SINGLE MOLECULE STUDIES OF DNA BINDING PROTEINS AND FOLDING OF RNase H PROTEINS USING OPTICAL TWEEZERS

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A Sneak Preview :

Optical tweezers were invented in the mid-1980s by Arthur Ashkin and co-workers at the Bell Telephone Laboratories[18]. Since then there has been a steady stream of developments and applications, particularly in the biological field. In the last five years, work using optical tweezers has increased significantly and they are becoming a mainstream tool within biological and nanotechnological fields. It is twenty years since Ashkin et al.[1] published their seminal paper 'Observation of a single-beam gradient force optical trap for dielectric particles'. The technique is now referred to as 'optical tweezers' or 'optical trapping' and their original paper has received 400 citations—half of these during the last five years. In essence, optical tweezers rely upon the extremely high gradient in the electric field produced near the waist of a tightly focused laser beam, creating a force sufficient to trap micron-sized dielectric particles in three dimensions. Commercial tweezers systems are now available, and although originally devised by physicists, it is mainly biologists who put optical tweezers to use. Optical tweezers can trap objects in the nanometer to micrometer size range, and manipulate trapped objects with sub-nanometer accuracy[3] although nanometer resolution is more typical. More importantly, optical tweezers are compatible with various types of light microscopy, such as bright field, differential interference contrast, phase contrast and fluorescence[4]. These features have allowed optical tweezers to become one of the most successful single-molecule techniques used in biological science.

Initially, optical tweezers were applied to the single-molecule investigation of cytoskeletal motor proteins[5, 6]. Recent

advances have made it possible to study DNA binding proteins at the single-protein level. This important group of proteins includes those which affect conformational changes in nucleic acids, as well as energy-fueled molecular motors. In this review, the principle of the optical trap is explained briefly. Then, the application of optical tweezers to the study of DNA binding proteins is presented.

However, technology does not stand still and tweezing techniques are presently undergoing a further spate of development leading to new possible applications. Recently, the study of proteins acting on DNA was aggressively undertaken at the single-molecule level. This paper gives a review of a most recent application of optical tweezers which has revealed the dynamic behavior of folding process of Escherichia coli ribonuclease H RNaseH protein [Cecconi et al. Science 309, 2057 (2005)] and also the single molecule studies of DNA binding proteins.

The Principle Behind Optical Tweezers:

The Basics

What is it? Optical Tweezers use light to manipulate microscopic objects as small as a single atom[2]. The radiation pressure from a focused laser beam is able to trap small particles. In the biological sciences, these instruments have been used to apply forces in the pN-range and to measure displacements in the nm range of objects ranging in size from 10 nm to over 100 mm.

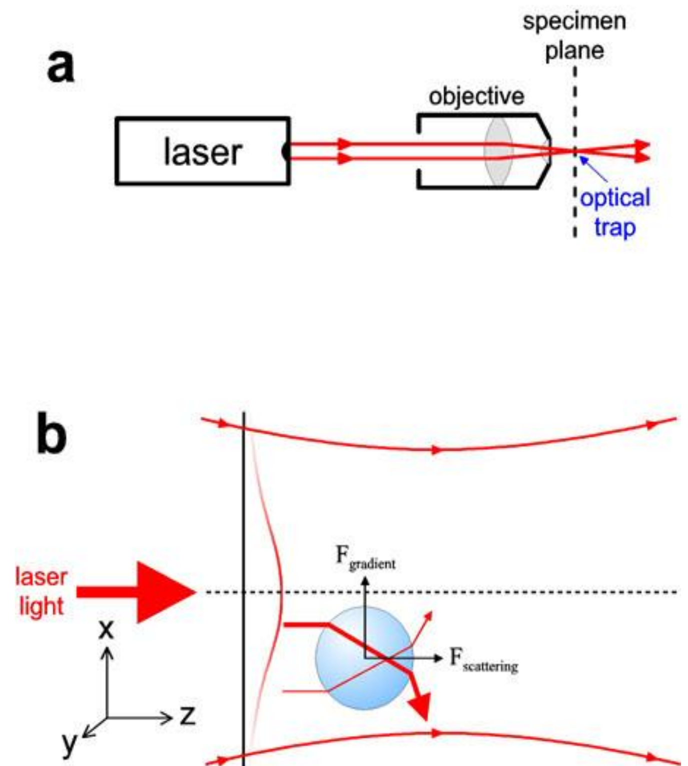

Figure 1. Optical Tweezers principles.

How does it work? The most basic form of an optical trap is diagramed in Fig 1a. A laser beam is focused by a high-quality microscope objective to a spot in the specimen plane[2]. This spot creates an "optical trap" which is able to hold a small particle at its center. The forces felt by this particle consist of the light scattering and gradient forces due to the interaction of the particle with the light (Fig 1b, see Details).

Most frequently, optical tweezers are built by modifying a standard optical microscope. These instruments have evolved from simple tools to manipulate micron-sized objects to sophisticated devices under computer-control that can measure displacements and forces with high precision and accuracy.

Applications

Optical Tweezers have been used to trap dielectric spheres, viruses, bacteria, living cells, organelles, small metal particles, and even strands of DNA. Applications include confinement and organization (e.g. for cell sorting), tracking of movement (e.g. of bacteria), application and measurement of small forces, and altering of larger structures (such as cell membranes). Two of the main uses for optical traps have been the study of molecular motors and the physical properties of DNA. In both areas, a biological specimen is biochemically attached to a micron-sized glass or polystyrene bead that is then trapped.

The ability to trap and manipulate small objects, such as polystyrene beads, results from light possessing momentum which is in the direction of propagation of the beam (Fig. 2). When the direction of light is altered by a particle *via* reflection or refraction, a corresponding change in momentum occurs. The law of conservation of momentum requires that the bead must undergo an equal and opposite momentum change, giving rise to forces acting on the particle.

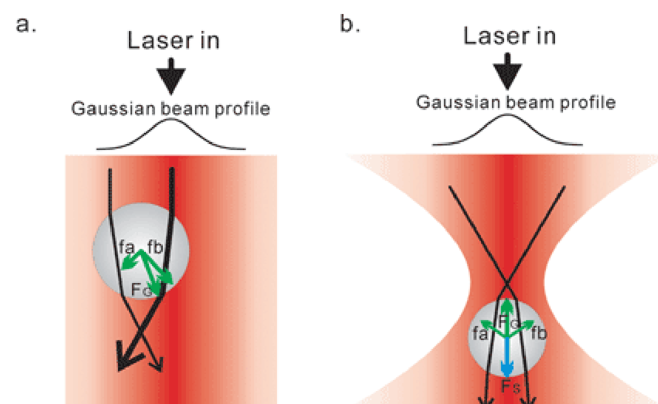

Fig. 2 Schematics showing the principle of optical tweezers based on ray optics. The representative laser paths are shown as black lines with arrows indicating the direction of beam propagation. The thickness of the black lines indicates the intensity of laser beam. The forces are shown as green and blue lines with arrows indicating the direction of forces. For detailed description refer to ref.4

Small particles (ranging in size from 10 nm to 10 µm in diameter) experience two types of forces near the focus which results in their stable three-dimensional trapping. Scattering forces arise from reflection of light at the surface of the particle, pushing the particles along the path of the laser beam in the direction of propagation of the light. In opposition to these, gradient forces tend to draw particles towards the center of the trap, thereby preventing their escape. Once the gradient forces dominate,

a stable three-dimensional trap results. Schematics showing these forces are presented in Fig. 2 and the reader is referred to ref. 4 for a more detailed description.

Science behind the Invention:

Over thirty years ago, Ashkin started experimenting with optical beams to manipulate objects[18]. He realized that an unfocused laser beam draws objects of high refractive index towards the centre of the beam and propels them in the direction of propagation. An arrangement of two counter-propagating beams allowed objects to be trapped in three dimensions. These experiments allowed him to observe the effects of radiation pressure and overcome the usually much larger radiometric (heating) effects of light by using relatively transparent objects in a transparent medium. He later discovered that a single, tightly focused, laser beam could be used to capture small dielectric particles in three dimensions. This technique enables small particles to be picked up and moved at will using a beam of visible light; hence the effect was christened optical tweezers. Forces acting within optical tweezers are understood either in terms of light momentum and ray optics or the force associated with the gradient in the optical electric field. For particles larger than the wavelength of the trapping light, ray optics analysis of the deviated light path gives the change in momentum flow and hence the reaction force acting on the object. For particles smaller than the wavelength, the ray optical approach is less satisfactory and it is better to consider the forces in terms of the electric field near the trapped particle. Forces can then be divided into those arising either from scattering of light or those arising from an intensity gradient. For particles of higher refractive index than the bathing medium (e.g. glass or polystyrene microspheres or bacteria in water), the gradient forces dominate and particles are drawn to the focal spot of a laser beam. For particles of low refractive index or strongly scattering material (air bubbles in water or metal particles), the gradient force is reversed and more complex beam arrangements are required if trapping is to be maintained (e.g. an annular beam).

1. Single molecule studies of DNA binding proteins using Optical Tweezers

1.1 Application to biological motors

The first biological motors studied at the single-molecule level with optical tweezers were cytoskeletal motor proteins[20]. These motor proteins (i.e.,

myosin, kinesin and dynein) generate mechanical force to move along protein tracks (for example, actin filaments and microtubules), using energy liberated from the hydrolysis of ATP. Force generation allows these motor proteins to engage in work inside cells, including that required to perform chromosome segregation, vesicle transport and muscle contraction. Using optical tweezers, precise measurement of the fundamental step size and maximum force generated by single molecules of these motor protein molecules has been achieved (for example, the step size and maximum force are 8 nm and 6 pN for kinesin, respectively).

For these studies, the optical tweezers were used to manipulate individual motor protein molecules via direct attachment to micrometre-sized polystyrene beads. The purpose of the beads is two-fold. First, they are used to deliver the motor to its tracks. Second, they are used to monitor the position of the bead attached motor proteins most recently with an accuracy of 0.1 nm, far beyond the diffraction limit of light. To track bead movement, the bright-field image of the bead is projected onto a photodetector, such as a quadrant photodiode or position sensitive detector, and the change in position of the image is measured (i.e., nanometry). Alternatively, the interference pattern of the bead can be projected onto a photodetector, where the change in the pattern is converted to a position signal to monitor bead movement (i.e., interferometry). Furthermore, and simultaneously, the measured displacement in each case can be converted to force exerted by the motor protein. As an application, a sensitive feedback system is being used to measure the position of a molecular motor under a constant load. When the bead attached to the molecular motor pulls with a force greater than a preset level, the position of the optical trap is moved to decrease the force. The error signal generated gives the position of the molecular motor[21].

1.1.1 Nucleic-acid polymerases

This family of DNA motor proteins plays essential roles in the life of an organism. While translocating along a DNA template (that is, a DNA track), DNA polymerases faithfully replicate DNA while RNA polymerases faithfully transcribe DNA into RNA. RNA polymerase (RNAP) is a highly processive motor, translocating thousands of base pairs without detaching from the DNA template. The energy used to drive RNAP translocation comes from

the nucleoside triphosphate addition to the 39-end of the nascent RNA molecule. During translocation, RNAP must accurately read the sequence of the template strand and transcribe mRNA that is subsequently translated into protein. Transcription occurs with high fidelity, and by comparison to DNA polymerase, must include a proofreading mechanism, as suggested by bulk-phase studies[22]. During proofreading, the enzyme would be expected to transiently pause and possibly even backtrack to allow removal of misincorporated bases.

To provide detailed insight into transcription fidelity, a series of studies were done using initially, the single-trap coverslip configuration and subsequently, the dual trap configuration to minimize noise (Fig. 2). The results revealed that the translocation velocity varied significantly between individual RNAP molecules. Second, for individual polymerase molecules, translocation was non-uniform, as traces of individual enzymes exhibited periods of constant velocity interspersed with several pauses of various duration. The pauses were classified based on their duration. Longer pauses had lifetimes greater than 20 seconds.11 They were distributed uniformly, occurring on average once every 1000 bp transcribed. Importantly, individual RNAP molecules were demonstrated to be capable of back tracking a distance of approximately 5 bp consistent with anticipated proofreading. In order to test whether backtracking was coupled to proofreading, GreA and GreB were added to the reaction. These proteins are transcription elongation factors which induce cleavage of nascent RNA. After backtracking, by cleaving the nascent RNA still bound to RNAP, the end of the RNA where transcription should be reinitiated is repositioned closer to the active site. This would be expected to enhance the rate of reinitiation and be observed as an overall decrease in pause duration. Consequently, in the presence of GreA and GreB, both the frequency and the duration of pauses were found to decrease. Therefore, the observed backtracking is consistent with a proofreading mechanism, which consists of rearward movement followed by nucleolysis

of RNA. The second class of pauses was shortlived, with a duration of 25 seconds or less, occurring with a frequency of y10 events every 1000 bp. These constituted > 95% of the pauses observed, with duration and frequency unaffected by load applied to the enzyme by the

optical tweezers, from 237 to +27 pN (negative and positive values indicates hindering and assisting force for

translocation, respectively). This is in contrast to the long-lived pauses described above, where frequency decreased from 1 event to 0.03 events every 1000 bp under load of +8 pN. The independence of the duration from load showed that motion accompanying the short pauses was as small as 0.06 bp, hence the short-lived pauses do not correspond to backtracking motion. Instead, the short-lived pause is a transient state, which could potentially precede long-lived pauses.

Most recently, the individual steps taken by RNAP during transcription were observed. The study demonstrated that RNAP advances predominantly in 3.7 A° (0.37 nm) increments along DNA. The distance of 3.7 A° is similar to that of one base pair in B-form DNA (i.e.3.4 A°), suggesting that RNAP advances one base pair each time a nucleotide is incorporated. Thus, during transcription, RNA polymerase translocates in increments of predominantly 1 base pair. When incorrect bases are incorporated, it pauses and backtracks to facilitate base removal, thereby ensuring transcription occur with high fidelity

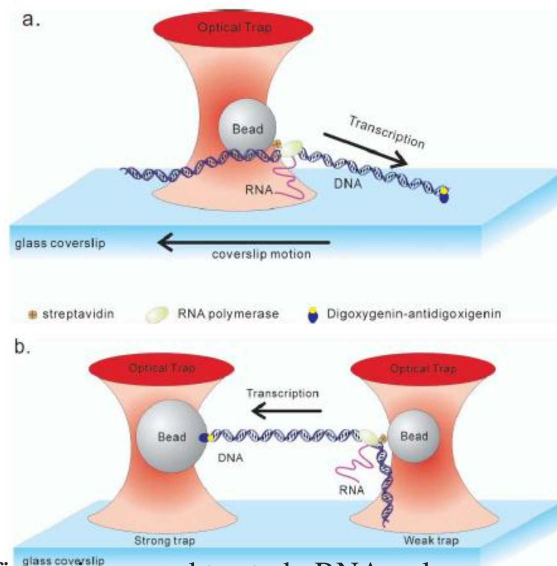

Fig. 3 Optical tweezers configurations used to study RNA polymerase. (a) A single trap configuration is shown. One end of the DNA is attached to a glass coverslip via a digoxigenin– antidigoxigenin linkage, while RNA polymerase is attached to an optically trapped bead via an avidin–biotin bond.7 The bead position is maintained by stage motion to provide constant tension (typically from right to left). (b) A schematic of a dual-trap configuration is shown. An RNA polymerase–DNA complex is trapped by two optical traps simultaneously. The left DNA end is manipulated via a digoxigenin–antidigoxigenin linkage, while RNA polymerase isattached via an avidin–biotin interaction. In this figure, the upstream end of DNA is linked to the left bead so that RNAP transcribes from right to left. The tension of the DNA–RNAP complex was kept constant during transcription by moving left stronger trap.

2.Folding of RNase H Protein:

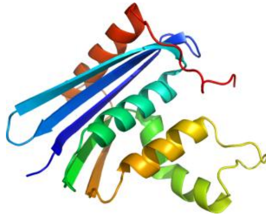

Fig. 4 RNase H Protein

Ribonuclease H or RNase H is a ribonuclease that cleaves the RNA in a DNA/RNA duplex to produce ssDNA. RNase H is a non-specific endonuclease and catalyzes the cleavage of RNA via a hydrolytic mechanism, aided by an enzyme-bound divalent metal ion. Importance of Ribonuclease H, or RNAse H, is that it is essential for HIV's function. Once HIV RNA has entered a cell and been copied into DNA by reverse transcriptase, it is no longer needed, since the DNA copy is what will be incorporated into the cell's genome. In fact HIV needs to get rid of the RNA so that the freshly-made DNA can get on and do its job. RNAse H helps by degrading the DNA-bound RNA. Hence it is an important target for antiretrovial drugs.Recently, Cecooni et al. studied the folding of RNaseH using single-beam optical tweezers. Force-induced unfolding of the molecule using optical tweezer was performed in order to probe the intermediate state. Two 500 base pairs DNA handles were attached to distinct positions on opposite sides of RNase H. Each handle was also independently attached to one of the two polystyrene beads. One of the beads was immobilized by a micropipette, and the other was trapped with a laser. Two different transitions were observed in the force extension curves when the protein was pulled apart two consecutive times. The high-force transition (19 pN) upon the first pull yielded the increase in extension of 50 nm; this matched the contour length of the unfolded protein and therefore was interpreted as complete unfolding of RNase H (N<-->U). After the protein is relaxed to a low force and is stretched again, reversible transitions of 40 nm in extension were observed at 5.5 pN (I<-->U); this implied that upon refolding, the protein does not completely return to the native state and that transitions between the folded state and a folding intermediate were occurring. This interpretation was supported by the fact that a longer wait in a low force, or a longer refolding time, restored the high-force transition. Such three-state behavior is also observed at a constant force for which the protein undergoes multiple transitions between the unfolded state and the intermediate state before finally settling on the native state.

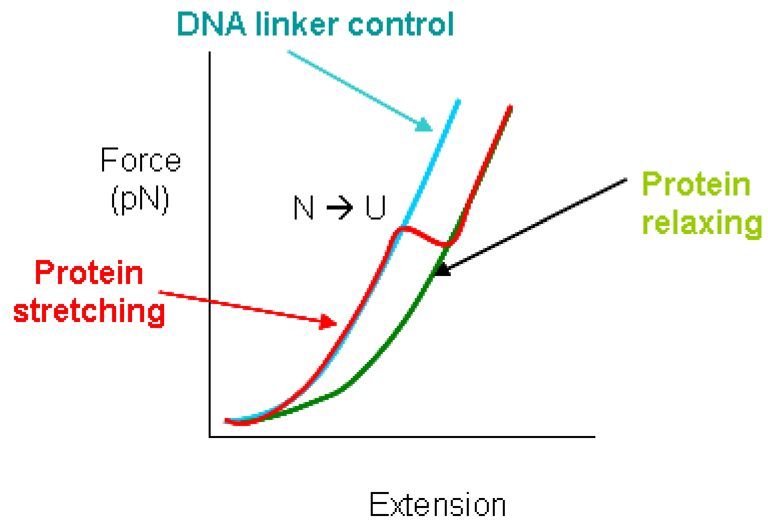

Fig. 5 RNase H folding transitions: Optical Trap

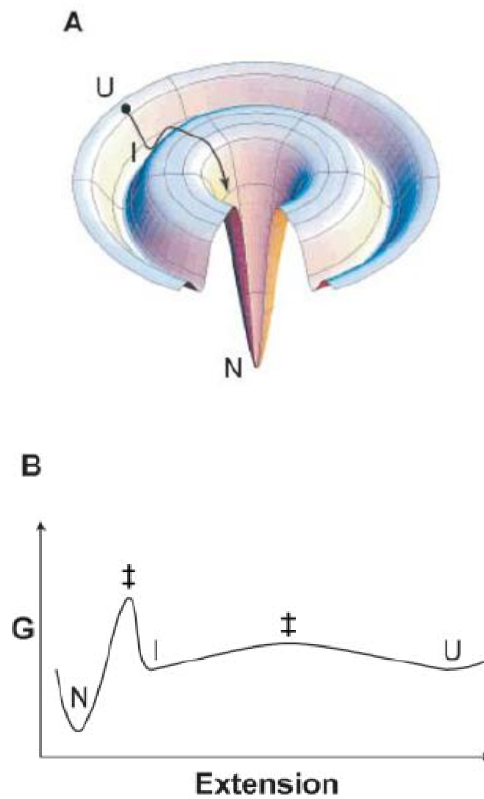

Fig. 6 Folding Landscape of RNase H protein

Conclusion:

Biological applications of optical tweezers are moving to a new level. Biologists thirst for more detailed mechanical and biochemical information on how single molecules work[19]. Recent advances in which optical tweezers have been combined with single molecule fluorescence imaging are a very exciting development. However, biologists also need advances in the tweezers design itself. They now want to grapple with single biological molecules and to sense the vibrations of individual domains and side chains with high time and high spatial resolution. The desire for higher-resolution data throws down the gauntlet to physicists to devise new breeds of optical tweezers that are sharper and more dextrous than those in use today.

As a model system, RNase H provides further incentive to investigate the folding pathway of more complex systems and potentially protein domains with strategically placed DNA handles. Further, a more accurate description of the folding landscape of proteins and transition states could be made, because single molecule methods can probe rare and potentially off pathway transient states.

In the coming years, further modifications to optical tweezer methods, capable of controlling the torque in the DNA or applying constant force using optical methods instead of a mechanical feed-back system, will also be applied to single-molecule studies of DNA binding proteins. Due to their broad adaptability, optical tweezers will undoubtedly continue to contribute significant insight to the understanding of protein–nucleic acid interactions, including those of ribosomes, DNA mismatch repair and recombination reactions.

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