A HaloTag Anchored Ruler for Week-Long Studies of Protein Dynamics

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ABSTRACT: Under physiological conditions, protein oxidation and misfolding occur with very low probability and on long times scales. Single-molecule techniques provide the ability to distinguish between properly folded and damaged proteins that are otherwise masked in ensemble measurements. However, at physiological conditions these rare events occur with a time constant of several hours, inaccessible to current single-molecule approaches. Here we present a magnetic-tweezers-based technique that allows, for the first time, the study of folding of single proteins during week-long experiments. This technique combines HaloTag anchoring, sub-micrometer positioning of magnets, and an active correction of the focal drift. Using this technique and protein L as a molecular template, we generate a magnet law by correlating the distance between the magnet and the measuring paramagnetic bead with unfolding/folding steps. We demonstrate that, using this magnet law, we can accurately measure the dynamics of proteins over a wide range of forces, with minimal dispersion from bead to bead. We also show that the force calibration remains invariant over week-long experiments applied to the same single proteins. The approach demonstrated in this Article opens new, exciting ways to examine proteins on the “human” time scale and establishes magnetic tweezers as a valuable technique to study low-probability events that occur during protein folding under force.

INTRODUCTION

Proteins inside the cell are constantly being recycled in order to maintain an adequate functionality of the entire proteome.1 Usually, proteins can reversibly unfold and then return to the functioning native state. However, certain environmental conditions such as redox stress or signaling can modulate the lifetime of proteins2 and subsequently alter their capability to carry out their function. It is thought that these conditions trap the protein in an irreversible unfolded or misfolded state, which is associated with cytotoxicity and numerous diseases. Single misfolding events are rare and occur on long time scales (days to weeks), making them difficult to observe in cellular or biochemical assays.3 Force spectroscopy is a technique that can distinguish between folded and unfolded states in a single protein domain, but advances in stability and attachment chemistry are needed to probe single molecules on the time scales associated with misfolding.

Force-clamp spectroscopy allows for the measurement of protein dynamics in response to well-defined force protocols. This technique, implemented with an atomic force microscope, has been used to measure the rates of protein folding and chemical reactions, such as thiol–disulfide exchange and disulfide isomerization.4,5 Force-clamp AFM makes use of an active feedback with a time constant that can approach 1 ms.5 The biggest limitation of this approach is the mechanical drift of the AFM instrument. Drift limits the duration of a single-molecule experiment to a few minutes and decreases accuracy at low forces. By contrast, magnetic tweezers is inherently stable and provides force-clamp conditions without feedback; therefore, it is an ideal instrument to study protein dynamics under force-clamp conditions in the low-force regime. Recent advances in tethering chemistry applied to force spectroscopy now allow for the study of covalently anchored proteins without the risk of detachment.6 The low-drift and high force resolution of magnetic tweezers combined with HaloTag anchored proteins constitutes an ideal approach to study protein dynamics at low forces. A limiting factor in the use of magnetic tweezers to study short proteins at low force is due to the difficulty in estimating precisely the applied force.7 Brownian fluctuations have been used to measure the force applied to short proteins tethered to paramagnetic beads.6,7 However, its direct use is questionable in the study of protein folding/unfolding reactions. Under mechanical force, proteins show folding/unfolding transitions as step-changes in the measured end-to-end length.8 These transitions dominate the motion of the paramagnetic bead at low forces, severely limiting the use of spectral analysis to measure the pulling force (Figure 1). Furthermore, owing to the proteins’ short length (20–300 nm), the measuring paramagnetic bead is forced to operate...
near a surface with its associated effects on viscosity and anisotropy. Here we use protein L folding dynamics \(^7\)\(^9\) to demonstrate a novel approach for calibrating the pulling force applied by a magnetic tweezers to short recombinant proteins. Once the calibration is established, it can be applied to different proteins and over extended periods of time with little variance. We demonstrate that the force-dependent step sizes of the folding/unfolding transitions \(^7\) can be used to derive a magnet law that precisely calculates the force applied to a protein, solely based on the distance between the magnets and the paramagnetic bead. The use of a magnet law demands precise and stable positioning of the magnets, in order to trust that the applied force is not drifting over time and can be reproduced from one experiment to the next. Furthermore, it is also essential that the magnets can be positioned fast and with high accuracy, and that the paramagnetic beads show a narrow dispersity in their magnetic properties. We demonstrate the remarkable stability of our magnetic tweezers instrument over hours-long recordings of protein L dynamics at low forces. We further expand the measuring time of these experiments to up to 2 weeks and measure the dynamics under force of the same protein with minimal drift.

### METHODS

1. **Magnetic Tweezers Setup.** Our custom-made setups are built on top of an inverted microscope (Olympus IX-71/Zeiss Axiovert S100) using 63× oil-immersion objective (Zeiss/Olympus), mounted on a nanofocusing piezo actuator (P-725; Physik Instrumente) and a 1.6× optivar lens. The fluid chamber was illuminated using a collimated cold white LED (Thor Labs). Images were acquired using a CCD Pike F-032b camera (Allied Vision Technologies) operating at 280 Hz or a Zyla 5.5 sCMOS camera (Andor), operating at up to 1030 Hz. Paramagnetic Dynabeads M-270 beads with a diameter of 2.8 µm (Invitrogen) were exposed to force using a pair of permanent neodymium grade N52 magnets (D33, K&J Magnetics), approaching the fluid cell from the top (Figure 1). The position of the magnets was controlled with a linear voice-coil (LFA-2010; Equipment Solutions), which is capable of moving 10 mm with \(\sim 0.7\) m/s speed and 150 nm position resolution. For long-term recordings, an xy-stage moving with \(\sim 100\) nm resolution (M-686, Physik Instrumente) was incorporated in order to address identical bead coordinates over separate days. The data acquisition and control of the voice-coil and piezo actuator were done using a multifunction DAQ card (NI USB-6341, National Instruments).

2. **Image Processing.** Image processing was done using custom-written software in Igor Pro (WaveMetrics), which is described in detail in the SI. In brief, the z-position displacement of a bead was...
determined in a three-step process: (1) Fourier transform (FFT) of the bead was acquired; (2) the radial profile was computed from the FFT with a pixel-addressing algorithm (FKA algorithm, see SI software); (3) radial profiles were correlated to a z-stack library of the bead acquired prior to the experiment (see SI; Figure S1). The z-position displacement was calculated for the paramagnetic bead tethered to protein, and for a local fixed reference bead used to correct for instrumental vibration and focal drift. With this procedure, frames rates of $\sim 1$ kHz are achievable.

3. Fluid Chamber Preparation. The single-molecule magnetic tweezers measurements were done in fluid chambers made of two sandwiched glass coverslips (Ted Pella) separated by strips of parafilm. The bottom surfaces were successively cleaned by sonication for 20 min in 1% Hellmanex (Hellma), acetone and ethanol (both from Sigma-Aldrich). After drying with air, the bottom surfaces were successively cleaned by sonication for 20 min in 1% Hellmanex, acetone and ethanol, the surfaces were cured at 100 °C for >1 h. The top glass surfaces were cleaned by sonication in 1% Hellmanex for 20 min and terminaled nonmagnetic polystyrene beads with diameter of 2.89 μm (Spherotech), diluted in PBS. After 10 min, the beads that did not adsorb were washed with 100 μL PBS buffer. The chambers were then reacted for >4 h with a solution of 10 μg/mL HaloTag amine (O4) ligand (Promega), diluted in the same PBS buffer. Finally, the fluid chambers were blocked for 12 h with TRIS blocking buffer: 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM MgCl₂, and 1% w/v sulfhydryl blocked-BSA (Lee Biosolutions).

4. Protein Expression, Purification, and Modification. Polypeptide constructs were engineered using a combination of BamHI, BglII, and KpnI restriction sites, as described previously. The protein constructs had eight repeats of protein L (B1 domain from Peptostreptococcus magnus), 127, or 9-domain ubiquitin, flanked by an N-terminal HaloTag enzyme (Promega) and a C-terminal AviTag for biotinylation. Proteins cross-linked with DNA had eight repeated domains of protein L or 127C47-63A engineered between a HaloTag at the N-terminus and a cysteine residue at the C-terminus. For purification purposes, a His₄-tag was also present before the AviTag or the cysteine residue. The multidomain proteins were expressed and purified as follows. BLR (DE3) or ERL competent cells containing the engineered pFN18A expression vector (Promega) were grown at 37 °C until OD₆₀₀ nm = 0.6–0.8. The protein overexpression was induced with 1 mM Isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) overnight at 25 °C. Cells were resuspended in 50 mM sodium phosphate buffer pH 7.0, 300 mM NaCl, 10% glycerol and 1 mM Dithiothreitol (DTT, Sigma), and disrupted by French press. The proteins were purified from the lysate following a two-step procedure: with a Ni–NTA affinity purification resin (GE), followed by size exclusion chromatography using a Superdex–200 HR column (GE) in 10 mM HEPES buffer pH 7.2, 150 mM NaCl, 10% glycerol and 1 mM DTT.
EDTA. The purified multidomain proteins were pooled and concentrated to 50–100 μM before biotinylation. Biotinylation was performed in 50 mM Bicine buffer pH 8.3, 10 mM magnesium acetate, 10 mM ATP, 100 μM biotin and 2.5 μg biotin ligase BirA enzyme (Avidity), at 30 °C, for 4 h. The biotinylation of the multidomain protein was confirmed through Western blotting, using conjugated streptavidin horseradish peroxidase (GE).

For the synthesis of DNA–protein constructs, a DNA segment from λ-phage DNA (Thermo Scientific) was amplified with amine and biotin primers (IDT) using a standard PCR mix (New England Biolabs), which resulted in a 605 bp DNA linker. The amine end of DNA was reacted with sulfo-SMCC (Thermo Scientific) for 1 h in a solution of Borax buffer pH 8.5 at room temperature. The sulfo-SMCC excess was removed using a PCR cleanup kit (NucleoSpin) and eluting with water. The reaction with the cysteine-terminated protein was done overnight in HEPES buffer, pH 7.2. The cross-linking reaction was confirmed using SDS polyacrylamide gels, stained with ethidium bromide (Figure S3).

5. Single-Molecule Measurements. The protein was freshly diluted in TRIS blocking buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM MgCl₂, and 1% w/v sulfhydril-blocked BSA) to ~0.01 μM and left to adsorb on the surface of the fluid chamber for 10 min. Chambers were then washed with TRIS blocking buffer, and streptavidin coated paramagnetic beads (Dynabeads M-270, Invitrogen) were added to bind with the protein for ~1 min, before approaching the magnets to a low-force position (4 mm). The protein experiments were performed in TRIS blocking buffer, while the protein–DNA experiments were performed in TRIS blocking buffer lacking MgCl₂. Experiments began after a z-stack library of both a protein-tethered paramagnetic bead and a fixed reference bead were acquired, and real-time z-position displacements were calculated using our image processing method (see SI, Figure S1).

The step sizes describing the unfolding and refolding of individual protein domains were measured from individual traces (Figure S4). For the low-force conditions, a boxcar filter (typically 50 points) was applied beforehand. Length histograms were obtained from the data corresponding to each step, and the absolute position was measured using a Gaussian fit. The size of each step was then obtained from the position of two consecutive histograms (Figure S4). The unfolding rates were calculated from the average traces assuming a single exponential unfolding kinetics.

RESULTS AND DISCUSSION

Our experimental approach is based on magnetic tweezers, a technique capable of applying mechanical force to single molecules via paramagnetic beads (Figure 1). This approach is now extended by the use of HaloTag-based covalent attachment chemistry, which allows for the exposure of single proteins to a mechanical force for extended periods of time.

Figure 3. Fiduciary marker for the magnet law, using the B–S overstretching transition. (A) A protein–DNA construct composed of HaloTag-(protein L)₈-cys protein cross-linked to a 605 bp DNA segment is tethered between a glass slide and a paramagnetic bead. The protein–DNA construct is first exposed to a constant force (MP = 1.4 mm), where protein L unfolds in steps of ~14 nm (blue), followed by a ramp-increase in force (from MP = 1.4 to 0.9 mm). (B) The B–S transition is observed at MP ≈ 0.99, marking the 65 pN point. (C) Similar experiment using the more stable titin I27 protein (HaloTag-I27-cys). In this case the I27 domains unfold at forces higher than the B–S transition. (D) Histogram of magnet position values where the B–S transition is measured in both I27 and protein L constructs. The line is a Gaussian fit with MP = 0.99 ± 0.05 mm (n = 34 traces, 10 different beads).
without detachment. In magnetic tweezers, the applied force is often calculated using a calibration curve that measures force as a function of the distance between the magnet and the paramagnetic bead tethered to the molecule under study. The applied force directly depends on the size and magnetic properties of the beads and the geometry and strength of the magnets. It was recently shown that a magnet law can accurately estimate the force on micrometer-long DNA molecules. Here we extend this approach by demonstrating a new method for obtaining the magnet law that applies to short proteins. We use the folding step sizes of protein L as a nanoscale ruler for the change in force with magnet position. When exposed to a constant force, protein L domains undergo unfolding transitions as step-size increases in the measured end-to-end length (Figure 2A). Protein L unfolds within several seconds in steps of 15.3 nm at magnet position 1.3 mm and within 2 min in steps of 12.3 nm at magnet position 2.5 mm. At magnet position 3.3 mm protein L reaches a steady state where unfolding and refolding steps of 8.7 nm are observed over an extended period of time (Figure 2A). The force scaling of the unfolding step sizes is also apparent in the length of the fully unfolded protein (eight domains in all cases; Figure 2A). Folding steps also scale with the pulling force and, for a given force, have the same size as the unfolding steps (Figure S4). This equation now gives a closed form for $MP(x)$, with $b$ as the only fitting parameter. We fit eq 4 to the data of Figure 2B using the Levenberg–Marquardt least orthogonal distance method with a confidence level of 95% (solid line, Figure 2B). From the fit we obtain $b = 0.90 \pm 0.03 \text{mm}^{-1}$, for $F_{B-S} = 65 \text{pN}$. The fit was weighted with the standard deviation of the measured extension steps (Figure 2B) and a standard deviation for the magnet position of 0.05 mm, as measured from the DNA–protein experiments (Figure 3D). The shading in Figure 2B marks the upper and lower confidence contours of the fit.

We now combine eq 3 with the WLC model to give

$$MP(x) = F_{B-S} - \frac{1}{b} \ln \left\{ \frac{1}{4} \frac{k_BT}{p} \left[ \frac{1}{4} \left( 1 - \frac{x}{\Delta L_c} \right)^{-2} - \frac{1}{4} + \frac{x}{\Delta L_c} \right] \right\}$$

where $MP$ is the magnet position, $x$ is the observed step size, and $a$ and $b$ are fitting parameters. Fitting the data of Figure 2B using eq 2 gives values of $a = 177 \pm 17 \text{pN}$ and $b = 1.07 \pm 0.05 \text{mm}^{-1}$. In order to refine the accuracy of this magnet law, we take advantage of a well-known force standard—the B–S overstretching transition of DNA molecules. This transition takes place at a well-defined force of 65 pN, independent of the loading rate. By including this fiduciary measurement, the magnet law becomes

$$F(MP) = F_{B-S} e^{\delta(MP_{B-S} - MP)}$$

where $F_{B-S}$ is the force at which the B–S transition is observed in DNA (65 pN), and $MP_{B-S}$ is the magnet position where the B–S transition is observed in our magnetic tweezers instrument. We measure $MP_{B-S}$ with the experimental approach shown in Figure 3. We engineered a polypeptide containing a HaloTag followed by eight domains of protein L and a terminal cysteine anchored to a 605 bp DNA linker (see Methods). When we apply a high force to this construct ($MP = 1.4 \text{mm}$; Figure 3A), we observe eight ~14 nm unfolding steps. As the magnet position is decreased linearly (increasing force), we then observe the overstretching transition as a sharp extension of 127 ± 28 nm (Figure 3A,B), at a magnet position of $MP_{B-S} = 0.99 \pm 0.05 \text{mm}$ ($n = 34$; Figure 3D). A similar construct composed of eight repeats of the more mechanically stable I27 protein and the same DNA segment first shows the overstretching transition at the same 0.99 mm magnet position, followed by eight ~25 nm steps, characteristic of the unfolding of I27 (Figure 3C).
A reference bead is maintained at a constant value (see also Methods and SI). Due to the exponential dependency of force with magnet position, we expect that errors will become more significant at high forces. As a proxy for errors in the high-force regime, we measure the unfolding rates of protein L over extended periods of time, which in this case is a better indicator for drift in force than the step size (which saturates at high forces, see discussion below) (Figure 4A). A single protein L construct was continuously exposed to unfolding-refolding cycles for >8 h (Figure 4A). These cycles consisted of unfolding the protein with 30-s pulses to 45 pN (MP = 1.4 mm), after which the protein was allowed to refold at 4.3 pN (MP = 4 mm). A moving average of 20 unfolding traces was fit with a single exponential to measure the unfolding rate over the 8-h-long experiment. Figure 4C shows the measured unfolding rates (mean rate of 0.20 ± 0.02 s⁻¹) over the full length of the trace. The mean unfolding rate of the first 100 boxes is not significantly different compared with the last 100 boxes (0.21 ± 0.02 s⁻¹ and 0.19 ± 0.02 s⁻¹, respectively). The observed rate variations, if arising solely from instrumental error, correspond to changes in force of ±2 pN (SD rates).

Another measure for the stability of our magnetic tweezers instrument is the distribution of unfolding and refolding step sizes, which were used as the basis for determining the magnet law (Figure 2B). Following the shape of the WLC model of polymer elasticity, the step sizes are relatively invariant at the high end of the force law. However, at forces below 20 pN, the step sizes change rapidly with force, hence providing a good measure of stability in this range. Figure 4B shows several 25-min-long unfolding pulses at 8 pN (MP = 3.3 mm) showing numerous unfolding and refolding steps as the protein equilibrates. From these data we measured the average step size in each pulse (Figure 4D), showing that these measurements are remarkably stable. The mean value of the step sizes at 8 pN is 9.0 ± 0.4 nm (Figure 4D), which is very close to the value predicted by the WLC model (8.9 nm). At this force, the domains are kept unfolded for longer times than at 45 pN, and after 3 h some domains failed to refold. Therefore, we have not included data longer than 3 h at this force.

The experiment shown in Figure 5 further extends the observations shown in Figure 4 to a single protein L construct experiment lasting 14 days. In this case, a single protein L construct is probed (afternoons) by unfolding at 45 pN for 45 s (MP = 1.4 mm; Figure 5A). For such long experiments, we exposed the molecules to high force only several times a day, so as to minimize the dissociation of the biotin–streptavidin and detachment of the paramagnetic bead. In between force pulses, the molecule was held at 4.3 pN (MP = 4.0 mm). Importantly, rates of unfolding were comparable between measuring days (Figure 5B). Moreover, the mean of the unfolding step sizes...
measured each day gave $15.3 \pm 0.7 \text{ nm}$, which is within the error of the predicted 14.7 nm calculated from the WLC model for a $MP = 1.4 \text{ mm}$ (eq 2); the average step sizes betrayed no directional drift (Figure 5C). We can thus conclude that our magnetic tweezers setup and magnet law are suitable for reliable long-term force measurements.

To resolve individually addressable HaloTag-protein L molecules, we modified the magnetic tweezers microscope stage with an $xy$-positioning stage with 100 nm resolution, and developed HaloTag-Ligand-functionalized fluid chambers having a $50 \mu m^2$ grid array (Figure S2). Using this combination, we could reliably return to individual beads on successive days. The molecule shown in Figure 5 was the longest lasting before detachment (14 days), from an initial group of 17 molecules that were tracked simultaneously and that lasted more than 1 day ($7 \text{ molecules, 1 day;} \ 3 \text{ days;} \ 1, \ 3 \text{ days;} \ 1, \ 4 \text{ days;} \ 1, \ 5 \text{ days;} \ 1, \ 6 \text{ days;} \ 1, \ 9 \text{ days;} \ 1, \ 10 \text{ days;} \ 1, \ 14 \text{ days}$). It is likely that the loss of anchoring occurred at the noncovalent streptavidin–biotin interface with the paramagnetic bead rather than the HaloTag-chloroalkane interface with the glass surface, which is covalent. Replacing the biotin–streptavidin interface with an orthogonal covalent HaloTag will solve this problem, allowing, in principle, for protein L constructs to be probed indefinitely.

Another potential source of error in the use of our magnet law results from bead-to-bead variations. However, our measurements indicate that for a given magnet position, M-270 paramagnetic beads generate a reproducible force. This is demonstrated in Figure 3D, where the overstretching B–S transition (65 pN) measured for 34 different traces from four different experiments, occurs over a very narrow distribution of magnet positions ($MP = 0.99 \pm 0.05 \text{ mm}$). Undoubtedly, it is important here to be able to position the magnets in a reproducible manner so that any variation results solely from the beads themselves. The voice-coil design with the submicron encoder solves this problem by giving a reliable positioning of the magnets between different experiments. Hence, we conclude that there is little variation in force among M-270 beads. Figure S7 compares our data and magnet law, with magnet laws obtained by others using thermal fluctuations of micrometer-long DNA tethers and the same paramagnetic beads (M-270; Dynabeads). It is clear that there is remarkably good agreement between these different methods at forces below 65 pN.$^{6c,14}$ While the change in force with magnet position has been shown to follow a single exponential,$^{20}$ several studies have proposed that a double exponential magnet law might be needed at higher forces.$^{13e,14}$ Magnetic tweezers, combined with HaloTag anchoring techniques, are opening up exiting possibilities to study protein dynamics with remarkable stability and accuracy under force clamp conditions. The methods demonstrated in this paper provide a robust approach to estimate the force applied to a protein in the biologically relevant low-force regime of 0–60 pN and for extended periods of time, comparable to protein turnover. These techniques complement force-clamp AFM spectroscopy that excels in speed and resolution at higher forces, but remains limited by mechanical drift in the low-force regime. To our knowledge, the mechanical study of a single protein over a period of 14 days is the longest yet reported. Such long-term recordings allow us to begin to study protein mechanics on the human time scale, where protein folding and misfolding occur over a lifetime, as, for example, with ocular cataracts or chronic traumatic brain injury. This long-term recording was enabled by the HaloTag covalent chemistry used for surface conjugation. Engineering of a second covalent anchor to replace the non-covalent biotin–streptavidin attachment to the magnetic bead would further improve the stability.
of the tethers, and could offer the potential for month-long or year-long single-molecule studies where rare events, such as those that take place in vivo, become detectable.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b05429.

Supplementary materials and methods, including Figures S1–S7 (PDF)

Magnetic Tweezers Software (TXT)

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#### Notes

The authors declare no competing financial interest.

### ACKNOWLEDGMENTS

This work was supported by NSF Grant DBI-1252857, and by NIH Grants GM116122 and HL061228. We would like to acknowledge Jie Yan, Hu Chen, and Mingxi Yao from the National University of Singapore for their help with the initial development of the instrument and for sharing the DNA–protein cross-linking protocol. D.J.E. were supported by the MSTP training grant to Columbia University. We thank also to Jaykar Nayeck for contributions to the development of the magnetic tweezers software.

### REFERENCES


