Single molecule force spectroscopy reveals engineered metal chelation is a general approach to enhance mechanical stability of proteins

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Significant mechanical stability is an essential feature shared by many elastomeric proteins, which function as molecular springs in a wide variety of biological machinery and biomaterials of superb mechanical properties. Despite the progress in understanding molecular determinants of mechanical stability, it remains challenging to rationally enhance the mechanical stability of proteins. Using single molecule force spectroscopy and protein engineering techniques, we demonstrate that engineered bi-histidine metal chelation can enhance the mechanical stability of proteins significantly and reversibly. Based on simple thermodynamic cycle analysis, we engineered a bi-histidine metal chelation site into various locations of the small protein, GB1, to achieve preferential stabilization of the native state over the mechanical unfolding transition state of GB1 through the binding of metal ions. Our results demonstrate that the metal chelation can enhance the mechanical stability of GB1 by as much as 100 pN. Since bi-histidine metal chelation sites can be easily implemented, engineered metal chelation provides a general methodology to enhance the mechanical stability of a wide variety of proteins. This general approach in protein mechanics will enable the rational tuning of the mechanical stability of proteins. It will not only open new avenues toward engineering proteins of tailored nanomechanical properties, but also provide new approaches to systematically map the mechanical unfolding pathway of proteins.

mechanical unfolding | rational design | stabilization | protein engineering | protein mechanics

Naturally occurring elastomeric proteins function as molecular springs in biological settings and exhibit mechanical properties that underlie the elasticity of natural adhesives (1), cell adhesion proteins (2), and muscle proteins (3–5). They are also potential building blocks for the bottom-up construction of functional nanomechanical devices and biomaterials with superb mechanical properties (6, 7). To use elastomeric proteins as building blocks for various applications, the ability to tailor the mechanical properties of elastomeric proteins at the molecular level is one of the essential requirements. The development of single molecule atomic force microscopy (AFM) as well as computer modeling has made it possible to examine the mechanical properties of proteins at the single molecule level *in vitro* and *in silico* (8–12), increasing the feasibility of elucidating the relationships between structural and mechanical properties of elastomeric proteins. Despite the tremendous progress in protein mechanics, it is generally not possible to enhance the mechanical stability of proteins in a rational fashion, with this being done in only a few isolated cases (13–17). Such a lack of knowledge has hindered the understanding of molecular design of naturally occurring elastomeric proteins and prevented rational design of novel protein-based materials.

Mechanical stability is an intrinsic property of proteins and is commonly defined as the force required to unfold a given protein. Mechanical stability is determined by the mechanical unfolding energy barrier and the distance between the native state and transition state (18, 19) and thus, is different from the thermodynamic stability of proteins (20). In contrast to the challenge in rationally enhancing the mechanical stability of proteins, many successful strategies to improve thermodynamic stability of proteins (21–28) have been unveiled by extensive experimental and computational enzyme engineering work. Engineered metal chelation is one such general approach for protein stabilization (22, 29–31). In this method, a bi-histidine (bi-His) motif, which consists of two histidines positioned to bind a bivalent metal ion (such as Ni^{2+}), can be easily engineered onto the surface of a protein. Due to preferential binding of the divalent metal ion to the native state over the denatured state, the protein can thus be stabilized (22). However, due to the difference between the thermodynamic and mechanical stability, strategies to enhance the thermodynamic stability of proteins cannot be readily used to enhance the mechanical stability.

Here, we carry out thermodynamic analysis to elucidate some general concepts on how to rationally improve the mechanical stability of proteins. As a proof of principle, we validate these concepts at the single molecule level in the small protein, GB1, by using engineered metal chelation. Our results demonstrate that engineered metal chelation is a general and effective approach to rationally enhance the mechanical stability of proteins in a fully reversible fashion. This general method in protein mechanics will enable the rational tuning of the mechanical stability of proteins, and we anticipate that it will find a wide range of applications in the engineering of diverse elastomeric proteins.

Results

Rationale for Enhancing the Mechanical Stability of Proteins. Thermodynamic stability is the free energy difference between the unfolded and folded state ($\Delta G_{U\text{-}N}$). In contrast, mechanical stability of proteins is determined by the mechanical unfolding energy barrier ($\Delta G_{\ddagger\text{-}N}$) and the distance between the native state and the mechanical unfolding transition state (Δx_u) (18, 19). To understand the general mechanism required to enhance the mechanical stability of proteins, we used the binding of metal ions as an example to carry out a simple thermodynamic cycle analysis for the mechanical unfolding reaction (Fig. 1*A*). Assuming that the unfolding distance does not change, the enhancement of the unfolding free energy barrier by metal ion binding ($\Delta\Delta G_{\ddagger\text{-}N}$) is equal to the difference in binding energy of the metal ion to the native state and the mechanical unfolding transition state ($\Delta G_{\text{bind}(\ddagger)} - \Delta G_{\text{bind}(N)}$). Therefore, if the metal ions bind more preferentially to the native state than the transition state, the native state will be preferentially stabilized by the binding of metal ions and thus, the unfolding energy barrier will be increased. In this way, the enhancement of the mechanical stability of the protein can be achieved. If the metal ion

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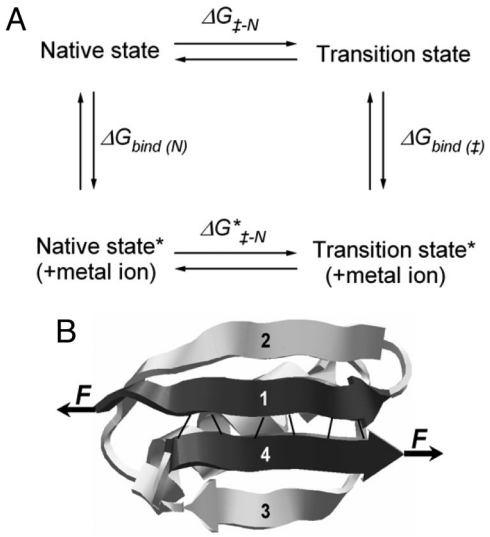

Fig. 1. Rationale for enhancing the mechanical stability of proteins. (*A*) Thermodynamic cycle analysis showing enhancement of the mechanical stability of a protein by preferential binding of a metal ion to the native state. The asterisks denote the protein in the metal ion-bound state (both native state and unfolding transition state). $\Delta\Delta G_{\ddagger\text{-}N}$ is defined as the change in the mechanical unfolding energy barrier caused by metal chelation and equals $\Delta G^*_{\ddagger\text{-}N} - \Delta G_{\ddagger\text{-}N}$. $\Delta\Delta G_{\text{bind}}$ is defined as $\Delta G_{\text{bind}(\ddagger)} - \Delta G_{\text{bind}(N)}$, where ΔG_{bind} is the Gibbs free energy for the binding reaction. Thermodynamic cycle analysis shows that $\Delta\Delta G_{\ddagger\text{-}N} = \Delta\Delta G_{\text{bind}}$. Therefore, the difference in mechanical unfolding free energy barrier upon chelation of metal ions is equal to the binding free energy difference between the mechanical unfolding transition state and the native state. (*B*) Mechanical topology of GB1. The two force-bearing β strands of GB1 (colored in dark gray) are the key region for the mechanical stability of GB1, as the rupture of the backbone hydrogen bonds (indicated by lines) connecting these two β strands are predicted to be the mechanical unfolding barrier for GB1. During the mechanical unfolding of GB1, there is slight sliding movement between the two force-bearing β strands.

stabilizes the transition state to the same degree as it does for the native state, the unfolding energy barrier will not change, and there will be no enhancement for the mechanical stability, although the thermodynamic stability of the protein will be enhanced. From this analysis, it becomes evident that enhancing the mechanical stability of a protein will be more demanding than enhancing thermodynamic stability, as the former involves not only stabilization of the native state, but also the unfolding transition state, which is difficult to study. Therefore, to enhance the mechanical stability of proteins, one will need to preferentially stabilize the native state over the mechanical unfolding transition state. For metal chelation, we will need to engineer a metal chelation site that will become somewhat disrupted in the mechanical unfolding transition state to achieve enhanced mechanical stability. As a proof of principle, we use the well-characterized small protein, GB1, as a model system to demonstrate the feasibility of realizing these general ideas.

GB1 is a small α/β protein that is composed of a β sheet packed against an α helix (32) (Fig. 1*B*). Its mechanical unfolding has been well characterized by single molecule AFM (33, 34). Molecular dynamics simulation predicts that the main mechanical unfolding event corresponds to the rupture of the backbone hydrogen bonds between the force-bearing β strands 1 and 4 (35, 36). In the mechanical unfolding transition state, the force-bearing strands 1 and 4 slide slightly against each other. Therefore, if we engineer a metal chelation site across the two force-bearing β strands, the slight sliding of β strand 1 against 4 may distort the metal chelation site in the mechanical unfolding transition state and result in the preferential binding of metal ions to the native state over the transition state, thus enhancing the unfolding energy barrier for GB1. This reasoning led us to engineer bi-His metal chelation sites into GB1 that are situated across the force-bearing β strands 1 and 4 and positioned to bind bivalent metal ions.

Binding of Metal Ions Significantly Enhances the Mechanical Stability of GB1 Bi-His Mutants. Using site-directed mutagenesis, we mutated residues 6 and 53 of GB1 to histidine to obtain the bi-His mutant, G6–53 (Fig. 2*A*). Equilibrium chemical denaturation studies showed that the presence of Ni^{2+} increased the thermodynamic stability of G6–53 in a Ni^{2+} concentration-dependent fashion, confirming the metal chelation capability of G6–53 [supporting information (SI) Fig. S1]. We then constructed polyprotein $(G6–53)_8$, which was composed of eight identical tandem repeats of G6–53, and used single molecule AFM to examine its mechanical stability. Stretching polyprotein $(G6–53)_8$ in the absence of metal ions resulted in force-extension curves with a characteristic sawtooth pattern, in which each individual sawtooth peak corresponded to the mechanical unfolding of the individual G6–53 domains in the polyprotein (Fig. 2*B*, black curve). The unfolding force peaks were equally spaced with a contour length increment (ΔL_c) of ~18.0 nm, as measured by fitting the Worm-Like Chain (WLC) model of polymer elasticity (37) to consecutive unfolding force peaks. The average unfolding force of G6–53 in the absence of metal ions was 119 ± 29 pN (average ± SD, n = 1,927) at a pulling speed of 400 nm/s (Fig. 2*C*, black histogram), which is lower than the unfolding force of WT GB1 (184 ± 41 pN) (34), indicating that the introduction of the bi-His site into the force-bearing region of GB1 destabilizes GB1 mechanically.

Stretching $(G6–53)_8$ in the presence of 4 mM Ni^{2+} resulted in sawtooth-like force-extension curves as the one shown in Fig. 2*B* (gray curve). The unfolding force peaks for G6–53 in the presence of 4 mM Ni^{2+} were equally spaced with a ΔL_c of ≈18 nm, which is identical to that for G6–53 in the absence of Ni^{2+}, suggesting that the unfolding force peaks indeed correspond to the mechanical unfolding of G6–53. However, the unfolding of G6–53 in the presence of 4 mM Ni^{2+} occurred at much elevated forces. The average unfolding force of G6–53 in 4 mM Ni^{2+} was 243 ± 49 pN (n = 2,226) (Fig. 2*C*, gray histogram), which is more than double that for G6–53 in the absence of Ni^{2+}. This unfolding force (≈243 pN) was also significantly higher than that for WT GB1 (≈180 pN). These results clearly indicate that the binding of Ni^{2+} to the engineered bi-His metal chelation site in GB1 significantly enhances the mechanical stability of G6–53, just as we predicted.

The Enhancement of Mechanical Stability by Metal Ion Binding Is Fully Reversible. The enhancement of mechanical stability by the binding of metal ions is fully reversible. Upon the addition of the Ni^{2+} competitive-binding agent imidazole to solution, the mechanical stability of the bi-His mutant, G6–53, was fully reversed. Fig. 3 shows an example of such experiments. The binding of 4 mM Ni^{2+} to G6–53 increased the mechanical unfolding forces of G6–53 from 110 pN to ≈240 pN, as evidenced by the shift of the unfolding force seen in the histogram in Fig. 3. Upon addition of 300 mM imidazole, imidazole will compete with histidine residues in GB1 to bind Ni^{2+}. This competitive binding will result in the dissociation of Ni^{2+} ions from G6–53. Accordingly, the unfolding forces of G6–53 were observed to drop back to ≈110 pN. This process is fully reversible and provides the possibility that the mechanical stability of G6–53 can be tuned using environmental stimuli in a fully reversible fashion.

The Enhancement in Mechanical Stability by Binding of Metal Ions Is Context Dependent. Having demonstrated that the binding of metal ions to engineered metal chelation sites can significantly enhance the mechanical stability of GB1, we investigated the influence of the location of metal chelation site on the mechanical stabilization effect. For this purpose, we engineered the bi-His mutants, G4–51 (Fig. 2*D*) and G8–55 (Fig. 2*G*), and their corresponding polyproteins, $(G4–51)_8$ and $(G8–55)_8$. The metal chelation properties of

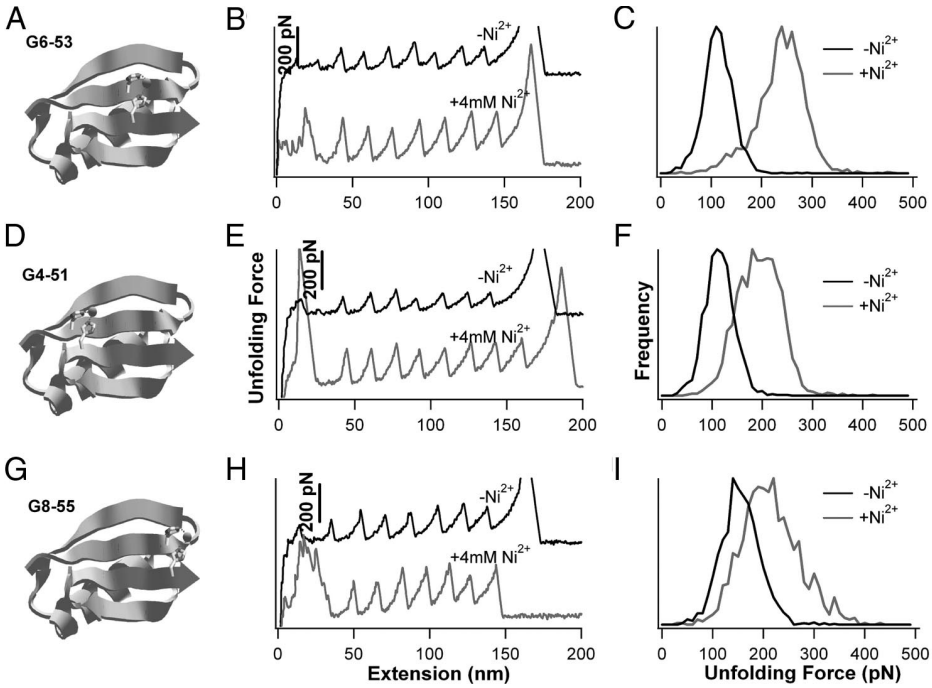

Fig. 2. The mechanical stability of GB1 bi-His mutants is enhanced by the binding of Ni^{2+}. (*A*, *D*, and *G*) Engineered bi-His metal chelation sites in GB1. The engineered bi-His sites are situated across the two force-bearing strands 1 and 4, and the binding of metal ions to the bi-His site will introduce a cross-strand bridge over the two force-bearing strands. (*B*, *E*, and *H*) Typical force-extension curves for the GB1 bi-His mutants G6–53, G4–51, and G8–55 in the absence of metal ions (black curves) and in the presence of 4 mM Ni^{2+} (gray curves). The mechanical unfolding forces of bi-His mutants increased dramatically upon binding of Ni^{2+} as compared with those in the absence of Ni^{2+}. (*C*, *F*, and *I*) Unfolding force histograms for the GB1 bi-His mutants G6–53, G4–51, and G8–55 in the absence (in black) and presence (in gray) of 4 mM Ni^{2+}, respectively. It is evident that, upon binding of Ni^{2+}, the mechanical unfolding force of bi-His mutants shifted toward higher forces, indicating that the mechanical stability of bi-His mutants are enhanced significantly by the binding of Ni^{2+}. The average unfolding forces are 119 pN (n = 1,927), 120 pN (n = 1,345), and 160 pN (n = 1,637) for G6–53, G4–51, and G8–55 in the absence of Ni^{2+}, respectively. In contrast, the average unfolding forces are 243 pN (n = 2,226), 198 pN (n = 1,609), and 219 pN (n = 1,098) for G6–53, G4–51, and G8–55 in the presence of 4 mM Ni^{2+}, respectively.

these bi-His mutants were confirmed by the observed increase in their thermodynamic stability upon binding of Ni^{2+} in chemical denaturation studies (Fig. S1). The thermal stabilization effect of bi-His mutants upon metal chelation depended upon the location of metal chelation site, as evidenced by differences in the increase in thermodynamic stability among the different bi-His mutants (Fig. S1, and Table S1). Using single molecule AFM, we measured the unfolding forces of G4–51 and G8–55 in the absence and presence of Ni^{2+}. As shown in Fig. 2 *E* and *F*, the introduction of the bi-His site in G4–51 resulted in a mechanical destabilization effect on GB1, and the mechanical unfolding of G4–51 occurred at ≈110 pN in the absence of metal ions. In contrast, the mechanical destabilization effect induced by the bi-His site was much milder in G8–55 than in G4–51 or G6–53, and the average unfolding force of G8–55 was about 160 pN in the absence of metal ions, only ~20 pN lower than that for WT GB1 (Fig. 2 *H* and *I*). It is interesting to note that, despite the general difference in the mechanical and thermodynamic stability of proteins, the mechanical unfolding force of bi-His mutants in the absence of Ni^{2+} correlates well with their thermodynamic stability (Table S1). The molecular origin of such a "coincidental" correlation remains to be examined.

Despite the mechanical destabilization effect of bi-His mutations, the binding of Ni^{2+} significantly enhanced the mechanical stability of both G4–51 and G8–55 (Fig. 2 *E* and *H*), and the mechanical unfolding of G4–51 and G8–55 in the presence of 4 mM Ni^{2+} occurred at ≈200 pN and ≈210 pN (Fig. 2 *F* and *I*), respectively.

It is evident that engineering a bi-His metal chelation site in the force-bearing region of GB1 offers an efficient approach to enhance the mechanical stability of GB1 through the binding of divalent metal ions. However, the amplitude of mechanical stability enhancement is not the same but depends on the context in which the metal chelation site is engineered. The metal chelation site in the very center of the force-bearing region (Site 6–53) had the

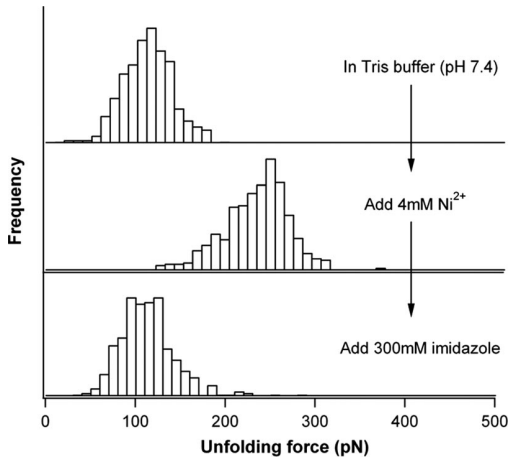

Fig. 3. The mechanical stability of G6–53 can be regulated reversibly by the binding of Ni^{2+} ions or its competitive binding reagent, imidazole. The unfolding force histogram for G6–53 is centered at ≈110 pN and was obtained in Tris buffer (10 mM, pH 7.4) (*Top*, n = 476). After addition of 4 mM Ni^{2+}, the unfolding force shifted toward a higher force with an average unfolding force of ≈250 pN (*Middle*, n = 567). Upon addition of 300 mM imidazole to the solution, the unfolding force shifted back to lower unfolding forces (*Bottom*, n = 429), generating a histogram that is indistinguishable from the unfolding force histogram obtained in the absence of Ni^{2+}. All these three unfolding force histograms were obtained from the same experiment.

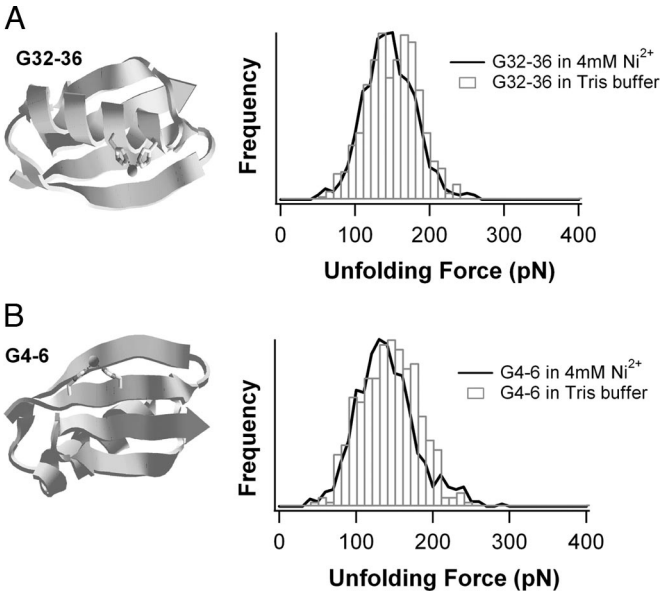

Fig. 4. Introduction of a metal chelation site outside the force-bearing region does not enhance the mechanical stability of the bi-His mutants G32–36 (*A*) and G4–6 (*B*), in which a metal chelation site was engineered into the α helix or the first β strand, respectively. The unfolding force histograms for the Ni^{2+}-bound (in black) and unbound forms (in gray) of bi-His mutants are indistinguishable for both G32–36 and G4–6. It is of note that, in comparison with WT GB1, the bi-His mutants G32–36 and G4–6 exhibit changes in mechanical stability due to the double histidine mutations. The unfolding force is 149 ± 37 pN (n = 832) and 142 ± 37 pN (n = 341) for G32–36 and G4–6, respectively.

strongest stabilization effect, while the site at the periphery of the force-bearing region had a weaker effect. It is of interest to note that a similar trend was observed on the relative increase in thermodynamic stability for the three bi-His mutants (Table S1).

Metal Chelation Sites Engineered Outside the Force-Bearing Region Do Not Affect the Mechanical Stability of GB1. As rationalized in the thermodynamic cycle analysis, preferential binding of metal ions to the metal chelation site in the native state over the transition state is the key to realizing the enhancement of mechanical stability. If the metal chelation provides similar stabilization to the mechanical unfolding transition state as it does to the native state, the unfolding free energy barrier will not change, and no net enhancement of mechanical stability will be achieved. To further validate this rationale, we engineered the control bi-His mutants, G32–36 and G4–6, in which a metal chelation site was engineered in the α-helix and the first β strand of GB1 (38), respectively. The metal chelation properties of G32–36 and G4–6 were confirmed by the increase in their thermodynamic stability in the presence of Ni^{2+} (Fig. S1). Since the α-helix is well within the core of GB1, it will not experience the mechanical stretching force until GB1 has completely unfolded. Therefore, the stretching force will not affect the binding affinity of the metal chelation site for metal ions in the mechanical unfolding transition state of G32–36. Fig. 4*A* shows the unfolding force histograms for G32–36 in the absence and presence of Ni^{2+}. As predicted, the binding of Ni^{2+} to G32–36 does not have any effect on the mechanical unfolding forces of G32–36, despite its clear effect in enhancing the thermodynamic stability of G32–36. Similarly, the binding of metal ions to the bi-His mutant G4–6 does not have any effect on its mechanical stability. These results clearly demonstrate that distorting the metal chelation site in the mechanical unfolding transition state is key to realizing the preferential binding of metal ions to the native state and the enhancement of mechanical stability.

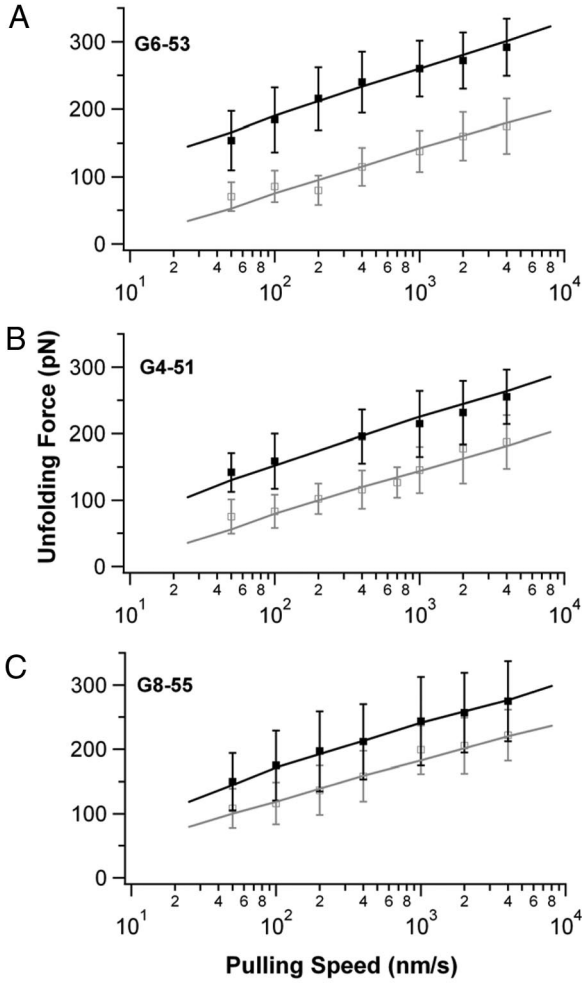

Fig. 5. The speed dependence of the mechanical unfolding forces of bi-His mutants in the absence and presence of 4 mM Ni^{2+}. The unfolding force of G6–53, G4–51, and G8–55 were measured at different pulling speeds in the absence (gray) and in the presence (black) of 4 mM Ni^{2+}. The solid lines correspond to Monte Carlo simulation fits to the experimental data using the parameters shown in Table 1. It is evident that the chelation of Ni^{2+} does not significantly change the slope of the speed dependence of unfolding forces of bi-His mutants. In contrast, the spontaneous unfolding rate constant, α_0, deceased by ≈2- to 20-fold upon binding of Ni^{2+}, indicating that the enhancement of mechanical stability by the binding of Ni^{2+} largely results from the increase in the mechanical unfolding free energy barrier.

Enhancing Mechanical Stability by Increasing the Free Energy Barrier. To confirm the mechanism for enhancing the mechanical stability of GB1, we needed to quantify the change in the unfolding free energy barrier upon the binding of Ni^{2+}. Toward this goal, we carried out single molecule AFM stretching experiments on $(G4–51)_8$, $(G6–53)_8$, and $(G8–55)_8$ at different pulling speeds. As shown in Fig. 5, the unfolding forces of bi-His mutants depend upon pulling speeds; the faster the pulling speed is, the greater the unfolding force. Using standard Monte Carlo simulation procedures (39, 40), we reproduced the force-extension curves of these three polyproteins. By fitting the unfolding force distributions and their dependence on pulling speeds (Fig. 5) simultaneously, we then estimated the mechanical unfolding rate constant at zero force (α_0) and the distance between the native state and transition state (Δx_u). We found that a Δx_u of 0.17 nm can describe the mechanical unfolding well for Ni^{2+}-bound bi-His GB1 mutants, while Δx_u of 0.20 nm is a good descriptor for the Ni^{2+}-free form of bi-His mutants. This result is consistent with the observation that the

Table 1. The summary of unfolding force, unfolding distance (Δx_u), and spontaneous unfolding rate constant α_0 at zero force for bi-His mutants G4–51, G6–53, and G8–55

	G4–51		G6–53		G8–55		
	$-Ni^{2+}$	$+Ni^{2+}$	$-Ni^{2+}$	$+Ni^{2+}$	$-Ni^{2+}$	$+Ni^{2+}$	wt GB1
Unfolding force (±SD), pN	120 ± 29	198 ± 43	119 ± 29	243 ± 49	160 ± 38	219 ± 57	184 ± 41
Δx_u, *nm*	0.20	0.17	0.20	0.17	0.20	0.17	0.17
α_0, s^{-1}	0.12	0.023	0.14	0.0071	0.029	0.014	0.039
$\Delta\Delta G_{\ddagger\text{-}N}$, kCal/mol ($\Delta\Delta RTln\alpha_0$)	0.99		1.79		0.46		—

unfolding force distributions for Ni^{2+}-bound bi-His mutants are somewhat broader than those for Ni^{2+}-free bi-His mutants (Fig. 2 *C*, *F*, and *I*). The measured α_0 and unfolding distance, Δx_u, for bi-His mutants are tabulated in Table 1, together with those for WT GB1. The increase in the mechanical unfolding free energy barrier ($\Delta\Delta G_{\ddagger\text{-}N}$) by metal chelation is equal to $RTln[\alpha_0(Ni^{2+}\text{-}bound)/\alpha_0(Ni^{2+}\text{-}free)]$, where R is the gas constant and T is temperature. Therefore, it can be calculated that $\Delta\Delta G_{\ddagger\text{-}N}$ ranges from 0.4 kCal/mol to 1.8 kCal/mol for bi-His mutants, indicating that the binding of Ni^{2+} stabilizes the native state more than the mechanical unfolding transition state, the very principle underlying the use of metal chelation to enhance mechanical stability. Of note, the binding of Ni^{2+} to the metal chelation site not only increased the mechanical unfolding energy barrier, but also reduced the unfolding distance, Δx_u. Both factors contributed to the enhancement of the mechanical stability. It seems that introduction of the bi-His site into GB1 leads to an increase in Δx_u from 0.17 nm for WT GB1 to 0.20 nm for bi-His mutants, but the binding of Ni^{2+} to the bi-His site brings Δx_u back to 0.17 nm. The underlying detailed molecular mechanism remains unclear.

Discussion

Tuning the mechanical stability of elastomeric proteins, especially enhancing the mechanical stability, has been a challenge in protein mechanics (11). Differing from thermodynamic stability (that is, the free energy difference between the unfolded state and native state), mechanical stability is "kinetic stability", in that it is directly related to the free energy difference between the native state and the mechanical unfolding transition state (20). Due to the involvement of the difficult-to-study mechanical unfolding transition state, it has not been possible to develop general and rational approaches toward enhancing the mechanical stability of proteins. Although there are many successful approaches in enzyme engineering to enhance the thermodynamic stability of proteins, such approaches cannot be directly applied to enhancing the mechanical stability of proteins. Engineered metal chelation, a robust approach to improve the thermodynamic stability of proteins, is such an example (22, 38).

Here, we have demonstrated a rational approach to enhance the mechanical stability of proteins via engineered metal chelation. Through a simple thermodynamic cycle analysis for the mechanical unfolding reaction, we discovered that the key to enhance the mechanical stability of proteins is the preferential stabilization of the native state over the mechanical unfolding transition state. Therefore, by engineering metal chelation bi-His sites across two force-bearing β strands, we successfully enhanced the mechanical stability of GB1 in a fully reversible fashion. The net mechanical stabilization achieved by metal chelation in bi-His mutants of GB1 was substantial and ranged from 60 pN to more than 120 pN. Such enhancement of mechanical stability is likely due to the distortion/disruption of the metal chelation site in the mechanical unfolding transition state by the stretching force, leading to the preferential stabilization of the native state over the transition state by the binding of divalent metal ions. The extreme case would be that the Ni^{2+} and bi-His coordination bond, resulting from the binding of Ni^{2+} to the bi-His metal chelation site, is fully ruptured in the mechanical unfolding transition state. In this case, the thermodynamic stabilization of the native state by metal binding can be fully converted into the increased mechanical unfolding energy barrier, leading to the maximum enhancement of the mechanical stability of proteins. Therefore, the larger the thermal stabilization effect is upon metal chelation, the larger the mechanical stabilization effect that can be potentially achieved. However, it is important to note that a larger thermodynamic stabilization effect only provides the possibility for achieving a larger mechanical stabilization effect. The actual amplitude of mechanical stabilization depends on the degree of preferential stabilization of the native state over the transition state.

In addition, it seems that metal chelation in the bi-His site across the force-bearing strands also helps to consolidate the force-bearing region and limit the shear-sliding movement of the two force-bearing strands. This effect is exemplified by the decrease in the mechanical unfolding distance, Δx_u, of the bi-His mutant upon the binding of metal ions.

Previous single molecule AFM studies have revealed that ligand binding (14) and protein-protein interactions (15, 41) can enhance the mechanical stability of some proteins. These methods are restricted to particular proteins that have unique ligand-binding properties and thus, cannot be easily generalized to other protein systems. In contrast, the bi-His based metal chelation site can be easily engineered into a wide range of proteins with little or no disruption of the native state and has been widely used in traditional enzyme engineering (38) as well as in protein folding studies (the so-called Ψ-value analysis) (42, 43). Therefore, the method of engineered metal chelation is not an approach unique to particular proteins. Instead, it can be used in a wide range of proteins and therefore represents a general approach in protein mechanics to rationally enhance the mechanical stability of elastomeric proteins. Moreover, different divalent metal ions, such as Cu^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}, exhibit different binding affinities to bi-His sites (44) and thus, may enable additional control over the enhancement of mechanical stability. Our preliminary data have shown that different metal ions can indeed lead to differential enhancement of mechanical stability in GB1.

The general ideas illustrated herein are not limited to metal chelation but constitute rather general principles underlying the enhancement of the mechanical stability of proteins. A wide variety of methodologies have been developed to improve the thermodynamic stability of proteins (21–28), and these methods are also potential routes that one can use to enhance the mechanical stability of proteins, as long as one can find effective ways to selectively stabilize the native state over transition state. The insights provided here will not only open up new avenues toward regulating the mechanical properties of elastomeric proteins in their biological settings, but also make it feasible to tailor the mechanical properties of elastomeric proteins for applications in bioengineering and material sciences.

Furthermore, the method of engineered metal chelation demonstrated here also has important implications in elucidating the structures of the unfolding transition state along its mechanical unfolding pathways. Metal chelation has been used to probe the structure of chemical folding/unfolding transition states in the so-called Ψ-value analysis (42, 43). Similarly, by engineering metal

chelation sites in different regions of the protein of interest, it is now feasible to use single molecule AFM to probe their effects on mechanical stability and deduce important information about the role of the mutated sites in the formation of the mechanical unfolding transition state. These studies will not only make it possible to map the mechanical unfolding transition state of proteins, just as the Ψ-value analysis does for classical protein folding/unfolding dynamics, but also provide an opportunity to directly compare the mechanical and chemical unfolding pathways and understand the differences between them.

Materials and Methods

Protein Engineering. Plasmids that encode WT GB1 were generously provided by Prof. David Baker of University of Washington. All of the bi-His mutants were constructed using the mega primer method with a sense primer comprising one His mutation and an anti-sense primer comprising the other His mutation. The gene sequences of all bi-His mutants were confirmed by direct DNA sequencing. All of the polyprotein genes were constructed as described previously (33, 34). The polyproteins were expressed in the DH5α strain, purified by Co^{2+} affinity chromatography, and eluted in PBS buffer with 300 mM NaCl and 150 mM imidazole. EDTA (20 mM) was added to the elution fractions to remove residual Co^{2+} that may exist in the elution fraction. The proteins were further dialyzed against Tris·HCl buffer (10 mM, pH 7.4, containing 100 mM NaCl) to completely remove EDTA and imidazole.

Single Molecule AFM Experiment. Single molecule AFM experiments were carried out on a custom-built AFM as described previously (33, 34). All of the force-extension measurements were carried out either in Tris·HCl buffer (10 mM, pH 7.4, containing 100 mM NaCl) or Tris·HCl plus 4 mM $NiCl_2$. The spring constant of AFM cantilevers (Si_3N_4 cantilevers from Veeco) was calibrated using the equipatition theorem before each experiment and typically had a value of 60 pN nm^{-1}. For Ni^{2+}-binding studies, we first deposited polyprotein and Ni^{2+} solution onto a glass coverslip containing 50 μl of Tris·HCl buffer and mixed them *in situ*. The AFM experiments were carried out after allowing the mixture to equilibrate for $\approx$30 min. The pulling speed was 400 nms^{-1} for all experiments, unless otherwise indicated.

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