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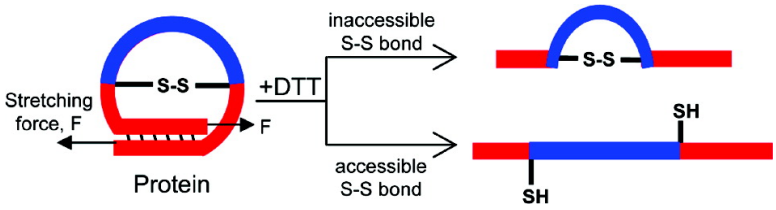

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A Single-Molecule Assay to Directly Identify Solvent-Accessible Disulfide Bonds and Probe Their Effect on Protein Folding

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Disulfide bonds are ubiquitous in proteins. According to a recent survey, there are 97 741 disulfide bonds in 121 779 protein structures available in the Protein Data Bank (PDB).[1] Native[2,3] as well as engineered[4−6] disulfide bonds have been shown to control the stability and function of proteins. The redox state of protein disulfide bonds in vivo, governing protein stability and function, depends on the disulfide bond accessibility to the surrounding reducing agent molecules in the intracellular environment as well as those present on the cell surface.[7,8] While structural techniques such as X-ray crystallography[6] can directly identify protein disulfide bonds, they only provide information on *static accessibility*[9,10] of the disulfide bonds to the surroundings. However, proteins are known to be dynamic in nature.[11] In the case of proteins for which structures are not available, indirect methods, such as measuring the changes in protein mobility by SDS-PAGE in different redox conditions,[4] analyzing digested protein fragments by mass spectrometry[12,13] and quantifying the thiols of reduced disulfide bonds by the Ellman's reagent,[4,6,14] have been implemented to identify protein disulfide bonds. While these assays are useful for identifying the presence of disulfide bonds in general, they often require protein denaturation and disulfide cleavage and are typically unable to identify intramolecular disulfide bonds in their oxidized state and directly measure their solvent accessibility.[15] Here, we present single-molecule force spectroscopy as a tool to directly identify intramolecular protein disulfide bonds in their oxidized state, measure their accessibility to small-molecule reducing agents in the bathing solution, and probe the differential folding kinetics of reduced and oxidized proteins at the single-molecule level.

We engineered a pair of cysteines (Cys24 and Cys55) into an 89-residue protein (I27, an immunoglobulin-like domain of titin) and produced a polyprotein, $(I27_{E24C-K55C})_8$, consisting of eight $I27_{E24C-K55C}$ domains.[16] Polyproteins are extremely useful for providing unambiguous molecular fingerprints in force spectroscopy experiments. As shown in Figure 1A, stretching a single $(I27_{E24C-K55C})_8$ molecule results in a sawtooth pattern of force peaks with a contour length (ΔL) of 17.7 nm that is consistent with the mechanical unfolding of the protein and extending 58 amino acids (i.e., 1−24 and 55−89). The Cys24−Cys55 disulfide bond acts as a mechanical barrier and protects 31 residues present between Cys24 and Cys55 from being stretched. However, in the presence of the reducing agent dithiothreitol (DTT), the disulfide bond is reduced and the unfolding of the reduced protein contains 28.4 nm force peaks (Figure 1A,B), which is consistent with stretching of 89 residues similar to the wild-type I27.[16] Moreover, when DTT is removed by buffer exchange, the disulfide bond re-forms, and once again, the sawtooth pattern consists of entirely 17.7 nm force peaks in agreement with the disulfide reoxidation (Figure 1A,B). The reduction and reoxidation kinetics are fitted with single-exponential functions, and the calculated bimolecular rate constant for the Cys24−Cys55 disulfide bond reduction is 0.06 M^{-1} s^{-1}. This direct assay demonstrates that the Cys24−Cys55 disulfide bond is accessible to the reducing agent in the solution. However, another polyprotein, $(I27_{G32C-A75C})_8$, containing a single Cys32−Cys75 disulfide bond in each I27 domain exhibited an extremely contrasting behavior. We measure a ΔL of 12.7 nm that is shorter than that of the wild-type, demonstrating the presence of the Cys32−Cys75 disulfide bond (see Supporting Information), which is not reduced even in 100 mM DTT. The reduction reaction (identified by force peaks with $\Delta L = 28.4$ nm) is very slow, and the reduced fraction of the disulfide bonds is always below 0.3 with an average of 0.07 over 5 h (Figure 1C). This direct assay demonstrates that the Cys32−Cys75 disulfide bond is not readily accessible to the reducing agent in the bulk, and it is exposed only upon mechanical unfolding of the protein. From these experiments, it is evident that the ΔL measurements not only unambiguously identify intramolecular protein disulfide bonds but also distinguish between solvent-accessible and sequestered disulfide bonds.

In addition, polyprotein stretching studies have other advantages compared to the bulk methods. For example, partially reduced polyprotein molecules offer a platform to simultaneously study the folding of oxidized and reduced domains by force spectroscopy. The reduction of the $(I27_{E24C-K55C})_8$ polyprotein in 10 mM DTT is very slow, and its force-extension trace consists of both reduced and oxidized $I27_{E24C-K55C}$ domains (Figure 2). Stretch−relax protein refolding[16] experiments were performed on this partially reduced polyprotein. As shown in Figure 2, a segment of the polyprotein picked by the AFM tip consisted of one reduced ($\Delta L = 28.4$ nm) and two oxidized ($\Delta L = 17.7$ nm) domains of $I27_{E24C-K55C}$. It has previously been shown that the Cys24−Cys55 disulfide bond accelerates the folding of $I27_{E24C-K55C}$ by >150 times compared to the reduced protein from experiments performed in different redox conditions.[16] In contrast, in the current experiment, both oxidized and reduced $I27_{E24C-K55C}$ domains are present in a single polyprotein molecule. The polyprotein is stretched to identify the number of oxidized and reduced domains, relaxed for $\Delta t = 100$ ms to refold, and then restretched to count the refolded domains (Figure 2). In this very short duration of relaxation, the oxidized domains refolded and regained mechanical stability, whereas the reduced domain remained unfolded. However, in the subsequent stretch−relax cycle, all three domains refolded when the relaxation time is increased to 10 s. Once again, in the subsequent stretch−relaxation cycle, the polypeptide is relaxed for 100 ms to confirm that the oxidized domains refold much faster than the reduced domain. This experiment demonstrates the potential of the single-molecule force spectroscopy to probe the folding kinetics of oxidized as well as reduced domains in single polyprotein molecules without invoking the particular need for making measurements in two separate different redox environments.

In conclusion, our experimental results demonstrate the capability of polyproteins in force spectroscopy to directly identify intramolecular protein disulfide bonds and determine their accessibility. This technique identifies disulfide bonds unambiguously at the

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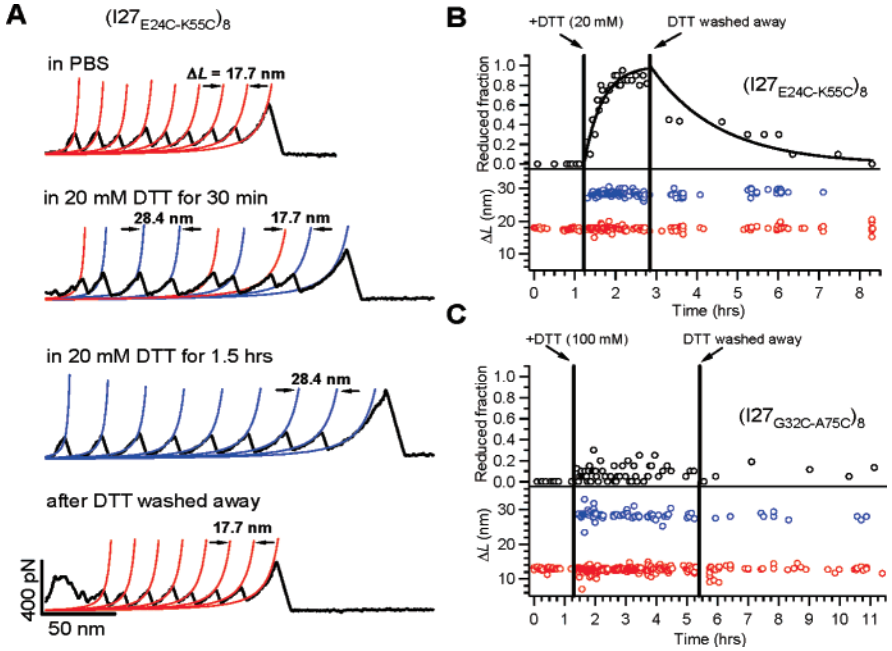

Figure 1. Identification and redox kinetics of solvent-accessible and sequestered disulfide bonds in I27 protein. (A) Force-extension trace of the $(I27_{E24C-K55C})_8$ polyprotein in the absence (top trace), presence (second and third traces), and removal (bottom trace) of DTT. Contour length changes (ΔL) measured by the worm-like chain model (WLC)[17] fits distinguish the oxidized protein ($\Delta L = 17.7$ nm) from the reduced protein ($\Delta L = 28.4$ nm). (B) Reduced fraction of the protein (top panel) defined as the ratio of the number of force peaks with $\Delta L = 28.4$ nm to the total number of force peaks (i.e., $\Delta L = 28.4$ and 17.7 nm). Each point is calculated from the force peaks of 3−5 force-extension traces obtained within 2−4 min interval during the reduction phase and within 30−60 min interval during the reoxidation phase. Single-exponential fits with rate constants 1.1×10^{-3} and 1.7×10^{-4} s^{-1} for the reduction and reoxidation processes, respectively, are also shown. The time distribution of the force peaks is shown in the lower panel. (C) The disulfide bonds in the $(I27_{G32C-A75C})_8$ polyprotein are sequestered and do not readily undergo chemical reduction. The disulfide reduction is a very slow process, and only 7% of the protein is reduced in 5 h after incubating in 100 mM DTT. The ΔL values for the oxidized and reduced forms the $I27_{G32C-A75C}$ protein are 12.7 and 28.4 nm, respectively.

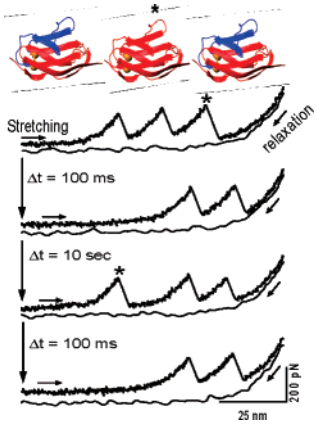

Figure 2. Stretch−relax refolding of the partially reduced $(I27_{E24C-K55C})_8$ polyprotein in 10 mM DTT. A segment of the polyprotein containing one reduced (marked with an asterisk) and two oxidized domains was stretched by the AFM tip. The oxidized domains fold much faster (<100 ms) compared to reduced domain (>100 ms) in agreement with previous experiments.[16]

single-molecule level compared to the bulk techniques. For example, X-ray crystallography has identified a disulfide bond in the I1 domain of titin.[18] However, a force spectroscopy study[19] on the $(I1)_8$ polyprotein has suggested that the formation of a disulfide bond in I1 is a relatively rare event in solution. Also, the single-molecule experiments proposed here require neither large quantities of proteins as in labeling methods nor protein crystallization as in crystallography. Moreover, it is possible to directly probe the folding of reduced and oxidized proteins simultaneously that is not currently possible in bulk studies.

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Supporting Information Available: Experimental methods and force-extension data of the $(I27_{G32C-A75C})_8$ polyprotein. This material is available free of charge via the Internet at http://pubs.acs.org.

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