Protein Engineering

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Nonmechanical Protein Can Have Significant Mechanical Stability**

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Many proteins function as complex mechanochemical machinery in living cells to constantly sense, generate, and bear mechanical forces.[1] In addition to their biological importance, these mechanical proteins also arouse tremendous interest in nanoscience and technology and have been incorporated into nanomechanical devices for well-defined applications.[2–4] It is clear that these mechanical proteins will find a wide range of applications as building blocks for the bottom-up construction of functional nanomechanical devices.[4,5] Elastomeric proteins are a special class of mechanical proteins.[6] They are placed under mechanical tension under physiological conditions and serve as molecular springs in a variety of biological machines and tissues to establish elastic connections and provide mechanical strength, elasticity, and extensibility.[6–13] Recent developments in single-molecule force spectroscopy have enabled the direct measurement of the mechanical stability and elasticity of elastomeric proteins at the single-molecule level.[7–10,13–16] Combined with molecular dynamics (MD) simulations, single-molecule atomic force microscopy (AFM) studies have revealed rich information about the mechanical architecture and design of elastomeric proteins.[14,17–23] Compared with proteins that commonly do not bear any mechanical functions under physiological conditions, mechanical proteins are just a small fraction of the total proteins inside cells. To construct multifunctional nanomechanical devices, it is desirable to extend single-molecule force-spectroscopic studies to include nonmechanical proteins (such as enzymes), as the abundant nonmechanical proteins may significantly expand the tool box of mechanical proteins, impart novel functionality, and enable applications such as force sensors[24] and switches. A prerequisite for these applications is that these nonmechanical proteins can withstand mechanical tension. It has been proposed that nonmechanical proteins may contain motifs of considerable mechanical stability;[25] however, most of the nonmechanical proteins that have been studied so far, such as barnase and carmodulin, are mechanically labile and only have marginal mechanical stability.[24,26–30] Green fluorescent protein (GFP) is the strongest nonmechanical protein to date and has a mechanical stability of approximately 100 pN.[24] It remains a question whether nonmechanical proteins can have sufficient mechanical stability and be used for mechanical applications. Herein, we use single-molecule AFM and protein engineering to demonstrate that nonmechanical proteins, with desired structure and topology, can have significant mechanical stability.

Extensive studies on the 27th immunoglobulin (I27) domain of the muscle protein titin have provided insight into how the I27 domain achieves high mechanical stability that is critical for the physiological function of titin.[14,17–22] The key structural feature of I27 is that its two terminus β strands are arranged in parallel and bonded by a series of backbone hydrogen bonds (Figure 1 A). Upon stretching from the N and C termini, a shear force is applied to the hydrogen-bonding network between the two terminal β strands, which form the strongest mechanically resistant region.[19,31] This shear topology of the hydrogen-bonding network seems to be a common feature among the stable mechanical proteins studied so far.[7,15,32–35] Based on this topological consideration, we have identified, from the pool of small model proteins that are commonly used in protein-folding studies, nonmechanical protein candidates that might be mechanically stable. The B1 immunoglobulin G (IgG) binding domain of streptococcal protein G is one of the smallest candidates.

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Protein G of streptococcal is a bacterial cell-wall protein that does not have known mechanical functions. The B1 IgG binding domain of protein G (referred to as GB1 thereafter) is a small α/β protein with only 56 amino acid residues and is composed of a four-strand β sheet packed against a long α helix.[36] The two central strands that comprise the N and C termini are parallel to each other (Figure 1B). An extensive hydrogen-bonding network among the β sheet exists and constitutes a potentially mechanically resistant structural motif. Based on this shear topology, we predict that GB1 will have considerable mechanical stability. This predication was supported by a similar predication made by Li and Makarov[25] based on MD simulation and the topological similarity between the third IgG binding domain of protein G and ubiquitin, which has been shown to be mechanically stable.[32]

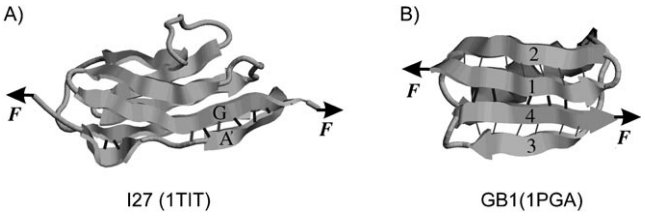

Figure 1. The nonmechanical protein GB1 is predicted to be mechanically stable based on protein topology. A) The topology of the mechanically stable protein I27. The two terminal β strands A′ and G are arranged in parallel and are bonded by a series of backbone hydrogen bonds (indicated by thick, dark lines). Upon stretching (indicated by arrows), the backbone hydrogen bonds between these two strands are subject to a shear force and form the mechanical resistance to stretching. B) The structure of GB1. The two terminal strands (strands 1 and 4) are arranged in a similar fashion to those of I27 and are potentially resistant to mechanical stretching force. GB1 is predicted to have significant mechanical stability. Backbone hydrogen bonds that link β strands 1 and 4 are indicated by thick, dark lines, whereas those linking the remaining part of the β sheet are indicated by thin lines.

We constructed a polyprotein that contains eight direct tandem repeats of the GB1 domain $(GB1)_8$. The polyprotein $(GB1)_8$ was deposited onto a clean glass cover slip, and stretched between the AFM tip and the glass substrate in phosphate buffer solution (PBS). Stretching polyprotein $(GB1)_8$ results in force-extension curves that show characteristic saw-tooth patterns, with as many as eight force peaks (Figure 2A). These force peaks are equally spaced with a peak-to-peak distance of approximately 15 nm, and their amplitudes vary around 180 pN (at a pulling speed of 400 nm s^{-1}). These equally spaced force peaks result from the sequential unfolding of individual GB1 domains in the polyprotein chain,[9,14] whereas the last peak in each force-extension curve corresponds to the extension of the unfolded polyprotein chain and its subsequent detachment from either the AFM tip or substrate. The amplitude of the last peaks varies greatly and can be as high as 1.5 nN, which is clearly different from the mechanical unfolding of the GB1 domains. The force-extension curves can be well described by the wormlike chain (WLC) model of polymer elasticity.[37] Fits of

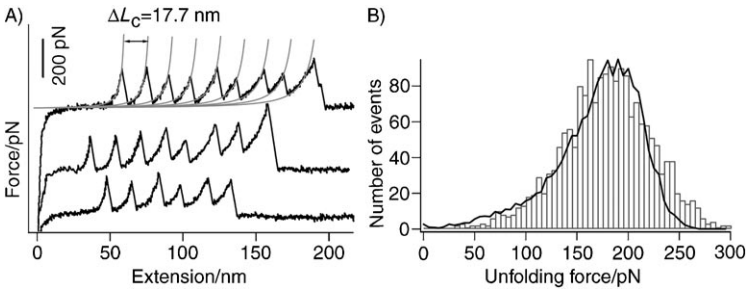

Figure 2. Force-extension measurements reveal that the GB1 protein is mechanically stable. A) Typical force-extension curves measured on $(GB1)_8$ polyproteins that show the characteristic saw-tooth pattern with equally spaced force peaks, which result from the mechanical unraveling of each individual GB1 domain in the polyprotein chain. The last peak in each force-extension curve corresponds to the detachment of the protein from either the AFM tip or substrate. The force-extension curves can be well described by the WLC model of polymer elasticity. The gray lines are WLC model fits to the individual unfolding force peaks with a contour length increment ΔL_c of 17.7 nm. B) Histogram of unfolding forces for the GB1 protein. The gray bars are experimental data measured from approximately 300 force-extension curves (as those shown in A) with a total of 1826 unfolding events at a pulling speed of 400 nm s^{-1}. The unfolding force histogram for GB1 peaks at approximately 178 pN and shows a broad distribution that spans a range of approximately 150 pN. This broad distribution reflects the intrinsic properties that underlie the energy profile of the mechanical unfolding of GB1. The solid, black line is a Monte Carlo simulation of the mechanical unfolding of $(GB1)_8$ polyprotein by using a spontaneous unfolding rate constant α_0 of 0.039 s^{-1}, an unfolding distance Δx_u of 0.17 nm, and a pulling speed of 400 nm s^{-1}.

the WLC model to the consecutive peaks measure a contour length increment of 18.0 ± 0.5 nm (see the Supporting Information), thus indicating that the GB1 protein will extend by approximately 18 nm upon mechanical unraveling.

The contour length increment upon the unfolding of GB1 was calculated to confirm that the saw-tooth patterns observed are due to the mechanical unraveling of an individual GB1 domain. A single GB1 domain is 20.2 nm (56 aa × 0.36 nm aa^{-1}; aa = amino acid residue) long when it is fully stretched, whereas the length of the folded GB1 domain (the distance between its N and C termini) is 2.6 nm.[36] Hence, the mechanical unfolding of a GB1 domain will lengthen the molecule by 17.6 nm, which is in good agreement with the contour length increment between consecutive peaks measured from the force-extension curves. This agreement corroborates that the saw-tooth patterns observed indeed result from the mechanical unfolding of individual GB1 domains in the polyprotein. Therefore, the average unfolding force reflects the average mechanical stability of the GB1 domain, which is approximately 178 ± 40 pN (average ± standard deviation (SD), $n = 1826$) at a pulling speed of 400 nm s^{-1}. This mechanical stability is comparable to that of the I27 domain,[14] a well-studied mechanical protein domain from the muscle protein titin. This result indicates that a nonmechanical protein can have significant mechanical stability, as do mechanical proteins.

The mechanical unfolding of proteins is a stochastic event;[38] therefore, the value of unfolding forces for GB1 vary randomly around the average value of approximately 180 pN. Figure 2B shows the unfolding-force histogram measured from approximately 300 force-extension curves with a total of 1826 unfolding events, such as those shown in Figure 2A. The

force histogram is centered at 178 pN and shows a broad distribution in unfolding forces, which spans a range of approximately 150 pN. The SD of the unfolding forces for GB1 is 40 pN, which is significantly larger than that of any other protein measured so far.[14,17,18,39] This large variation in unfolding forces is much greater than the experimental uncertainty associated with the force measurements (≈20 pN), thus reflecting the intrinsic properties associated with the underlying energy landscape[38] for the mechanical unfolding of the GB1 domain.

The WLC model fits the force-extension curves of GB1 well and no detectable unfolding intermediate state is observed (see Figure 2A). Hence, the mechanical unfolding of the GB1 domain can be modeled as a two-state process with force-dependent unfolding and folding rate constants.[40] Based on a standard procedure,[14] Monte Carlo simulation was used to reproduce the unfolding-force histogram shown in Figure 2B and extract the information about the unfolding rate constant at zero force α_0 and the unfolding distance Δx_u, which is the distance between the folded state and the transition state along the reaction coordinate. We found that the unfolding-force histogram can be reproduced adequately by using an unfolding rate constant of $0.039\ s^{-1}$ and an unfolding distance of 0.17 nm. The unfolding distance for GB1 is smaller than the typical values (0.25–0.4 nm) of Δx_u for β-sheet proteins,[8,9,14,22] thus suggesting that the transition state for the mechanical unfolding of GB1 is highly nativelike. The unfolding rate constant α_0 is one order of magnitude smaller than the chemical unfolding rate constant at zero denaturant concentration for GB1.[41]

Similar to elastomeric proteins, the unfolding forces of GB1 are found to depend upon the pulling speed at which the protein is stretched and unraveled. The faster the pulling speed is, the higher the unfolding force is required to unravel the GB1 domain. The average unfolding force versus the pulling speed (20–3500 $nm\,s^{-1}$) for GB1 is plotted in Figure 3, and for comparison, the speed dependence of the unfolding force for I27 is also plotted.[14] The data show that the unfolding force for GB1 has a much stronger dependence on the pulling speed than that of the I27 protein.

Monte Carlo simulation was used to fit the speed dependence of the unfolding forces. We found that the speed dependence of the unfolding forces can be well described with $\alpha_0 = 0.039\ s^{-1}$ and $\Delta x_u = 0.17$ nm, which is in good agreement with the estimate from Figure 2.

Compared with the I27 protein,[8,9,14,15,22] the unfolding rate constant α_0 for GB1 is two orders of magnitude bigger than that of I27, yet GB1 is as mechanically stable as I27, which is due to the unusually small unfolding distance Δx_u for GB1. If the distance to the transition state for GB1 were similar to that of I27 (0.25 nm), a mechanical unfolding rate constant of $0.039\ s^{-1}$ would cause the GB1 domain to unfold at an average force of approximately 100 pN at a pulling speed of $400\ nm\,s^{-1}$. A shorter unfolding distance effectively compensates the effect of the faster unfolding rate constant on the mechanical stability, thus illustrating the vital importance of the unfolding distance to the mechanical stability of proteins. It is possible that the small unfolding distance Δx_u directly results from the shear topology and the extensive hydrogen-bonding network of the GB1 protein, thus revealing the physical basis that underlies the hypothesis that the β-sheet proteins of shear topology tend to be mechanically stable.

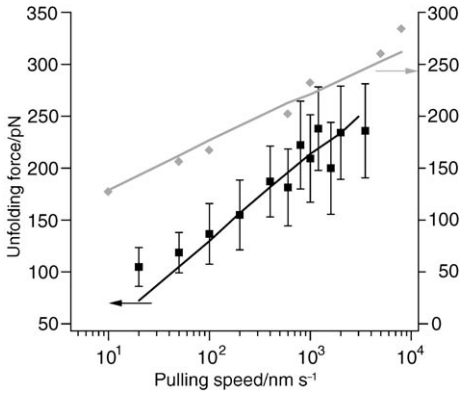

Figure 3. Unfolding kinetics of the GB1 protein. The unfolding forces of GB1 (black squares) strongly depend on the pulling speed at which the polyprotein was stretched and unraveled. The measured unfolding forces at high pulling speed were corrected for the hydrodynamic drag force that acts directly on the AFM cantilever.[44] The unfolding kinetics of GB1 can be reproduced adequately by Monte Carlo simulations with $\alpha_0 = 0.039\ s^{-1}$ and $\Delta x_u = 0.17$ nm (black, solid line). The unfolding distance for GB1 is much smaller than that of other proteins. For comparison, the unfolding kinetics of I27 protein, as well as its Monte Carlo fit, are also plotted (gray diamonds and gray line). The data was taken from ref. [14].

The experimental data reported herein closely matches the prediction made by Li and Makarov for a similar domain from protein G (the third IgG binding domain from streptococcal protein G).[25] Although the structural details differ between these two domains, the mechanical unfolding of both domains involves the separation of the two parallel terminal β strands, which are bonded by a network of hydrogen bonds and constitute the mechanical resistance. A recent study[42] demonstrated that protein L, a structural homologue of GB1, is also mechanically stable and unfolds at approximately 130 pN at a pulling speed of $400\ nm\,s^{-1}$, thus providing additional supporting evidence of the importance of the shear β-sheet topology on the mechanical stability. It is likely that proteins homologous to GB1 share the same feature and constitute a family of proteins that have remarkable mechanical stability. Furthermore, this study can also aid future experimental efforts to search for potential mechanical functions for proteins in vivo.

In summary, we have demonstrated that a nonmechanical protein, such as GB1, can have significant mechanical stability. Our findings corroborate the importance of protein topology for mechanical stability, and we anticipate that by using simple topological considerations more proteins with considerable mechanical stability can be discovered and the tool box of elastomeric proteins will be expanded. This approach will pave the way for the exploitation of nonmechanical proteins with defined functionalities for various nanomechanical applications, such as force sensors and switches.

Experimental Section

The plasmid that encodes the GB1 protein was generously provided by David Baker of the University of Washington. The GB1 monomer, flanked with a 5′ *BamH*I restriction site and 3′ *Bgl*II, *Kpn*I restriction sites, was amplified by the polymerase chain reaction and subcloned into the pQE80L expression vector. The $(GB1)_8$ polyprotein gene was constructed by using a previously described method[14] based on the identity of the sticky ends generated by the *BamH*I and *Bgl*II restriction enzymes. The $(GB1)_8$ polyproteins were expressed in the DH5α strain and purified by Ni^{2+}-affinity chromatography. The polyprotein was kept at 4 °C in PBS buffer at a concentration of 740 $\mu g\,mL^{-1}$.

Single-molecule AFM experiments were carried out on a custom-built atomic force microscope, which was constructed as described.[43] All the force-extension measurements were carried out in PBS buffer. In a typical experiment, $(GB1)_8$ polyprotein sample (1 μL) was deposited onto a clean glass cover slip covered by PBS buffer (50 μL) and was allowed to adsorb for approximately 5 min. The spring constant of each individual cantilever (Si_3N_4 cantilevers from Vecco, with a typical spring constant of 40 $pN\,nm^{-1}$) was calibrated in solution using the equipartition theorem before and after each experiment.

The mechanical unfolding of the GB1 protein was described as a two-state Markovian process with force-dependent rate constants. Monte Carlo simulations of the stretching of the GB1 polyprotein was carried out according to reported procedures[14] to estimate the unfolding rate constant at zero force α_0 and the distance of the native state to the transition state Δx_u along the reaction coordinate of the mechanical unfolding reaction.

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