Protein Folding

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# Reversible Unfolding–Refolding of Rubredoxin: A Single-Molecule Force Spectroscopy Study**

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***Abstract:*** *In metalloproteins, metal centers serve as active sites for a range of functional purposes and as important structural elements to facilitate protein folding and assembly. It is challenging to observe the reversible unfolding and refolding of metalloproteins because of a loss or decomposition of the metal center. Here, the reversible unfolding–refolding of the iron–sulfur protein rubredoxin was observed directly using single-molecule force spectroscopy. The results demonstrate that the iron can remain attached to the CXXC motif when rubredoxin is unfolded. Upon relaxation, the unfolded rubredoxin can refold into its native holo state with the reconstituted $FeS_4$ center. The possible loss of iron from the unfolded protein prevents rubredoxin from refolding into its native holo state. These results demonstrated that unfolding of rubredoxin is reversible, as long as the iron remains attached, and provide experimental evidence for the iron-priming mechanism for the folding of rubredoxin.*

Metal ions play important roles in a variety of biological processes. They are often incorporated into metal centers within metalloproteins by coordinating to amino acid residues within the backbone of the protein.[1] The incorporation of metal ions significantly increases the functionality of protein by creating enzymatic active sites, and facilitating protein folding and assembly by acting as a structural site.[2] Thus, it is important to understand the role metal ions play in the folding process of metalloproteins. Previous studies suggest that binding of the metal ion to the unfolded protein chain can serve as a nucleation site to guide the folding of certain Cu- and Fe-containing proteins.[3] Thus, studies on how metal centers initially form are critical to understanding the overall metalloprotein folding process.[4]

Protein folding and unfolding is typically reversible under given experimental conditions, such as thermal or chemical denaturation. However, experimental observation of the reversible unfolding–refolding within metalloproteins is challenging. Even for the simplest iron–sulfur protein, rubredoxin, reversible complete unfolding and refolding has not been experimentally observed.[3a] It is believed that either the loss of iron or decomposition of the iron–sulfur cluster during unfolding prevents the protein from refolding.[5] Thus, attempts to study the reversible folding of iron–sulfur proteins mostly rely on the addition of an excess amount of iron when the protein is chemically unfolded. However, direct experimental evidence of the successful reconstitution of iron–sulfur sites during protein refolding has been difficult to obtain.

In order to directly examine the reversibility of unfolding–refolding of rubredoxin, we utilized atomic force microscopy (AFM) to directly probe the unfolding–refolding at the single-molecule level. Using AFM, it is possible to directly stretch a protein from its N and C termini, and force the protein to unfold (Figure 1 A).[6] In addition, the enclosed $FeS_4$ center can also be ruptured during unfolding by breaking ferric–thiolate bonds.[7] Here, we provide the first experimental evidence of a reversible rubredoxin unfolding–refolding process through a metastable Fe-$(SCys)_2$ intermediate in aqueous solution without addition of any iron source.

Rubredoxin is a small monomeric metalloprotein, consisting of 53 residues. It contains a $FeS_4$ center, in which an iron is coordinated by two bi-cysteine–iron chelation loops (C5XXC8 and C38XXC41,[8] Figure 1 A).

In order to investigate whether the unfolding–refolding of rubredoxin is reversible, we first stretched rubredoxin (RD) to a denatured and extended state. We used polyprotein $(RD\text{-}GB1)_n$ in such experiments, where GB1 domains serve as fingerprints for identifying single-molecule stretching events.[9] Stretching $(RD\text{-}GB1)_n$ results in typical sawtooth-like force-extension curves (Figure 1 B), where each individual force peak corresponds to the unfolding of either rubredoxin (with a contour length increment $\Delta Lc$ of around 13 nm, in red) or GB1 (with a $\Delta Lc$ of around 18 nm, in black).[9] The $\Delta Lc$ of about 13 nm results from the unfolding of rubredoxin, rupture of the $FeS_4$ center, and subsequent extension of the 37 amino acid residues between Cys5 and Cys41. The complete mechanical unfolding of rubredoxin requires the rupture of at least two ferric–thiolate bonds within the same iron-chelating CXXC loop.[7] However, it is unknown whether the ferric ion dissociates from rubredoxin

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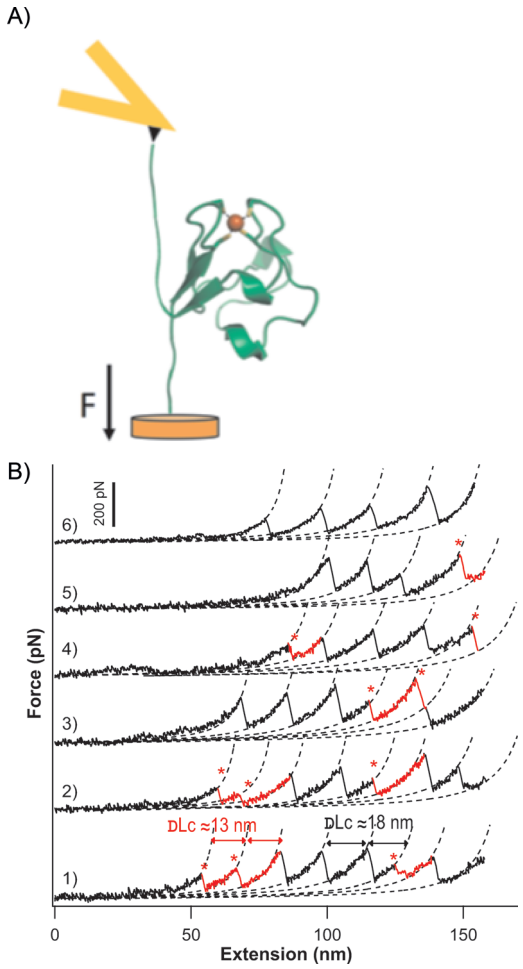


***Figure 1.*** A) AFM experiments performed on rubredoxin. B) Six consecutive force-extension curves of the polyprotein $(RD\text{-}GB1)_n$ using AFM. Unfolding events in red show a ΔLc of around 13 nm, corresponding to the unfolding of rubredoxin. Observing holo-rubredoxin unfolding events in consecutive stretching–relaxation cycles is indicative of the successful reconstitution of $FeS_4$ and the refolding of holo-rubredoxin. In addition, fewer rubredoxin refolding events were observed (from curves 1 to 6) when rubredoxin was subjected to more stretching–relaxation cycles.

right after the unfolding of rubredoxin, or whether it remains bound to the other CXXC loop.

In order to investigate whether the unfolded and extended rubredoxin can refold into holo-rubredoxin with the $FeS_4$ center fully reconstituted, we relaxed the unfolded polypeptide chain quickly to zero force.[10] After waiting for 10 seconds, we stretched the polyprotein again to probe whether rubredoxin could fold back to its native holo state. Since GB1 is a fast folder,[11] we observed the complete refolding of all GB1 domains in almost all the curves, as evidenced by characteristic unfolding events with ΔLc of around 18 nm (curves 2–6). To our surprise, we also observed unfolding events with ΔLc of about 13 nm (curves 2–5). As the unfolding of apo-rubredoxin does not generate measurable unfolding force peaks,[7] the unfolding event of ΔLc of approximately 13 nm must correspond to the unfolding of refolded rubredoxin with a reconstituted $FeS_4$ center. Since there is no ferric ion in the buffer, this result indicates that the ferric ion is still attached to one CXXC loop as $Fe(SCys)_2$ (or less likely Fe(SCys)) in the unfolded and extended rubredoxin chain after the $FeS_4$ center ruptures, and the two CXXC binding loops are able to coordinate the ferric ion and reconstitute the $FeS_4$ center again after the polypeptide chain has been relaxed. The formation of this intermediate species has been proposed as an important step in the folding of rubredoxin (the iron-priming mechanism), but evidence on its existence is lacking.[5a,12] Here, our results clearly show the existence of such an intermediate for the first time, and provide supporting evidence for the mechanism.

To further characterize this intermediate, we stretched and relaxed the same rubredoxin molecule over multiple cycles. Most proteins, such as GB1, are able to undergo hundreds of unfolding–refolding cycles without showing fatigue. In contrast, we found that rubredoxin fatigues quickly, relatively quickly losing its ability to refold as stretching–relaxation cycles are repeated. As shown in Figure 1 B, in the very first stretching force-extension curve, four GB1 and three rubredoxin unfolding events were observed. After relaxation for 10 seconds at zero force, all these unfolding events were observed again (curve 2), suggesting that all these domains refolded. However, only one or two rubredoxin unfolding events were observed in curves 3–5, while almost all GB1 refolded during cycles 2–5. From cycle 6 onwards, no rubredoxin unfolding events were observed and only GB1 unfolding events were present.

The more times rubredoxin is unfolded, the less probable is its refolding (Figure 2 A). After about 6 cycles, most rubredoxin domains cannot refold, and the $FeS_4$ center

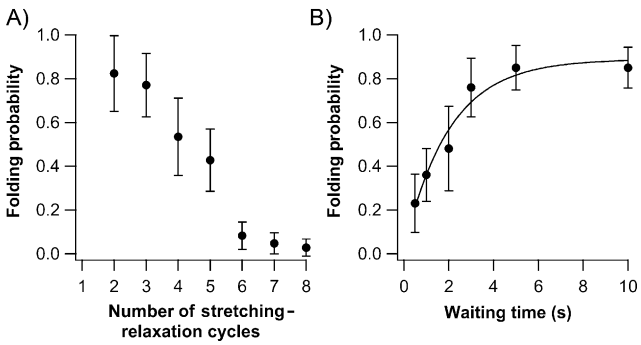


***Figure 2.*** A) The relationship between the refolding probability of rubredoxin and the number of times rubredoxin is unfolded. Each dot represents the refolding probability after a certain number of stretching/relaxation cycles. This result shows that the probability of refolding decreases when rubredoxin is continually unfolded, almost losing its ability to refold after six cycles. B) The relationship between the refolding probability of rubredoxin and the waiting time at which rubredoxin is relaxed at zero force.

cannot be reformed. To the best of our knowledge, this is the first time that this fatigue behavior has been observed in any protein at the single—molecule level, let alone an iron–sulfur protein.

To eliminate the possibility that this fatigue behavior is simply due to slow refolding kinetics, we measured the

  

refolding kinetics of rubredoxin at zero force using a well-established double-pulse protocol (Figure S1 in the Supporting Information).[10b] Folding kinetics can be fitted with a first-order rate law, with a folding rate constant $\beta_0$ of $0.50 \pm 0.02\ s^{-1}$ at zero force (Figure 2B). It is evident that a waiting time of 10 seconds is sufficient to allow refolding to occur, suggesting that the folding fatigue exhibited by rubredoxin is not due to slow refolding kinetics, but is an intrinsic property of ferric holo-rubredoxin.

Based on the folding kinetics of rubredoxin, we interpret the folding fatigue as the result of a loss of iron from the unfolded rubredoxin. When rubredoxin is unfolded and extended, the $Fe(SCys)_2$ intermediate is exposed to the solution, which does not contain additional ferric ions other than the endogenous iron coordinated in holo-rubredoxin. As a result, the iron may dissociate from the CXXC binding loop to the aqueous solution. After several stretching–relaxation cycles, the iron may eventually detach from the protein, leading to a loss of iron. Our results indicate that the iron is bound to the CXXC loop in the unfolded state in solution as metastable $Fe(SCys)_2$ or $Fe(SCys)$.

This study indicates that when a ferric ion is present as $Fe(SCys)_2$ in the unfolded rubredoxin, rubredoxin can readily refold and the $FeS_4$ center can be reconstituted. If the ferric ion dissociates from rubredoxin, the $FeS_4$ center can no longer be reconstituted. This finding provides evidence for the iron-priming mechanism, in which the binding of an iron to chemically denatured rubredoxin is critical for rubredoxin folding.[5a,12] Based on our single-molecule unfolding–refolding experiments on rubredoxin, we described the formation mechanism of the $FeS_4$ center in Figure 3. First, by mechanically stretching rubredoxin, an unfolded and extended state with the iron bound to one CXXC loop is naturally achieved, resulting in a *meta*-stable $Fe(SCys)_2$ intermediate. While this species has been predicted, this study is the first confirmation of its existence. The protein can then collapse upon relaxation, where the other CXXC loop can chelate $Fe(SCys)_2$ to form the $FeS_4$ center, initiating the complete refolding of holo-rubredoxin. It is of note that in this mechanism, the lifetime of $Fe(SCys)_2$ and the dissociation rate of the iron remain unknown. Combined AFM and UV-Vis spectroscopy will be required to determine these key parameters (see the Supporting Information).

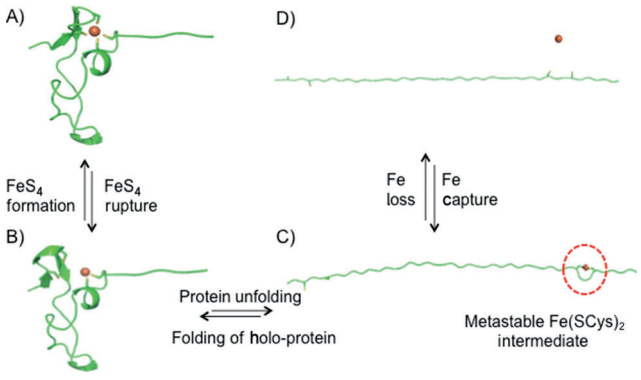


***Figure 3.*** Proposed unfolding and refolding mechanism of holo-rubredoxin, based on single-molecule force spectroscopy experiments. The mechanical unfolding of holo-rubredoxin involves the rupture of ferric–thiolate bonds, the complete unfolding, and the extension of the rubredoxin chain. A metastable $Fe(SCys)_2$ intermediate is likely to form before the ferric ion is completely dissociated from unfolded rubredoxin. For the refolding of holo-rubredoxin, the formation of the $Fe(SCys)_2$ intermediate is a critical step. This mechanism corraborates the iron-priming mechanism proposed for the folding of holo-rubredoxin.

In addition to the folding mechanism of rubredoxin, our observation of a metastable $Fe(SCys)_2$ species may also help explore how Fe clusters are transferred into iron–sulfur proteins. Recent studies show that the biosynthesis of iron–sulfur proteins requires a delicate machinery in which a scaffolding protein is able to form the initial iron–sulfur cluster.[13] However, how the iron–sulfur cluster is transferred to the target apo-protein is largely unknown. The experimentally observed metastable Fe–CXXC species, in which the iron can easily exchange with the surrounding environment, suggests that this may be an intermediate step toward incorporating the FeS cluster into metalloproteins when the metal cluster is transferred from the scaffolding protein to the apo-protein.

The CXXC motif is present extensively in a wide variety of metalloproteins, including zinc finger proteins, and many metal centers are attached to protein structures through this unique sequence. Thus, our study may open a new avenue toward understanding how the CXXC loop interacts with the metal center, which may provide important information regarding metalloprotein folding.

## *Experimental Section*

Polyprotein Engineering: The gene of protein chimera cys-Rubredoxin-GB1-cys was constructed as described previously.[7a,9b] Cys-RD-GB1-Cys was overexpressed in *Escherichia coli* strain DH5α and purified using $Co^{2+}$-affinity chromatography based on TALON resins (Clontech). The protein was kept in Tris buffer in pH 7.4 at a concentration of around $2\ mg\,mL^{-1}$. The protein was further purified using ion-exchange chromatography to produce pure ferric-RD-GB1 protein. Then, cys-RD-GB1-cys was reacted with a stoichiometric amount of $BM(PEO)_3$ (1,8-bis-maleimido-$(PEO)_3$, Molecular Biosciences), through a thiol–maleimide coupling reaction, to produce the polyprotein $(RD\text{-}GB1)_n$.[7a,9b]

Single-molecule AFM: Single molecule unfolding and refolding experiments on rubredoxin were performed on a home-built AFM as described previously.[10a] Each $Si_3N_4$ AFM cantilever (MLCT, Bruker, Santa Barbara, CA) was calibrated in solution before each experiment, showing a spring constant of around $40\ pN\,nm^{-1}$. All experiments were performed in Tris buffer (pH 7.4) at room temperature. In a typical experiment, 2 μL of the polyprotein $(RD\text{-}GB1)_n$ solution were deposited on a clean glass coverslip covered with about 50 μL of Tris buffer, and allowed to absorb on the surface for around 15 min. The protein was stretched under a constant pulling speed of $400\ nm\,s^{-1}$.

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[1] S. J. Lippard, J. M. Berg, *Principles of bioinorganic chemistry*, University Science Books, Mill Valley, **1994**.
[2] R. H. Holm, P. Kennepohl, E. I. Solomon, *Chem. Rev.* **1996**, *96*, 2239 – 2314.
[3] a) I. Bertini, J. A. Cowan, C. Luchinat, K. Natarajan, M. Piccioli, *Biochemistry* **1997**, *36*, 9332 – 9339; b) I. Pozdnyakova, J. Guidry, P. Wittung-Stafshede, *J. Am. Chem. Soc.* **2000**, *122*, 6337 – 6338.
[4] P. Wittung-Stafshede, *Acc. Chem. Res.* **2002**, *35*, 201 – 208.
[5] a) A. Morleo, F. Bonomi, S. Iametti, V. W. Huang, D. M. Kurtz, *Biochemistry* **2010**, *49*, 6627 – 6634; b) S. Prakash, M. Sundd, P. Guptasarma, *PLoS One* **2014**, *9*, e89703.
[6] a) M. Rief, M. Gautel, F. Oesterhelt, J. M. Fernandez, H. E. Gaub, *Science* **1997**, *276*, 1109 – 1112; b) S. R. K. Ainavarapu, J. Bruji, H. H. Huang, A. P. Wiita, H. Lu, L. Li, K. A. Walther, M. Carrion-Vazquez, H. Li, J. M. Fernandez, *Biophys. J.* **2007**, *92*, 225 – 233.
[7] a) P. Zheng, H. Li, *J. Am. Chem. Soc.* **2011**, *133*, 6791 – 6798; b) P. Zheng, S.-i. J. Takayama, A. G. Mauk, H. Li, *J. Am. Chem. Soc.* **2012**, *134*, 4124 – 4131.
[8] a) M. K. Eidsness, K. A. Richie, A. E. Burden, D. M. Kurtz, Jr., R. A. Scott, *Biochemistry* **1997**, *36*, 10406 – 10413; b) P. R. Blake, J. B. Park, F. O. Bryant, S. Aono, J. K. Magnuson, E. Eccleston, J. B. Howard, M. F. Summers, M. W. Adams, *Biochemistry* **1991**, *30*, 10885 – 10895.
[9] a) Y. Cao, C. Lam, M. Wang, H. Li, *Angew. Chem. Int. Ed.* **2006**, *45*, 642 – 645; *Angew. Chem.* **2006**, *118*, 658 – 661; b) P. Zheng, Y. Cao, H. Li, *Langmuir* **2011**, *27*, 5713 – 5718.
[10] a) J. M. Fernandez, H. Li, *Science* **2004**, *303*, 1674 – 1678; b) M. Carrion-Vazquez, A. F. Oberhauser, S. B. Fowler, P. E. Marszalek, S. E. Broedel, J. Clarke, J. M. Fernandez, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 3694 – 3699; c) A. Borgia, P. M. Williams, J. Clarke in *Annu. Rev. Biochem.*, *Vol. 77*, Annual Reviews, Palo Alto, **2008**, pp. 101 – 125.
[11] Y. Cao, H. Li, *Nat. Mater.* **2007**, *6*, 109 – 114.
[12] F. Bonomi, S. Iametti, P. Ferranti, D. Kurtz, Jr., A. Morleo, E. Ragg, *J. Biol. Inorg. Chem.* **2008**, *13*, 981 – 991.
[13] a) R. Lill, *Nature* **2009**, *460*, 831 – 838; b) B. Py, F. Barras, *Nat. Rev. Microbiol.* **2010**, *8*, 436 – 446.

  