Enhancing the Mechanical Stability of Proteins through a Cocktail Approach

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ABSTRACT Rationally enhancing the mechanical stability of proteins remains a challenge in the field of single molecule force spectroscopy. Here we demonstrate that it is feasible to use a “cocktail” approach for combining more than one approach to enhance significantly the mechanical stability of proteins in an additive fashion. As a proof of principle, we show that metal chelation and protein-protein interaction can be combined to enhance the unfolding force of a protein to ~450 pN, which is ~3 times of its original value. This is also higher than the mechanical stability of most of proteins studied so far. We also extend such a cocktail concept to combine two different metal chelation sites to enhance protein mechanical stability. This approach opens new avenues to efficiently regulating the mechanical properties of proteins, and should be applicable to a wide range of elastomeric proteins.

INTRODUCTION

Elastomeric proteins serve as the basic building blocks in a wide variety of mechanical machineries in cells as well as biomaterials of superb mechanical properties (1). Understanding the design principles of elastomeric proteins will not only help us understand the working mechanism of biological machineries, but may also improve our abilities to design novel elastomeric proteins with tailored mechanical properties for constructing smart materials for applications in material sciences and nanotechnology (2). Over the last decade, single-molecule force spectroscopy studies and molecular dynamics simulations have provided tremendous insights into the molecular design of elastomeric proteins at the single molecule level.

As an ultimate test of the understanding of molecular determinants of mechanical stability and an essential step toward tailor designing elastomeric proteins, rationally enhancing the mechanical stability of elastomeric proteins remains a challenging task and research focus. Several strategies have been developed successfully to rationally regulate the mechanical stability of proteins. These strategies include rational control of the unfolding pathway by disulfide bond formation (3), improving hydrophobic packing (4,5), reconstruction of the force-bearing region of proteins (6,7), ligand binding (8–11), and engineered metal chelation (12,13).

However, compared with the well-developed methods used to enhance the thermodynamic stability of proteins/enzymes (14–17), these methods to enhance the mechanical stability remain limited. Furthermore, these methods are only used one at a time, and the resulted enhancement of protein mechanical stability is also rather limited. To our knowledge, possible synergetic effects from more than one method remain largely unexplored.

Here we report a “cocktail approach” in which two methods are used simultaneously to efficiently enhance the mechanical stability of proteins in an additive fashion. As proofs-of-principle, we demonstrate the feasibility of such a cocktail approach by combining metal chelation and protein-protein interaction approaches as well as combining two independent metal chelation approaches.

MATERIALS AND METHODS

Protein engineering

Plasmid that encodes wild-type GB1 was a generous gift from Prof. David Baker of the University of Washington (Seattle, WA). All of the bi-His and tetra-His mutants were constructed using mega primer approaches with a sense primer for the first histidine mutation (or the first two histidine mutations in the case of tetra-His mutant) and an anti-sense primer comprising the second His mutation (or the last two His mutations).

The gene sequences of all bi-His or tetra-His mutants were confirmed by DNA sequencing. All of the polyprotein genes were constructed as described previously. The polyproteins were expressed in the DH5α strain, purified by Co²⁺ affinity chromatography, and eluted in phosphate-buffered saline buffer with 300 mM NaCl and 150 mM imidazole. EDTA (20 mM) was added to the elution fractions to remove residual Co²⁺ that may exist in the elution fractions. The proteins were further dialyzed against Tris-HCl buffer (10 mM, pH 7.4, containing 100 mM NaCl) to remove completely EDTA and imidazole. All proteins were stored in the dialysis buffer in frozen forms at −80°C before use.

Single-molecule atomic force microscopy

Single-molecule force spectroscopy experiments were carried out on a homebuilt atomic force microscope (AFM) as described previously (18). All of 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 or in 11.3 μM Human Fc fragment of IgG antibody (hFc). The spring constant of AFM cantilevers (Si₃N₄ cantilevers from Veeco,
Plainview, NY) was calibrated using the equipartition theorem before each experiment and typically had a value of ~60 pN nm\(^{-1}\). For the experiments in the presence of Ni\(^{2+}\) and hFc, we first deposited polyprotein, Ni\(^{2+}\), and/or hFc solution onto a freshly cleaned 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 ~30 min. The pulling speed was 400 nm s\(^{-1}\) for all experiments.

**Circular dichroism**

Circular dichroism spectra were recorded on a model No. J810 spectropolarimeter (JASCO, Oklahoma City, OK) with a 0.2-cm path-length cuvette and a scan rate of 50 nm min\(^{-1}\) under nitrogen gas flush. The protein samples were measured in 0.5 x phosphate-buffered saline at pH 7.4. The concentration of the protein samples is ~0.1–0.3 mg/mL. For each protein sample, an average of three scans is reported. The reported spectra have corrected buffer contributions.

**Surface plasmon resonance**

HBS-N (GE Healthcare, Kings Park, NY) aqueous buffer containing 10 mM HEPES pH 7.4, 150 mM NaCl was used for surface plasmon resonance (SPR) experiments, which were carried out on a Biacore 3000 system (GE Healthcare). Human Fc fragment of IgG antibody (hFc) was immobilized on a CM5 chip (GE Healthcare) until the SPR signal reached ~1000 RU (resonance units). Different concentrations of analytes, monomeric G6-53 proteins both in the presence and in the absence of nickel ions, were then passed through the CM5 chip to measure the binding of G6-53 to hFc under these two different situations. The corresponding dissociation steps of G6-53 from hFc were monitored by passing buffer alone through the CM5 chip.

**Equilibrium chemical denaturation measurement**

Chemical denaturation experiments were carried out on an Eclipse Fluorescence Spectrophotometer (Varian, Cary, NC). Tryptophan fluorescence of all GB1 mutants was excited at 280 nm and the emission spectra were monitored at 360 nm to probe the unfolding process. The data were fitted to the following equation to measure the thermodynamic stability of the given protein:

\[
F = \frac{\exp\left(m \cdot [D] - \Delta G_{D,N}^{H0}/RT\right)}{1 + \exp\left(m \cdot [D] - \Delta G_{D,N}^{H0}/RT\right)}. \tag{1}
\]

Here \(F\) is the fraction of proteins in denatured state, \(m\) is the slope of the transition, \([D]\) is the concentration of the denaturant, \(\Delta G_{D,N}^{H0}\) is the free energy of unfolding in the absence of denaturant, \(R\) is the gas constant, and \(T\) is the absolute temperature in Kelvin.

**RESULTS AND DISCUSSION**

It has been shown that engineered metal chelation (12,13) and protein-protein interactions (11) are two effective methodologies to enhance the mechanical stability of proteins. Here we attempt to combine these two independent methodologies to enhance the mechanical stability of proteins in an additive fashion. We use a bi-histidine (bi-His) mutant G6-53 of the B1 IgG binding domain of protein G from *Streptococcus* (GB1) as our model system, in which residues 6 and 53 on the force-bearing strands 1 and 4 were mutated to histidines. In one of our previous studies (12), we showed that bi-His mutant G6-53 can chelate divalent metal ions, such as Ni\(^{2+}\), with a high affinity. Metal chelation resulted in a significant increase in the mechanical stability of G6-53. As shown in Fig. 1 A, stretching polyprotein (G6-53)\(_h\) results in characteristic sawtooth-like force-extension curves, where an individual sawtooth peak corresponds to a single mechanical unfolding event of an individual G6-53 domain. The average unfolding force of G6-53 in the absence of Ni\(^{2+}\) is 120 ± 1.1 pN (mean ± standard error of the mean).

The binding of Ni\(^{2+}\) resulted in a significant increase of the mechanical stability of G6-53 to 260 ± 2.1 pN. In addition to metal chelation, it is well known that GB1 has a high binding affinity to the Fc fragment of IgG antibody (19) and the binding of Fc can significantly increase the mechanical stability of GB1 (11). The Fc binding epitope of GB1 is on the α-helix side, distant from the force-bearing β-strands 1 and 4. Hence, the bi-His mutation should not have any major influence on the Fc binding capability of G6-53. Indeed, SPR spectroscopy results (see Fig. S1 A in the Supporting Material) show that G6-53 can bind Fc fragment of human IgG antibody (hFc) with high affinity (\(K_D = 24\) nM). This result provides the possibility of using both metal chelation and Fc binding to enhance the mechanical stability of G6-53. Next, we use single-molecule AFM to examine the effect of the binding of hFc fragment on the mechanical stability of G6-53. Stretching polyprotein (G6-53)\(_h\) in the presence of 11.3 µM hFc resulted in sawtooth-like force-extension curves showing the same contour length increment \(\Delta L_c\) of ~18 nm, but the unfolding force peaks occurred at higher amplitude (Fig. 1 A). The average unfolding force of G6-53 in the presence of hFc is 260 ± 4.6 pN, an increase of ~140 pN compared with the unfolding force of G6-53 in the absence of hFc (Fig. 1 B).

Having established the enhancement effects of Ni\(^{2+}\) and hFc on the mechanical stability of G6-53, we then examined the possibility of simultaneously using both metal chelation and hFc binding to enhance the mechanical stability of G6-53. Stretching polyprotein (G6-53)\(_h\) in the presence of 4 mM Ni\(^{2+}\) and 11.3 µM hFc resulted in force-extension curves similar to those of apo-G6-53, but the unfolding of G6-53 occurred at much elevated forces (Fig. 1 A). Unfolding force histogram of G6-53 in the presence of Ni\(^{2+}\) and hFc showed a clear bimodal distribution, with the first peak centering at 260 ± 6.3 pN and the second peak centering at 450 ± 5.2 pN (Fig. 1 B). The position of the first force peak is similar to that of Ni\(^{2+}\)-bound G6-53 or hFc-bound G6-53, whereas the second force peak is completely new. We attributed the lower unfolding force peak to the unfolding of Ni\(^{2+}\)-bound or hFc-bound G6-53, and the higher unfolding force peak to the unfolding of G6-53 with both Ni\(^{2+}\) and hFc bound.

A bimodal distribution in the unfolding force histogram of G6-53 with both ligands present is surprising, as the concentration of both ligands is close to the saturating threshold.
concentration for the binding of a single ligand to G6-53. One plausible explanation for this result is that the binding of Ni\(^{2+}\) may decrease the hFc binding affinity of G6-53. To examine this possibility, we used SPR to measure the binding of hFc to G6-53 in the presence of Ni\(^{2+}\) ions (Fig. S1 B). Our SPR results show that the dissociation constant \(K_d\) for hFc/G6-53 complex increased by ~4 times with the addition of Ni\(^{2+}\) ions (\(K_d = 98\) nM). This increase in \(K_d\) is qualitatively consistent with the observed bimodal distribution shown in Fig. 1 B (see also the Supporting Material), confirming that the unfolding force peak at ~450 pN is indeed due to the unfolding of G6-53 with both Ni\(^{2+}\) and hFc bound.

It is clear that the binding of both ligands (Ni\(^{2+}\) and hFc) significantly increases the mechanical unfolding force of G6-53. In fact, the unfolding force of G6-53 with both ligands bound (~450 pN) is 3.7 times of the original unfolding force of G6-53, highlighting the significance of combining two stabilization methods in enhancing protein mechanical stability. Evidently, enhancements of mechanical stability via the binding of Ni\(^{2+}\) and hFc can be combined to obtain an additive effect, although due to the higher \(K_d\) and relatively low statistics, the measured unfolding force of G6-53 with both ligands bound is affected by the Gaussian fits. Thus, the existence of a possible synergistic effect needs to be further validated in our future endeavors.

To further test the cocktail concept to enhance the mechanical stability of proteins, we also test the use of two independent metal chelation sites to realize the mechanical stability enhancement. Our previous single-molecule AFM studies showed that bi-his mutants G4-51, G6-53, and G8-55 can bind Ni\(^{2+}\) ions and the binding of Ni\(^{2+}\) can significantly increase their mechanical stability (12). Hence, bi-histidine sites 4-51, 6-53, and 8-55 are suitable to be combined to achieve additive stabilization effect. However, His-X-His motif, such as position 4-6, on the same \(\beta\)-strand also constitutes good metal chelation sites (14). To avoid such unnecessary cross talking, we engineered a tetra-histidine mutant G4-8-51-55, which contains two bi-his metal chelation sites 4-51 and 8-55. Because the separation distance between two metal chelation sites is sufficiently large, crosstalk between the two metal chelation sites can be avoided. Far-UV circular dichroism
measurements indicate that the tetra-histidine mutant G4-8-51-55 largely retained its \( \alpha+\beta \) structure (Fig. 2 A), despite the introduction of tetra-histidine mutations into GB1 which resulted in reduced molar ellipticity. This reduced molar ellipticity is likely the result of the low \( \beta \)-sheet forming propensity of His residues (20).

To verify that G4-8-51-55 can chelate Ni\(^{2+} \) ions, we first measure the thermodynamic stability of G4-8-51-55 in the absence and in the presence of Ni\(^{2+} \) ions. As shown in Fig. 2 B, upon binding of Ni\(^{2+} \), the midpoint of denaturation, \( [D]_{30\%} \), shifts slightly toward higher \( [D] \) and the \( m \) value also shows significant change upon the binding of Ni\(^{2+} \). Fitting the chemical denaturation curves to Eq. 1 shows that the binding of Ni\(^{2+} \) to G4-8-51-55 resulted in an increase in thermodynamic stability \( \Delta \Delta G_{U,N} \) of G4-8-51-55 by \(-2.06 \) kcal/mol, which is very close to the sum of the \( \Delta \Delta G_{U,N} \) due to the binding of Ni\(^{2+} \) to G4-51 and G8-55 alone (12). This result indicates that the thermody-
219 ± 1.7 pN in the presence of 4 mM Ni²⁺, respectively (Fig. 3, B and D). Their unfolding forces increase by ~80 pN and ~60 pN upon metal chelation for G4-51 and G8-55, respectively. We then measured the mechanical stability of G4-8-51-55 in the absence and presence of Ni²⁺. The unfolding forces of G4-8-51-55 in the absence of Ni²⁺ are ~110 ± 1.6 pN, whereas in the presence of 4 mM Ni²⁺, the unfolding forces increases dramatically to 247 ± 2.8 pN, which is 140 pN higher than that of apo-G4-8-51-55 (Fig. 3, E and F). The increase in unfolding force of G4-8-51-55 upon metal chelation is very close to the sum of the increase of the unfolding forces of both bi-His mutants (80+60 pN)—clearly showing that the mechanical stabilization by two metal chelation is additive, indicating the successful implementation of the cocktail approach.

It was previously shown that proteins’ mechanical stability can be predicted reasonably well from their topology (21–23). Here we have demonstrated that by simply combining two different stabilization methods, we are able to enhance the mechanical stability of a protein dramatically. Even with the topology of a protein maintained, there is still much room to improve its mechanical stability. The average unfolding force of G6-53 in the presence of both Ni²⁺ and hFc is ~450 pN, which is comparable to the unfolding forces of the most stable proteins (24) or protein complexes (25) reported to date. These results suggest that it is possible to modulate the mechanical stability of proteins, which is governed by noncovalent interactions inside the proteins, in a broad range with its upper limit being the mechanical stability of covalent bonds (26).

We anticipate that such a cocktail approach to enhance the mechanical stability of proteins can be easily extended to other proteins, for which mechanical stability is essential for their function and application. Further development of such a cocktail approach will explore the use of other strategies, including utilizing specific metal binding motifs as seen in metal containing proteins (for example, carbonic anhydrase (27)), to make this method more robust and powerful.

It is also important to note that successful implementation of the cocktail approach relies on the careful design of a protein and its stabilization strategy. Mechanical stability is a kinetic stability and it depends on the free energy barrier between the native state and the mechanical unfolding transition state. We have shown that the key to enhancing the mechanical stability is to stabilize preferentially the native over the mechanical unfolding transition state (12). To use the cocktail approach to enhance the mechanical stability of proteins in an additive fashion, it is also important to ensure that the mechanical unfolding under the influence of two different stabilization mechanisms follow the same unfolding pathway. In such a case, the increasing in the mechanical unfolding energy barrier by two different methods can be additive. If the two unfolding pathways are different, additive effects may not be obtained.

For example, in a pioneering experiment, it was shown that the binding of small ligands methotrexate (MTX) or nicotinamide adenine dihydrophosphate (NADPH) to dihydrofolate reductase (DHFR) can increase the mechanical stability of DHFR by ~50 pN, respectively (8). However, simultaneous binding of the MTX and NADPH did not result in any additive enhancement of the mechanical stability, despite that the binding of MTX and NADPH to DHFR are additive in terms of thermodynamic stability (8). One possible explanation is that DHFR unfolds via two different pathways upon binding of MTX and NADPH. Therefore, careful analysis of the mechanical unfolding pathways under the influence of ligand binding is crucial for the successful implementation of the cocktail approach.

In summary, we have demonstrated the proof-of-principle of the cocktail approach as an effective method to enhance the mechanical stability of proteins. We believe this to be a novel approach that opens new avenues to efficiently regulating the mechanical properties of proteins and should be applicable to a wide range of elastomeric proteins.

SUPPORTING MATERIAL

One figure is available at http://www.biophysj.org/biophys/supplemental/S0006-3495(11)00248-7.

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REFERENCES


