Single-Molecule Manipulation

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Detecting Force-Induced Molecular Transitions with Fluorescence Resonant Energy Transfer**

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Single-molecule techniques have been responsible for substantial advances in the field of biophysics. Among these approaches, single-molecule fluorescence resonant energy transfer (FRET) spectroscopy provides an experimental view of the structural properties of individual molecules, whereas optical-tweezers force microscopy allows direct manipulation of the reaction coordinate of a single molecule. However, the simultaneous application of these techniques is complicated by optical-trap-induced photobleaching, which substantially reduces fluorophore longevity to unacceptably short timescales. Herein, we describe a general solution to this problem and apply it to a novel force sensor based on a DNA hairpin, in the first successful combination of optical trapping and FRET. By alternately exposing the sample molecule to the optical-trapping and fluorescence-excitation lasers, we demonstrate the ability to reversibly manipulate a single molecule while simultaneously monitoring its structural configuration. This integrated measurement provides high-resolution mechanical control over molecular conformation with fluorescence-based structural reporting. The application of this technique for single-molecule exploration will lead to new experiments that employ combined optical trapping and single-molecule fluorescence for the simultaneous and active manipulation and monitoring of molecular structure in real time.

Single-molecule force microscopy and fluorescence spectroscopy reveal individual molecular properties that are clouded by the inherent averaging of ensemble methods. However, the individual approaches of these techniques often fail to uncover the interplay between applied mechanical forces and structural changes. A single measurement of a force-sensing molecule connects these two perspectives by directly manipulating a molecular reaction coordinate while simultaneously detecting localized structural effects.

Among the biophysical techniques capable of probing single-molecule properties, optical-tweezers force microscopy operates at piconewton force levels that are optimal for the detection of nanometer-scale conformational transitions. Likewise, single-molecule FRET spectroscopy provides complementary information about dynamic structural properties, including environment, orientation, and proximity, with comparable spatial resolution.[1] Previous efforts to combine these two techniques for a single, coincident measurement have been complicated by accelerated photobleaching rates induced by the high-intensity optical trap. Because of this effect, which is especially pronounced in common single-molecule FRET donor labels such as the dyes Cy3 and Alexa 555,[2] previous advances towards combining these techniques have spatially separated the fluorescent markers from the optical trap[3] or have employed uniquely robust chromophores.[4] We recently described a broadly applicable solution to this problem by alternately modulating the fluorescence-excitation and optical-trapping beams, which dramatically reduced this phenomenon without compromising trap integrity.[5] Herein, we show that such an optical modulation can be adapted to extend the emission times of FRET-paired labels without otherwise affecting their photophysical properties. To demonstrate this technique, we describe the first combination of optical-tweezers force microscopy with the single-molecule FRET detection of a novel force-sensing molecule into a single, integrated method capable of actively controlling molecular structure while simultaneously monitoring the conformational state of a single DNA hairpin molecule.

The mechanics of DNA hairpins have been studied at the single-molecule level and, thus, offer a benchmark for examining optical tweezers and single-molecule FRET in a combined arrangement. These structures, which are commonly used to model secondary structure in nucleotides, are

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readily adapted for the mechanical exploration of conformational dynamics, as they undergo a sequence-dependent, reversible unzipping transition.[6,7] In addition, alternate constructs have been adapted for force-sensing applications.[8] The structure used in this work, which contains a 20-base-pair hairpin stem, is flanked by noncomplimentary sequences annealed to oligonucleotides functionalized with the fluorophores Cy3 and Alexa 647 (Figure 1). Complexes exhibiting

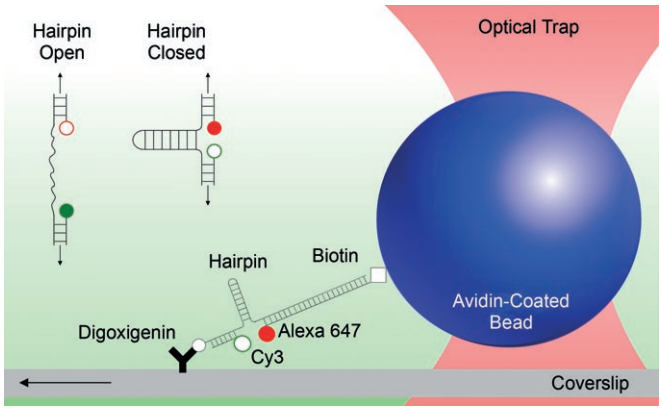

Figure 1. Experimental assay design (see Experimental Section for details). DNA hairpin complexes, labeled with opposing Cy3 and Alexa 647 fluorophores, were mechanically loaded by translating the coverslip, as the position of the trapped bead and the emission of the fluorophores were simultaneously monitored. The optical trap and the evanescent fluorescence-excitation field are depicted in the background in red and green, respectively. The inset cartoons show detail of the hairpin conformational change and the expected fluorophore emission (filled circles).

single-molecule FRET emission were mechanically loaded with the optical trap, effectively reducing the energetic barrier to hairpin opening. This unzipping transition, which occurs at a force of approximately 18 pN, comparable to other similar measurements,[7] was reflected by the displacement of the bead toward the center of the trap. The conformational transition was accompanied by a simultaneous reduction in FRET efficiency caused by the increased physical separation of the Cy3 donor and the Alexa 647 acceptor, which indicated the precise location of the structural change caused by the translation of the mechanical load between the low-force (ca. 6 pN) and high-force (ca. 24 pN) states (Figure 2). The DNA complexes were moved through several transitions in a process corresponding to the reversible opening and closing of the hairpin segment, which demonstrated both the high degree of mechanical control and the simultaneous reporting by FRET emission. Furthermore, in the representative trace, single-step photobleaching of the donor after approximately 65 s verified the single-molecule measurement.

This combination of optical-tweezers force microscopy and single-molecule FRET detection represents a significant advance for measuring the effects of structural changes on molecular function in a single molecule. By mechanically altering the conformational energy landscape, we actively induced a structural rearrangement pinpointed by strategically placed fluorescence labels. With minor modifications to

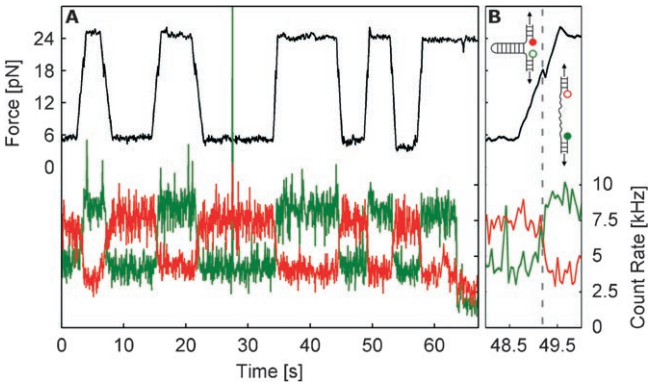

Figure 2. Mechanically induced conformational changes monitored with FRET spectroscopy. A) A DNA hairpin was manipulated with optical tweezers between open or closed conformational states (black) that transition at loads of approximately 18 pN. The state of the hairpin was revealed by FRET between the donor Cy3 (green) and the acceptor Alexa 647 (red). The donor photobleaches at approximately 65 s, confirming that a single FRET pair was monitored. B) Detail of a single hairpin opening transition accompanied by a simultaneous change in FRET, as highlighted by the gray dashed line. The inset cartoons depict the state of the hairpin.

existing assays, this approach can be extended beyond this model system to provide important new insight into the localized effects of mechanical force in biomolecular systems. For example, this combined technique can be adapted to monitor the intermolecular processes involved in the formation of a mechanically loaded protein complex,[9] the effects of mechanical deformation on single-enzyme catalysis,[10] or the intramolecular movements involved in biological-motor motility.[11,12] In addition, the presence of quantized single-molecule fluorescence signals can provide unambiguous verification of the size and location of a mechanical event, a critical tool for the design of often complex single-molecule assays. The new perspective that arises from this ability to physically deform single molecules while simultaneously measuring structural changes will allow the design of novel force-sensing molecules and will permit a new class of experiments for probing the interrelationship between molecular structure and biochemical function.

Experimental Section

A digoxigenin-labeled segment of single-stranded DNA with a 44-base self-complementary internal sequence (digoxigenin-GATGATGGTAGATGATGTATTGTTGTTTCGCCGCGGGCCGGCGCGCGGTTTTCCGCGCGCCGGCCCGCGGCGTTTGTGGAGCTGAGATGAGATGGTACTG; Integrated DNA Technologies, Coralville, IA (USA); detailed in reference [7]) was annealed at its ends to oligonucleotides labeled with Cy3 (Cy3-CAACAATACATCATCTACCATCATC; Integrated DNA Technologies) and Alexa 647 (GGATCCAGTACCATCTCATCTCAGCTCCAC-Alexa 647; Integrated DNA Technologies). This complex was then phosphorylated at its 5′ end with polynucleotide kinase (New England Biolabs, Ipswich, MA (USA)) and ligated with T4 ligase (New England Biolabs) to a biotinylated 1007-base-pair segment of double-stranded DNA (PABX4T-fimbrin; primer 1: biotin-CAAATCATCTGTTTCATTGAAACCTGACATG, primer 2: GATCC-abasic-ATGGATGAGATGGCTACCACTCAGATTTCC; Inte-

grated DNA Technologies). Low concentrations of hairpin complexes were incubated with 750-nm avidin-coated polystyrene beads (Bangs Laboratories, Fishers, IN (USA)) and immobilized on an antidigoxigenin (Roche Applied Science, Indianapolis, IN (USA))-coated glass coverslip (Corning Life Sciences, Inc., Acton, MA (USA); Figure 1). Other assay conditions and force-fluorescence instrumentation were as previously described.[5] Briefly, the instrumentation was carefully aligned to ensure coincident illumination of the sample plane by optical-trapping (1064 nm; Coherent, Santa Clara, CA (USA)), position-detection (975 nm; Corning Lasertron, Bedford, MA (USA)), and fluorescence-excitation (532 nm; World Star Tech, Toronto, ON (Canada)) lasers. To confirm the integrity of the two functional attachment points and separate fluorophore labels, the FRET activity on individual slides was verified through wide-field imaging on an intensified camera, translation of a single chromaphore to a predefined pinhole region, and acquisition on two separate avalanche photodiodes (Perkin Elmer Optoelectronics, Fremont, CA (USA)). After slide verification, tethered beads were prepositioned in a 0.204-pN nm^{-1} optical trap using a custom automated centering routine (Labview, National Instruments Corporation, Austin, TX (USA)), and individual hairpins were loaded to estimate the conformational transition force. The fluorescence excitation, set to 532 nm and 500 μW, was then uncovered, and the individual hairpins were loaded at 250 nm s^{-1} back and forth through several unzipping transitions. During this movement, the bead-position signals were filtered through an in-line anti-aliasing filter at 200 Hz (Krohn Hite, Brockton, MA (USA)) and then acquired at 20 Hz (Labview). The donor and acceptor fluorescence signals, which were also sampled at 20 Hz, were spatially isolated through a 200-μm pinhole, spectrally separated by a 628-nm dichroic mirror (Chroma Technologies, Rockingham, VT (USA)), and focused through 5-cm focal-length lenses onto separate avalanche photodiodes.

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