$. The lack of a saw-tooth pattern suggests that DHFR is mechanically weak and unfolds at forces below the detection limits of single molecule AFM. (B) Force-extension curve of the polyprotein $(DHFR)_8$ in the presence of 1.2 mM MTX shows a clear saw-tooth pattern of unfolding events at an average force of 78 pN. Fits of the WLC model of polymer elasticity (thin lines) reveal a contour increment between unfolding events of $\Delta L_C$ of 67.3 ± 0.5 nm, which is in close agreement with the expected length gained by unfolding a DHFR molecule (65 nm). This result indicates that the binding of MTX significantly improves the mechanical stability of DHFR. Adapted from S. R. Ainavarapu, L. Li, C. L. Badilla and J. M. Fernandez, *Biophys. J.*, 2005, **89**, 3337.

Although this methodology depends on specific protein–ligand systems and may not be universal,[65–68] ligand-binding represents an attractive way to modulate the mechanical stability of proteins and has great potential in engineering proteins that are sensitive to and can be modulated by environmental stimuli, such as ligands.

## Anisotropy of proteins′ mechanical stability offers the potential to entail new mechanical stability of proteins

Different from thermodynamic stability, mechanical stability of proteins is an anisotropic property. Independent studies by Fernandez's group[69] and Radford's group[70] demonstrated that the mechanical stability of the same protein depends on the pulling direction: the same protein can exhibit drastically different mechanical stability if the protein is pulled from different directions *via* different pairs of residues. For example, ubiquitin unfolds at ~200 pN when it is pulled from its N- and C-termini. In contrast, the same ubiquitin will unfold at a much lower force of ~80 pN when pulled from its C-terminus and residue Lys48. The anisotropic nature of the mechanical response of the protein offers unique possibilities to explore diverse mechanical properties from the same protein. Using GFP as a model system, Rief and coworkers have exploited this idea systematically.[71] They substituted a pair of residues in GFP at selected locations with cysteine

 

residues and used them to connect several GFPs into a polyprotein *via* oxidizing cysteine residues. The disulfide linkage established upon oxidation allows the stretching force to be applied to GFP along the direction pre-determined by the two cysteine residues. As shown in Fig. 4, the mechanical unfolding force of GFP exhibits great diversity and anisotropy depending on the pulling direction. The unfolding forces of GFP range from ~100 pN to more than 500 pN, the latter representing the highest unfolding force of any protein mediated by non-covalent interactions. In addition, each individual GFP polyprotein also showed different spring constants. This study elegantly demonstrates the feasibility that one protein building block can be used for multiple purposes involving different mechanical stability, entailing the perspective of multi-purpose nanomechanical protein building blocks.

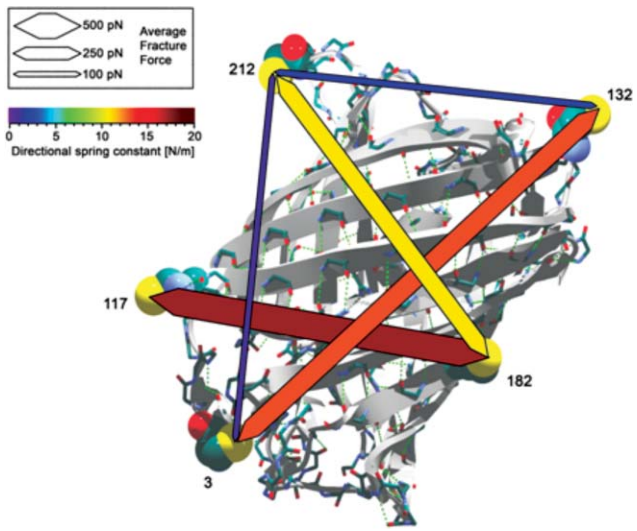


**Fig. 4 Directional deformation response of the GFP fold**. The width of the strings connecting points of force application (space-filled residues) represents the average unfolding force in that particular direction. The color of the strings encodes the directional spring constant, *i.e.*, protein rigidity in the respective direction. Depending upon the pulling directions, the mechanical unfolding forces of GFP range from 100 pN to more than 500 pN. Adapted from H. Dietz, F. Berkemeier, M. Bertz and M. Rief, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 12724. © 2006 by The National Academy of Sciences of the USA.

## Engineering novel proteins by recombining protein fragments

Since the molecular determinants of proteins′ mechanical stability are yet to be fully established, the rational design of proteins with tailored mechanical properties remains a great challenge. This situation resembles the scenario encountered in the field of enzyme engineering. To circumvent this difficulty, directed evolution of enzymes has been an efficient way to engineer enzymes with improved or even novel enzymatic activity. Different from rational design, directed evolution allows one to evolve particular enzymatic properties by building a large enough library that contains rare beneficial mutations. Although different from rational design, directed evolution samples a large sequence space that allows one to screen proteins of improved functionality.

Recombination is an important approach used in laboratory-based directed evolution. Recombination is an important mechanism in nature for proteins to acquire novel functions. Recombination offers the advantage of combining beneficial mutations from multiple parents into a single offspring and has been exploited extensively by nature during evolution in improving protein traits such as enzymatic activity. This method has also been used extensively in the directed-evolution of proteins in the laboratory and has become one of the most important strategies in engineering proteins with novel functions.[72,73] My laboratory has explored the use of recombination of protein fragments to engineer proteins of novel mechanical stability.[53]

Using the 27$^{th}$ and 32$^{nd}$ immunoglobulin domains, the two well-characterized domains from muscle protein titin,[35] as model systems, we demonstrated the feasibility of using protein recombination to engineer proteins of novel mechanical properties. I27 and I32 share high sequence homology and are ideal systems for protein recombination. We interchanged two structural fragments between the two parent proteins and constructed four hybrid daughter proteins: I27-A′G-I32, I32-A′G-I27, I27-CDE-I32 and I32-CDE-I27 (Fig. 5). In the first two hybrid proteins, the force-bearing A′ and G strands (mechano-active site) are interchanged between I27 and I32, and in the latter two, the non-force-bearing C, D and E β strands are interchanged between I27 and I32, respectively. We found that all four hybrid daughter proteins fold into well-defined three-dimensional structures. Compared with the mechanical stability of the parent proteins, the four hybrid

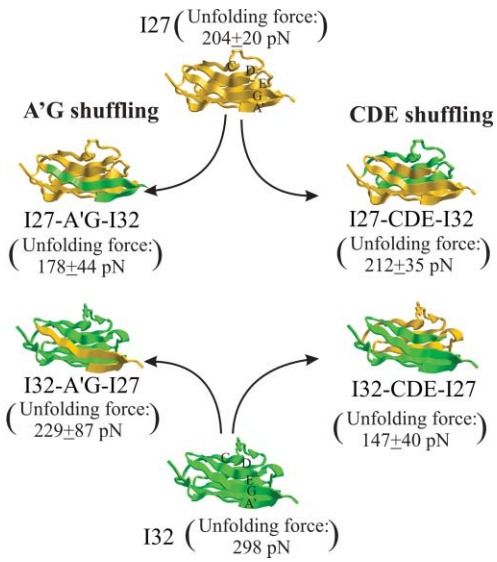


**Fig. 5 Engineering novel mechanical proteins by recombination of protein fragments from I27 and I32**. The middle column shows the three dimensional structures of I27 (yellow) and I32 (green). The structure of I32 was obtained by homology modeling. By interchanging the A′ and G β strands between I27 and I32, hybrid proteins I27-A′G-I32 and I32-A′G-I27 were engineered (left column). Interchanging the C, D, and E β strands between I27 and I32 resulted in hybrid proteins I27-CDE-I32 and I32-CDE-I27 (right column). In the hybrid proteins, the fragments coming from the wild type I27 are shown in yellow, while those from wild-type I32 are shown in green. The hybrid daughter proteins exhibit mechanical properties that are distinct from those of parent proteins. Adapted from D. Sharma, Y. Cao and H. Li: Engineering Proteins with Novel Mechanical Properties by Recombination of Protein Fragments. *Angew. Chem., Int. Ed.*, 2006, **45**, 5633. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

daughter proteins exhibit mechanical properties that are distinct from their parents (Fig. 5). It is noted that two daughter proteins, I27-CDE-I32 and I32-A′G-I27, exhibit mechanical stability that is higher than the parent protein I27, but lower than I32. It is conceivable that if one can build a library large enough, it is possible to obtain daughter proteins that are mechanically stronger than both parent proteins. Such a library will also entail proteins with diverse mechanical stability and make it possible to use statistical analysis to help decipher the molecular determinants of mechanical stability.

This study demonstrated the great potential of shuffling protein fragments among homologous parent proteins to engineer novel mechanically stable proteins. Currently, the main challenge in this methodology is the lack of efficient screening methods to select hybrid mutants with desirable mechanical properties, due to the lack of full knowledge of molecular determinants of mechanical stability of proteins. Determining the mechanical stability of the resultant hybrid mutants will still require single molecule AFM. Therefore, developing efficient methods to screen proteins of desirable mechanical stability will be an important task for future endeavors.

## Rational tuning of the mechanical stability of proteins by redesigning unfolding pathways

Since the molecular determinants for mechanical stability of proteins are not fully understood, it remains a great challenge to modulate the mechanical properties of proteins in a rational fashion. Despite the progress in this field, most efforts in tuning proteins' mechanical stability are largely trial-and-error in nature. Recently, based on a model system Top7, we have developed a new strategy to tune the mechanical stability of proteins in a rational fashion.[43] This strategy is based upon redesigning the mechanical unfolding pathways of the protein to achieve predefined mechanical stability.

Combining single molecule AFM and SMD, we discovered that Top7 unfolds *via* a novel substructure-sliding mechanism. Due to the symmetry of the structure, Top7 could unfold in two potential unfolding pathways with apparently different heights of the energy barrier (Fig. 6A): one being the sliding of substructure A against B/C, and the other one being the sliding of substructure C against A/B. SMD simulations revealed that the unfolding of Top7 is dominated by the pathway of sliding A against B/C, suggesting that this is the pathway of the lower energy barrier. Using computationally designed disulfide mutants, we were able to specifically block one unfolding pathway and forced Top7 to unfold *via* the pathway of the higher energy barrier, thereby increasing the mechanical stability of Top7. Lowering the mechanical stability of a given protein is well within the reach of current knowledge,[37,56,57,60] however, it remains challenging to rationally increase the mechanical stability of a protein. The successful example on Top7 represents a unique approach towards this challenge: tuning the mechanical stability of the protein *via* regulating its mechanical unfolding pathway. This method illustrates the great potential of employing simulation and computational biology methods in tailoring the mechanical properties

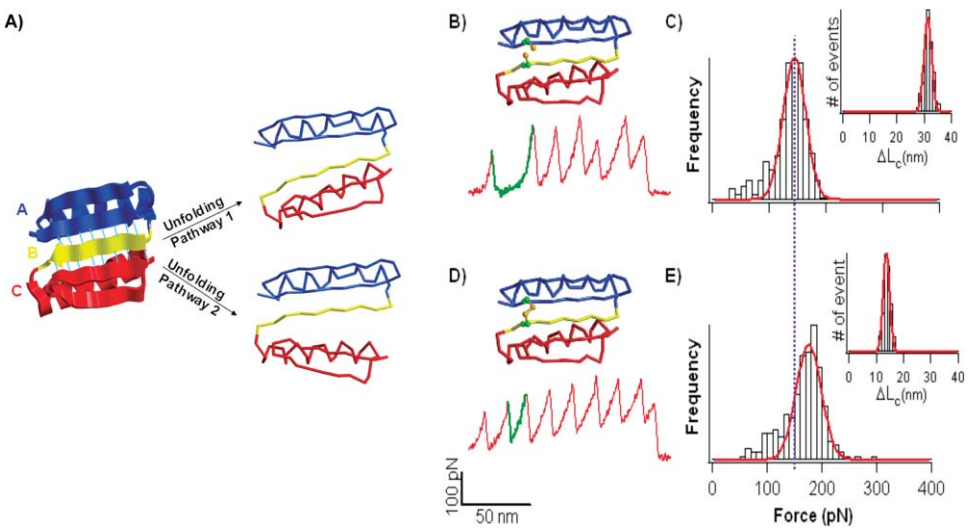


**Fig. 6 Tuning the mechanical stability of Top7 by redesigning its mechanical unfolding pathway**. (A) There are two potential unfolding pathways for Top7: the first one corresponds to the sliding of substructure A against B/C, while the second one corresponds to the sliding of substructure C against A/B. SMD simulations showed that the first unfolding pathway dominates the unfolding of Top7. (B–E) The formation of a disulfide bond modulates the mechanical unfolding pathway of Top7 and its mechanical stability. Force-extension curves and cartoon representations of designed Top7 mutants are shown in (B) and (D). (B) Mechanical properties of reduced Q3C/T51C-Top7. In the presence of DTT, the disulfide bond does not form. The force-extension curves show unfolding events of reduced Q3C/T51C with $\Delta L_C$ of ~30 nm (green). (C) The average unfolding force of reduced Q3C/T51C is 140 pN and $\Delta L_C$ is 31.0 ± 2.0 nm (inset). (D) The mechanical stability of oxidized Q3C/T51C increased due to the shifting of the unfolding pathway. Upon oxidation, 3C and 51C form a disulfide bond that covalently links strands 1 and 3, blocking the unfolding pathway of sliding substructure A against B/C. The unfolding of oxidized Q3C/T51C results in unfolding events with $\Delta L_C$ of ~13 nm. (E) The average unfolding force of oxidized Q3C/T51C is 172 pN, a ~30 pN increase as compared with the reduced Q3C/T51C, and $\Delta L_C$ is 13.5 ± 1.7 nm (inset). Red lines in (C) and (E) are Gaussian fits. Adapted from D. Sharma, O. Perisic, Q. Peng, Y. Cao, C. Lam, H. Lu and H. Li, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 9278. © 2007 by The National Academy of Sciences of the USA.

 

of proteins in a systematic and rational way. It is anticipated that this new strategy will serve as one of the important criteria to computationally design novel proteins with tunable mechanical stability that can be further modulated *via* environmental stimuli, such as redox potential.

## Outlook

Single protein mechanics and engineering has made tremendous progress over the last decade. However, this is still a new burgeoning field of inquiry. Many fundamental questions regarding the molecular determinants of proteins' mechanical stability remain to be answered. The lack of full understanding of such molecular interactions has become a significant hurdle to rationally engineer proteins of tailored mechanical stability, which limits the further exploration of elastomeric proteins for nanomechanical applications. Therefore, efforts to dissect the molecular determinants of proteins' mechanical stability will continue to be of critical and immediate importance. Towards this goal, new methodologies, both experimental and computational, will be required to thoroughly examine the contribution of different non-covalent interactions to the mechanical stability of proteins. Methodologies developed in biochemistry and protein/peptide chemistry will be directly beneficial to such efforts. For example, the recently developed amide-to-ester mutagenesis[74,75] can be employed to directly probe the role of backbone hydrogen bonds in determining the mechanical stability of proteins. Furthermore, it is also important to continue exploring new approaches to use external means to modulate the mechanical stability of proteins. Recent noteworthy examples include the use of light to modulate the structure and mechanical stability of a bacterial blue light receptor protein Per-Arnt-Sim[44] and the use of oligomerization domains to construct mechanical proteins of higher order structures.[76] These combined efforts will uncover the design principles of mechanical proteins and enable the rational design of proteins with well-defined and tailored nanomechanical properties that can be utilized as novel biomaterials and components in nanomechanical and biomedical devices.[77,78]

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