Engineered Bi-Histidine Metal Chelation Sites Map the Structure of the Mechanical Unfolding Transition State of an Elastomeric Protein Domain GB1

Tao Shen, Yi Cao, Shulin Zhuang, and Hongbin Li*
Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada

ABSTRACT Determining the structure of the transition state is critical for elucidating the mechanism behind how proteins fold and unfold. Due to its high free energy, however, the transition state generally cannot be trapped and studied directly using traditional structural biology methods. Thus, characterizing the structure of the transition state that occurs as proteins fold and unfold remains a major challenge. Here, we report a novel (to our knowledge) method that uses engineered bi-histidine (bi-His) metal-binding sites to directly map the structure of the mechanical unfolding transition state of proteins. This method is adapted from the traditional $\psi$-value analysis, which uses engineered bi-His metal chelation sites to probe chemical (un)folding transition-state structure. The $\phi^{\text{Mz}}/\psi$-value is defined as $\Delta G_{U-N}/\Delta \Delta G_{U-N}$, which is the energetic effects of metal chelation by the bi-His site on the unfolding energy barrier ($\Delta G_{U-N}$) relative to its thermodynamic stability ($\Delta \Delta G_{U-N}$) and can be used to obtain information about the transition state in the mutational site. As a proof of principle, we used the small protein GB1 as a model system and set out to map its mechanical unfolding transition-state structure. Using single-molecule atomic force microscopy and spectrofluorimetry, we directly quantified the effect of divalent metal ion binding on the mechanical unfolding free energy and thermodynamic stability of GB1, which allowed us to quantify $\phi^{\text{Mz}}/\psi$-values for different sites in GB1. Our results enabled us to map the structure of the mechanical unfolding transition state of GB1. Within GB1’s mechanical unfolding transition state, the interface between force-bearing $\beta$-strands 1 and 4 is largely disrupted, and the first $\beta$-hairpin is partially disordered while the second $\beta$-hairpin and the $\alpha$-helix remain structured. Our results demonstrate the unique application of $\psi$-value analysis in elucidating the structure of the transition state that occurs during the mechanical unfolding process, offering a potentially powerful new method for investigating the design of novel elastomeric proteins.

INTRODUCTION

Understanding how proteins fold and unfold remains one of the central challenges in the field of life sciences (1–3). The transition state, in which the protein is equally likely to fold or unfold, is critical for determining the kinetics of protein folding and unfolding. However, due to its high free energy, the transition state cannot be trapped and studied directly using traditional structural biology methods. Thus, characterizing the structure of the protein folding/unfolding transition state remains challenging. Over the last two decades, $\phi$-value analysis has become a major experimental method used to determine the structure of the transition state, and has served as a benchmark for molecular dynamics (MD) simulations of protein folding/unfolding (4,5). In this method, conserved point mutations are introduced throughout the sequence of the protein of interest one at a time, and thus certain side-chain interactions are selectively deleted without causing major perturbations to the overall protein structure. The $\phi$-value, defined as the ratio of energetic effect of such point mutations on the (un)folding energy barrier relative to its thermodynamic stability, can identify the degree to which the deleted interactions are present in the transition state. If the deleted interactions are fully formed in the transition state as in the native state, $\phi = 0$, and if the deleted interactions are fully disrupted in the transition state, $\phi = 1$. In this way, the $\phi$-value analysis can yield valuable information about the transition-state structure at the mutational site (2,4,5). Another method, termed $\psi$-value analysis, was developed to map the transition-state structure and investigate the heterogeneity of protein folding pathways. In this method, single bi-histidine (bi-His)-based divalent metal ion binding sites are engineered one at a time into different regions of a given protein (6–8). The $\psi$-value, which is the ratio of the energetic effect of metal chelation on the (un)folding energy barrier relative to its thermodynamic stability, can identify the degree to which the bi-His metal chelation site forms in the transition state, providing structural information about the mutated site in the (un)folding transition state. In both methods, the validity of the analysis depends on the assumption that the point mutation or the pairwise mutation to His does not alter the folding pathway and the transition state. A unique advantage of $\psi$-value analysis is that it allows for measurement of the flux between different folding/unfolding pathways, resolving the question of whether a fractional $\phi$-value is due to partial breakage of an interaction or the presence of additional pathways.

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*Correspondence: hongbin@chem.ubc.ca
Yi Cao’s present address is Department of Physics, Nanjing University, Nanjing, People’s Republic of China.
Shulin Zhuang’s present address is College of Environmental and Resource Sciences, Zhejiang University, Hangzhou, People’s Republic of China.
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Over the last decade, the development of single-molecule force spectroscopy techniques has provided a new way to probe protein folding/unfolding dynamics along a well-defined reaction coordinate set by the vector of the applied stretching force, and yielded new and difficult-to-obtain insights into how proteins fold and unfold at the single-molecule level (1,9–13). Just as in classical ensemble protein folding/unfolding studies, however, mapping the mechanical unfolding/folding transition state remained challenging. Despite the development of mechanical \( \psi \)-value analysis to characterize the mechanical unfolding transition state of proteins, only three proteins to date have been characterized by that method (14–17). Experimental efforts to characterize the structure of the mechanical unfolding transition state remain limited, and MD simulations remain the best source of information about the mechanical unfolding transition state (18–20). Based on the principles of \( \psi \)-value analysis, we developed what to our knowledge is a new method that uses engineered bi-His metal chelation sites to track how a protein unfolds under mechanical force, and thus allows the structure of its mechanical unfolding transition state to be mapped. As a proof of principle, we applied this new method to map the mechanical unfolding transition state structure of a small protein, GB1, experimentally.

**Principles of Mechanical \( \psi \)-Value Analysis**

It has been shown that the binding of metal ions to engineered bi-His metal chelation sites with the appropriate geometry can lead to an increase in the measured thermodynamic stability of proteins (21,22). The binding affinity of a bi-His site to a metal ion depends on the geometry/conformation of the bi-His site in the protein. Thus, the binding affinity between the two can be used as a probe to obtain information about the conformation of a specific bi-His metal chelation site.

Based on a thermodynamic cycle analysis for mechanical unfolding (Fig. 1A), we show that the change of the mechanical unfolding energy barrier upon metal ion binding

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**FIGURE 1** Principle of mechanical \( \psi \)-value analysis as used to map the mechanical unfolding transition state of proteins. (A) Thermodynamic cycle analysis for the mechanical unfolding of proteins in the presence of metal ions. The asterisks denote the protein in the metal ion-bound state (both native state and unfolding transition state). \( \Delta \Delta G_{1,N} \) is the change in the mechanical unfolding energy barrier caused by metal chelation, where \( \Delta G_{1,N}^{*} - \Delta G_{1,N} \), \( \Delta G_{\text{bind}} \) is defined as \( \Delta G_{\text{bind}(1)} - \Delta G_{\text{bind}(N)} \), where \( \Delta G_{\text{bind}} \) is the Gibbs free energy for the binding reaction. Thermodynamic cycle analysis shows that the change in the mechanical unfolding energy barrier upon metal ion binding (\( \Delta \Delta G_{1,N} \)) is equal to the difference in binding energy of the metal ion to the native state and the mechanical unfolding transition state (\( \Delta G_{\text{bind}(1)} - \Delta G_{\text{bind}(N)} \)). (B) Free-energy diagram for the two extreme \( \psi \)-values observed in mechanical protein unfolding. If the metal chelation site is formed in the mechanical unfolding transition state as in the native state, \( \Delta \Delta G_{1,N} = 0 \) and \( \psi^{M2+} = 0 \). If the metal chelation site is completely disrupted in the mechanical unfolding transition state, \( \Delta \Delta G_{1,N} = \Delta \Delta G_{U,N} \) and \( \psi^{M2+} = 1 \).
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\( \Delta \Delta G_{J,N} \) equals the difference in binding energy of the metal ion to the native state and the mechanical unfolding transition state \( \Delta G_{\text{bind}(J)} - \Delta G_{\text{bind}(N)} \). Thus, \( \Delta \Delta G_{J,N} \) will provide information as to whether the bi-His site is formed or not in the mechanical unfolding transition state. Following the concepts of ψ-value and ψ-value analyses \((5,8)\), we can now describe the energetic effect of metal binding to the bi-His site on the mechanical unfolding energy barrier relative to its effect on the thermodynamic stability as \( \phi^{M2+}\psi = \Delta \Delta G_{J,N} / \Delta \Delta G_{U,N} \).

If the metal chelation site in the mechanical unfolding transition state is formed in the same way as in the native state, \( \Delta \Delta G_{J,N} = 0 \) and \( \phi^{M2+}\psi = 0 \). If the metal chelation site is completely disrupted in the mechanical unfolding transition state, \( \Delta \Delta G_{J,N} = \Delta \Delta G_{U,N} \) and \( \phi^{M2+}\psi = 1 \). If the metal chelation site is partially disrupted, we expect that the transition state will have a lower binding affinity to metal ions, and \( 0 < \phi^{M2+}\psi < 1 \). Therefore, we can use \( \phi^{M2+}\psi \) to readily map the structure of the mechanical unfolding transition state around the bi-His site.

It is important to note that in this analysis, we assume that there is only one homogeneous mechanical unfolding pathway. This assumption is largely valid for the mechanical unfolding of GB1, because the same unfolding pathway is observed within the range of pulling speeds examined. For proteins with multiple unfolding pathways, a mechanical ψ-value analysis (similar to that described previously \((6–8)\)) can be used to map out the heterogeneity of the mechanical unfolding pathways.

It is worth noting that because a continuous change in metal ion concentration leads to a continuous increase in the thermodynamic stability of the specific bi-His region, the ψ-value in chemical protein (un)folding studies is defined as

\[
\psi_{U} = \frac{\partial (\Delta \Delta G_{J,N})}{\partial (\Delta \Delta G_{U,N})}
\]

In this case, \( \psi \) is the infinitesimal change in activation energy for unfolding relative to the infinitesimal change in equilibrium thermodynamic stability \((7,8)\). However, in previous mechanical unfolding studies, we showed that the metal-bound and metal-free forms of bi-His mutants have a distinct mechanical stability that does not change as a function of metal ion concentration \((23,24)\). Thus, the mechanical ψ-value reduces to \( \psi_{U} \). In this work, we use \( \phi^{M2+}\psi \) to map the structure of the mechanical unfolding transition state.

To determine \( \phi^{M2+}\psi \), one needs to experimentally determine \( \Delta \Delta G_{J,N} \), \( \Delta \Delta G_{U,N} \), and \( \Delta \Delta G_{\text{bind}(J)} - \Delta \Delta G_{\text{bind}(N)} \). While this can be measured readily using single-molecule atomic force microscopy (AFM), mechanical unfolding and chemical unfolding may follow different pathways involving different transition states, \( \Delta \Delta G_{J,U,N} \) should be the same for the two different pathways provided that the mutation does not affect the mechanical and chemical unfolded states differently \((15)\). Therefore, one can use bulk chemical denaturation to determine \( \Delta \Delta G_{J,U,N} \) for the analysis of the mechanical unfolding pathway. This was previously used in mechanical ψ-value analysis to probe the mechanical unfolding transition-state structure \((14,17,28)\).

### MATERIALS AND METHODS

**Protein engineering**

The plasmid encoding wild-type (WT) GB1 was a generous gift from Professor David Baker (University of Washington). All of the bi-His mutants were constructed using megaprimer approaches with a sense primer for the first His and an anti-sense primer comprising the second His mutation. The DNA sequences of all bi-His mutants were confirmed by direct DNA sequencing. All polypeptide genes, which consist of eight identical tandem repeats of the bi-His mutant, were constructed as described previously \((24,29)\). The polypeptides were expressed in the DH5α Escherichia coli strain, purified by Co²⁺ 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 any residual Co²⁺ that might exist in the elution fractions. The mutant proteins were dialyzed against Tris-HCl buffer (10 mM, pH 7.4, containing 100 mM NaCl) to completely remove EDTA and imidazole. All proteins were stored in the dialysis buffer at −80°C.

**Single-molecule AFM**

Single-molecule force spectroscopy experiments were carried out on an in-house-built atomic force microscope as described previously \((24,29)\). All of the force extension measurements were carried out in Tris-HCl buffer (10 mM, pH 7.4, containing 100 mM NaCl or Tris-HCl plus 4 mM NiCl₂). We calibrated the spring constant of the atomic force microscope cantilevers (Si₃N₄ cantilevers; Bruker, Camarillo, CA) before each experiment using the equipartition theorem (typically ~60 pN nm⁻¹). For experiments in the presence of Ni²⁺, we first deposited polypeptide and Ni²⁺ solution onto a freshly cleaned glass coverslip containing 50 mL of Tris-HCl buffer and mixed in situ. AFM experiments were carried out after the mixture was allowed to equilibrate for ~30 min. The pulling speed was 400 nm s⁻¹ for all experiments except pulling speed dependence measurements.

**Equilibrium chemical denaturation measurements**

Chemical denaturation experiments were carried out on a Cary Eclipse fluorescence spectrophotometer. The tryptophan fluorescence of all bi-His mutants was excited at 280 nm and the emission spectra were monitored
at 360 nm to probe the unfolding process. Data were fitted to the following equation to measure the thermodynamic stability of the given protein:

\[
F = \frac{\exp\left[\left(m[D] - \Delta G_{\text{U}}^{\text{H2O}}\right)/RT\right]}{1 + \exp\left[\left(m[D] - \Delta G_{\text{U}}^{\text{H2O}}\right)/RT\right]},
\]

(1)

where \( F \) is the fraction of proteins in the denatured state, \( m \) is the slope of the transition, \([D]\) is the concentration of the denaturant, \( \Delta G_{\text{U}}^{\text{H2O}} \) is the free energy of unfolding in the absence of denaturants, \( \Delta G_{\text{U}} \) is the free energy barrier.

### RESULTS AND DISCUSSION

#### Design of GB1 bi-His mutants

Having defined the mechanical \( \phi_{\text{M2+U}} \) value for mechanical protein unfolding, we applied this method to experimentally map the mechanical unfolding transition state of a small protein, GB1. GB1, the B1 IgG binding domain of streptococcal protein G, is an \( \alpha/\beta \) protein (32) that has been used extensively as a model system for protein (un)folding studies (33–37). In addition, GB1 has been a paradigm for single-molecule AFM (23,29) and MD simulations (38–40) aimed at elucidating how proteins unfold under force, and how the mechanical stability of a protein is determined by detailed molecular interactions within its structure. Here, we introduced bi-His motifs into different regions of GB1, and used single-molecule AFM techniques to evaluate the effects of metal binding on the mechanical unfolding energy barrier.

The design of bi-His metal chelation sites in proteins is well established (21,22). In a \( \beta \)-strand, His-x-His should constitute a metal chelation site, and His-x-x-x-His in a \( \alpha \)-helix should constitute a metal chelation site (where x refers to any nonproline residue). Across two neighboring \( \beta \)-strands, surface-exposed adjacent residues in the two strands can form a metal chelation site. Following these general rules and the x-ray crystal structure of GB1 (PDB code 1PGA) (41), we designed nine bi-His mutants in different regions of GB1 to probe their structure in the mechanical unfolding transition state. According to the location of the bi-His substitution in GB1, engineered bi-His mutants were classified into three categories (Fig. 2): 1), mutants to probe the structure of force-bearing \( \beta \)-strands 1 and 4 (G4-51, G6-53, and G8-55); 2), mutants to probe the structure of the two \( \beta \)-hairpins (\( \beta \)-hairpin 1 and \( \beta \)-hairpin 2) in the mechanical unfolding transition state (G4-17, G6-15, G44-53 and G42-55); and 3), mutants to probe the structure of the \( \alpha \)-helix in the transition state (G32-36 and G24-28).

Bi-His mutant sequences are shown in Table S1 of the Supporting Material. Because all bi-His mutations are located in the solvent-exposed region of GB1, bi-His mutants should not affect the structure of GB1. Indeed, all mutants showed circular dichroism (CD) spectra very similar to that of WT GB1 (see Fig. S1).

#### Equilibrium binding of Ni\(^{2+} \) to bi-His mutants of GB1

We first performed chemical denaturation experiments to quantify the effect of metal binding (Ni\(^{2+} \)) on the thermodynamic stability of bi-His GB1 mutants in the presence of Ni\(^{2+} \) sufficient to achieve saturation. Representative chemical denaturation curves are shown in Fig. 3, and the equilibrium free energy in the absence and presence of Ni\(^{2+} \) is shown in Table 1. [GdmCl]\(_{0.5} \) is the concentration of GdmCl at which 50% of protein is unfolded, and can be used as a measure of the thermodynamic stability of the protein. It is clear that the thermodynamic stability of all bi-His

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**FIGURE 2** Bi-His mutants of GB1 are designed to probe different regions of GB1 in its mechanical unfolding transition state. Mutants G4-51, G6-53, and G8-55 are designed to probe the structure of the force-bearing strands in the transition state; G4-17, G6-15, G44-53, and G42-55 are designed to probe the structure of the two \( \beta \)-hairpins in the transition state; and mutants G24-28 and G32-36 are designed to probe the structure of the \( \alpha \)-helix in the transition state.
two exceptions. G44-53 does not show obvious binding affinity (Fig. S2).

Mutants G6-15 and G44-53 do not confer significant metal binding behavior upon binding of Ni\textsuperscript{2+} that is not found in the other proteins investigated: binding of Ni\textsuperscript{2+} to G6-15 leads to a decrease in thermodynamic stability, rather than the stabilization effect that is usually observed upon binding of Ni\textsuperscript{2+} (Fig. S2 B). This abnormal behavior suggests that the binding of Ni\textsuperscript{2+} destabilized the native state of G6-15. Within the sequence and structure of GB1, it is noted that a positively charged Lys-4 is adjacent to the bi-His-Ni\textsuperscript{2+} site of mutant G6-15. Therefore, it is possible that repulsive electrostatic interactions between Lys-4 and Ni\textsuperscript{2+} may be responsible for destabilization upon Ni\textsuperscript{2+} binding.

TABLE 1 Thermodynamic, kinetic, and mechanical stabilities of GB1 bi-His mutants in the absence and presence of Ni\textsuperscript{2+}

| Mutant   | Bi-His site location | D_1/2 (apo) (M) | D_1/2 (Ni\textsuperscript{2+}) (M) | ΔΔG_N-U (kCal/mol) | F_U (apo) (pN) | F_U (Ni\textsuperscript{2+}) (pN) | a_0 (apo) (s\textsuperscript{-1}) | a_0 (Ni\textsuperscript{2+}) (s\textsuperscript{-1}) | Δx_0 (apo) (nm) | Δx_0 (Ni\textsuperscript{2+}) (nm) | ΔΔG_{U,N} (kCal/mol) | φ_{M2+U} \textsuperscript{5} |
|----------|---------------------|----------------|-----------------------------------|-------------------|----------------|------------------|-----------------|-----------------|----------------|----------------|----------------|-----------------
| WT       | –                   | 2.51           | 2.51                              | –                 | 180            | 180              | 0.039           | 0.17            | 0.039          | 0.17           | 0              | –              |
| G4-51\textsuperscript{4} | Force-bearing       | 1.21           | 1.93                              | 1.47 ± 0.03       | 120 ± 0.8      | 198 ± 1.1        | 0.12            | 0.20            | 0.20           | 0.17           | 0              | 0.99 ± 0.18     |
| G6-53\textsuperscript{4} | β-strands           | 1.34           | 2.01                              | 1.59 ± 0.03       | 119 ± 0.7      | 243 ± 1.0        | 0.14            | 0.20            | 0.0071         | 0.17           | 0              | 1.79 ± 0.32     |
| G8-55\textsuperscript{4} | –                   | 2.21           | 2.41                              | 0.36 ± 0.02       | 160 ± 0.9      | 219 ± 1.7        | 0.029           | 0.20            | 0.14           | 0.17           | 0.44 ± 0.08     | 1.22 ± 0.26     |
| G6-15     | β-Hairpin 1         | 1.78           | 1.36                              | –1.04 ± 0.05      | 142 ± 0.9      | 140 ± 0.8        | 0.07            | 0.20            | 0.07           | 0.20           | 0              | –              |
| G4-17     | –                   | 2.36           | 3.10                              | 1.48 ± 0.06       | 177 ± 2.7      | 187 ± 2.1        | 0.07            | 0.17            | 0.07           | 0.17           | 0              | 0              |
| G42-55    | β-Hairpin 2         | 1.96           | 2.74                              | 1.39 ± 0.03       | 160 ± 1.6      | 207 ± 2.6        | 0.05            | 0.20            | 0.02           | 0.17           | 0.54 ± 0.10     | 0.39 ± 0.25     |
| G44-53    | –                   | 1.49           | 1.54                              | 0.12 ± 0.04       | 131 ± 0.8      | 131 ± 0.8        | 0.08            | 0.20            | 0.08           | 0.20           | 0              | –              |
| G24-28    | α-Helix             | 1.72           | 2.73                              | 1.39 ± 0.06       | 155 ± 1.6      | 148 ± 1.3        | 0.08            | 0.20            | 0.08           | 0.20           | 0              | –              |
| G32-36\textsuperscript{5} | –                   | 2.08           | 2.63                              | 1.02 ± 0.03       | 155 ± 2.2      | 146 ± 1.4        | 0.06            | 0.20            | 0.06           | 0.20           | 0              | –              |

\textsuperscript{4}F_U (apo) and F_U (Ni\textsuperscript{2+}) are presented as average ± standard error of the mean.

\textsuperscript{5}a_0(apo) and a_0(Ni\textsuperscript{2+}) are estimated using Monte Carlo simulations; the error is estimated to be ±30%.

\textsuperscript{6}ΔΔG_{U,N} is calculated using the equation ΔΔG_{U,N} = RTln(a_0(apo)/a_0(Ni\textsuperscript{2+})), and the error is determined assuming an 30% error for a_0(apo) and a_0(Ni\textsuperscript{2+}).

\textsuperscript{7}φ_{M2+U} is calculated using ΔΔG_{U,N} as calculated above.

\textsuperscript{8}Unfolding force and unfolding rate constant data are taken from Cao et al. (23).
binding. Although the effect of Ni\(^{2+}\) binding on G6-15 is destabilizing, its thermodynamic and mechanical effects can nonetheless be used to evaluate the structure of the mechanical unfolding transition state using \(\phi_{M2^+}^{U}\)-value analysis.

It is important to note that the equilibrium free-energy change (\(\Delta\Delta G_{U,N}\)) for bi-His mutants upon binding of Ni\(^{2+}\) as reported in Table 1 is at 14.3 mM Ni\(^{2+}\). Because \(\Delta G_{U,N}\) is dependent on the concentration of Ni\(^{2+}\), \(\Delta\Delta G_{U,N}\) should also depend on the concentration of Ni\(^{2+}\). Our previous results showed that 14.3 mM Ni\(^{2+}\) is close to saturating the binding site located on the bi-His mutant (24), and thus 14.3 mM Ni\(^{2+}\) should be close to the saturated concentration of Ni\(^{2+}\). For simplicity, we use \(\Delta\Delta G_{U,N}\) at 14.3 mM Ni\(^{2+}\) as the thermodynamic stability difference caused by the binding of Ni\(^{2+}\).

**Effect of Ni\(^{2+}\) binding on the mechanical unfolding of GB1 bi-His mutants**

Single-molecule force spectroscopy techniques were used to investigate the effect of Ni\(^{2+}\) binding on the mechanical stability of GB1 bi-His mutants. Stretching mutant polyproteins (bi-His-mutant) resulted in force-extension curves with a characteristic sawtooth pattern appearance, where individual sawtooth peaks correspond to the mechanical unfolding of individual domains in the polyprotein chain, and the last peak generally corresponds to the detachment of the polyprotein chain from either the AFM tip or substrate. Fig. 4 shows typical force-extension curves of representative bi-His mutants in the absence and presence of 4 mM Ni\(^{2+}\). The unfolding force histograms of each mutant in the presence and absence of 4 mM Ni\(^{2+}\) are also shown in Fig. 4 and Fig. S3.

We have previously shown that the metal-bound and metal-free forms of bi-His mutants have a distinct mechanical stability that does not change as a function of metal ion concentration (23,24). In the presence of 4 mM Ni\(^{2+}\), >85\% of bi-His mutants are in the Ni\(^{2+}\)-bound form, which allows us to measure the mechanical stability of Ni\(^{2+}\)-bound forms of the bi-His mutant with sufficient accuracy. Thus, we carried out AFM experiments in the presence of 4 mM Ni\(^{2+}\) to determine the mechanical stability of Ni\(^{2+}\)-bound bi-His mutants.

It is clear that the force at which different mutants unfold is affected differently by the binding of Ni\(^{2+}\). The unfolding force of some bi-His mutants (e.g., G6-53) can increase as much as 100\%, whereas others (e.g., G4-17) do not change at all, suggesting that Ni\(^{2+}\) binding has different effects on the mechanical unfolding energy barrier for different bi-His mutants.

To quantify the change in mechanical unfolding energy barrier upon Ni\(^{2+}\) binding, we carried out force-extension experiments at different pulling speeds. The representative dependence of unfolding force on the pulling speed is shown in Fig. S4. Using well-established Monte Carlo simulation procedures (42), we fitted the unfolding force distribution and dependence between the unfolding force and pulling speed simultaneously to estimate the spontaneous unfolding rate constant (\(\alpha_0\)) at zero force, as well as the distance between the native state and the mechanical unfolding transition state (\(\Delta_x\)). Based on \(\alpha_0\), we calculated the change in mechanical unfolding free energy \(\Delta\Delta G_{U,N}\) using the following relationship: 

\[
\Delta\Delta G_{U,N} = -RT\ln[\alpha_0(Ni^{2+})/\alpha_0(\text{apo})]
\]

The results are shown in Table 1.

Having measured the equilibrium free-energy change (\(\Delta\Delta G_{U,N}\)) and the mechanical unfolding free energy change (\(\Delta\Delta G_{U,N}^{M}\)), we determined the mechanical \(\phi_{M2^+}^{U}\)-value for the mechanical unfolding of GB1 and used it to map the mechanical unfolding transition state. It is evident that the three structural regions of GB1 show different mechanical \(\phi_{M2^+}^{U}\)-values (Fig. 5 and Table 1).
The interface between the two force-bearing \( \beta \)-strands is largely disrupted in the mechanical unfolding transition state of GB1

Bi-His mutants in force-bearing \( \beta \)-strands 1–4 show high \( \phi^{M2+U} \)-values that are close to one, suggesting that bi-His sites that span the two force-bearing strands are disrupted significantly in the mechanical unfolding transition state, and thus the binding affinity to Ni\(^{2+} \) in the transition state is significantly reduced or completely eliminated. This conclusion is consistent with MD simulations (38,39) showing that the key event that occurs as GB1 mechanically unfolds corresponds to the rupture of force-bearing \( \beta \)-strands 1 and 4. This structural element was previously used to rationally enhance the mechanical stability of GB1 (24). Of note, \( \phi^{M2+U} \) for G4-51 is significantly smaller than that for G6-53 and G8-55, suggesting that the N-terminus of the force-bearing strands is likely more structured than the C-terminus of the force-bearing strands in the mechanical unfolding transition state. This result also implies that the shearing and separation of the two force-bearing \( \beta \)-strands may initiate from the C-terminus.

The first \( \beta \)-hairpin is largely intact in the mechanical unfolding transition state, and the second \( \beta \)-hairpin is partially unstructured

The binding of Ni\(^{2+} \) does not lead to any change in the mechanical stability of G4-17 and G6-15, suggesting that the mechanical unfolding transition state for these mutants has the same binding affinity to Ni\(^{2+} \) as the native state, resulting in \( \phi^{M2+U} \)-values close to zero. This indicates that the first \( \beta \)-hairpin remains largely intact in the mechanical unfolding transition state. In contrast, G42-55 (where the bi-His site is located in the second hairpin) shows a different behavior. The binding of Ni\(^{2+} \) to mutant G42-55 results in an increase in mechanical unfolding force, giving rise to a \( \phi^{M2+U} \)-value of 0.39. This fractional \( \phi^{M2+U} \)-value suggests that the structure around residues 42 and 55, which is at the end of the second \( \beta \)-hairpin, is partially disrupted in the mechanical unfolding transition state, thus showing a reduced binding affinity to Ni\(^{2+} \). Unfortunately, because the bi-His mutant G44-53 does not bind Ni\(^{2+} \) in the native state, G44-53 cannot be used to probe the structure of the mechanical unfolding transition state of the entire second \( \beta \)-hairpin.

The \( \alpha \)-helix is intact in the mechanical unfolding transition state

The binding of Ni\(^{2+} \) to bi-His mutants in the \( \alpha \)-helix does not change the mechanical stability, giving rise to a \( \phi^{M2+U} \)-value of zero. This result suggests that the \( \alpha \)-helix is intact in the mechanical unfolding transition state. In summary, in the mechanical unfolding transition state of GB1, the \( \alpha \)-helix and the first \( \beta \)-hairpin remain largely intact. Mutations in the second \( \beta \)-hairpin show intermediate \( \phi^{U,M2+} \)-values, suggesting that the second \( \beta \)-hairpin is partially disrupted and mutants in this region show reduced binding affinity in the transition state. Mutations in force-bearing \( \beta \)-strands 1 and 4 are close to one, indicating that the force-bearing strands are deformed such that the bi-His metal chelation sites engineered across the two strands are largely disrupted and no longer bind Ni\(^{2+} \) in the mechanical unfolding transition state (Fig. 5). These results provide the first (to our knowledge) experimental picture of GB1’s transition state as it unfolds under an applied mechanical force.

Comparing the unfolding transition-state structure for mechanical and chemical unfolding of GB1

The folding and unfolding of GB1 has been studied extensively with the use of classical ensemble chemical denaturation methods, and the structure of the unfolding transition state was mapped previously (34). In the chemical unfolding transition state, the second \( \beta \)-hairpin is fully structured and the first \( \beta \)-hairpin is largely unstructured. The formation of the second hairpin is critical for reaching the transition state along the folding pathway. Moreover, it was found that the second hairpin is stable in isolation (34,36). This picture of the chemical unfolding transition state is drastically different from that of mechanical unfolding. In the mechanical unfolding transition state, the second hairpin is partially unstructured and unravels first while the first hairpin remains structured. The two transition-state structures attained during either chemical or
mechanical unfolding are drastically different, which suggests that chemical and mechanical unfolding of GB1 follows very different pathways. It is worth noting that during mechanical unfolding, the thermodynamically more-stable second hairpin unravels first, again demonstrating that mechanical and thermodynamic stabilities do not necessarily correlate (43). The reasons for this somewhat paradoxical behavior remain to be determined. It is likely that the interactions between the $\alpha$-helix and the two terminal $\beta$-hairpins further modify the relative mechanical stability of the two hairpins.

**Comparison between single-molecule AFM studies and MD simulations**

The mechanical unfolding of GB1 has been simulated in MD simulations (38–40). MD simulations predict that the forced unfolding of GB1 proceeds through two highly similar pathways (38,39). In the first unfolding pathway, $\beta$-hairpins 1 and 2 slide concurrently against each other along the $\alpha$-helix. In the second unfolding pathway, the second $\beta$-hairpin detaches from the rest of the GB1 and unravels, resulting in an intermediate state consisting of the intact $\beta$-hairpin 1 and $\alpha$-helix. The differences between the two pathways are subtle, and the main divergence lies in the time at which contacts between the two terminal $\beta^\prime$-hairpins and the $\alpha$-helix break.

The structure of GB1’s mechanical unfolding transition state as mapped by our experimentally determined $\phi^{M2+U}$ is largely consistent with MD simulation trajectories, where the first $\beta$-hairpin and $\alpha$-helix remain largely intact while the contacts in the two force-bearing strands are completely disrupted.

MD simulations also raise the possibility for an alternative interpretation of the partial $\phi^{M2+U}$-value observed for G42-55. We interpret the partial $\phi^{M2+U}$-value observed for G42-55 as an indication that the bi-His site 42-55 in the mechanical unfolding transition state is partially disrupted to a structure with a lower Ni$^{2+}$ binding affinity, suggesting that the C-terminal $\beta$-hairpin is partially unstructured in the mechanical unfolding transition state. However, MD simulations predicted two different unfolding pathways (38), in which the rupture of the C-terminal $\beta$-hairpin occurs either simultaneously with or after the disruption of the contacts between strands 1 and 4. In this vein, the partial $\phi^{M2+U}$-value observed for G42-55 could be interpreted as a measure of the heterogeneity of the unfolding pathways, as originally proposed by Krantz and Sosnick (7) and Sosnick et al. (8) in their development of the $\psi$-value analysis method. Different unfolding pathways should exhibit different unfolding signatures in force-extension curves at different pulling speeds. A recent MD simulation study predicted that at very low pulling forces in constant force experiments, GB1 would unfold following an alternative unfolding pathway (37). However, within the range of the pulling speeds we used (50–4000 nm/s) and the range of unfolding forces we observed in constant velocity experiments (100–200 pN), we did not observe any sign of multiple unfolding pathways for GB1. Moreover, because the mechanical stability of G42-55 shows a distinct mechanical stability in the apo- or Ni$^{2+}$ form, it is not possible to tune the mechanical stability continuously as in a chemical $\psi$-value analysis (7,8). Thus, it is difficult to distinguish between the two scenarios in attempting to explain the partial $\phi^{M2+U}$-value observed for G42-55.

Nonetheless, an engineered metal chelation-based $\phi^{M2+U}$-value analysis, as demonstrated here, provides an excellent experimental means of mapping the mechanical unfolding transition state, as well as a benchmark for MD simulations, just as $\phi^\prime$ and $\psi$-value analyses can be used to achieve a synergistic understanding of protein folding/unfolding mechanisms by combining experiments and simulations.

**Comparison of the mechanical unfolding mechanism for GB1 and protein L**

Although their sequence identity is low, protein L and GB1 share a similar overall topology (34). However, protein engineering studies revealed that these proteins exhibit very different chemical folding transition states: for protein L, the first $\beta$-turn folds first in the transition state while the second $\beta$-turn remains disrupted, whereas for GB1, it is the second $\beta$-turn that folds first (34). Despite this distinction, MD simulations predicted a similar unfolding mechanism for both proteins, i.e., that the second $\beta$-hairpin unravels first in the mechanical unfolding transition state (38). Results from a mechanical $\phi^\prime$-value analysis of protein L are consistent with those obtained in MD simulations (17). Together with the outcomes presented here, these results suggest that protein L and GB1 share a similar mechanical unfolding mechanism. It remains to be determined whether this is true for other proteins with similar $\beta$-grasp fold structures, such as ubiquitin.

**CONCLUSION**

Using engineered bi-His mutants, we were able to directly map the mechanical unfolding transition-state structure of protein GB1 and thus provide the first (to our knowledge) experimental picture of GB1’s mechanical unfolding transition state. Our results show that contacts between force-bearing $\beta$-strands 1 and 4 are largely disrupted in the transition state, whereas the first $\beta$-hairpin and $\alpha$-helix are largely intact. The second hairpin is partially disrupted at this point of unfolding under force. Our results are in good agreement with MD simulations, and thus provide a critical validation of MD simulation results. We anticipate that this method can be widely used to probe the mechanical unfolding transition state of elastomeric proteins under
a stretching force, as well as the possible heterogeneity of multiple mechanical unfolding pathways.

**SUPPORTING MATERIAL**

A table and four figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(12)00794-1.

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