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Single Molecule Force Spectroscopy Reveals Critical Roles of Hydrophobic Core Packing in Determining the Mechanical Stability of Protein GB1

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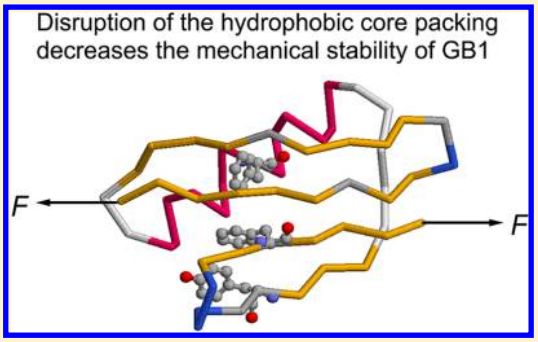
Supporting Information

ABSTRACT: Understanding molecular determinants of protein mechanical stability is important not only for elucidating how elastomeric proteins are designed and functioning in biological systems but also for designing protein building blocks with defined nanomechanical properties for constructing novel biomaterials. GB1 is a small α/β protein and exhibits significant mechanical stability. It is thought that the shear topology of GB1 plays an important role in determining its mechanical stability. Here, we combine single molecule atomic force microscopy and protein engineering techniques to investigate the effect of side chain reduction and hydrophobic core packing on the mechanical stability of GB1. We engineered seven point mutants and carried out mechanical ϕ-value analysis of the mechanical unfolding of GB1. We found that three mutations, which are across the surfaces of two subdomains that are to be sheared by the applied stretching force, in the hydrophobic core (F30L, Y45L, and F52L) result in significant decrease in mechanical unfolding force of GB1. The mechanical unfolding force of these mutants drop by 50−90 pN compared with wild-type GB1, which unfolds at around 180 pN at a pulling speed of 400 nm/s. These results indicate that hydrophobic core packing plays an important role in determining the mechanical stability of GB1 and suggest that optimizing hydrophobic interactions across the surfaces that are to be sheared will likely be an efficient method to enhance the mechanical stability of GB1 and GB1 homologues.



INTRODUCTION

Mechanical properties of proteins are essential in a wide range of biological processes, ranging from cell−cell adhesion, muscle contraction to protein degradation and translocation.[1,2] Understanding how proteins are designed in nature to achieve desired mechanical stability not only is important for understanding biological processes but also holds the promise to design novel protein-based biomaterials for biomedical and material science applications. Over the past decade, single molecule atomic force microscopy (AFM),[3−8] in combination with protein engineering and molecular dynamics simulations,[9,10] has enabled the characterization of mechanical properties of proteins at the single molecule level in great detail, and some general molecular determinants of protein mechanical stability have emerged.[4,11−14] Protein topology has been recognized as an important determinant for the mechanical stability of proteins, and proteins of a shear topology often exhibit high mechanical stability.[4,12,13,15,16] In the shear topology, two force-bearing β-strands are arranged in parallel and form the so-called mechanical clamp to provide mechanical resistance to the applied stretching force. Backbone hydrogen bonds connecting the two force-bearing strands have been shown to be critical for the mechanical stability of proteins. In addition to protein topology, detailed molecular interactions within proteins must also make important contributions to protein mechanical stability,[12,17,18] as proteins of the same fold can exhibit vastly different mechanical stability.[19,20] For example, immunoglobulin-like domains of the giant muscle protein titin share the same protein fold but exhibit a large range of mechanical stability spanning from 150 pN to more than 300 pN.[3,20]

Elucidating the role of these molecular interactions, such as hydrophobic interactions within the hydrophobic core, in determining protein mechanical stability requires detailed characterization of the structure of the mechanical unfolding transition state of proteins. Because of its high free energy, transition state is short-lived and does not populate in experiments, making it difficult to characterize using traditional structural biology methods. ϕ-value analysis has evolved into one major experimental tool to experimentally map the structure of the transition state and serves as a benchmark for molecular dynamics (MD) simulations of protein folding/unfolding.[21−23] In this method, the energetic effect of conserved point mutation on the (un)folding energy barrier relative to its thermodynamic stability is used to obtain

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structural information on the transition state in the mutational site. This method has been used to map the mechanical unfolding transition state of proteins and provided insights into molecular determinants of protein mechanical stability. However, only three proteins, I27 domain of titin,[24] TNfn3[25] from tenascin-C, and protein L,[26] have been examined using this method to date. Experimental observations on I27 and TNfn3 suggested that residues in the hydrophobic cores play a minor role in modulating protein mechanical stability,[24,25,27] while AFM experiments on protein L showed that mutation I60V led to a significant decrease of the mechanical stability of protein L,[26] suggesting that hydrophobic core packing plays potentially important roles in the mechanical unfolding of protein L. To further investigate the effect of hydrophobic core packing and side chain reduction on protein mechanical stability, here we combine single molecule AFM and ϕ-value analysis to investigate the mechanical unfolding of a small protein GB1.

GB1, the B1 IgG binding domain of protein G from *Streptococcus*, contains 56 amino acid residues and is an α/β protein consisting of a four-strand β-sheet packed against an α-helix.[28] Although GB1 and protein L share low sequence identity, they belong to the same β-grasp fold and are structural homologues.[29] In our previous work, we have used GB1 as a model elastomeric protein domain for extensive protein mechanics studies.[30−32] The mechanical properties and folding/unfolding dynamics of GB1 have been characterized in great detail,[31] and based on GB1, methodologies have been developed to rationally enhance the mechanical stability of proteins.[32,33] In this work, we carry out mechanical ϕ-value analysis of the mechanical unfolding of GB1 and map the structure of its mechanical unfolding transition state. Our results show that mutations in the hydrophobic core of GB1 can lead to significant decrease in the mechanical stability of GB1, suggesting that hydrophobic core packing plays important roles in determining the mechanical stability of GB1.

MATERIALS AND METHODS

Construction of Polyproteins. The plasmid encoding wild-type GB1 was generously provided by David Baker of the University of Washington. All the point mutants (L7A, T11A, T16A, F30L, Y45L, D46A, and F52L) were constructed using standard PCR-based site-directed mutagenesis techniques and confirmed by DNA sequencing. Using well-established procedures,[31,34] we constructed genes of polyprotein chimera $(GB1mutant\text{-}I27)_4$, where the well-characterized I27 domains serve as internal fingerprints. Using the same strategy, we also constructed homopolyprotein genes of these GB1 mutants. At the C-terminus of polyproteins, there are two engineered cysteine residues. It is possible that some polyprotein molecules can be oxidized to form dimers via the formation of a disulfide bond. Polyproteins were overexpressed in the DH5α strain, purified by using Co^{2+} affinity chromatography and then stored at 4 °C in PBS buffer with 300 mM NaCl and 150 mM imidazole.

Single-Molecule Force Spectroscopy Experiments. Single-molecule AFM experiments were carried out on a custom-built AFM as described previously.[31,35] All the force−extension measurements for both homopolyproteins and heteropolyproteins were carried out in PBS buffer (pH 7.4). Around 1 μL of protein solution was deposited onto a clean glass coverslip covered by about 50 μL of PBS and was allowed to adsorb onto the glass coverslip for ∼5 min before the force−extension measurements. Experiments were performed at room temperature. The spring constant of each individual cantilever (Si_3N_4 cantilevers from Veeco, with a typical spring constant of 70 pN/nm) was calibrated in PBS buffer using the equipartition theorem before each experiment. The pulling speed used in the AFM experiments was 400 nm/s, unless otherwise indicated. Data analysis was done using custom-written software in Igor Pro.

Monte Carlo Simulation. In the Monte Carlo simulation,[36−38] the unfolding of GB1 and I27 domains was modeled as two-state processes with force-dependent rate constants: $\alpha(F) = \alpha_0 \exp(F\Delta x_u/k_BT)$, where k_B is the Boltzmann constant, T is the temperature in kelvin, $\alpha(F)$ is the unfolding rate constant under a stretching force of F, α_0 is the unfolding rate constant at zero force, and Δx_u is the distance between the folded state and the transition state.[39,40] Monte Carlo simulations were carried out according to published procedures[19,33] to reproduce force−extension curves of $(GB1mutant\text{-}I27)_4$ polyprotein chimera and estimate the unfolding rate constant at zero force (α_0) and the distance between the folded state and the transition state (Δx_u) along the reaction coordinate of the mechanical unfolding.

ϕ-Value Analysis. ϕ-value analysis[24,3−5,34,41] was used to map the structure of the mechanical unfolding transition state of GB1. The ϕ-value for the mechanical unfolding is defined as

$$\phi_U = \Delta\Delta G_{TS-N}/\Delta\Delta G_{D-N}$$

where $\Delta\Delta G_{D-N}$ is the difference of the thermodynamic stability between the wild-type (wt) and mutant GB1 and $\Delta\Delta G_{TS-N}$ is the difference of the mechanical unfolding free energy between the wt GB1 and mutant, respectively. $\Delta\Delta G_{TS-N}$ is calculated using the relationship

$$\Delta\Delta G_{TS-N} = -RT \ln(\alpha_0^{wt}/\alpha_0^{mut})$$

where the values of α_0 were obtained from Monte Carlo simulations.

RESULTS

Design of GB1 Variants To Probe the Effect of Point Mutations on the Mechanical Stability of GB1. We used mechanical ϕ-value analysis to map the structure of the mechanical unfolding transition state and evaluate the contribution of specific side-chain interactions to the mechanical unfolding force of GB1. We chose seven different positions throughout GB1 to make conservative side-chain deletion (Figure 1). These positions are located in different

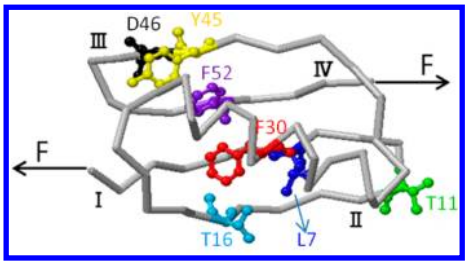

Figure 1. Three-dimensional structure of GB1 (PDB code: 1PGA). Residues that are chosen for mechanical ϕ-value analysis are displayed in ball-and-stick representations.

secondary structural regions of GB1 and were chosen to probe their roles on the mechanical unfolding of GB1. In addition, some of these point mutants have been shown to significantly affect the thermodynamic or kinetic stability of GB1.[29] For example, it was shown that mutation F52L results in an acceleration of the chemical unfolding kinetics by 100 times over wt GB1.[29] Such dramatic effects offer the highest possibility for us to observe the mechanical phenotypic effect of point variants, if any. Of these variants, L7A, T11A, and T16A are located in the first β-hairpin, F30L is located in the α-helix, and Y45L, D46A, and F52L are located in the second β-hairpin. It is of note that T11A and D46A are located at the two β-turns of GB1, and we intend to use these two mutations to

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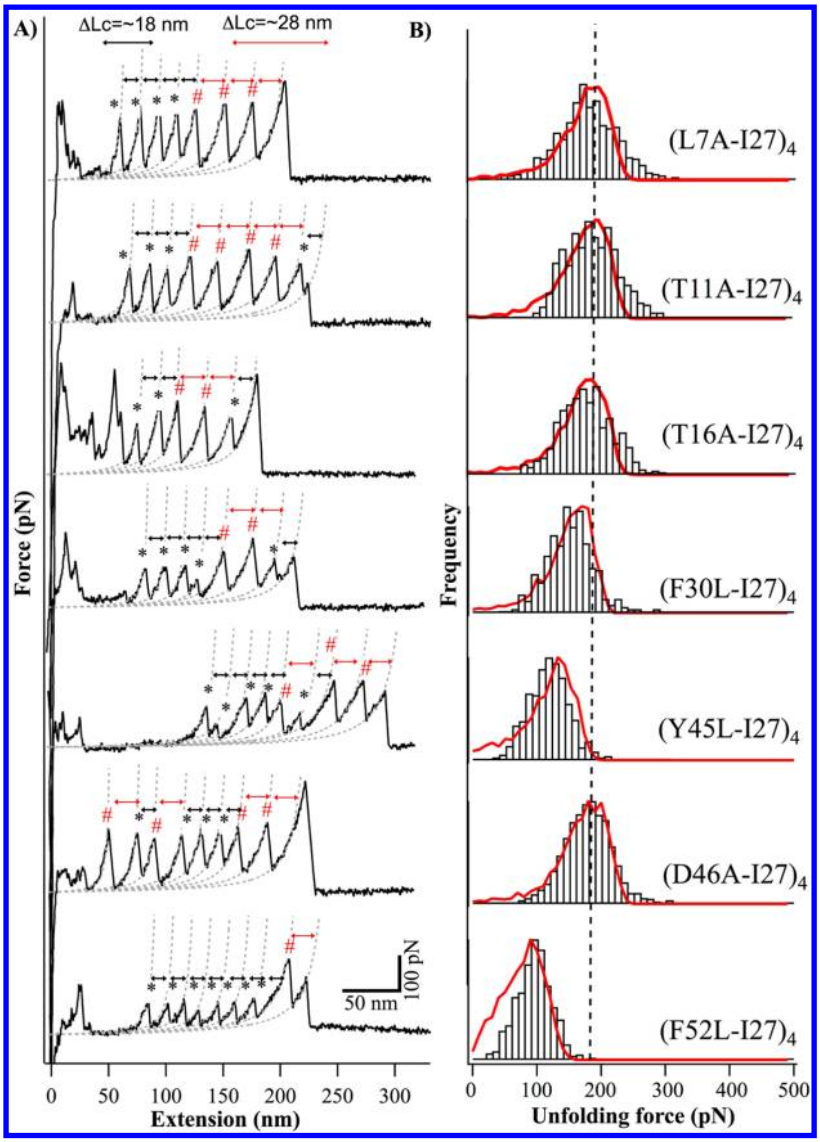

Figure 2. Single molecule AFM experiments reveal the effect of side-chain reduction on the mechanical stability of GB1. (A) Representative force–extension curves of $(GB1mutant\text{-}I27)_4$ polyprotein chimera. Dotted lines are WLC fits to the force–extension curves. Unfolding events of GB1 mutants are indicated by an ∗ and show contour length increment of 18 nm, while unfolding event of I27 domains are indicated by # and show ΔLc of 28 nm. It is of note that the force–extension curve shown for $(F52L\text{-}I27)_4$ displays seven F52L unfolding events and corresponds to the unfolding of a dimer of $(F52L\text{-}I27)_4$, which formed due to the oxidation of C-terminal cysteine residues into a disulfide bond between two $(F52L\text{-}I27)_4$. In principle, at least six I27 unfolding events should be observed in this molecule. The fact that only one I27 unfolding event was observed is likely due to the detachment of the polyprotein chain from either the AFM tip or substrate prior to the unfolding of the rest I27 domains in the polyprotein chain. (B) Unfolding force histograms of GB1 mutants measured at a pulling speed of 400 nm/s. Solid lines correspond to Monte Carlo simulation results generated using parameters shown in Table 1. The dotted line indicates the average unfolding force of wt GB1 at the same pulling speed (400 nm/s). The number of events for each histogram is shown in Table 1.

evaluate the role of the β-turn in the mechanical unfolding of GB1.[29] Out of the seven variants, four are Ala substitutions, while F30L, Y45L, and F52L are Leu substitutions. To use single molecule AFM to investigate the mechanical properties of these variants, we used the well-characterized I27 domain as a fingerprint and constructed polyprotein chimera $(GB1variant\text{-}I27)_4$. In these chimera polyproteins, the unfolding force of I27 serves as an internal calliper[34,42] which allows the comparison of the mechanical effect of each point mutation on the mechanical stability of GB1 without any ambiguity. In addition, the use of the fingerprint domain I27 ensures that the mechanical unfolding signatures of the thermodynamically significantly destabilized mutants, such as F52L, can be identified without any ambiguity. As a control, we also constructed polyproteins that are composed of eight tandem repeats of a specific mutant to eliminate the possible effect of interactions between I27 and GB1. Both homopolyproteins and chimera polyproteins are expressed as soluble proteins in *E. coli* in high yield and purified using standard Co^{2+} affinity chromatography.

Effect of Side-Chain Reduction on the Mechanical Stability of GB1. We used single molecule AFM to stretch polyprotein chimera $(GB1mutant\text{-}I27)_4$ to measure their mechanical unfolding forces. Stretching polyprotein chimera resulted in force–extension curves of characteristic sawtooth pattern appearance (Figure 2A), where each individual unfolding force peak corresponds to the mechanical unfolding event of individual GB1 variant or I27 domain. Fitting

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Table 1. Thermodynamic, Kinetic, and Mechanical Stability of GB1 and Its Mutants

	unfolding force (pN)[a]	location	k_u^{4M} (s^{-1})[b]	$\Delta\Delta G_{D-N}$ (kcal/mol)[c]	$\phi_u^{chemical}$ [g]	Δx_u (nm)[d]	α_0 (s^{-1})[e]	$\Delta\Delta G_{TS-N}$ (kcal/mol)[f]	$\phi_u^{mechanical}$ [h]
WT	183 ± 40		1.22			0.17	0.039		
L7A	181 ± 47 (n = 571)	β-strand I	10.72	1.85	0.68	0.17	0.04	0.015	0.01
T11A	182 ± 40 (n = 474)	β-strand II	3.4	0.60	0.98	0.17	0.039	0	0
T16A	172 ± 46 (n = 627)	β-strand II	2.34	0.38	1	0.17	0.06	0.26	0.67
F30L	145 ± 37 (n = 400)	α-helix	12.53	1.42	0.95	0.19	0.1	0.58	0.39
Y45L	115 ± 32 (n = 519)	β-strand III	67.52	3.34	0.7	0.2	0.25	1.10	0.33
D46A	178 ± 38 (n = 1394)	β-strand III	1.38	1.74	0.04	0.17	0.06	0.26	0.15
F52L	95 ± 29 (n = 656)	β-strand IV	173.55	3.54	0.81	0.22	0.3	1.21	0.34

[a]The data are presented as average ± standard deviation (no. of events). [b]Chemical unfolding rate constant in 4 M GuHCl. Taken from ref 29. [c]$\Delta\Delta G_{D-N} = \Delta G_{D-N}(wt) - \Delta G_{D-N}(mutant)$. Taken from ref 29. [d]Unfolding distance, obtained from Monte Carlo simulations. [e]Mechanical unfolding rate constant at zero force, obtained from Monte Carlo simulations. [f]$\Delta\Delta G_{TS-N} = \Delta G_{TS-N}(wt) - \Delta G_{TS-N}(mutant)$ for mechanical unfolding. The data are calculated using $\Delta\Delta G_{TS-N} = -RT \ln(\alpha_0^{wt}/\alpha_0^{mut})$. [g]$\phi_u^{chemical}$: ϕ_u value for chemical unfolding of GB1. Taken from ref 29. [h]$\phi_u^{mechanical}$: ϕ_u value for mechanical unfolding of GB1. The data are calculated using $\phi_U = \Delta\Delta G_{TS-N}/\Delta\Delta G_{D-N}$.

wormlike chain (WLC) model of polymer elasticity to consecutive unfolding force peaks revealed two populations of unfolding events: one with a contour length increment (ΔLc) of ∼28 nm, which corresponds to the unfolding of the fingerprint domains I27, and the other one with a ΔLc of ∼18 nm, which corresponds to the unfolding of GB1 mutants (highlighted by an asterisk). ΔLc of GB1 mutants is identical to that of wt GB1, suggesting that these point mutations did not cause major change to the folded structure of GB1.

The unfolding force histograms for each GB1 variant are shown in Figure 2B. Of all the variants analyzed (Table 1 and Figure 2), four variants (L7A, T11A, T16A, and D46A) unfold at forces similar to that of wt GB1, i.e., ∼180 pN at a pulling speed of 400 nm/s, and exhibit similar broad distribution of unfolding forces as wt GB1. In contrast, variants F30L, Y45L, and F52L unfold at significantly lower forces than wt GB1 (ΔF_{WT-MUT} varies from 50 to 90 pN at a pulling speed of 400 nm/s) as well as other variants. These results indicated that point mutations F30L, Y45L, and F52L lead to a significant reduction of the mechanical stability of GB1, while other point mutations do not affect the mechanical stability of GB1 considerably. Force−extension measurements using homopolyproteins yield similar results as chimera polyproteins, suggesting that the use of I27 domain does not affect the mechanical stability of GB1 mutants. These results clearly indicated that interactions mediated by residues in the hydrophobic core can play important roles in determining the mechanical stability of GB1.

Furthermore, it is of note that the unfolding force distributions of F30L, Y45L, and F52L are narrower than those of wt GB1 as well as variants L7A, T11A, T16A, and D46A. Since the width of the unfolding force distribution is related to the unfolding distance Δx_u, the distance between the native state and the transition state,[40] the narrower distribution suggests that the mechanical unfolding transition state for variants F30L, Y45L, and F52L is shifted as compared with wt GB1.

To quantify the effect of point mutations on the unfolding free energy barrier (ΔG_{TS-N}) and the unfolding distance Δx_u, we carried out mechanical unfolding experiments of GB1 mutants at different pulling speeds. As shown in Figure 3, the pulling speed dependences of unfolding forces for variants L7A, T11A, T16A, and D46A are almost identical to that of wt. However, the pulling speed dependences for variants F30L, Y45L, and F52L show a clear shift with respect to that of wt. And the slopes for variants F30L, Y45L, and F52L are also

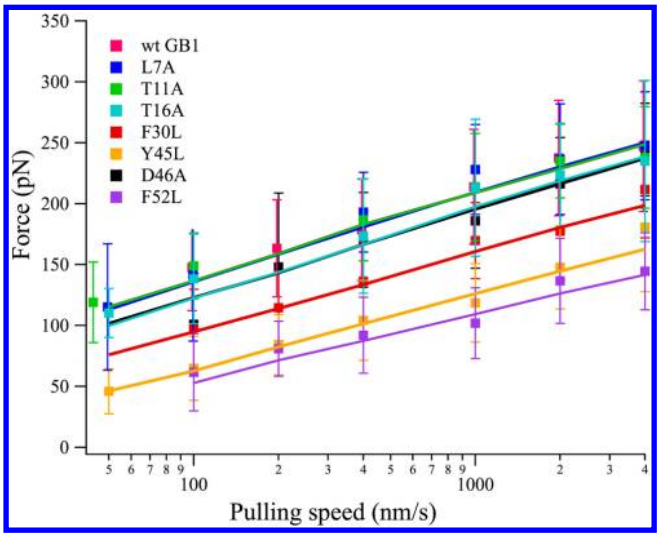

Figure 3. Pulling speed dependency of the unfolding force of GB1 mutants. Experimental data are shown as symbols, while solid lines correspond to Monte Carlo simulations results using the parameters shown in Table 1. Error bars shown for each data point correspond to the standard deviation of the measured unfolding forces. The average number of events for each data point at pulling speeds other than 400 nm/s is 66 for L7A, 60 for T11A, 60 for T16A, 78 for F30L, 73 for Y45L, 80 for D46A, and 49 for F52L.

slightly different from that of wt GB1. These results suggest that the reduced mechanical unfolding force for F30L, Y45L, and F52L is due to the decrease of the mechanical unfolding barrier ΔG_{TS-N} as well as slight shift of the unfolding transition state.

Since the unfolding of GB1 and its variants appear as two-state processes, we used the well-established Monte Carlo simulation procedures[34,36] to estimate the intrinsic unfolding rate constant at zero force (α_0) and Δx_u. We found that the unfolding force distribution and its pulling speed dependence can be described adequately using parameters tabulated in Table 1. It is of note that Δx_u for variants F30L, Y45L, and F52L increased from 0.17 nm (wt) to 0.19−0.22 nm, suggesting a shift of the transition state toward the unfolded state.

Mechanical ϕ_u-Value Analysis Maps the Mechanical Unfolding Transition State of GB1. ϕ-value analysis is a powerful experimental method to probe transition state structure of proteins during their folding−unfolding reactions

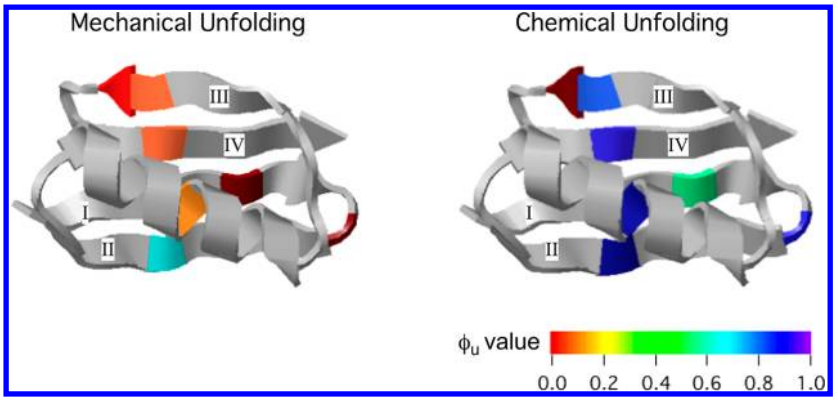

Figure 4. Comparison of ϕ-values for mechanical and chemical unfolding of GB1 suggests that mechanical and chemical unfolding of GB1 follows different pathways.

at near atomic resolution.[21,22] Recently, ϕ-value analysis has also been applied to map the mechanical unfolding transition state of proteins.[24] By comparing the relative energetic effect of mutations on the native and transition state of mechanical unfolding, one can calculate the mechanical unfolding ϕ-value, which is defined as $\Delta\Delta G_{TS-N}/\Delta\Delta G_{U-N}$, to estimate the extent of preservation of interactions in the mutated site in the mechanical unfolding transition state. A mechanical unfolding ϕ-value of zero suggests that the mutated site is as structured in the mechanical unfolding transition state as in the native state, while a ϕ-value of one suggests that the mutated site is fully disrupted in the mechanical unfolding transition state as compared with the native state. A fractional ϕ-value would suggest that the mutated site is partially structured/disrupted in the transition state.

On the basis of the measured mechanical unfolding barrier ΔG_{TS-N} and thermodynamic stability ΔG_{D-N} reported in the literature,[29] we have calculated the mechanical unfolding ϕ_u-values for different residues of GB1, which allowed us to map and visualize the structure of the mechanical unfolding transition state of GB1 (Figure 4).

Residues L7, T11, and D46 showed low mechanical ϕ_u-values that are close to zero, suggesting that in the mechanical unfolding transition state, side-chain interactions mediated by these residues are not affected by the stretching force and remain largely intact. Surprisingly, residue T16 showed a high mechanical ϕ_u-value of 0.7. In contrast, residues F30, Y45, and F52 showed medium ϕ_u-values (>0.3), suggesting that interactions mediated by side chains of these residues are partially disrupted in the mechanical unfolding transition state by the stretching force. The map of mechanical unfolding ϕ_u-values for GB1 is consistent with the mechanical unfolding transition state structure predicted by molecular dynamics simulations:[43] the first β-hairpin and α-helix remain largely structured, while the second β-hairpin is partially disrupted in the mechanical unfolding transition state.

It is worth pointing out that the decrease of the unfolding force for a given mutant depends on the ϕ-value as well as $\Delta\Delta G_{N-D}$. Although GB1 has a high ϕ-value at position T16, T16A has a small $\Delta\Delta G_{N-D}$ (0.38 kcal/mol), leading to a small decrease of unfolding force by only ~11 pN. It is also important to note that the structure of the mechanical unfolding transition state is different from that of the chemical unfolding transition state, as evidenced from the comparison of the mechanical and chemical ϕ-values (Figure 4 and Table 1). This observation is similar to those observed on protein L,[26] I27,[17] and TNfn3[25,44] and corroborates the idea that the protein unfolds via distinct pathways in mechanical and chemical experiments, as mechanical unfolding is along a predefined reaction coordinate set by the stretching force while chemical unfolding is largely a global event.

DISCUSSION

GB1 is a α/β protein, and its structure belongs to the β-grasp fold. The two force-bearing β-strands are arranged in parallel, constituting a shear topology, which is a typical feature of mechanically stable proteins. Proteins belonging to this family have been shown, by both simulation[43,45] and single molecule AFM experiments, that they are mechanically stable. Backbone hydrogen bonds have been identified as a key feature in contributing to their significant mechanical stability. Since structural homologous proteins can exhibit very different mechanical stability, it is also recognized that side chain interactions are also important for determining protein mechanical stability.[26] In particular, it has been suggested that protein mechanical stability also depends on interactions across the surfaces in a shear topology that are to be sheared upon forced unfolding.[26] Consistent with this proposal, single molecule AFM experiments on protein L revealed that mutation I60V did result in destabilization of mechanical stability of protein L.[26] Our results on GB1 provided another system to corroborate this proposal.

Similar to protein L, molecular dynamics simulations on GB1 suggested that the mechanical unfolding of GB1 involved the shearing of two structural motifs against each other: the second β-hairpin against the rest of GB1, the first β-hairpin and the α-helix.[43,45] Close inspection of GB1 structure reveals that residues F30, Y45, and F52 are all at the interface connecting the two structural motifs through hydrophobic interactions. Our results show that the reduction of hydrophobic core packing upon mutation F30L, Y45L, or F52L results in significant decrease of the mechanical stability of GB1, providing strong evidence that interactions mediated by these hydrophobic residues at the interface of the two shearing motifs play critical roles in determining the mechanical stability of GB1. We propose that these hydrophobic interactions are serving as an internal "mechanical clamp", in addition to the patch of backbone hydrogen bonds connecting the two force-bearing strands, that "glues" the two structural motifs together to resist the mechanical shear and prevent GB1 from mechanical unfolding. Disruption of these interactions will loosen up the surfaces that are to be sheared by the stretching

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force and give rise to the accelerated mechanical unfolding kinetics and longer unfolding distance Δx_u.

Because of the importance of these hydrophobic interactions between the two shearing motifs, optimizing hydrophobic core packing should in principle allow the modulation of the strength of this mechanical clamp and help tailor protein mechanical stability. Indeed, we found that a GB1 mutant, Gc3b4, which was computationally designed to improve hydrophobic core packing by the Mayo group[46] and contains seven mutations in the core of GB1, exhibits improved mechanical stability (unfolding force increased by 30 pN).[33] However, Gc3b4 was not specifically designed for the purpose of improving the mechanical stability of GB1. In a recent elegant design effort, Brockwell's group identified a rheostat in protein L.[26] Using a single point mutation I60F to improve the hydrophobic core packing, Brockwell and co-workers successfully increased the mechanical stability of protein L by 80 pN.

Furthermore, MD simulations revealed very similar mechanical unfolding pathways for protein L[12,43] and GB1,[43] and the rheostat identified in protein L is also located at the interface between the two shearing motifs.[12] These results raised the question if this additional "mechanical clamp" between the two shearing motifs is a general feature among proteins belong to the β-grasp fold. Future experimental efforts will be required to systematically test this hypothesis by investigating other proteins within this protein fold family, such as ubiquitin.[47,48]

■ ASSOCIATED CONTENT

Supporting Information

Force−extension curves and unfolding force histograms of homopolyproteins of GB1 mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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