Modulating the Mechanical Stability of Extracellular Matrix Protein Tenascin-C in a Controlled and Reversible Fashion

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Received 10 March 2009; received in revised form 14 May 2009; accepted 19 May 2009
Available online 27 May 2009

Stretching force can induce conformational changes of proteins and is believed to be an important biological signal in the mechanotransduction network. Tenascin-C is a large extracellular matrix protein and is subject to stretching force under its physiological condition. Regulating the mechanical properties of the fibronectin type III domains of tenascin-C will alter its response to mechanical stretching force and thus may provide the possibility of regulating the biological activities of tenascin-C in living cells. However, tuning the mechanical stability of proteins in a rational and systematic fashion remains challenging. Using the third fibronectin type III domain (TNfn3) of tenascin-C as a model system, here we report a successful engineering of a mechanically stronger extracellular matrix protein via engineered metal chelation. Combining steered molecular dynamics simulations, protein engineering and single-molecule atomic force microscopy, we have rationally engineered a bihistidine-based metal chelation site into TNfn3. We used its metal chelation capability to selectively increase the unfolding energy barrier for the rate-limiting step during the mechanical unfolding of TNfn3. The resultant TNfn3 mutant exhibits enhanced mechanical stability. Using a stronger metal chelator, one can convert TNfn3 back to a state of lower mechanical stability. This is the first step toward engineering extracellular matrix proteins with defined mechanical properties, which can be modulated reversibly by external stimuli, and will provide the possibility of using external stimuli to regulate the biological functions of extracellular matrix proteins.

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Keywords: mechanical unfolding; tenascin; single-molecule force spectroscopy; mechanical stability; rational design

Introduction

Stretching force is believed to be an important biological signal in the mechanotransduction network and can induce conformational changes of proteins, including force-induced unfolding, to regulate a broad range of cellular processes.1-5 Tenascin-C is a large extracellular matrix protein that plays important roles in regulating cell–matrix interactions.6 Tenascin-C is subject to significant stretching forces under physiological conditions and is mainly expressed in regions that are subject to heavy tensile load7 or in tissues that undergo extensive structural remodeling processes.8-10 The mechanical properties of tenascin-C have been investigated in detail with the use of single-molecule atomic force microscopy (AFM).11-13 It was revealed that the fibronectin type III (FnIII) domains of tenascin-C can unfold under a stretching force to extend the contour length of tenascin-C to several times its resting length when tenascin-C is fully folded. It was proposed that force-induced unfolding of FnIII domains of tenascin-C can effectively prolong the lifetime of a ligand–receptor bond mediated by tenascin-C.11 If one can rationally...
regulate the mechanical unfolding behaviors of FnIII domains by controlling their mechanical stability, it may become possible to regulate the response of tenascin-C to stretching force and thus to regulate the biological activities of the extracellular matrix proteins in vivo. As the first step, here we report our endeavor to engineer a mechanically stronger mutant of the third FnIII domain of tenascin-C (TNfn3), which can be modulated by metal ions between two mechanical states that exhibit distinct high and low mechanical stability.

The mechanical properties of TNfn3 have been studied in detail with the use of single-molecule AFM and steered molecular dynamics (SMD) simulations. These studies revealed that TNfn3 unfolds in a complex fashion involving multiple intermediate states and several energy barriers. Such complex unfolding behaviors are in sharp contrast with the simple two-state unfolding behaviors exhibited by many proteins, such as ubiquitin, protein L, and protein G, and represent a technical challenge for rational modulation of the mechanical stability of TNfn3.

Mechanical unfolding force is a measure of the mechanical stability of a given protein and is directly related to the height of the mechanical unfolding energy barrier and the distance to the transition state. Increasing the mechanical unfolding energy barrier will generally lead to enhanced mechanical stability. However, tuning the mechanical stability of proteins in a rational and systematic fashion remains challenging despite the significant progress in the field of single protein mechanics and engineering over the last decade. Only a few examples of successfully enhancing the mechanical stability of proteins were reported on a few isolated cases. Recently, using a small nonmechanical protein, GB1, we have developed an engineered metal-chelation-based method to rationally enhance the mechanical stability of proteins. The key to the use of biHis-based metal chelation approach to enhance the mechanical stability of proteins is to preferentially stabilize the native state over the mechanical unfolding transition state. For proteins that are simple two-state unfolders, such as GB1, the design of a metal chelation site to realize preferential stabilization of the native state seems relatively straightforward. However, for proteins such as TNfn3 that involve complex unfolding behaviors, use of engineered metal chelation to enhance their mechanical stability is much more challenging because it requires selective and preferential stabilization of a particular intermediate state over the transition state during the rate-limiting step.

The TNfn3 domain has a typical β-sandwich structure and contains an integrin-binding Arg-Gly-Asp (RGD) loop. The mechanical unfolding of TNfn3 and its structural homologous FnIII domains from fibronectin have been investigated in detail using single-molecule AFM and SMD simulations. Although the sequence homology between TNfn3 and its homologous FnIII domains from fibronectin is low, SMD simulations predicted that the mechanical unfolding of both FnIII domains follows similar molecular events. Figure 1 shows our constant force SMD simulation results on both TNfn3 and the 10th FnIII domain from fibronectin (FNfn10). Similar to previous SMD simulation results, our SMD simulations showed that the native state of both FNfn10 and TNfn3 domains are not stable upon stretching. When subjected to stretching force, both domains enter into a stable intermediate state, I1 (referred to as twist intermediate state I1), in which the tertiary structure of both domains remains largely intact, by slightly straightening the disordered N-terminal end of both domains. After this pre-elongation event, the subsequent mechanical unfolding of TNfn3 and FNfn10 is characterized by two distinct barriers: the first barrier corresponds to the transition from the twist intermediate state I1 to an aligned intermediate state I2, and the second barrier corresponds to the transition from the aligned intermediate state I2 to a partially unfolded intermediate I3. Both SMD simulations and single-molecule AFM experiments suggest that barrier I (the transition from I1 to I2) is the rate-limiting step, and the mechanical unfolding force peaks observed in single-molecule AFM experiments on TNfn3 and FNfn10 correspond to the barrier crossing in this rate-limiting step. During this transition, the N-terminal end of strand A is straightened by breaking key backbone hydrogen bonds between strands A and B and the two β-sheets rotate and align relative to each other. For FNfn10, the breaking of the backbone hydrogen bonds between residues 6 and 23 was observed to be a key event coinciding with the transition from I1 to I2 for FNfn10. In contrast, the backbone hydrogen bonds between Ser6 and Phe23 in TNfn3 are unstable. They break and reform during the intermediate state I1 (as evidenced by the fluctuation in interaction energy between Ser6Phe23 in Fig. 1d) and break before crossing barrier I in most trajectories. Therefore, the interactions between Ser6 and Phe23 do not make a significant contribution to

Results

Rational design of the engineered metal chelation site in TNfn3

The key to the use of biHis-based metal chelation approach to enhance the mechanical stability of proteins is to preferentially stabilize the native state over the mechanical unfolding transition state. For proteins that are simple two-state unfolders, such as GB1, the design of a metal chelation site to realize preferential stabilization of the native state seems relatively straightforward. However, for proteins such as TNfn3 that involve complex unfolding behaviors, use of engineered metal chelation to enhance their mechanical stability is much more challenging because it requires selective and preferential stabilization of a particular intermediate state over the transition state during the rate-limiting step. The TNfn3 domain has a typical β-sandwich structure and contains an integrin-binding Arg-Gly-Asp (RGD) loop. The mechanical unfolding of TNfn3 and its structural homologous FnIII domains from fibronectin have been investigated in detail using single-molecule AFM and SMD simulations. Although the sequence homology between TNfn3 and its homologous FnIII domains from fibronectin is low, SMD simulations predicted that the mechanical unfolding of both FnIII domains follows similar molecular events. Figure 1 shows our constant force SMD simulation results on both TNfn3 and the 10th FnIII domain from fibronectin (FNfn10). Similar to previous SMD simulation results, our SMD simulations showed that the native state of both FNfn10 and TNfn3 domains are not stable upon stretching. When subjected to stretching force, both domains enter into a stable intermediate state, I1 (referred to as twist intermediate state I1), in which the tertiary structure of both domains remains largely intact, by slightly straightening the disordered N-terminal end of both domains. After this pre-elongation event, the subsequent mechanical unfolding of TNfn3 and FNfn10 is characterized by two distinct barriers: the first barrier corresponds to the transition from the twist intermediate state I1 to an aligned intermediate state I2, and the second barrier corresponds to the transition from the aligned intermediate state I2 to a partially unfolded intermediate I3. Both SMD simulations and single-molecule AFM experiments suggest that barrier I (the transition from I1 to I2) is the rate-limiting step, and the mechanical unfolding force peaks observed in single-molecule AFM experiments on TNfn3 and FNfn10 correspond to the barrier crossing in this rate-limiting step. During this transition, the N-terminal end of strand A is straightened by breaking key backbone hydrogen bonds between strands A and B and the two β-sheets rotate and align relative to each other. For FNfn10, the breaking of the backbone hydrogen bonds between residues 6 and 23 was observed to be a key event coinciding with the transition from I1 to I2 for FNfn10. In contrast, the backbone hydrogen bonds between Ser6 and Phe23 in TNfn3 are unstable. They break and reform during the intermediate state I1 (as evidenced by the fluctuation in interaction energy between Ser6Phe23 in Fig. 1d) and break before crossing barrier I in most trajectories. Therefore, the interactions between Ser6 and Phe23 do not make a significant contribution to
the mechanical resistance for TNfn3. This finding has been confirmed by our single-molecule AFM experiments on a proline mutant of TNfn3, S6P, in which mutation S6P does not result in a noticeable change in the mechanical unfolding force and mechanical unfolding kinetics of TNfn3.

It is clear that breaking the interactions between residues 6 and 23 can occur concurrently with (for FNfn10) or before (for TNfn3) the rate-limiting β-sheet-aligning process. Therefore, we reason that if we can strengthen the interactions between Ser6 and Phe23 of TNfn3 in the native state and twist state I1 to delay their breaking to coincide with the β-sheet-aligning process, the seemingly unimportant interactions between residues 6 and 23 may be incorporated as part of the unfolding energy barrier. In this way, we should be able to increase the energy barrier for the rate-limiting step for the mechanical unfolding of TNfn3 and hence increase its mechanical stability.

Since the backbone hydrogen bonds between Ser6 and Phe23 are unstable, we attempt to mutate both residues 6 and 23 to histidines so that the binding of bivalent metal ions, such as Ni$^{2+}$, to the engineered biHis metal chelation site can enhance the interactions between residues 6 and 23. The relative orientation of both residues in the three-dimensional structure of TNfn3 makes the engineering of such a metal chelation site plausible.

Metal ion Ni$^{2+}$ can bind to biHis6–23 and increase its thermodynamic stability

We engineered a biHis mutant of TNfn3, biHis6–23, and used equilibrium chemical denaturation to examine its metal chelation capability. Figure 2a shows the equilibrium chemical denaturation curves of biHis6–23 in the absence and presence of Ni$^{2+}$. It is evident that upon addition of 14.3 mM Ni$^{2+}$, the equilibrium denaturation curve shifts toward higher guanidinium chloride (GdmCl) concentration. The increase in [GdmCl]$_{0.5}$, at which 50% of the protein is unfolded, suggested that the thermodynamic stability of mutant biHis6–23 increases upon the binding of Ni$^{2+}$. The shift in [GdmCl]$_{0.5}$ (2.41 M for biHis6–23 in the absence of Ni$^{2+}$ versus 2.80 M in the presence of 14.3 M Ni$^{2+}$, respectively) corresponds to an increase of 0.88 kcal/mol in thermodynamic

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**Fig. 1.** Mechanical unfolding pathways of FNfn10 and TNfn3 predicted by SMD simulations. (a and c) SMD simulations show that mechanical unfolding of FNfn10 (a) and of TNfn3 (c) follow similar molecular events and exhibit two dominant barriers: barrier I is for the transition from twist intermediate state I1 to aligned state I2, and barrier II is for the transition from aligned state I2 to partially unfolded intermediate state I3. Crossing barrier I is the rate-limiting step. (b and d) Extension–time plots (top) and the interaction energy–time plots (bottom) for the mechanical unfolding of FNfn10 (b) and TNfn3 (d). The rupture of backbone hydrogen bonds between residues 6 and 23 is a necessary step for crossing barrier I. The rupture of hydrogen bond Arg$^{6\text{HN}}$–Asp$^{23\text{O}}$ of FNfn10 coincides with crossing barrier I, while the hydrogen bond Ser$^{6\text{HN}}$–Phe$^{23\text{O}}$ in TNfn3 is unstable and breaks and reforms in intermediate I1, as evidenced by its energy fluctuation (d).
stability for biHis6–23 upon the addition of 14.3 mM Ni^{2+} (see Materials and Methods). This result confirms that the engineered biHis site at position 6–23 indeed has metal chelation capability. For convenience, biHis6–23 without binding of metal ions is referred to as the apo form of biHis6–23.

The binding of Ni^{2+} can enhance the mechanical stability of biHis6–23

Using single-molecule AFM, we constructed a polyprotein, (biHis6–23)_8, which is composed of eight identical tandem repeats of biHis6–23, to examine the mechanical stability of biHis6–23. The construction of a polyprotein is a necessary step in single-molecule AFM to measure the mechanical stability of proteins, as the unfolding of polyproteins provides unambiguous fingerprints in identifying single-molecule stretching and unfolding events.

Stretching polyprotein (biHis6–23)_8 in its apo form results in force–extension curves of characteristic sawtooth pattern appearance (Fig. 3a, black curve), in which the individual force peak, with the exception of the last one, corresponds to the mechanical unfolding of the individual biHis6–23 domains. The last peak corresponds to the detachment of the polyprotein chain from either the AFM tip or glass substrate. Fitting the force–extension curves using the worm-like-chain model of polymer elasticity measures the contour length increment \( \Delta L_c \) of \( \sim 30 \) nm upon the unfolding of biHis6–23, which is identical to that of wild-type (wt) TNfn3. The unfolding forces show a narrow distribution with an average value of 121 ± 14 pN at a pulling speed of 400 nm/s (average ± SD, \( n = 580 \), Fig. 3b), which is identical, within experimental error, to the unfolding force of wt TNfn3 (125 pN at the same pulling speed). To investigate whether metal chelation can enhance the mechanical stability of biHis6–23, we carried out force–extension measurements of biHis6–23 in the presence of 4 mM Ni^{2+}. As shown in Fig. 3a (gray curve), the stretching of polyprotein (biHis6–23)_8 in the presence of 4 mM Ni^{2+} results in force–extension curves of similar sawtooth appearance. Compared with that of apo biHis6–23, the unfolding events of Ni^{2+}-bound form of biHis6–23 exhibit similar contour length increment \( \Delta L_c \) of \( \sim 30 \) nm. However, the unfolding of Ni^{2+}-bound biHis6–23 occurs at much elevated forces as compared with that of apo form. The average unfolding force of biHis6–23 in the presence of 4 mM Ni^{2+} is \( 157 \pm 19 \) pN (\( n = 1132 \), Fig. 3b), which is a 36-pN increase compared with that of the apo form of biHis6–23 and a 32-pN increase over wt TNfn3. The amplitude of mechanical stability enhancement is significantly higher than the uncertainty of the unfolding force measurements, clearly demonstrating that the binding of metal ions to the biHis metal chelation site significantly enhances the mechanical stability of biHis6–23.

This enhancement effect on mechanical stability by metal chelation is intrinsically due to the binding of Ni^{2+} to the TNfn3 biHis mutant, and not to the nonspecific binding of Ni^{2+} to TNfn3. To confirm this point, we carried out a control experiment on wt TNfn3 polyprotein. As shown in Supplementary Fig. S1, the mechanical unfolding force of wt TNfn3 remained the same before and after the addition of Ni^{2+}, suggesting that nonspecific binding of Ni^{2+} does not alter the mechanical stability of TNfn3. Therefore, the mechanical stabilization effect is due to the specific binding of nickel ions to the engineered biHis site.

Binding and unbinding of Ni^{2+} ions enables the modulation of the mechanical stability of biHis6–23 in a reversible fashion

The enhanced mechanical stability of biHis6–23 is the result of the binding of Ni^{2+} ions to the protein. Hence, modulating the binding and unbinding of Ni^{2+} ions to biHis6–23 should provide a convenient
means to modulate the mechanical stability of biHis6–23 in a reversible fashion. It is well known that a strong metal chelator, such as EDTA (ethylenediaminetetraacetic acid), has much higher binding affinity to Ni\(^{2+}\) than to biHis-based binding sites; thus, we can use EDTA to induce the dissociation of Ni\(^{2+}\) from biHis6–23. As shown in Fig. 4, binding of Ni\(^{2+}\) results in an increase in mechanical stability for biHis6–23, from \(\sim 120\) to \(\sim 150\) pN (Fig. 4a and b).

After the addition of EDTA, the mechanical unfolding force of biHis6–23 reduces back to \(\sim 120\) pN (Fig. 4c). This modulation is fully reversible, allowing us to use environmental stimuli, in this case Ni\(^{2+}\) and EDTA, to selectively modulate the mechanical stability of biHis6–23 between its two distinct mechanical forms. Therefore, the biHis6–23 represents a novel elastic protein that is responsive to environmental stimuli.

The key to enhancement of mechanical stability is to selectively enhance the energy barrier for the rate-limiting step

The enhancement of the mechanical stability of biHis6–23 by metal chelation suggests that coordination bonds between Ni\(^{2+}\) ion and histidine residues 6 and 23 stabilize the interactions between residues 6 and 23, and the breaking of the coordination bond as well as the backbone hydrogen bonds between 6 and 23 is part of the rate-limiting step during the transition from twist intermediate state I\(_1\) to the aligned state I\(_2\). Thus, the seemingly unimportant interactions between residues 6 and 23 for wt TNfn3 have been engineered to make important contributions to the energy barrier for the rate-limiting step. After this step, the structures of the aligned intermediate state I\(_2\) should be the same for both wt TNfn3 and biHis6–23.

The key to the enhancement of mechanical stability is selectively enhancing the energy barrier for the rate-limiting step, e.g., preferential stabilization of the native state over the mechanical unfolding transition state. If the aligned intermediate state I\(_2\) is stabilized to the same degree as the twist intermediate state I\(_1\), the energy barrier for the rate-limiting step will not change. To confirm this reasoning, we engineered biHis mutant biHis71–85, in which residues 71 and 85 were mutated to histidines. The biHis metal chelation site 71–85 is located across FG\(_{\beta}\)-hairpin and its metal chelation capability is confirmed by equilibrium chemical denaturation studies (Fig. 2b). SMD simulation of the mechanical unfolding of TNfn3 showed that in the aligned state I\(_2\) after crossing barrier I, the structural change in FG\(_{\beta}\) strands is minimum and the backbone hydrogen bonds between \(\beta\) strands F and G remain intact. Therefore, the metal chelation should stabilize the mechanical unfolding transition.
state the same amount as it does on the native state. There should be no net change in the free-energy barrier for unfolding, and hence there should be no enhancement effect on the mechanical stability of TNfn3. Indeed, single-molecule AFM experiments on polyprotein (biHis71–85)$_8$ showed that the unfolding force of biHis71–85 remains the same before and after the addition of 4 mM Ni$^{2+}$: 110±13 pN ($n=356$) for the apo form of biHis6–23. All the three unfolding force histograms were obtained from the same experiment.

Here we showed that despite the complex nature of the mechanical unfolding pathway of TNfn3, it is possible to use engineered metal chelation to selectively increase the unfolding energy barrier for the rate-limiting step, thus demonstrating the robustness of this method in such a complex unfolding scenario. The key to this success is the detailed knowledge about the unfolding pathways of TNfn3 revealed by SMD simulations, revealing the power of the synergetic combination of engineered metal chelation approach with SMD simulations. We anticipate that such powerful combinations will enable the engineered metal chelation approach to be readily applied to modulate the mechanical properties of a broad range of naturally occurring elastomeric proteins, such as fibronectin, that are important for mechanotransduction.

Compared with other methods used to enhance the mechanical stability of proteins, engineered metal chelation is also efficient. By using bivalent metal ions and mutating only two key residues, we have increased the mechanical stability of TNfn3 by 36 pN, which is significantly higher than the increase of 20 pN achieved in a similar effort to enhance the mechanical stability of an FnIII domain by mutating as many as 15 residues.$^{14}$ More importantly, the modulation of the mechanical stability by metal chelation is not a permanent change, but rather fully reversible, providing the possibility of modulating the mechanical response of extracellular matrix proteins with the use of environmental stimuli in a fully reversible fashion.

**Residues 6 and 23 represent a fine regulator for the mechanical stability of TNfn3**

The rate-limiting step for the mechanical unfolding of TNfn3 is the transition from the twist state I1 to the aligned state I2. For wt TNfn3, interactions between residues 6 and 23 break before the β-sheet-aligning process and thus do not contribute to the unfolding energy barrier. For Ni$^{2+}$-bound biHis6–23, breaking the interactions between residues 6 and 23 seems to get delayed and occurs concurrently with the aligning process of the two β sheets, thus contributing to the unfolding energy barrier. Hence, regulating the timing of breaking the interactions between residues 6 and 23 can lead to the modulation of the unfolding energy barrier of TNfn3.

However, it is important to note that the rate-limiting step remains to be the transition from twist state I1 to the aligned state I2 for TNfn3, and the interactions between residues 6 and 23 are broken in the aligned state I2. Hence, in order to enhance the mechanical stability of TNfn3, it is important not to overstabilize the interactions between 6 and 23, because the overstabilized interactions between 6 and 23 may become too strong to break in the aligned state I2 and thus become irrelevant to the mechanical unfolding barrier for TNfn3. Such a scenario will result in the change of the mechanical unfolding pathway of TNfn3. To illustrate this point, we have engineered a disulfide mutant biCys6–23,

**Discussion**

**Engineered metal chelation is an efficient and robust method to modulate the mechanical stability of proteins**

In our previous work,$^{28}$ we successfully employed engineered metal chelation approach to enhance the mechanical stability of a simple two-state unfoldor GB1. However, its application to proteins of complex unfolding behaviors has not been attempted.

**Fig. 4.** The mechanical stability of biHis6–23 can be modulated reversibly by the binding of Ni$^{2+}$ ions and metal chelator EDTA. The unfolding force histogram of apo biHis6–23 is centered on ~120 pN (top, $n=476$). After the addition of 8 mM Ni$^{2+}$, the average unfolding force of biHis6–23 increases to ~150 pN (middle panel, $n=567$). Upon the addition of 80 mM EDTA to the solution, the average unfolding force reduces back to a lower value of ~120 pN (bottom, $n=429$), which is indistinguishable from that of apo biHis6–23. All the three unfolding force histograms were obtained from the same experiment.
in which residues 6 and 23 are mutated to cysteines. Since the disulfide bond will not break until the stretching force reaches 1.5 nN or higher, the disulfide bond between 6 and 23 will not break when biCys6–23 passes the main unfolding energy barrier, e.g., aligning the two β sheets during the mechanical unfolding process of biCys6–23. Therefore, the formation of a disulfide bond between residues 6 and 23 will not play a role in the rate-limiting step of the mechanical unfolding of biCys6–23. Instead, the mechanical stability of biCys6–23 will still be determined by aligning the two β sheets. In this case, the mechanical unfolding pathway has been altered because the interactions between residues 6 and 23 are no longer broken in the aligned Ι2 state. Single-molecule AFM results showed that this is indeed the case. Figure 6a shows typical force–extension curves of polypeptide (biCys6–23)8 in the reduced (in black) and oxidized forms (in gray). The mechanical unfolding of oxidized biCys6–23 is characterized by a much shortened ΔLc of ∼23 nm compared with that of the reduced form (∼29 nm). This shortening in ΔLc is indicative of the formation of a disulfide bond between residues 6 and 23 and is consistent with sequestration of 18 residues by the disulfide bond 6–23. However, the mechanical unfolding force of oxidized biCys6–23 does not increase compared with that of the reduced form biCys6–23 (111 ± 14 pN for oxidized biCys6–23 versus 120 ± 15 pN for the reduced one, Fig. 6b). Instead, the mechanical unfolding force of oxidized biCys6–23 is slightly lower than that of the reduced form biCys6–23. SMD simulations showed similar behaviors during the mechanical unfolding of the oxidized biCys6–23 involving intermediates similar to that of the wt TNfn3: twist intermediate state Ι1 and aligned intermediate state Ι2 (Fig. 6c), with the exception that residues 6 and 23 remain bonded in the aligned state Ι2. Therefore, despite the similarity in trajectories, the mechanical unfolding pathway of the oxidized biCys6–23 is altered as compared with the wt TNfn3. Moreover, the formation of the disulfide bond affects the unfolding behavior of biCys6–23 after the intermediate state Ι2. Since the disulfide bond prevents the separation of the A-strand from the β sandwich structure, the commonly observed “A-strand separates first” pathway for wt TNfn3 is blocked. Instead, the unfolding of biCys6–23 is now characterized by the separation of the G strand from the β sandwich structure.

Conclusion

Combining SMD simulations, protein engineering and single-molecule AFM, we have designed a biHis-based metal chelation site into the TNfn3 domain and used bivalent metal ions to modulate the mechanical stability of TNfn3 in a controlled and reversible manner.
fashion. This is the first demonstration of using a metal chelation-based method to enhance the mechanical stability of an extracellular matrix protein domain that is of biological significance in mechanotransduction under physiological conditions. We anticipate that this method can be readily applied to modulate the mechanical properties of many other extracellular matrix proteins, such as fibronectin. Such applications will likely open the door to modulate the mechanical response of mechanical proteins during mechanotransduction in living cells in a well-defined and controlled fashion.

Materials and Methods

Protein engineering

TNfn3 biHis mutants biHis6–23 and biHis71–85 and bicysteine mutant biCys6–23 were constructed using wt
TNfn3 (a generous gift from Prof. Harold Erickson) as a template via mega primer method with a sense primer comprising one mutation and an antisense primer comprising the other mutation. The gene sequences of both biHis and biCys mutants were confirmed by direct DNA sequencing. Genes of polyproteins (biHis6–23), (biHis71–85) and (biCys6–23), were constructed using well-established methods as described previously. Polyproteins were expressed in DH5α strain, purified by Co2+–affinity chromatography, and eluted in phosphate-buffered saline with 300 mM NaCl and 250 mM imidazole. EDTA (20 mM) was added to the elution fractions to buffered saline with 300 mM NaCl and 250 mM imidazole. The oxidation of biHis71–85 and (biCys6–23) may exist in the elution fraction. The proteins were further dialyzed against Tris buffer (10 mM, pH 7.4, containing 100 mM NaCl) to completely remove EDTA and imidazole. The oxidation of biCys6–23 mutant was achieved at room temperature by ambient O2. The reduced biCys6–23 was obtained by incubating the protein with 20 mM dithiothreitol at room temperature.

Single-molecule AFM

Single-molecule AFM experiments were carried out on a custom-built atomic force microscope as described previously. All the force–extension measurements were carried out either in Tris–HCl buffer (10 mM, pH 7.4, containing 100 mM NaCl) or in Tris–HCl plus 4 mM NiCl2. The spring constant of AFM cantilevers (Si3N4 cantilevers from Veeco) was calibrated using equipartition theorem before each experiment with a typical value of 60 pN/nm. The pulling speed was 400 nm/s for all experiments.

Chemical denaturation experiments

Chemical denaturation experiments were carried out on a Cary Eclipse fluorescence spectrophotometer. Trypophan fluorescence of TNfn3 mutants was monitored at 360 nm to probe the unfolding process. The excitation wavelength was 280 nm. The chemical denaturation data were fitted to the following equation to measure the thermodynamic stability of the given protein:11

\[
F = \frac{\exp\left(\frac{(m \cdot |D| - \Delta G^\text{HLO})}{RT}\right)}{1 + \exp\left(\frac{(m \cdot |D| - \Delta G^\text{HLO})}{RT}\right)}
\]

where \(F\) is the fraction of unfolded proteins, \(m\) is the slope of the transition, \(|D|\) is the concentration of the denaturant, \(\Delta G^\text{HLO}\) is the thermodynamic stability of the protein in the absence of denaturant, \(R\) is the gas constant and \(T\) is the absolute temperature in kelvins. The thermodynamic stability, \(\Delta G^\text{HLO}\), of the protein can be measured using \(\Delta G^\text{HLO} = m \cdot |D|_{\text{LO}}\), where \(|D|_{\text{LO}}\) is the concentration of denaturant at which 50% of the protein is unfolded.

SMD simulations

Constant-force SMD simulations of the mechanical unfolding of TNfn3 and FNfn10 were carried out at 500 pN with the program NAMD 2.6 and with CHARMM22 force field following the protocol described previously. Crystal structures of TNfn3 (Protein Data Bank accession code 1TEN) and FNfn10 (1FNF, residues Val1416 to Thr1509) were used as the starting conformation for simulated equilibration. The initial structures of TNfn3 and FNfn10 were equilibrated for 1 ns at 300 K using explicit water model. The interaction energy between residues was calculated using VMD.44

Acknowledgements

We thank Prof. Hui Lu for helpful discussion and Prof. Stephen Withers and his group for their generous help in chemical denaturation experiments. This work is supported by Canadian Institutes of Health Research (CIHR) Operating Grant MOP-81225, Michael Smith Foundation for Health Research and Canada Research Chair program. H.L. is a Michael Smith Foundation for Health Research Career Investigator. Q.P. is supported by a Pacific Century Graduate Scholarship from the Province of British Columbia.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.05.057

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