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Single molecule force spectroscopy: a new tool for bioinorganic chemistry

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Metalloproteins are essential in biology. The incorporation of metal ion into metalloproteins significantly expands protein functionality and enhances protein stability. Over the last few years, atomic force microscopy-based single molecule force spectroscopy (SMFS) has evolved into a unique tool allowing for probing metalloproteins and metal–ligand bonds one molecule/bond at a time. Mechanical strength of a wide variety of metal–ligand bonds has been measured in metal–ligand complexes as well as in metalloproteins, providing detailed information of their underlying free energy profiles and the influence of the protein environment on the bond strength. SMFS experiments have directly demonstrated the effect of the metal binding on the mechanical stability of proteins. Moreover, SMFS has enabled the direct observation of the unfolding and folding of metalloproteins, revealing detailed mechanistic insight into the unfolding pathways modulated by the metal center.

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Introduction

Metalloproteins are ubiquitous in nature and play essential roles in a wide variety of biological processes. In metalloproteins, metal ions coordinate with amino acid residues to constitute the metal center, which serves as the enzymatic active center to entail the functionality of proteins or as structural sites to facilitate protein folding and assembly [1]. To understand the structure, function and dynamics of metalloproteins, a suite of methodologies have been developed to tackle specific issues surrounding metalloproteins, and insights obtained using these tools have advanced our understanding of metalloproteins significantly [2–4]. Most of these methodologies provide ensemble average information about the given physical and chemical properties of the metalloprotein/metal center. Moreover, due to the complexity of metalloproteins, developing new tools remains an important task in the field. Over the last two decades, the development of single molecule techniques has provided new tools to probe metalloproteins at the single molecule level, in particular, single molecule fluorescence and single molecule force spectroscopy (SMFS) techniques [5,6]. This article will be focused on SMFS.

Over the last two decades, atomic force microscopy-based SMFS has evolved into a powerful technique in the field of single molecule biophysics and chemistry [7•]. SMFS has enabled the direct measurement of the inter/intramolecular interactions (such as ligand–receptor interaction [8,9] and covalent bonds [10•,11,12]) as well as the mechanical/elastic properties of a wide range of macromolecules, ranging from polysaccharides, DNA, synthetic polymers and all the way to proteins [13••,14–17,18•], at the single molecule level with an unprecedented pico-Newton resolution. These measurements have provided a rich wealth of information about the underlying free energy profiles and molecular mechanisms of these inter/intra-molecular interactions as well as the force-induced conformational transitions in macromolecules, which is otherwise difficult to obtain using traditional ensemble methods. In particular, SMFS has become an indispensable tool to study protein folding-unfolding dynamics as well as protein elasticity at the single molecule level [13••,18•,19,20••]. In combination with steered molecular dynamics simulations [21], SMFS studies have provided detailed mechanistic insights into the protein folding-unfolding mechanism, including those under the influence of ligand binding, as well as the elastic properties of elastomeric proteins and their regulation. Excellent reviews have been published to cover these diverse topics of SMFS and interested readers are referred to these reviews [14,16,17,22].

Here we will provide a brief review of the use of SMFS to probe the metal–ligand interactions, and the folding-unfolding dynamics of metalloproteins at the single molecule level. Compared with the extensive use of SMFS in folding-unfolding studies of non-metalloproteins, the use of SMFS to probe metalloproteins and metal–ligand interactions is a new emerging area that has experienced significant progress over the last few years. We will

highlight these key developments and offer a future perspective.

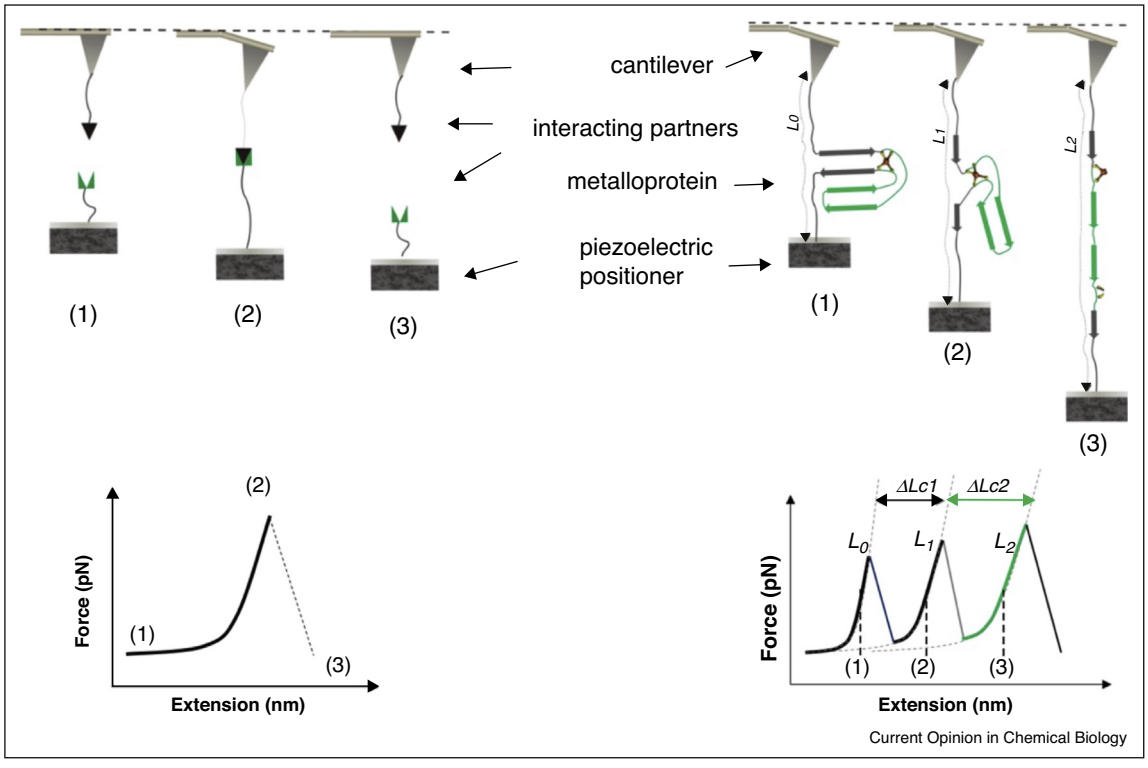
Measuring metal coordinate bond strength: from non-biological systems to metalloproteins

The development of SMFS allowed the direct measurement of the mechanical strength of metal–ligand bonds, ranging from relatively weak ones to very strong ones. Such direct measurements of chemical bond strength by single molecule AFM started from non-biological model systems. In these experiments, the interacting partners are immobilized on the AFM tip and substrate, respectively. To avoid short range non-specific interactions between the AFM tip and substrate, a polymer linker is often used as a spacer to immobilize the interacting partners [23] (Figure 1). Upon stretching, the polymer spacer extends and an entropic force develops, which directly acts upon the metal–ligand bond. When the metal–ligand bond ruptures, the rupture force can be directly recorded from the force–extension curves. And subsequent analysis will provide detailed information about the mechanical strength, free energy profile (including both free energy barrier for rupture and the width of the potential well) of the metal–ligand bond.

Au–S bond is one of the first metal–ligand bonds that have been studied using SMFS. In a pioneering SMFS study, the mechanical strength of the Au–S bond was measured using the polysaccharide amylose as a molecular linker and fingerprint for single molecule identification. It was found that the rupture force of Au–S bond is ~1.4 nN, which is consistent with the covalent nature of the Au–S bond [10•]. This result was confirmed by other studies on the mechanical strength of Au–S bond [24,25•]. The high mechanical strength of Au–S bond was also corroborated by quantum chemistry calculations [26].

Using similar strategies, a range of metal coordination bonds/interactions were measured at the single molecule

Figure 1



Principles of the AFM-based SMFS measurements of metal–ligand bond strength. Left panel: SMFS measurements of the metal–ligand bond strength. The interacting partners are immobilized onto the AFM tip and substrate via a polymer spacer. Stretching the metal–ligand bond leads to the stretching of the polymer spacer and the metal–ligand bond strength can be determined from the rupture force of the force–extension curve. Right panel: Schematics of the SMFS measurements of the mechanical strength of metal–ligand bonds in a metalloprotein and the resultant force–extension curve. *L* represents the contour length of the protein and can be measured by fitting the force–extension curve to the Worm-like chain model of polymer elasticity (dotted line). Stretching a metalloprotein containing a mechanically stable metal center will lead to the unfolding of the protein sequence outside the metal center first, giving rise a contour length increment of ΔLc1 (ΔLc1 = $L_1 - L_0$). Further stretching will lead to the rupture of the metal center, and the elongation of the polypeptide sequence sequestered by the metal center, giving rise to a contour length increment of ΔLc2 (ΔLc2 = $L_2 - L_1$). ΔLc2, which can be calculated from the number of amino acid residues sequestered by the metal center, provides an unambiguous fingerprint for identifying the metal–ligand rupture event.

level. First such studies were performed on the well-known NTA (nitrilotriacetate)/His(histidine)-tag system which is widely used for protein purification [27–29]. Different metal ion–NTA complexes were tested. The rupture force between $Histag_6$ and NTA-Ni(II), Co(II), Zn(II) and Cu(II) were measured. However, it is important to note that although these studies dealt with similar (even identical) metal–ligand systems, the measured rupture force in different studies showed relatively large variation, ranging from ~50 pN to more than 200 pN. The exact reason for this difference remains largely unknown. One possible reason may lie in the difference of the environment in which the metal–ligand bond is located in these studies. Another possible reason is the potential convolution of the single molecule rupture events with non-specific interactions. Developing more robust identification method of single molecule rupture events remains much needed.

The metal–terpyridine complex is another widely studied system involving metal–N interaction as the nitrogen atom from pyridine can coordinate with many different transitional metal ions. For example, the rupture force of ruthenium(II) bisterpyridine complex was determined to be ~95 pN [30]. A recent experiment on osmium (II) bisterpyridine system showed a similar rupture of 100 pN under normal condition. Interestingly, the rupture force varied from 80 pN to 130 pN under different reduction potential [31••]. It is suggested the modification of bond strength is due to the change of redox state of Os from II to III. This study demonstrates the unique modification effect from electrochemical properties of metal–ligand bond system. Moreover, SMFS experiments on ligand substitution reaction of a Pd–pyridine complex revealed that the applied mechanical force on the complex can accelerate the substitution reaction rate [32].

Direct measurement of metal–ligand bond strength in metalloproteins

Single molecule AFM provides a novel tool to study these metal–ligand interactions in their native biological environment: metalloproteins. To fully unfold and extend a metalloprotein, the rupture of the metal–ligand bonds in the metal center is a necessary step. Thus, the bond rupture can now be unambiguously identified from the mechanical unfolding event of a metalloprotein, as the contour length increment of the metalloprotein upon the rupture of the metal center can provide a fingerprint for identifying the mechanical rupture of the metal–ligand bonds. Moreover, the wide variety of metalloproteins can provide a rich platform that allows for a systematic and comprehensive measurement of the bond strength of a wide variety of metal–ligand bonds.

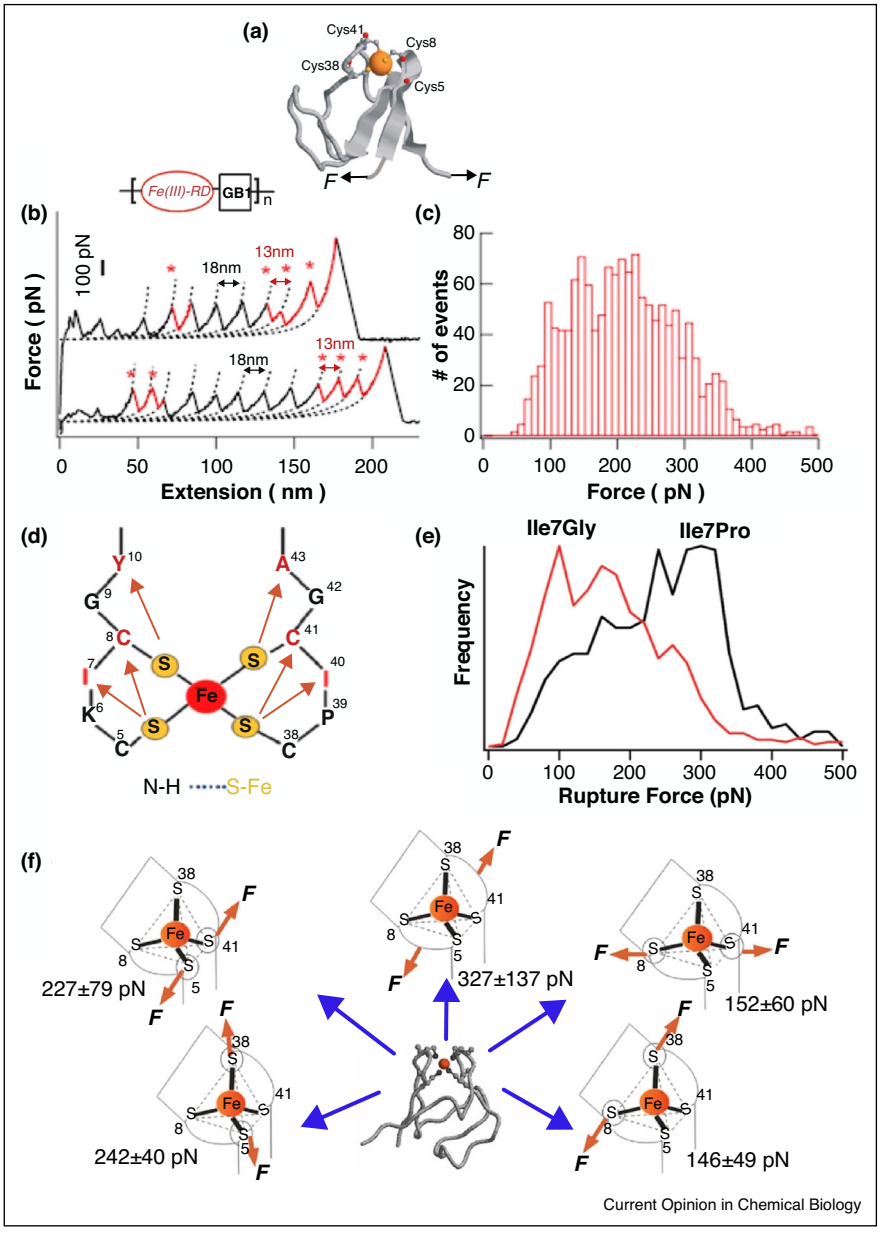
The first such single molecule AFM experiment was carried out to measure the strength of ferric–thiolate bond in a native metalloprotein rubredoxin [33••]. Rubredoxin is a small iron–sulfur protein, in which a ferric ion is coordinated by four cysteine residues to constitute the iron center (Figure 2). The rupture of the ferric–thiolate bonds in the metal center will lead to the stretching of the polypeptide sequence (residues 5–41) sequestered by the metal center, giving rise to an unfolding event of the contour length increment of ~13 nm (37 aa × 0.36 nm/aa). In the AFM experiment, the unfolding event of a single rubredoxin displays a contour length increment of 13 nm, thus this unfolding event can be attributed to the rupture of the ferric–thiolate bond in an unambiguous way. In this manner, the force required to rupture ferric–thiolate bond was measured to be ~210 pN at a pulling velocity of 400 nm/s. Although the ferric–thiolate bond is highly covalent (i.e. containing a large covalent nature), its rupture force is comparable to the unfolding force of some of the stable non-metalloproteins, but much smaller than that of Au–S bonds. Dynamic force spectroscopy studies allowed the determination of the spontaneous dissociation rate of the ferric–thiolate bond, as well as the distance between the ground and mechanical rupture transition state of the ferric–thiolate bond. These results were later corroborated by quantum chemical calculations [34•].

This study paved the way to directly measuring the strength of metal–ligand bonds in metalloproteins in an unambiguous way. Following a similar strategy, the mechanical rupture force of ferrous–thiolate bond, zinc–thiolate bond in rubredoxin as well as ferric–thiolate bond in a plant type [2Fe–2S] ferredoxin from *Cyanobacteria anabaena* were measured [33••,35,36•]. Using a blue copper protein azurin as a model protein, the bond strength of Cu–S bond as well as Cu–N bond was measured [37•]. It is interesting that despite the difference of the covalency of Cu–S and Cu–N bonds, the rupture force of both bonds are quite similar (55 pN versus 46 pN).

It has long been recognized that the protein environment can have a strong influence on the thermodynamic stability and physical properties, such as redox potential, of the metal center [38,39]. Similarly, the mechanical bond strength of metal–ligand is also strongly affected by the protein environment. For example, the ferric–thiolate bond from *Clostridium pasteurianum* rubredoxin is mechanically stronger than that from *Pyrococcus furiosus* rubredoxin, although the three dimensional structures of both rubredoxin are very similar [33••]. Tuning the protein environment can also help tune the mechanical strength of the metal–ligand bond. Using protein engineering techniques, the strength of N–H···Sγ hydrogen bonds in the secondary coordination sphere was modulated by site directed mutagenesis, which in turn affects the mechanical stability of Fe(III)–thiolate bonds [40•].

Recently, the bond strength of Au–S bond in a gold-specific binding protein, GoIB was directly measured

Figure 2



Single molecule AFM studies of ferric–thiolate bond rupture in rubredoxin. **(a)** The three dimensional structure of rubredoxin. **(b–c)** Typical force–extension curves of (rubredoxin-GB1)$_n$ clearly shows the unfolding events with ΔLc of ~13 nm, which correspond to the rupture of iron–sulfur center. The rupture force of ferric–thiolate bonds shows a broad distribution. **(d–e)** The strength of N–H···Sγ hydrogen bonds in the secondary coordination sphere affects the mechanical stability of Fe(III)–thiolate bonds of rubredoxin. These N–H···Sγ hydrogen bonds, which are formed between protein backbone amides with cysteinyl Sγ atoms in the secondary coordination sphere, are proposed to play important roles in modulating the functional and structural properties of the iron–sulfur center. **(f)** Mechanical anisotropy of the rupture force of the iron–sulfur center in rubredoxin. When pulled along different directions, the ferric–thiolate bonds rupture at different forces. (b–c), (d–e) and (f) are adapted from Refs. [33••,40•,42•], respectively.

[41•]. The rupture force of the Au–S bond in GoIB is surprisingly low (~165 pN), much lower than that Au–S bonds measured on different gold surfaces (~1000 pN). From the crystal structure of Au(I)-GoIB, it was found that the average Au–S bond length in GoIB is much longer than the reported average length of Au–S bonds. The primary reason for the much lower mechanical stability of the Au–S bond in GoIB was attributed to its longer bond length. This result again highlighted the influence of the unique biological environment on the

stability and strength of metal coordination bonds in proteins. However, the origin of this unusually long bond length for the Au–S bond in GoIB remains unknown and deserves further studies, both computational and experimental ones.

Another unique yet unrecognized aspect in other spectroscopic studies is the anisotropy of metal center in metalloproteins. Depending on the direction along which the force is applied, the same iron–sulfur center in rubredoxin ruptures at different forces, revealing the mechanical anisotropy of a metal center at the molecular scale [42•].

The binding of metal cofactors modulates the mechanical unfolding and mechanical stability of metalloproteins

The binding of metal cofactors to metalloproteins generally leads to thermodynamic stabilization of the protein. This effect is evident in many metalloproteins in which the metal center serves as a structural purpose rather than a functional purpose. This effect has been also used to enhance the thermodynamic stability of enzymes by engineering bi-histidine-based metal binding site [43], and has been used to develop methods to map the folding/unfolding transition state of proteins (the so-called ψ-value analysis) [44]. However, due to the difference between thermodynamic stability and mechanical stability, the effect of binding of metal cofactor may not necessarily correlate with their thermodynamic effect.

Using a series of engineered bi-His mutants of GB1, the effect of Ni^{2+} binding on the mechanical stability of GB1 was investigated in detail [45••]. It was discovered that depending on the site of the engineered bi-His site, the binding of metal cofactors can either stabilize protein mechanically or have no influence on the mechanical unfolding. If the metal binding site becomes disrupted, either partially or completely, in the mechanical unfolding transition state, the native state will be stabilized preferentially over the transition state, leading to an increase of the mechanical unfolding free energy barrier and increase of mechanical unfolding forces. Otherwise, the binding of metal cofactors will not bear any effect on the mechanical unfolding energetics or mechanical unfolding force. This conceptual understanding has led to the rationalization of the effect of metal cofactor binding on mechanical unfolding for various proteins. Mechanical stabilization effect has been observed on metalloproteins (such as calmodulin [46••] and rubredoxin [33••]) as well as metal-binding proteins, such as the calcium binding proteins D-crystallin [47] and cadherin [48], as well as BetP, a trimeric Na^+ coupled betaine symporter [49].

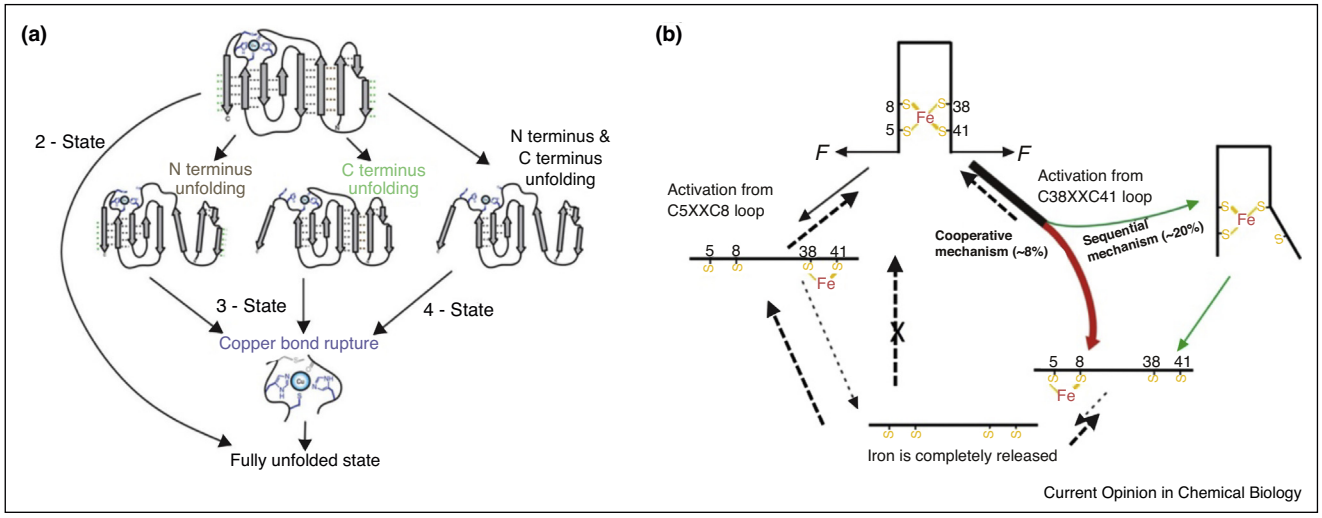
Moreover, this effect also led to the development of the so-called mechanical ψ-value analysis method to map the mechanical unfolding transition state of proteins, as demonstrated for a model protein GB1 [50•].

It is of note that many of the metal cofactors can only form relatively weak metal–ligand bond in metalloproteins, thus their rupture or reformation does not produce distinct mechanical signature during the mechanical unfolding/folding of metalloproteins [45••,46••,47–49]. Thus, the modulation of the mechanical unfolding/folding pathways by the binding of metal cofactor can only be deduced indirectly [46••,51]. However, if the metal–ligand bonds are strong, stable intermediate state(s) will appear in the unfolding process, and give rise to unfolding pathways that are distinct from apo-proteins.

Rubredoxin provides an excellent proto-typical model system in this regard [33••,52,53•]. Due to the high mechanical stability of ferric–thiolate bonds, the iron center in rubredoxin serves as a roadblock to resist mechanical unfolding. After the protein structure outside the metal center has unfolded (residues 1–5 and 41–56), the polypeptide sequence between Cys5 and Cys41 sequestered by the metal center forms an unfolding intermediate state. Further stretching of rubredoxin leads to the rupture of the metal center and the complete unfolding of rubredoxin. Thus, the metal center modulates the unfolding free energy profile of rubredoxin and results in a stable intermediate state. The mechanical unfolding of ferric–rubredoxin is thus a three-state unfolding process, Native-Intermediate-Unfolded. This modulation effect is a general feature among metalloproteins carrying stable metal–ligand bonds and has been observed in numerous model metalloproteins [35,37•,53•,54].

If the force required to rupture metal–ligand bond is comparable to that of protein secondary structures, further modulation of the unfolding pathway can then be observed. The mechanical unfolding of azurin provides a unique example in this aspect [37•]. It was discovered that, after initial unfolding, the metal center gives rise to a stable intermediate state. The competition between unraveling of the metal center and unraveling protein secondary structure resulted in a bifurcation of the unfolding pathways of azurin starting from its intermediate state (Figure 3). Moreover, how the metal center ruptures under a stretching force can provide further modulation of the unfolding pathway. For example, in a loop insertion mutant of rubredoxin, it was found that despite of its simple tetrahedral geometry, the rupture of FeS_4 center is stochastic and follows multiple complex pathways (Figure 3): concurrent rupture of two ferric–thiolate bonds versus sequential rupture [52]. Distinct rupture mechanism gives rise to different unfolding signatures in the observed force–extension curves. Similarly, the unfolding of DnaJ Hsp40 chaperone, which contains two consecutive highly conserved zinc finger domains,

Figure 3



Mechanical unfolding pathways of azurin and rubredoxin. **(a)** The mechanical unfolding of azurin follows multiple pathways and involves the rupture of the Cu–metal center. **(b)** The mechanical rupture of the iron–sulfur center in rubredoxin follows multiple, complex pathways that include concurrent rupture of multiple ferric–thiolate bonds as well as sequential rupture of ferric–thiolate bonds, which leads to the formation of intermediate species. (a) and (b) are adapted from Refs. [37•,42•], respectively.

also shows a complex multistate unfolding pathway, which is dictated by the sequential rupture of the two ZnS_4 centers in the protein [54]. Collectively, these studies illustrate the intricate effect that a metal center can have in modulating the mechanical unfolding pathway of a metalloprotein.

Directly monitoring the folding of metalloproteins

In classical ensemble experiments, the folding of metalloproteins *in vitro* often goes to two extremes: for calmodulin-like metalloproteins, folding can be fast and an easy task; for other metalloproteins such as rubredoxin, reversible folding-unfolding can be challenging, due to the loss or disintegration of the metal center. SMFS provides a unique tool that can potentially address both.

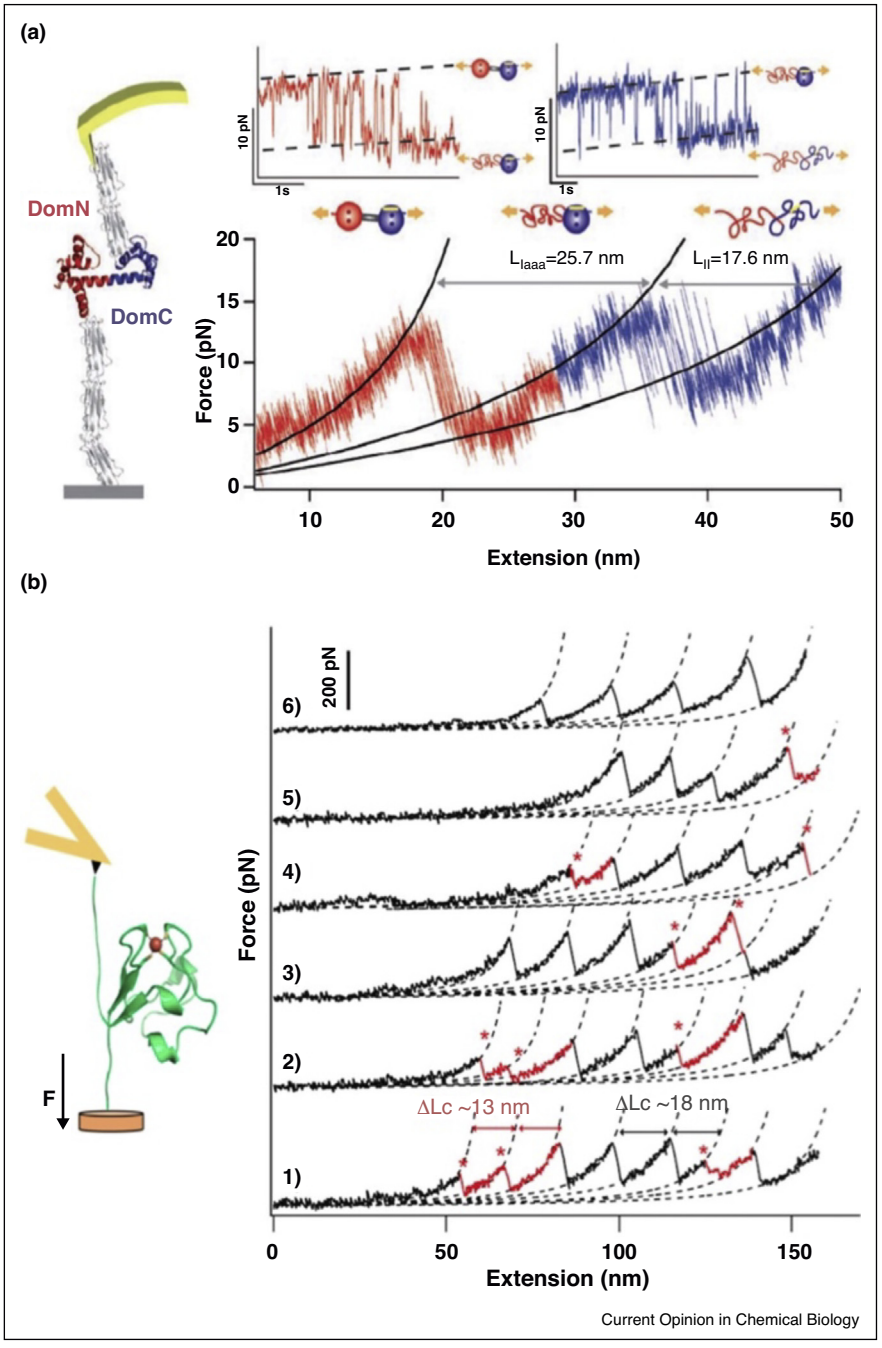
The unfolding–folding of single calmodulin was directly monitored using a low drift AFM [46••]. Under a pulling speed of 1 nm/s, which provides a force resolution of 2 pN, the folding and unfolding transitions of the two domains of calmodulin can be directly observed in real time (Figure 4) in the force–extension measurements. The effect of ligand binding (both Ca^{2+} and a small peptide ligand) on the folding was directly measured in these SMFS experiments, allowing for the reconstruction of the ligand-dependence of the free energy landscape. More complex folding pathways of calmodulin was later observed using a more sensitive force spectroscopy tool optical trapping [55].

For many other metalloproteins, *in vitro* reversible unfolding–folding experiments have been challenging. Using small iron–sulfur proteins as a model system, the folding of these metalloproteins [36•,53•] was probed directly via SMFS. In both rubredoxin and ferredoxin, it was observed that upon complete unfolding of the metalloprotein and the disruption of the iron–sulfur center, unfolded proteins can refold into their native holoform with the iron–sulfur center fully reconstituted. In the case of rubredoxin, it was found that after unfolding, ferric ion can remain bounded to the cysteine residues in the unfolded polypeptide chain for a short period of time, which can serve as a nucleation site to facilitate the folding of rubredoxin into its holo form. Dissociation of the ferric ion from the unfolded rubredoxin will lead to the disappearance of the folding events of rubredoxin in such experiments (Figure 4), providing supporting evidence to the proposed iron priming mechanism for the folding of rubredoxin. These studies demonstrate that the unfolding and folding of these metalloproteins (rubredoxin and ferredoxin) can be reversible at the single molecule level. However, in these studies, it was not possible to observe the folding of apo-form of the metalloproteins, thus the complete folding pathway of these metalloproteins remains to be established. The question that binding of the ferric ion occurs before or after the folding of apo-rubredoxin remains an open one.

Conclusions and further perspective

Due to their critical importance in biology, metalloproteins remain the focus of intense studies, from both

Figure 4



Single molecule AFM studies allow for the monitoring of the folding of metalloproteins. **(a)** The equilibrium unfolding–folding transitions of individual calmodulin in the presence of Ca^{2+} can be directly observed at low pulling speeds (1 nm/s). **(b)** Unfolded rubredoxin can refold back to its native holo-form when the ferric ion remains associated with the unfolded rubredoxin chain. The dissociation of ferric ion renders rubredoxin unable to refold to its native holo-form. (a) and (b) are adapted from Refs. [46••,53•], respectively.

functional and structural/physical perspectives. New experimental tools are needed to further advance our understanding of metalloproteins. As a new member of the tool box in studying metalloproteins, single molecule AFM has demonstrated its great potential in revealing new and complementary insights into metalloproteins that are otherwise difficult to obtain. Although significant progress has been made, using SMFS to study metalloproteins remains a new emerging area. More opportunities and challenges are still ahead.

Using SMFS, it is possible to obtain key parameters characterizing the bonding potential of the metal–ligand bonds in metalloproteins, including free energy barrier for rupture and the distance between the bound state to the transition state. Systematic studies will be required to investigate the bond strength of different metal–ligand bonds and reveal general rules governing the mechanical strength of such metal–ligand bonds. When combined with other experimental techniques, such as Raman spectroscopy and cyclic voltammetry, as well as more efficient quantum computation, these measurements will likely provide a more complete characterization of the free energy profile of the metal–ligand bonds at greater details.

SMFS has provided a unique tool to probe the folding/unfolding dynamics of metalloproteins. However, the complete folding pathway of metalloproteins remains to be observed and characterized due to the challenges associated with establishing the clear mechanical signatures of folding/unfolding of the apo-metalloproteins. To tackle these challenges, it is key to further improve the force resolution as well as time resolution of the AFM-based SMFS technique, as well as exploiting other SMFS techniques for studying metalloproteins, such as the optical tweezers and magnetic tweezers. These efforts will prepare SMFS for investigating folding/misfolding mechanisms of disease-causing metalloproteins, such as superoxide dismutase, a Zn–Cu metalloprotein that is directly linked to amyotrophic lateral sclerosis [56], and even probing metalloprotein folding in a more biologically relevant context, for example, in the presence of iron–sulfur biogenesis proteins.

Moreover, the use of SMFS allows one to manipulate the structure or environment around the metal center in a given metalloprotein. This unique feature may provide opportunities for probing the metal center in a more functional context, such as the chemical reactivity of the metal center, in a more biologically relevant environment. A recent experiment that probes the protonation and ligand substitution of the iron–sulfur center in rubredoxin is an endeavor along this line [57•]. To accomplish this goal in a more general context, coupling SMFS with other techniques, such as electrochemistry and single molecule fluorescence methods, will be essential. Such combined techniques will surely provide a new angle to study metalloproteins in a functional context. Certainly more exciting results are yet to come!

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Holm RH, Kennepohl P, Solomon EI: **Structural and functional aspects of metal sites in biology**. *Chem Rev* 1996, **96**:2239-2314.

2. Scott RA, Lukehart CM: *Applications of Physical Methods to Inorganic and Bioinorganic Chemistry*. Wiley-Interscience; 2007.

3. *Physical Methods in Bioinorganic Chemistry*. Sausalito, CA: University Science Books; 2000.

4. Lippard SJ, Berg JM: *Principles of Bioinorganic Chemistry*. Mill Valley, CA: University Science Books; 1994.

5. Zhuang X, Rief M: **Single-molecule folding**. *Curr Opin Struct Biol* 2003, **13**:88-97.

6. Chen P, Keller AM, Joshi CP, Martell DJ, Andoy NM, Benitez JJ, Chen TY, Santiago AG, Yang F: **Single-molecule dynamics and mechanisms of metalloregulators and metallochaperones**. *Biochemistry* 2013, **52**:7170-7183.

7. • Alsteens DGHE, Newton R, Pfreundschuh M, Gerber C, Müller DJ: **Atomic force microscopy-based characterization and design of biointerfaces**. *Nat Rev Mater* 2017, **2**:17008.
An excellent review article on the general aspects of the atomic force microscopy.

8. Florin E, Moy V, Gaub H: **Adhesion forces between individual ligand–receptor pairs**. *Science* 1994, **264**:415-417.

9. Dammer U, Hegner M, Anselmetti D, Wagner P, Dreier M, Huber W, Güntherodt HJ: **Specific antigen/antibody interactions measured by force microscopy**. *Biophys J* 1996, **70**:2437-2441.

10. • Grandbois M, Beyer M, Rief M, Clausen-Schaumann H, Gaub HE: **How strong is a covalent bond?** *Science* 1999, **283**:1727-1730.
The first demonstration of the bond strength measurement using SMFS, including covalent bond and gold–thiolate bond.

11. Schmidt SW, Beyer MK, Clausen-Schaumann H: **Dynamic strength of the silicon–carbon bond observed over three decades of force-loading rates**. *J Am Chem Soc* 2008, **130**:3664-3668.

12. Ribas-Arino J, Shiga M, Marx D: **Understanding covalent mechanochemistry**. *Angew Chem Int Ed* 2009, **48**:4190-4193.

13. •• Rief M, Gautel M, Oesterhelt F, Fernandez JM, Gaub HE: **Reversible unfolding of individual titin immunoglobulin domains by AFM**. *Science* 1997, **276**:1109-1112.
First AFM-based SMFS experiment demonstrating the force-induced unfolding and folding of titin domains.

14. Marszalek PE, Dufrene YF: **Stretching single polysaccharides and proteins using atomic force microscopy**. *Chem Soc Rev* 2012, **41**:3523-3534.

15. Rief M, Oesterhelt F, Heymann B, Gaub HE: **Single molecule force spectroscopy on polysaccharides by atomic force microscopy**. *Science* 1997, **275**:1295-1297.

16. Puchner EM, Gaub HE: **Force and function: probing proteins with AFM-based force spectroscopy**. *Curr Opin Struct Biol* 2009, **19**:605-614.

17. Javadi Y, Fernandez JM, Perez-Jimenez R: **Protein folding under mechanical forces: a physiological view**. *Physiology (Bethesda)* 2013, **28**:9-17.

18. • Carrion-Vazquez M, Oberhauser AF, Fowler SB, Marszalek PE, Broedel SE, Clarke J, Fernandez JM: **Mechanical and chemical unfolding of a single protein: a comparison**. *Proc Natl Acad Sci U S A* 1999, **96**:3694-3699.
First demonstration of the polyprotein approach to study mechanical unfolding–folding of proteins.

19. Kosuri P, Alegre-Cebollada J, Feng J, Kaplan A, Inglés-Prieto A, Badilla Carmen L, Stockwell Brent R, Sanchez-Ruiz Jose M,

Holmgren A, Fernández Julio M: **Protein folding drives disulfide formation**. *Cell* 2012, **151**:794-806.

20. •• Fernandez JM, Li H: **Force-clamp spectroscopy monitors the folding trajectory of a single protein**. *Science* 2004, **303**:1674-1678.
Force-clamp spectroscopy was demonstrated to monitor the folding trajectories of single proteins.

21. Sotomayor M, Schulten K: **Single-molecule experiments in vitro and in silico**. *Science* 2007, **316**:1144-1148.

22. Li HB: **'Mechanical engineering' of elastomeric proteins: toward designing new protein building blocks for biomaterials**. *Adv Funct Mater* 2008, **18**:2643-2657.

23. Kienberger F, Ebner A, Gruber HJ, Hinterdorfer P: **Molecular recognition imaging and force spectroscopy of single biomolecules**. *Acc Chem Res* 2006, **39**:29-36.

24. Skulason H, Frisbie CD: **Detection of discrete interactions upon rupture of Au microcontacts to self-assembled monolayers terminated with –S(CO)CH_3 or –SH**. *J Am Chem Soc* 2000, **122**:9750-9760.

25. • Xue Y, Li X, Li H, Zhang W: **Quantifying thiol–gold interactions towards the efficient strength control**. *Nat Commun* 2014, **5**:4348.
SMFS measurements showed that the rupture force of the Au–S bond can be regulated by the oxidation state and the environment pH.

26. Konopka M, Rousseau R, Stich I, Marx D: **Detaching thiolates from copper and gold clusters: which bonds to break?** *J Am Chem Soc* 2004, **126**:12103-12111.

27. Schmitt L, Ludwig M, Gaub HE, Tamper R: **A metal-chelating microscopy tip as a new toolbox for single-molecule experiments by atomic force microscopy**. *Biophys J* 2000, **78**:3275-3285.

28. Kienberger F, Kada G, Gruber HJ, Pastushenko VP, Riener C, Trieb M, Knaus H-G, Schindler H, Hinterdorfer P: **Recognition force spectroscopy studies of the NTA–His6 bond**. *Single Mol* 2000, **1**:59-65.

29. Conti M, Falini G, Samori B: **How strong is the coordination bond between a histidine tag and Ni-nitrilotriacetate? An experiment of mechanochemistry on single molecules**. *Angew Chem Int Ed* 2000, **39**:215-218.

30. Kudera M, Eschbaumer C, Gaub HE, Schubert US: **Analysis of metallo-supramolecular systems using single-molecule force spectroscopy**. *Adv Funct Mater* 2003, **13**:615-620.

31. •• Hao X, Zhu N, Gschneidtner T, Jonsson EO, Zhang JD, Moth-Poulsen K, Wang HD, Thygesen KS, Jacobsen KW, Ulstrup J, Chi QJ: **Direct measurement and modulation of single-molecule coordinative bonding forces in a transition metal complex**. *Nat Commun* 2013, **4**:10.
A novel electrochemistry AFM method to study the mechanical rupture force of Os–N bond under different reduction potentials.

32. Kersey FR, Yount WC, Craig SL: **Single-molecule force spectroscopy of bimolecular reactions: system homology in the mechanical activation of ligand substitution reactions**. *J Am Chem Soc* 2006, **128**:3886-3887.

33. •• Zheng P, Li H: **Highly covalent ferric–thiolate bonds exhibit surprisingly low mechanical stability**. *J Am Chem Soc* 2011, **133**:6791-6798.
The first SMFS measurement of the bond strength of a metal–ligand bond in a metalloprotein. It was shown that the highly covalent ferric–thiolate bond rupture at around 200 pN, significantly lower than the Au–S bond.

34. • Arantes GM, Bhattacharjee A, Field MJ: **Homolytic cleavage of Fe–S bonds in rubredoxin under mechanical stress**. *Angew Chem Int Ed Engl* 2013, **52**:8144-8146.
A multiscale modeling approach was used to investigate the dissociation mechanism of the force-induced rupture of the Fe–S bond in rubredoxin.

35. Zheng P, Li HB: **Direct measurements of the mechanical stability of zinc–thiolate bonds in rubredoxin by single-molecule atomic force microscopy**. *Biophys J* 2011, **101**:1467-1473.

36. • Lei H, Guo YB, Hu XD, Hu CG, Hu XT, Li HB: **Reversible unfolding and folding of the metalloprotein ferredoxin revealed by single-molecule atomic force microscopy**. *J Am Chem Soc* 2017, **139**:1538-1544.
A direct demonstration of the reversible unfolding and folding of a [2Fe–2S] plant type ferredoxin. It was shown that the complex [2Fe–2S] iron–sulfur center can be reconstituted after the rupture of the metal–thiolate bonds.

37. • Beedle AEM, Lezamiz A, Stirnemann G, Garcia-Manyes S: **The mechanochemistry of copper reports on the directionality of unfolding in model cupredoxin proteins**. *Nat Commun* 2015, **6**:7894.
A SMFS study on copper-containing metalloproteins azurin and plastocyanin. It is shown that the rupture of copper center can serve as a reporter for the protein unfolding pathway.

38. Marshall NM, Garner DK, Wilson TD, Gao YG, Robinson H, Nilges MJ, Lu Y: **Rationally tuning the reduction potential of a single cupredoxin beyond the natural range**. *Nature* 2009, **462**:113-116.

39. Lin IJ, Gebel EB, Machonkin TE, Westler WM, Markley JL: **Changes in hydrogen-bond strengths explain reduction potentials in 10 rubredoxin variants**. *Proc Natl Acad Sci U S A* 2005, **102**:14581-14586.

40. • Zheng P, Takayama SJ, Mauk AG, Li H: **Hydrogen bond strength modulates the mechanical strength of ferric–thiolate bonds in rubredoxin**. *J Am Chem Soc* 2012, **134**:4124-4131.
A study showing the backbone amide NH–S hydrogen bond in the secondary coordination sphere of FeS_4 center can modify the mechanical strength of the ferric–thiolate bonds in rubredoxin.

41. • Wei W, Sun Y, Zhu M, Liu X, Sun P, Wang F, Gui Q, Meng W, Cao Y, Zhao J: **Structural insights and the surprisingly low mechanical stability of the Au–S bond in the gold-specific protein GolB**. *J Am Chem Soc* 2015, **137**:15358-15361.
A SMFS study of the Au–S bond strength in a protein environment. The Au(I)–S bond in the protein GolB ruptures at only ~160 pN, significantly lower than Au–S bonds measured on different gold surfaces.

42. • Zheng P, Chou CC, Guo Y, Wang Y, Li H: **Single molecule force spectroscopy reveals the molecular mechanical anisotropy of the FeS_4 metal center in rubredoxin**. *J Am Chem Soc* 2013, **135**:17783-17792.
The mechanical anisotropy of the iron–sulfur center is directly demonstrated in rubredoxin.

43. Kellis JT Jr, Todd RJ, Arnold FH: **Protein stabilization by engineered metal chelation**. *Biotechnology (N Y)* 1991, **9**:994-995.

44. Krantz BA, Sosnick TR: **Engineered metal binding sites map the heterogeneous folding landscape of a coiled coil**. *Nat Struct Biol* 2001, **8**:1042-1047.

45. •• Cao Y, Yoo T, Li HB: **Single molecule force spectroscopy reveals engineered metal chelation is a general approach to enhance mechanical stability of proteins**. *Proc Natl Acad Sci U S A* 2008, **105**:11152-11157.
An engineered bi-histidine metal chelation method was demonstrated as a general approach to enhance proteins' mechanical stability.

46. •• Junker JP, Ziegler F, Rief M: **Ligand-dependent equilibrium fluctuations of single calmodulin molecules**. *Science* 2009, **323**:633-637.
The equilibrium unfolding–folding transitions of single calmodulin molecules were observed directly using a custom built low drift AFM. And the ligand dependence of the full energy landscape was reconstructed.

47. Ramanujam V, Kotamarthi HC, Ainavarapu SRK: **Ca^{2+} binding enhanced mechanical stability of an archaeal crystallin**. *PLOS ONE* 2014, **9**:e94513.

48. Oroz J, Valbuena A, Vera AM, Mendieta J, Gómez-Puertas P, Carrión-Vázquez M: **Nanomechanics of the cadherin ectodomain: "canalization" by Ca^{2+} binding results in a new mechanical element**. *J Biol Chem* 2011, **286**:9405-9418.

49. Park PS-H, Sapra KT, Koliński M, Filipek S, Palczewski K, Muller DJ: **Stabilizing effect of Zn^{2+} in native bovine rhodopsin**. *J Biol Chem* 2007, **282**:11377-11385.

50. • Shen T, Cao Y, Zhuang S, Li H: **Engineered bi-histidine metal chelation sites map the structure of the mechanical unfolding transition state of an elastomeric protein domain GB1**. *Biophys J* 2012, **103**:807-816.

An engineered bi-histidine metal chelation-based method to map the mechanical unfolding transition state of proteins.

51. Cao Y, Li H: **Dynamics of protein folding and cofactor binding monitored by single-molecule force spectroscopy**. *Biophys J* 2011, **101**:2009-2017.

52. Zheng P, Takayama SJ, Mauk AG, Li H: **Single molecule force spectroscopy reveals that iron is released from the active site of rubredoxin by a stochastic mechanism**. *J Am Chem Soc* 2013, **135**:7992-8000.

53. • Zheng P, Wang YY, Li HB: **Reversible unfolding-refolding of rubredoxin: a single-molecule force spectroscopy study**. *Angew Chem Int Ed* 2014, **53**:14060-14063.
A direct demonstration of the reversible unfolding–refolding of a simple iron–sulfur protein rubredoxin.

54. Perales-Calvo J, Lezamiz A, Garcia-Manyes S: **The mechanochemistry of a structural zinc finger**. *J Phys Chem Lett* 2015, **6**:3335-3340.

55. Stigler J, Ziegler F, Gieseke A, Gebhardt JCM, Rief M: **The complex folding network of single calmodulin molecules**. *Science* 2011, **334**:512-516.

56. Rakhit R, Chakrabartty A: **Structure, folding, and misfolding of Cu,Zn superoxide dismutase in amyotrophic lateral sclerosis**. *Biochim Biophys Acta* 2006, **1762**:1025-1037.

57. • Zheng P, Arantes GM, Field MJ, Li H: **Force-induced chemical reactions on the metal centre in a single metalloprotein molecule**. *Nat Commun* 2015, **6**:7569.
A direct demonstration of the force-induced chemical reactions on the metal center in metalloproteins.