Single Molecule Force Spectroscopy Reveals the Molecular Mechanical Anisotropy of the FeS₄ Metal Center in Rubredoxin

Peng Zheng,† Chih-Chung Chou,† Ying Guo,† Yanyan Wang,†‡ and Hongbin Li†‡

†Department of Chemistry, University of British Columbia Vancouver, British Columbia V6T 1Z1, Canada
‡State Key Laboratory of Precision Measurements Technology and Instruments, School of Precision Instrument and Opto-Electronics Engineering, Tianjin University, Tianjin, 30072 P. R. China

ABSTRACT: Mechanical anisotropy is an important feature of materials. Depending on the direction it is pulled, a material can exhibit very different mechanical properties. Mechanical anisotropy on the microscopic scale has also been observed for individual elastomeric proteins. Depending upon the direction along which it is stretched, a protein can unfold via different mechanical unfolding pathways and exhibit vastly different mechanical stability. However, it remains to be demonstrated if the concept of mechanical anisotropy can be extended to the molecular scale for small molecular objects containing only a few chemical bonds. Here, we choose the iron–sulfur center FeS₄ in the simplest iron–sulfur protein rubredoxin as a model system to demonstrate the molecular level mechanical anisotropy. We used single molecule atomic force spectroscopy to investigate the mechanical rupture of the FeS₄ center along different pulling directions. The FeS₄ cluster is a simple molecular object with defined three-dimensional structure, where a ferric ion and four coordinating cysteinyl ligands are arranged into a distorted tetrahedral geometry. Mutating two specific residues in rubredoxin to cysteines provides anchoring points that enable us to stretch and rupture the FeS₄ center along five distinct and precisely controlled directions. Our results showed that the mechanical stability as well as the rupture mechanism and kinetics of the FeS₄ center are strongly dependent upon the direction along which it is stretched, suggesting that the very small and simple FeS₄ center exhibits considerable mechanical anisotropy. It is likely that structural asymmetry in the FeS₄ cluster and the modulation of the local environment due to partial unfolding of rubredoxin are responsible for the observed mechanical anisotropy. Our results suggest that mechanical anisotropy is a universal feature for any asymmetrical three-dimensional structure, even down to a molecular scale, and such mechanical anisotropy can be potentially utilized to control the mechanochemical reactivity of molecular objects.

INTRODUCTION

Due to structural anisotropy, mechanical properties of macroscopic materials often exhibit anisotropy, where a material can exhibit vastly different mechanical properties depending on the direction in which it is pulled, which in turn determines the specific applications of these materials.1,2 Graphite is a well-known case of anisotropy, showing a vastly different stability when stretched along the direction that is parallel or perpendicular to its molecular ring structure. Over the past decade, the development of single molecule force spectroscopy techniques has made it possible to study mechanical anisotropy on the microscopic scale on individual elastomeric proteins.3−10 Such mechanical anisotropy on the microscopic scale was first observed on a small all-β protein E2lip38 and a small α/β protein ubiquitin,5 and later in many other proteins.4,5,7,8,10−12 By selecting two well-defined residues, a protein can be stretched along different directions in single molecule force spectroscopy experiments. In these experiments, the pulling direction is determined by the two anchoring points, and the protein chain (including folded and unfolded sequences) serves as a rope to deliver the force to the protein via the two anchoring points. As a consequence, a protein can unfold along different pathways and exhibits different mechanical stability depending on the direction along which it is stretched.3−5,10,12,13 Molecular dynamics simulations corroborated such mechanical anisotropy and revealed that the molecular origin of mechanical anisotropy lies in different interactions that are to be ruptured as the protein is stretched along different pulling directions.5,5−7,10 These results provide a reliable means to manipulate single protein along different directions using single molecule force spectroscopy techniques. Although microscopic mechanical anisotropy is observed in individual proteins, hundreds of chemical bonds and non-covalent interactions are involved during the mechanical unfolding process, even for small proteins with <100 amino acid residues. It remains to be established whether mechanical anisotropy can be observed on the molecular scale for small molecular objects containing only a few chemical bonds, such as metal clusters.14,15 Moreover, it is yet to be demonstrated...
how a stretching force can be applied to small molecular objects along distinct and well-controlled directions due to the size of such small molecular objects. To address these questions, we have combined single molecule atomic force microscopy (AFM) and protein engineering techniques to investigate how a naturally occurring metal cluster responds to mechanical stretching forces that are applied in several distinct and precisely controlled directions.

The FeS₄ metal center in the simplest iron–sulfur protein *Pyrococcus furiosus* rubredoxin (pf-RD) was chosen as the experimental subject due to its small size and structural simplicity. Metal cluster FeS₄ occurs naturally in rubredoxins, and its inorganic analogues have also been previously synthesized. The FeS₄ cluster is a simple molecular object with defined three-dimensional (3D) structure, where a ferric ion is coordinated by four cysteinyl ligands arranged into a tetrahedral geometry (Figure 1A).²²

![Figure 1](image.png)

Figure 1. Details of the rubredoxin structure. (A) The 3D structure of rubredoxin, which contains a FeS₄ center where a ferric ion is coordinated by four sulfur atoms from cysteine residues. The bottom is a schematic of rubredoxin with the tetrahedron FeS₄ center highlighted. (B) The side-view of rubredoxin. The bottom is a simple line drawing schematic showing the overall structure of rubredoxin. Dotted lines indicate the backbone hydrogen bonds connecting β-strands. Residues that are outside the FeS₄ center are colored in blue, while residues that are sequestered by the FeS₄ center are colored in red.

Depending on how force is applied to this geometry, the FeS₄ cluster can be stretched along a maximum of six distinct pulling directions, thus offering an ideal model system to study potential molecular level mechanical anisotropy.

For the FeS₄ cluster in rubredoxin, two CXXC chelation loops coordinate the ferric ion by forming four ferric–thiolate bonds (for simplicity, these ferric–thiolate bonds are abbreviated as Fe−S(Cys5); Fe−S(Cys8); Fe−S(Cys38); and Fe−S(Cys41) bond in this study), and the center itself assumes a pseudotetrahedral geometry. The FeS₄ center divides rubredoxin into two parts: residues 1–4 and 42–53 are outside the center, while residues 5–41 are trapped inside the center (Figure 1B). In our previous work, we have shown that it is possible to mechanically unfold rubredoxin and rupture the FeS₄ center using the AFM by stretching the wild-type rubredoxin along its N- and C-termini.²⁷⁻²⁸ In this pulling geometry, after structural elements outside of the FeS₄ center have been unfolded, the stretching force will be applied to the FeS₄ center along the direction defined by Cys5 and Cys41. These observations have been validated recently by quantum mechanics/molecular dynamics calculations of the mechanical unfolding of rubredoxin and the rupture of FeS₄ center.²⁴ In this study, we will investigate how the FeS₄ center responds to the stretching force that is applied to the center along different pulling directions. Although the FeS₄ center is the subject of interest in this study, the protein framework surrounding the metal center is also critical for discerning the mechanical response of FeS₄ when stretched along different directions. On the one hand, rubredoxin framework provides an indispensable handle to apply force to the FeS₄ center. On the other hand, the unique contour length increment from the partial unfolding of rubredoxin and the rupture of metal center under different pulling direction provide an unambiguous signature to validate our designed pulling direction on the FeS₄ center of rubredoxin.

### METHODS AND MATERIALS

**Protein Engineering.** The genes encoding bicycisteine variants RDI149, RD154, RD153, RD135, RD135 and RD6,40 were engineered using standard site-directed mutagenesis methods based on wild-type rubredoxin gene. The numbers in each variant indicate the residues that are mutated to cysteines. The two cysteine substitutions were introduced into the rubredoxin gene in two sequential steps. DNA sequences were confirmed by DNA sequencing. All proteins were overexpressed in the DH5α strain of *E. coli* and purified by Co⁺⁺−affinity chromatography using TALON His-Tag purification resins (Clontech). Using a 3K MWCO Amicon ultra centrifugal filter (Millipore), the protein solution was exchanged into Tris buffer (pH 8.5, 10 mM). The Fe form rubredoxin variants were then separated using ion exchange chromatography. Finally, polyproteins were obtained using a thiol-maleimide coupling reaction between the cysteines of rubredoxin and BM(PEO)₃. All the rubredoxin mutants show characteristic UV−vis spectra that are comparable to that of wild-type rubredoxin.

**Single Molecule AFM Experiments.** A custom-built AFM as described previously was used for all single-molecule AFM experiments reported here. The spring constant (typically around 40 pN/nm) of each Si₃N₄ cantilever (MLCT probe, Bruker Corp.) was obtained in solution using the equipartition theorem before each experiment. Data acquisition and analysis were done using custom-written codes in Igor Pro (WaveMetrics, Lake Oswego).

Single molecule AFM experiments were performed following well-established method. In a typical experiment, ~2 μL of the polypeptide solution at a concentration of ~2 mg/mL was added to a clean glass coverslip covered by ~50 μL of Tris buffer (pH ~7.5). The protein was allowed to absorb for ~5 min before force–extension measurements. During AFM experiments, the cantilever was brought into contact with the substrate at a contact force of ~1 nN and then retracted away from the surface to pick up and stretch polypeptide molecules. A typical pulling speed used in our force–extension measurements is 400 nm/s. On average, about 2% of trials lead to the picking up and stretching of single polypeptide molecules.

Individual force peaks in the sawtooth-like force–extension curves were fitted using the worm-like chain (WLC) model of polymer elasticity to measure the contour length increment upon domain unfolding and the rupture of FeS₄ center. The persistence length used in the WLC fitting is ~0.4 nm, which is typical for polypeptides with a long spacer or unfolded domain. The mechanical rupture process of the FeS₄ center can be modeled as a two-state rupture process with force–dependent rate constants:³⁶⁻³⁸

\[
k(\bar{F}) = k_{eq} \times \exp \left( \frac{\Delta F - F}{k_B T} \right)
\]

where \(k(\bar{F})\) is the rate constant for dissociation at a stretching force \(F\), \(k_{eq}\) is the spontaneous dissociation rate constant at zero force, \(\Delta F\) is the distance between the bound and transition states, \(k_B\) is the Boltzmann constant, and \(T\) is the absolute temperature. Since the force–extension
measurements were done on polypeptides at a constant velocity, the force evolves in a complex manner in the sawtooth-like force-extension curves, making it difficult to derive analytical solutions for the relationship between the rupture force and pulling velocity.\textsuperscript{39,40} Thus we used well-established Monte Carlo simulations procedure\textsuperscript{35,41} to estimate the dissociation rate constant \( k_d \) at zero force and \( \Delta x \) by including similar experimental conditions. Briefly, in such Monte Carlo simulations, we stretch a virtual polypeptide made of folded rubredoxin domains of an initial contour length of 30 nm at a certain pulling speed from zero extension. The persistence length of the unfolded polypeptide chain is taken as 0.4 nm. The contour length of the polypeptide chain will change upon the unfolding of rubredoxin domains. In every time step (typically 0.1 ms), the extension \( x \) is increased, and the entropic force acting on the polypeptide chain at the current extension \( x \) is then calculated using the interpolation formula of WLC model of polymer elasticity:\textsuperscript{34} \( F(x) = \left( k_BT/p \right) (x/L + 0.25 \cdot \left( 1 - x/L \right)^{-2} - 0.25) \). The probability of observing the rupture of any Fe\textsubscript{S}\textsubscript{4} center in rubredoxin was calculated using the following equation: \( P_u = N_f k_f \Delta t \), where \( P_u \) is the rupture probability of any Fe\textsubscript{S}\textsubscript{4} center, \( N_f \) is the number of folded rubredoxin domains, \( k_f \) is the force-dependent rate constant of the Fe\textsubscript{S}\textsubscript{4} center, and \( \Delta t \) is polling time interval. Then each domain was polled to determine the status of the Fe\textsubscript{S}\textsubscript{4} center,\textsuperscript{35} following the Monte Carlo approach. Based on this method, force-extension curves for different rubredoxin polypeptides were generated (typically ~100 runs), and the average rupture force at each pulling speed was calculated and compared with experimental values.\textsuperscript{35}

\section*{RESULTS}

\textbf{Design Principle for Probing Mechanical Anisotropy of the Fe\textsubscript{S}\textsubscript{4} Center.} The first step in exploring the molecular mechanical anisotropy of the Fe\textsubscript{S}\textsubscript{4} center is choosing residues, or anchoring points, at which the stretching force can be applied to the Fe\textsubscript{S}\textsubscript{4} cluster along the polypeptide rope. Thanks to the simple structure of the metal center, the four ferric-thiolate bonds naturally allow the metal center to be stretched in different directions. Thus, selecting different combinations of the two individual ferric-thiolate bonds enables us to fully explore the mechanical anisotropy of the target. Based on the structure of the Fe\textsubscript{S}\textsubscript{4} center in rubredoxin, four different bond combinations were chosen: Fe\textsuperscript{2+}–S(Cys5)/Fe\textsuperscript{2+}–S(Cys41), Fe\textsuperscript{2+}–S(Cys8)/Fe\textsuperscript{2+}–S(Cys41), Fe\textsuperscript{2+}–S(Cys8)/Fe\textsuperscript{2+}–S(Cys38), and Fe\textsuperscript{2+}–S(Cys5)/Fe\textsuperscript{2+}–S(Cys38).

The Fe\textsubscript{S}\textsubscript{4} center is covalently enclosed in the protein structure, making it challenging to directly apply the stretching force to the metal center along different directions. Since the stretching force is transmitted from one anchoring point to the other through the polypeptide chain, we can use the polypeptide chain of rubredoxin as a handle to apply force to the Fe\textsubscript{S}\textsubscript{4} center along a defined pulling direction. For example, stretching rubredoxin along its N- to C-termini will first lead to the unfolding of structural elements that are outside of the Fe\textsubscript{S}\textsubscript{4} center (blue residues in Figure 1B), then the stretching force can be applied to the Fe\textsubscript{S}\textsubscript{4} center via Cys5 and Cys41, thus defining the direction along which the Fe\textsubscript{S}\textsubscript{4} center is stretched.
Using similar strategies, we can stretch the FeS₄ center along the other three distinct directions. To do so, we adapted a cysteine tether approach, which has been used extensively in investigating the mechanical anisotropy of proteins, 4,5,7,8 to study the molecular level mechanical anisotropy of the FeS₄ center. In this strategy, a pair of residues outside the metal center in rubredoxin are mutated to cysteines, which serve as the anchoring points for the stretching force to be applied to different parts of rubredoxin and eventually to the FeS₄ center along different directions17 (Supporting Information) (Figure 2). In addition, stretching rubredoxin along the direction set by these two exogenous cysteine residues forces rubredoxin to unfold in a specific pathway, giving rise to a specific contour length increment upon the rupture of the FeS₄ center and subsequent unfolding of rubredoxin.17 Such specific contour length increments allow for unambiguous identification of the metal cluster rupture events during single molecule stretching experiments.

Following this design principle, we engineered four different bicysteine rubredoxin variants. Individual variants were chemically linked through the two engineered cysteines through a maleimide–thiol coupling reaction, resulting in a polypeptide (RD-variant), composed of identical tandem repeats of the rubredoxin variant with specific linkages. These introduced cysteine residues serve as the anchoring sites, where force used to ultimately unfold the metal center will be applied.

**Mechanical Anisotropy of the FeS₄ Center at the Single Bond Level.** We first stretched the FeS₄ center along the direction of Fe–S(Cys5) and Fe–S(Cys41) bonds using RD1,49 variant. Stretching the (RD1,49)polypeptide results in characteristic sawtooth-like force–extension curves resulted from unfolding of rubredoxin.17,28 Fitting the force–extension trace using the WLC model of polymer elasticity25 reveals force peaks with two different contour length increments (ΔLc): ~4.5 and ~13.0 nm (Figure S1). This result supports a two-step unfolding scenario for RD1,49 (Figure 2B). Residues 1–4 and 42–49, located outside the metal center, unfold first at ~62 pN (Figure S1A, Table S1), and their extension leads to force peaks with a ΔLc of 4.4 ± 1.0 nm (Figure S1B) (colored in blue in Figure 2), which is close to the expected value of 3.95 nm (12aa*0.365 nm/aa + 0.65 nm − 1.08 nm = 3.95 nm, where 0.365 nm/aa is the length per amino acid, 0.65 nm is the through space distance between Cys5 and Cys41, and 1.08 nm is the through space distance between residues 1 and 49 in folded rubredoxin). The subsequent rupture of the FeS₄ center leads to force peaks with a ΔLc of 13.0 ± 0.8 nm (colored in red in Figure 2), close to the expected contour length increment (37aa (residues 5–41)*0.365 nm/aa − 0.65 nm = 12.9 nm, where 0.65 nm is the through space distance between Cys5 and Cys41). Thus, the 227 ± 79 pN (n = 1063) force arising from peaks with a ΔLc of ~13 nm can be unambiguously assigned as the rupture force of the FeS₄ center as it is stretched across Fe–S(Cys5) and Fe–S(Cys41) bonds. The rupture of FeS₄ center was then investigated as force is applied through Fe–S(Cys8) and Fe–S(Cys41) bonds within the (RD1,49)polypeptide (Figures 3A,B and S2). Similar to the two-step unfolding scenario observed for RD1,49, the AFM results from RD1,49 show that RD15–49 also unfolds in a two-step manner (Figures 3C and S2A). The first step corresponds to the rupture and extension of the protein structure outside the metal cluster (residues 49–41) as well as the alignment of residues 8–15 (colored in blue) and results in force peaks with an average unfolding force of 85 ± 30 pN (Figure S2B) and a ΔLc of 4.5 ± 0.8 nm (Figure S2C), which is close to the expected value of 4.6 nm ((8 + 9)aa*0.365 nm/aa − 1.62 nm, where 1.62 nm is the through space distance between residues 15 and 49) (Figure S2). After the rupture of these secondary structures, the stretching force will be applied...
to the metal cluster directly via the Fe–S(Cys8) and Fe–S(Cys41) bonds. Rupture of the FeS4 cluster results in a force peak with a ΔLc of 6.4 ± 0.7 nm (Figure S2C). The average rupture force of the FeS4 cluster along the Fe–S(Cys8) and Fe–S(Cys41) direction is 152 ± 60 pN (n = 708) (Figure 3D), which is less than that observed along the Fe–S(Cys5) and Fe–S(Cys41) direction of RD1,49. This result clearly indicates that the stability of the FeS4 center varies considerably when stretched from different directions, providing experimental evidence for the mechanical anisotropy of FeS4 center in rubredoxin.

Following similar strategies, we also measured the mechanical stability of the FeS4 cluster along two additional pulling directions (Fe–S(Cys38)–Fe–S(Cys8); Fe–S(Cys5)–Fe–S(Cys38)) using rubredoxin variants (RD15,35) and (RD1,35)n. Stretching the metal center from Fe–S(Cys5) to Fe–S(Cys38) is achieved using RD1,35 (Figures 4 and S3). Stretching RD1,35 results in an one-step rupture of the FeS4 center. The rupture of FeS4 center and unfolding of rubredoxin result in force peaks with a ΔLc of ~11 nm, which arise from the rupture of the FeS4 center and unfolding of rubredoxin. A histogram of rupture force shows an average unfolding force of 242 ± 40 pN (n = 1340). Solid line is the Gaussian fit to the experimental data.

To the metal cluster directly via the Fe–S(Cys8) and Fe–S(Cys41) bonds. Rupture of the FeS4 cluster results in a force peak with a ΔLc of 6.4 ± 0.7 nm (Figure S2C). The average rupture force of the FeS4 cluster along the Fe–S(Cys8) and Fe–S(Cys41) direction is 152 ± 60 pN (n = 708) (Figure 3D), which is less than that observed along the Fe–S(Cys5) and Fe–S(Cys41) direction of RD1,49. This result clearly indicates that the stability of the FeS4 center varies considerably when stretched from different directions, providing experimental evidence for the mechanical anisotropy of FeS4 center in rubredoxin.

Following similar strategies, we also measured the mechanical stability of the FeS4 cluster along two additional pulling directions (Fe–S(Cys38)–Fe–S(Cys8); Fe–S(Cys5)–Fe–S(Cys38)) using rubredoxin variants (RD15,35) and (RD1,35)n. Stretching the metal center from Fe–S(Cys5) to Fe–S(Cys38) is achieved using RD1,35 (Figures 4 and S3). Stretching RD1,35 results in an one-step rupture of the FeS4 center. The rupture of FeS4 center and unfolding of rubredoxin result in force peaks with a ΔLc of ~11 nm, which arise from the rupture of the FeS4 center and unfolding of rubredoxin. A histogram of rupture force shows an average unfolding force of 242 ± 40 pN (n = 1340). Solid line is the Gaussian fit to the experimental data.

Figure 4. Mechanical stability of FeS4 as it is stretched from Fe–S(Cys5) and Fe–S(Cys8). (A) Schematic showing the stretching scenario of RD1,35. Left panel: line drawing schematic; right panel: 3D structure representation with the same coloring scheme. Upon mechanical rupture of the FeS4 center, structural elements colored in red will be extended and contribute to the observed ΔLc, while sequences colored in black do not contribute to ΔLc. (B) A schematics shows how the tetrahedron FeS4 center is stretched in RD1,35 via anchoring ferric–thiolate bonds Fe–S(Cys5) and Fe–S(Cys38). (C) A typical force extension curve from the mechanical unfolding of RD1,35 shows force peaks with a ΔLc of ~11 nm, which arise from the rupture of the FeS4 center and unfolding of rubredoxin. (D) A histogram of rupture force shows an average unfolding force of 242 ± 40 pN (n = 1340). Solid line is the Gaussian fit to the experimental data.

Figure 5. Mechanical stability of FeS4 as it is stretched from Fe–S(Cys8) and Fe–S(Cys38). (A) Schematic showing how RD15,35 is stretched. Left panel: line drawing schematic; right panel: 3D structure representation with the same coloring scheme. (B) A schematics shows how the tetrahedron FeS4 center is stretched in RD15,35 via anchoring ferric–thiolate bonds Fe–S(Cys8) and Fe–S(Cys38). (C) A typical force extension curve from the mechanical unfolding of RD15,35. The rupture of FeS4 center and unfolding of rubredoxin result in force peaks with a ΔLc of ~3 nm. Dotted lines are WLC fits to the data. (D) Histogram of the rupture force shows an average value of 146 ± 49 pN (n = 985). Solid line is the Gaussian fit to the data.
Δ bonds are ruptured, which leads to the extension of six residues (colored in green). Sulfur atoms in the anchoring ferric and the small length gain upon the rupture of a single ferric length resolution of the AFM in stretching When Stretched from Cys5 to Cys38. thiolate bond in the FeS4 center in rubredoxin, it has been di ther measured and used for the displacement of the AFM AFM tip and the determination of the mechanical properties of the rubredoxin protein. However, the pulling direction of Cys5-Fe-Cys38 at the given pulling velocity of 400 nm/s. 5−35 (Figure S3). The rupture force measured along the direction of Cys5-Fe-Cys38 is 242 ± 40 pN (n = 1340) (Figure 4D). Similarly, the mechanical rupture of the FeS4 cluster along the direction of Cys8-Fe-Cys38 using RD15,35 also follows an one-step mechanism (with a ΔLc of ~ 2.7 ± 0.4 nm), giving an average mechanical rupture force of 146 ± 49 (n = 985) (Figures S4). From these results, it is evident that the very small molecular object FeS4 cluster exhibits strong mechanical anisotropy when stretched. In particular, the metal cluster is most resistant along the pulling direction of Cys5-Fe-Cys38 at the given pulling velocity of 400 nm/s.

Mechanical Rupture Mechanisms of the FeS4 Center When Stretched from Cys5 to Cys38. Due to the limited length resolution of the AFM in stretching flexible polymers and the small length gain upon the rupture of a single ferric–thiolate bond in the FeS4 center in rubredoxin, it has been difficult to discern the detailed mechanical rupture mechanism of the FeS4 center. Using a loop elongation variant of rubredoxin, we have shown that the mechanical rupture of the FeS4 center follows a stochastic mechanism involving concurrent rupture of multiple ferric–thiolate bonds as well as a stepwise rupture mechanism with the concurrent rupture mechanism being dominant. However, such mechanisms are yet to be confirmed in wild-type rubredoxin.

A close analysis of the force peaks in the force–extension curves of RD1,35 provides valuable insights into the mechanical rupture mechanism of the FeS4 center in wild-type rubredoxin. We found that the majority of mechanical rupture events of the FeS4 center in RD1,35 occurs in a single step fashion and results in a single force peak, suggesting that the rupture of the FeS4 center involves concurrent rupture of multiple ferric–thiolate bonds. In addition, a small population (~10%) of rupture events of the FeS4 center appears to occur in a two-step fashion (Figure 6A,B), resulting in clearly resolvable twin force peaks (circled in Figure 6A, also see Figure S6). The first step results in a rupture event with a ΔLc of ~2 nm, and the second step results in a rupture event with a ΔLc of ~9 nm, with the sum of the two ΔLc being 11 nm, which agrees well with the ΔLc observed for the one-step rupture of the FeS4 center (Figure 4). Fitting the twin peaks using WLC model with the same persistence length yields ΔLc1 of 1.9 ± 0.3 nm and ΔLc2 of 9.0 ± 0.3 nm (Figure 6C). The corresponding force value for the twin force peaks is 235 ± 35 pN (n = 177) and 239 ± 38 pN (n = 173), respectively (Figure 6D,E). This first step (ΔLc of ~2 nm) is likely due to the rupture of Fe–S(Cys5) and Fe–S(Cys38) thiolate bonds (with Fe–S(Cys8) and Fe–S(Cys41) remain intact) and the subsequent extension of residues 5−8 and 38−41 (6aa*0.365 nm/aa). The second step (ΔLc of 9

Figure 6. The mechanical rupture of the FeS4 center can occur in a stepwise fashion when it is stretched from Cys5-Fe-Cys38 direction. (A) A typical force–extension curve reveals a two-step rupture mechanism. Circled force peaks are clearly resolvable twin peaks with ΔLc of ~2 and 9 nm, respectively. (B) A simple schematics of FeS4 shows how Fe–S(Cys8) and Fe–S(Cys38) bonds are stretched after Fe–S(Cys5) and Fe–S(Cys38) bonds are ruptured, which leads to the extension of six residues (colored in green). Sulfur atoms in the anchoring ferric–thiolate bonds are circled. (C) ΔLc histogram of the twin force peaks. The first peak is with a ΔLc of 1.9 ± 0.3 nm (green), and the second peak is with ΔLc of 9.0 ± 0.3 nm (blue). (D,E) Histogram of the rupture forces from peaks with a ΔLc = 1.9 ± 0.3 nm (D) and ΔLc = 9.0 ± 0.3 nm (E). The average rupture force for the two rupture steps is 235 ± 35 pN (n = 173) and 239 ± 38 pN (n = 173), respectively.
have investigated, the metal center is always stretched by subjecting two ferric–thiolate bonds to the stretching force. The FeS\textsubscript{4} center in rubredoxin is constituted by two CXXC chelation loops such that the two neighboring cysteinyl ligands (Cys5- and Cys8- or Cys38- and Cys41) are connected by the CXXC loops, which is different from synthetic inorganic FeS\textsubscript{4} analogues (in which the cysteinyl ligands are monodentate). This unique arrangement provides additional possible geometries for probing the mechanical resistance of the FeS\textsubscript{4} center in \textit{pf}-rubredoxin (Figure 8A,B). Mutating residues 6 and 40 inside the chelation loops to cysteines enabled us to mechanically stretch the FeS\textsubscript{4} center along a unique direction such that ferric–thiolate bonds Fe–S(Cys5) and Fe–S(Cys8) bear the stretching force simultaneously on the side of the C5XXC8 loop while Fe–S(Cys38) and Fe–S(Cys41) bonds bear the load on the C38XXC41 loop simultaneously, due to the fact that the two anchoring residues are in the middle of the two CXXC loops, respectively. This pulling geometry will likely offer much higher mechanical resistance for the FeS\textsubscript{4} center when compared to the other four possible pulling geometries, since the four ferric–thiolate bonds will be subject to the stretching force simultaneously.

To experimentally investigate this possibility, we designed bicysteine variant RD6,40. Stretching the (RD6,40)\textsubscript{n} polypeptide results in sawtooth-like force–extension curves with a contour length increment \( \Delta L \) of 12.5 nm (Figures 8C and S5), resulting from the rupture of the FeS\textsubscript{4} center and subsequent unfolding of rubredoxin. As anticipated, the mechanical rupture force of 327 ± 137 pN (\( n = 130 \)) is much higher than that exhibited by other pulling geometries. In fact, this rupture force almost doubles that observed for RD15,35 where the FeS\textsubscript{4} center is ruptured along the direction of Fe–S(Cys8)–Fe–S(Cys38). This suggests that loading four ferric thiolate bonds directly and simultaneously offers the highest mechanical stability for the FeS\textsubscript{4} center.

\section*{Discussion}

The Molecular-Scale FeS\textsubscript{4} Cluster Exhibits Mechanical Anisotropy. By stretching the FeS\textsubscript{4} center from four different directions using single molecule AFM, we discovered that the mechanical stability of the metal center is depended on the direction of the applied force. The rupture force of the FeS\textsubscript{4} cluster measured from different pulling directions ranges from approximately 150 to 250 pN at a pulling speed of 400 nm/s. Compared with macromolecules such as proteins, the very small and simple FeS\textsubscript{4} center studied here exhibits considerable mechanical anisotropy. Our results suggest that this mechanical anisotropy may be a universal feature for any asymmetrical 3D structure, even down to a molecular scale. In addition, the mechanical rupture of the FeS\textsubscript{4} center exhibits different rupture kinetics and mechanism when the FeS\textsubscript{4} center is stretched along different directions. This observation suggests that such mechanical anisotropy can be potentially useful in controlling the mechanochemical reactivity of metal centers as well as other small molecular objects.

Furthermore, the metal center motif is a critical part of metalloproteins.\textsuperscript{14,15} Besides their functional roles, metal centers can play important structural roles in the (un)folding and stability of metalloproteins. For example, metal centers mediated by iron and zinc play important structural roles for iron–sulfur proteins and zinc-finger proteins.\textsuperscript{47–49} Thus, from the perspective of metalloproteins, the knowledge of stability of metal centers is important for understanding how metal-
FeS$_4$ cluster will have a Td symmetry. Therefore, all six pulling elements outside of the FeS$_4$ center unfolds at forces that are atoms with four corresponding ferric stretched, the FeS$_4$ cluster can be stretched along six distinct into a tetrahedron. Depending on which two ligands are
rubredoxin protein framework on the FeS$_4$ center should be
mechanically isotropic. Such molecular objects should be
mechanically anisotropic, although protein’s mechanical anisotropy is not the focus of this study. For example, upon stretching from its N–C-termini, structural elements outside of the FeS$_4$ center unfolds at forces that are below our detection limit, no unfolding force peaks were observed. In contrast, stretching rubredoxin via residue 1 and 49 results in clear unfolding force peaks of the structural elements outside of the FeS$_4$ center ($\Delta L_c$ of $\sim$4.5 nm and unfolding force of $\sim$60 pN (Figure 2 and Figure S1)). Thus, rubredoxin is a great example combining the mechanical anisotropy at the protein level as well as the level of the metal center.

Molecular Origin of Mechanical Anisotropy in the FeS$_4$ Cluster in Rubredoxin. The FeS$_4$ cluster consists of five atoms with four corresponding ferric–thiolate bonds arranged into a tetrahedron. Depending on which two ligands are stretched, the FeS$_4$ cluster can be stretched along six distinct pulling directions. If the four cysteinyl ligands are identical (such as the chemically synthesized analogue Fe(SPh)$_4$), the FeS$_4$ cluster will have a $T_d$ symmetry. Therefore, all six pulling directions should be equivalent due to the structural symmetry of the tetrahedron. Such molecular objects should be mechanically isotropic.

For the FeS$_4$ cluster in rubredoxin, all four ligands are cysteines; thus the cluster itself has high symmetry, raising the question as to why the FeS$_4$ in rubredoxin is not mechanically isotropic. To address this question, the influence of the rubredoxin protein framework on the FeS$_4$ center should be taken into account. On the one hand, the two CXXC chelation loops in rubredoxin are different, and amide backbone hydrogen bonds from secondary coordination sphere modify the ferric–thiolate bonds to different extents. All these effects lead to a distorted tetrahedral geometry of the FeS$_4$ center in rubredoxin and render an asymmetric protein environment for the FeS$_4$ center. The distorted tetrahedral geometry of the FeS$_4$ center has been demonstrated by X-ray and NMR studies, which have shown slightly different ferric–thiolate bond length. Such molecular asymmetry likely underpins the observed molecular mechanical anisotropy for the FeS$_4$ center in rubredoxin. Furthermore, when pulled from different directions in our experimental design, different portions of rubredoxin are ruptured. The partial unfolding of rubredoxin likely changes the local environment of the FeS$_4$ cluster, including the degree of solvent exposure. It is likely that mechanical anisotropy arises from structural asymmetry in the FeS$_4$ cluster and the modulation of the local environment due to partial unfolding of rubredoxin. Detailed computational studies, as those recently carried out for wild-type rubredoxin, should provide further insights into the contribution of these mechanisms and how different molecular interactions define mechanical anisotropy.

CONCLUSION

Combining protein engineering and single molecule AFM techniques, we have stretched and ruptured the FeS$_4$ metal center in rubredoxin along five distinct pulling directions. In these experiments, the protein structure of rubredoxin serves as an indispensable signature for identifying the mechanical rupture of the FeS$_4$ center. Our results showed that the mechanical stability and rupture mechanism of this small FeS$_4$ center, which consists of only four ferric thiolate bonds, strongly depend on the pulling direction along which it is stretched, suggesting considerable mechanical anisotropy for this small molecular object. Such mechanical anisotropy likely originates from the structural asymmetry of the metal center and the influence caused by the local protein environment. Our study extends the concept of mechanical anisotropy to the molecular level and suggests that mechanical anisotropy may be...
rupture of the FeS$_4$ center via force-bearing bonds Fe−S(Cys4) and Fe−S(Cys15) in RD1,35; mechanical rupture of the FeS$_4$ center via force-bearing bonds Fe−S(Cys35) and Fe−S(Cys38) in RD1,35; mechanical rupture of the FeS$_4$ center via anchoring residues 6 and 40; differences between the single-step and two-step rupture events of FeS$_4$ center in RD1,35. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

Corresponding Author
Hongbin@chem.ubc.ca

Notes
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We thank Ashlee Jollymore for critical reading of the manuscript. This work is supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), Canada Research Chairs Program and Canada Foundation for Innovation.

**REFERENCES**


(36) Bell, G. I. Science 1978, 200, 618.
(38) Evans, E.; Ritchie, K. Biophys. J. 1997, 72, 1541.
(40) Cao, Y.; Li, H. Langmuir 2011, 27, 1440.
(43) It is of note that although the number of data points in the second peak of the twin peaks is limited, we can use WLC fits to readily estimate the contour length increment during the first rupture event in the twin peak. Similar scenarios were also observed and treated in the mechanical unfolding of proteins, where unfolding intermediate states only have marginal kinetic stability or have short contour length increment (refs 57–59).
(44) It is important to note that current AFM techniques cannot directly resolve length changes due to the rupture of a single ferric–thiolate bond in a soft polypeptide chain. The fact that we can resolve the stepwise rupture mechanism in RD1,35 benefits from the extension of six residues (residue 8−and 38−41 upon the rupture of ferric–thiolate bonds Fe−S(Cys5) and Fe−S(Cys8)) in RD1,35; mechanical rupture of the FeS$_4$ center via force-bearing bonds Fe−S(Cys4) and Fe−S(Cys15) in RD1,35; mechanical rupture of the FeS$_4$ center via anchoring residues 6 and 40; differences between the single-step and two-step rupture events of FeS$_4$ center in RD1,35. This material is available free of charge via the Internet at http://pubs.acs.org.

a universal feature for any asymmetrical 3D structure, from macroscopic scale down to molecular scale.
(56) Blake, P. R.; Park, J.-B.; Zhou, Z. H.; Hare, D. R.; Adams, M. W. W.; Summers, M. F. Protein Sci. 1992, 1, 1508.