DRUG-DNA INTERACTIONS AT SINGLE MOLECULE LEVEL

A VIEW WITH OPTICAL TWEEZERS

A dissertation presented by

Thayaparan Paramanathan

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In partial fulfillment of the requirements for the degree of

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In the field of

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ABSTRACT OF DISSERTATION

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Abstract

Studies of small molecule–DNA interactions are essential for developing new drugs for challenging diseases like cancer and HIV. The main idea behind developing these molecules is to target and inhibit the reproduction of the tumor cells and infected cells. We mechanically manipulate single DNA molecule using optical tweezers to investigate two molecules that have complex and multiple binding modes. Mononuclear ruthenium complexes have been extensively studied as a test for rational drug design. Potential drug candidates should have high affinity to DNA and slow dissociation kinetics. To achieve this, motifs of the ruthenium complexes are altered. Our collaborators designed a dumb-bell shaped binuclear ruthenium complex that can only intercalate DNA by threading through its bases. Studying the binding properties of this complex in bulk studies took hours. By mechanically manipulating a single DNA molecule held with optical tweezers, we lower the barrier to thread and make it fast compared to the bulk experiments. Stretching single DNA molecules with different concentration of drug molecules and holding it at a constant force allows the binding to reach equilibrium. By this we can obtain the equilibrium fractional ligand binding and length of DNA at saturated binding. Fitting these results yields quantitative measurements of the binding thermodynamics and kinetics of this complex process. The second complex discussed in this study is Actinomycin D (ActD), a well studied anti-cancer agent that is used as a prototype for developing new generations of drugs. However, the biophysical basis of its activity is still unclear. Because ActD is known to intercalate double stranded DNA (dsDNA), it was assumed to block replication by stabilizing
dsDNA in front of the replication fork. However, recent studies have shown that ActD binds with even higher affinity to imperfect duplexes and some sequences of single stranded DNA (ssDNA). We directly measure the on and off rates by stretching the DNA molecule to a certain force and holding it at constant force while adding the drug and then while washing off the drug. Our finding resolves the long lasting controversy of ActD binding modes, clearly showing that both the dsDNA binding and ssDNA binding converge to the same single mode. The result supports the hypothesis that the primary characteristic of ActD that contributes to its biological activity is its ability to inhibit cellular replication by binding to transcription bubbles and causing cell death.
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“The heights by great men reached and kept
were not attained by sudden flight,
but they, while their companions slept,
were toiling upward in the night.”

-Henry Wadsworth Longfellow
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<th>Deoxyribonucleic Acid</th>
<th>AFM</th>
<th>Atomic Force Microscopy</th>
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<td>Ribonucleic Acid</td>
<td>WLC</td>
<td>Worm Like Chain</td>
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<td>Adenine</td>
<td>FJC</td>
<td>Freely Jointed Chain</td>
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<td>Thymine</td>
<td>EtBr</td>
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<td>YO</td>
<td>Oxazole Yellow</td>
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<td>U</td>
<td>Uracil</td>
<td>ActD</td>
<td>Actinomycin D</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
<td>phen</td>
<td>1,10-phenanthroline</td>
</tr>
<tr>
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<td>Messenger RNA</td>
<td>dppz</td>
<td>dipyrido[3,2-a2’,3’]phenazine</td>
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<td>Transfer RNA</td>
<td>ΔΔ-P</td>
<td>Δ,Δ- [μ-bidppz(phen)4Ru2]</td>
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Chapter 1: Introduction

1.1 Deoxyribonucleic Acid (DNA)

1.2 DNA Replication and Transcription

1.3 Interactions of Small Molecules with DNA

1.4 Single Molecule Force Spectroscopy

1.5 Models of Polymer Elasticity
1.1 Deoxyribonucleic Acid (DNA)

Deoxyribonucleic acid, generally referred to as DNA by its acronym, is the basis of life, which contains the information for the development and functioning of all the living organisms. DNA was extracted from the cell in its crude form as a mixture with proteins and ribonucleic acid (RNA) in 1869 by Friedrich Miescher. In 1919 Phoebus Levene suggested that the DNA nucleotide is made of three elements phosphate, deoxyribose sugar and four nitrogenous bases which made up the four building blocks of the DNA namely adenine (A) thymine (T), guanine (G) and cytosine (C).

![Building Blocks of DNA - Adenine, Thymine, Guanine and Cytosine](image)

Figure 1.1.1: Building Blocks of DNA - Adenine, Thymine, Guanine and Cytosine

Friedrich Miescher (1844-1895) collected bandages used in local hospitals and extracted the pus from it using salt water and precipitated the nuclei using an alkaline solution. He named the unique chemical substance he found as “Nuclein”, which contained nucleic acids and proteins. He has discovered the crude extract of DNA at his age of 25 without even knowing that was the first step towards tracing the heredity.

Source: Dolan DNA Learning Center, Cold Spring Harbor Laboratory
Levene was the first to identify two different sugars associated with nucleic acids ribose and deoxyribose, which distinguished the RNA containing ribose sugar and DNA containing deoxyribose. He also numbered the carbon atoms in the pentapeptide sugar from 1-5, starting from the carbon, where the nitrogenous base was bound. The phosphate was found to bind the carbon#5 (Figure 1.2).

![Figure 1.2: Carbons in the pentapeptide ribose sugar (Blue) are numbered starting from the carbon where nitrogenous base (Cytosine) binds (Left). Nucleotides linked through phosphodiester bond to form chain (Right)](image)

Four different nitrogenous bases formed four nucleotides. These nucleotides are linked through a phosphodiester bond by the phosphate group of one nucleotide bonding through the hydroxyl group of the carbon#3 of the sugar in the other nucleotide. The end of the chain containing hydroxyl group is known as the 3’ end and the other end is known as the 5’ end.

Phoebus Levene (1869-1940) is well known for his incorrect tetra-nucleotide form of DNA despite of the important concepts of DNA he discovered, such as the DNA nucleotide is made of three elements phosphate, deoxyribose sugar and four nitrogenous bases. He published 700 papers (33 in Science) in his life time.

Source: Dolan DNA Learning Center, Cold Spring Harbor Laboratory
Levene thought that all four nucleotides were found in the same quantity, predicted that these four different nucleotides (A, T, C and G) were joined to form a tetra-nucleotide, and that DNA was a repeat of these tetra-nucleotides. Also until the 1940s many scientists, including Levene, believed that proteins carry the heredity information from generation to generation.

In 1951 Erwin Chargaff showed that all species have the same amount of Adenine (A) as Thymine (T) and same amount of Guanine (G) as Cytosine (C) in their DNA. This disproved widely accepted tetra-nucleotide model of Levene, which predicted equal amount of A, C, T and G. This gave the idea of base pairing in DNA strands and helped to end the race that was going on in determining the structure of DNA.

Figure 1.1.3: Base pairing in DNA- Cytosine (Orange) pairs up with Guanine (Purple) and Thymine (Red) pairs up with Adenine (Green) through hydrogen bonds (Maroon broken lines)

Erwin Chargaff (1905-2002) is well known for his rules about the DNA bases known as Chargaff’s ratio which lead to the discovery of the DNA double helix structure. He published 30 papers before he reached the age of 30.

Source: American Philosophical Society, Dolan DNA Learning Center, Cold Spring Harbor Laboratory
James Watson and Francis Crick at Cavindish Laboratory in Cambridge came up with the double helix structure of the DNA in 1953. This structure envisions two strands of DNA base pairing, where Adenine pairs up with Thymine and Guanine pairs up with Cytosine to form a twisted ladder called double helix. The above model was based on the X-ray diffraction image obtained by Rosalind Franklin and Raymond Gosling.

Backbone of the helix was made up with the deoxyribo sugar-phosphate bonds where each phosphate group carried a negative charge, which gives rise to a highly negatively charged backbone. The backbones are formed with two anti-parallel helical chains one running from 3′ to 5′ and the other running from 5′ to 3′ (Figure 1.4).

The base pairs stacked on top of each other to form the double helix structure (Figure 1.4), which has a wider groove and a narrow groove known as major and minor grooves respectively. The base stacking and the two different grooves are the significant characters of the DNA that is responsible for different types of DNA-small molecule interactions.

The most common form of DNA is known as the B-DNA, which is right-handed double helix which is the normal conformation of DNA in vivo. B form of DNA (Figure 1.4) has a diameter of 2 nm and base pair (bp) separation of 0.34 nm and the structure repeats every 10 base pair.
Francis Crick (1916-2004), James Watson and Maurice Wilkins (1916-2004) were awarded the 1962 Nobel Prize in Physiology or Medicine for their contribution towards the discovery of the DNA structure.

Source: Official Website of Nobel Prize (http://nobelprize.org)
1.2 DNA Replication and Transcription

Although the details of replication and transcription are much more complicated, I have outlined the process for non-biological readers to have a picture of these processes.

During cell division all living organisms have to produce two identical copies of the DNA from the parent cell. This process, known as replication, is essential to pass on biological heredity. During replication the dsDNA strands are unwound and separated by the enzyme helicase and single strand binding proteins binds to the separated strands to prevent them from re-annealing. The DNA polymerase, which reads the genetic codes to produce a complimentary copy, binds at the fork created by the helicase, known as the replication fork (Figure 1.2.1).

The DNA polymerase can move only in one direction (from 3’ to 5’) along the strand. Therefore replication from one of the strand known as the leading strand is not a problem and happens continuously. The other strand known as the lagging strand forms a loop (not shown in

Figure 1.2.1: Simplified Cartoon of Replication Fork
the diagram for simplicity) and is replicated in small fragments known as okazaki fragments. (For more details watch the video at www.dnai.org produced by Cold Spring Harbor Laboratory).

On the other hand, producing the complementary RNA copy of DNA is known as transcription. During transcription RNA polymerase binds to the DNA and produces an anti-parallel RNA strand, where all Thymine (T) nucleotides are replaced by its RNA complement Uracil (U). The stretch of RNA that is transcribed from DNA is known as the transcription unit, which expresses at least one gene (region of genomic sequence that carries specific information about heredity). Depending on the purpose of this unit the resulting RNA is named mRNA, tRNA, rRNA etc. The messenger RNA (mRNA) as they are named, carry the message code to produce proteins. RNA polymerase binds DNA at the promoter region and moves along DNA in the 3’ to 5’ direction by unwinding and forming a bubble in the DNA so the RNA transcription can be done at the bubble.

![Figure 1.2.2: Schematic diagram of transcription bubble](image)

**Figure 1.2.2: Schematic diagram of transcription bubble**
1.3 Interactions of Small Molecules with DNA

DNA interactions with small molecules are relevant to fundamental intracellular processes such as DNA replication, transcription, and the regulation of gene expression. Small molecules that bind DNA can interfere with these processes, and thus play a key role in rational drug design for complex diseases such as cancer and AIDS. Furthermore, a detailed understanding of small molecule binding to DNA may provide insight into the DNA binding properties of larger, more complex molecules such as proteins.

Small molecules may bind DNA covalently, which is an essentially irreversible interaction, or non-covalently in a reversible process. Our focus has been on reversible binding of small molecules since they can be potential drug candidates and can be easily characterized with single molecule experiments. Reversible binding modes can be categorized into three major groups, namely intercalation, single strand binding and groove binding.

![Diagram of DNA interactions with small molecules]

Figure 1.3.1: Schematic diagram illustrating the three major reversible binding modes of small molecules with DNA – intercalation, ssDNA binding and groove binding.
Intercalators have flat aromatic rings that slide between adjacent dsDNA base pairs for \( \pi \)-electron system interactions, lengthening and unwinding dsDNA\(^7\). Intercalators stabilize dsDNA, and are known to bind at the front of replication fork to prevent replication when used as drugs. Daunomycin, used in chemotherapy\(^8\), Proflavin, used against psoriasis and herpes virus,\(^9\) and ethidium are a few of the intercalators that are currently used as drugs for different diseases.

Only a few small molecules bind to single stranded DNA. Glyoxal (C\(_2\)H\(_2\)O\(_2\)) is one of them. It binds to exposed Guanine bases of the ssDNA. ssDNA binding is a more common mode for proteins.

The majority of molecules that bind in dsDNA grooves are positively charged, and thus binding is dominated by electrostatic interaction with the negatively charged backbone and assisted by hydrogen bonds and van der Waals interactions\(^ {10-11}\). Distamycin-A, netropsin (a naturally occurring antibiotic)\(^9\) and Hoechst 33259 are a few of the DNA groove binders.

Small molecules such as berenil and psoralen bind DNA with multiple binding modes, while molecules such as binuclear ruthenium complexes and YOYO have complex modes such as threading intercalation and bis-intercalation.

Berenil binds into the minor groove, favoring AT-rich regions, at low concentrations, but intercalates within the base pairs at high concentrations\(^ {10}\). Psoralen is a drug used in Psoralen Ultra Violet A (PUVA) therapy, which involves exposure of administered Psoralen to Ultra Violet (UV) A light as treatment of specific skin diseases\(^ {12}\). The drug intercalates with dsDNA, then forms a covalent bond with pyrimidine in one DNA strand to form a mono-adduct. Exposure to UV A light results in a covalent bond on the other DNA strand for formation of a
cross-link. Although exposure to UV B light may break cross-links, it does not affect the mono-adducts.

YOYO is a bridged oxazole yellow (YO) dimer which stacks two aromatic ring systems into two intercalating sites, causing a clamp-like binding known as bis-intercalation. It can also bind in the DNA major groove at high concentrations. This dye is particularly useful to study the properties of DNA, since it is non-fluorescent in solution and highly fluorescent upon binding to dsDNA.

Binuclear ruthenium complexes are two covalently linked Ru(phen)$_2$dpdz$^{2+}$ moieties, and they initially bind in the major grooves of dsDNA. These complexes then thread through the DNA bases, and the bi-dppz bridge intercalates between the dsDNA base pairs. In order for this dumb-bell shaped binuclear ruthenium complex to thread, the dsDNA must melt so that the bulky end can slide through the unpaired bases and adopt the final threaded conformation.

Actinomycin D is an antibiotic and anti cancer agent, recently shown to inhibit HIV replication is the most studied antibiotic, which has a long lasting controversial issue regarding its binding to DNA. It is believed to bind to both ssDNA and dsDNA.

We have studied the interactions of the Bimolecular Ruthenium complex (chapter 3) and Actinomycin D (chapter 4) using optical tweezers (chapter 2). The details are discussed in chapters 3 and 4.
1.4 Single Molecule Force Spectroscopy

Single molecule methods have provided a clearer understanding of a wide range of fundamental biological processes, including DNA replication, transcription, and repair. Single molecule force spectroscopy began with the capture and manipulation of single DNA molecules. Techniques such as optical tweezers, magnetic tweezers, and atomic force microscopy (AFM) apply forces to single molecules, probing conformational changes and structural dynamics in a variety of conditions. Such measurements explore the interactions of DNA with molecules ranging from small ligands to complex proteins. Quantifying the thermodynamics and kinetics of these interactions leads to substantial insights into DNA binding mechanisms in important biological systems.

Single-beam optical tweezers instruments focus a high power laser through a high numerical aperture microscope objective to form an optical trap. Dual-beam optical tweezers instruments use microscope objectives to bring two counter-propagating laser beams to an overlapping focus to form an optical trap. The trap captures one typically streptavidin-coated polystyrene bead, while a second bead is attached to a micropipette tip fixed to a flow cell or is held in another optical trap. A single biotin-labeled DNA molecule is tethered to the beads through a biotin-streptavidin linkage or some other attachment method that can withstand the forces to be applied. Translation of the flow cell or optical trap pulls the bead affixed to the pipette tip or held in the trap, resulting in extension of the captured DNA molecule. This displaces the bead in the optical trap, which provides a measurement of the force on the DNA molecule with piconewton (pN) accuracy \(^26\).
Magnetic tweezers use a glass slide and magnetic bead, both coated with streptavidin or another attachment ligand, in order to capture a biotin-labeled DNA molecule. Translation of the glass slide through a magnetic field gradient results in a force on the DNA molecule, measured as three-dimensional motion of the magnetic bead in video acquisition. Advantages of this technique include single molecule manipulation in three dimensions and detection of forces as low as 0.05 pN\textsuperscript{27}.

Although AFM is predominantly used in imaging applications, the technique may be used for single molecule force spectroscopy. A single DNA molecule is immobilized between the surface and the AFM tip, and force is measured as a function of extension and relaxation. A typical DNA attachment technique functionalizes opposite ends of the molecule with thiol and biotin. The thiolated end binds covalently to a gold surface, while the streptavidin-coated AFM tip captures the biotin-labeled end of a single DNA molecule\textsuperscript{28}. Resolution of the DNA stretching curves is on the order of 5-10 pN\textsuperscript{29-30}.

Steven Chu (the current energy secretary of the USA) was awarded the 1997 Nobel Prize in Physics for his contribution towards the optical trapping of small molecules. The first demonstration was actually done by Arthur Ashkin in 1970 was published in PRL.

Source: Official Website of Nobel Prize (http://nobelprize.org)
1.5 Models of Polymer Elasticity

Polymer models of dsDNA and ssDNA effectively characterize DNA force-extension curves obtained in single molecule force spectroscopy measurements.

The Worm-Like Chain (WLC) model used to describe stiffer polymers, assumes a smooth distribution of bending angles. For a polymer of length (L), the tangent vector \( \vec{t}(S) \) at position \( \vec{r}(S) \) at distance S along the chain can be written as:

\[
\vec{t}(S) = \frac{d\vec{r}(S)}{dS}
\]  

(1.5.1)

It is shown that the orientation correlation function for WLC model decays exponentially\(^3^1\):

\[
\langle \vec{t}(0) \cdot \vec{t}(S) \rangle = \exp \left( -\frac{S}{P} \right)
\]

(1.5.2)

where \( P \) is known as the persistence length, which reflects the bend stiffness of the polymer. WLC model describes dsDNA in terms of observed length \( b_{ds} \) of an elastic polymer under the influence of tension \( F \).\(^3^2\)-\(^3^6\). Though no exact solutions to this model are known, an approximate solution is appropriate for high forces:
where $P_{ds}$ is the persistence length, $B_{ds}$ is the end-to-end or contour length of dsDNA, and a stretch modulus $S_{ds}$ is added to account for backbone extensibility. Here $k_B$ is Boltzmann’s constant and $T$ is temperature.

On the other hand the Freely Jointed Chain (FJC) model describes the as a collection of independent monomers with varying bond angles connected together by free hinges.

![Figure 1.5.2: Freely Jointed Chain Model](image)

FJC model is used to describe more flexible polymers like ssDNA:

$$b_{ss}(F) = B_{ss} \left[ \coth \left( \frac{2P_{ss}F}{k_BT} \right) - \frac{1}{2} \frac{k_BT}{P_{ss}F} \right] \left[ 1 + \frac{F}{S_{ss}} \right].$$

where $P_{ss}$ is the persistence length, $B_{ss}$ is the end-to-end or contour length of ssDNA, and a stretch modulus $S_{ss}$ is added to account for backbone extensibility.
Chapter 2: Methodology

2.1 Optical Tweezers

2.2 Stretching Single DNA Molecule

2.3 Force Induced Melting Transition

2.4 Viewing DNA Interactions with optical tweezers

Portions of this chapter are from the publication:

Biophysical Characterization of DNA Binding from Single Molecule Force Measurements

Kathy Chaurasiya, Thayaparan Paramanathan, Micah J. McCauley and Mark C. Williams

Physics of Life Reviews (In Press Vol 7 Issue 3)

Impact Factor: 3.773
2.1 Optical Tweezers

A strongly focused laser beam has the ability to catch and hold particles of dielectric material in a size range from nm to µm. This technique used in optical tweezers makes it possible to study and manipulate particles like atoms, molecules and small dielectric spheres. Optical tweezers have been proven as a powerful tool to study biological systems since it was introduced to study biological molecules in 1987. Optical tweezers are capable of measuring forces of the order of 0.01 to 300 piconewtons.

The nature of the force is predicted by the trap stiffness. The force exerted on the bead is expected to be proportional to the displacement. The proportionality constant $k$ is known as the trap stiffness.

$$ F = kx $$

(2.1.1)

Physics behind the tweezing is due to the dipole-force (gradient-force) of light incident on a dielectric object. The dipole force is due to the interaction between the dipole induced by the electric field from the laser and the electric field itself. The force is proportional to the gradient of the intensity of the laser field $E$, thus leading to the name "gradient force".

$$ F = \frac{1}{2} \alpha \nabla E^2 $$

(2.1.2)

where $\alpha$ is the proportionality constant between the polarization of dipole and electric field.
If the diameter of trapped particle (5µm in our case) is significantly larger than the wavelength of the laser used (830nm in our case) the trapping can be explained by ray optics. The particle will experience two types of force one is the scattering force which tends to push the particle in the direction of laser propagation and the other is the gradient force that tends to push it towards the center of the beam.

For simplicity let’s consider a particle on the center of the beam to understand the physics behind the scattering force (Figure 2.1.1). With two symmetric rays (blue and red) we can calculated the net momentum change on the photons in the ray due to the refraction of light and attribute equal and opposite momentum to the bead.

![Figure 2.1.1: Scattering Force on the bead](Image)
It is clear that the bead is pushed in the direction of the beam by the scattering force. Now to understand the gradient force let’s consider a particle that is slightly displaced from the center of the beam (Figure 2.1.2). Gaussian profile of the laser beam now plays a huge role in trapping. The more intense beam (maroon) push the particle towards the center and the less intense beam (pink) push the particle away from the center. Since the momentum change caused by the intense beam is much higher the net force due to gradient of the laser will be towards the center.

![Gaussian Profile](image)

**Figure 2.1.2: Gradient Force on the bead**

In the dual beam optical tweezers set up there are two counter propagating laser beams focused tightly to trap the particle. In this case scattering force from the counter propagating beams cancel out each other.
The dual beam optical tweezers (Figure 2.1.3) used in my experiments, built by Dr. Micah J. McCauley, uses two 200 mW 830 nm continuum wave diode lasers. The beam (red lines in Figure 2.1.3) from these lasers are well collimated and tightly focused into a flow cell by two microscopic objectives which have a working distance of 2 mm.

![Dual beam optical tweezers setup in Williams Lab](image)

Figure 2.1.3: Dual beam optical tweezers setup in Williams Lab

The tightly focused laser beam is capable of catching small polystyrene beads of diameter around 5 µm. When a force is exerted on the bead, it gets displaced. As a result, the laser is deflected and the deflection of laser is detected with the help of position sensing detectors. This deflection is proportional to the force exerted on the bead and hence the force on the bead can be calibrated to the deflection measured.
In a real experiment streptavidin-coated beads are flown into the flow cell of volume around 200 µL. Once a bead is trapped with the tweezers (Figure 2.1.4a) it is attached to the micro pipette which is a part of the flow cell. Then the pipette is moved away so that another bead can be trapped by the laser tweezers. Once we have two beads, one attached to the tip of the micropipette and the other trapped by the laser (Figure 2.1.4b) all the excess beads are rinsed away with the buffer flow. Then DNA (λ phage with 48500 bp) which is labeled with biotin at the opposite strand 3’ ends is flowed through the flow cell. The DNA gets attached to the bead in the trap (Figure 2.1.4c) by biotin-streptavidin bonds, which are strong enough to hold the link up to the force limit used in our experiments. The free end of the DNA, which floats with the flow of the cell, is then fished by the bead attached to the tip by moving the tip back and forth. Once we catch the other end we rinse the excess DNA with the buffer flow and now we have a single DNA molecule attached between the beads (Figure 2.1.4d). The force-extension curves obtained by stretching this single DNA molecule have provided a new way of looking at the interactions of DNA at single molecule level.

Figure 2.1.4: Fishing for single DNA molecule
2.2 Stretching single DNA molecules

Single molecule DNA stretching experiments determine force as a function of extension (Figure 2.2.1). At low extensions, the measured tension increases gradually as the duplex uncoils in what is known as the entropic regime. As extension approaches the dsDNA contour length, the backbone resists further extension and the force increases dramatically in an elastic response. At ~65 pN, dsDNA undergoes an overstretching transition, increasing to ~1.7 times its contour length at nearly constant force.

![Force-Extension curve of single DNA molecule stretched and relaxed in optical tweezers experiment](Figure from McCauley & Williams, Biopolymers 85 (2007))
There is a second transition at the end of this overstretching plateau, near the contour length of ssDNA. If the extension is reduced at this point, the relaxation curve will match the stretching curve. Some hysteresis, where the relaxation curve (blue open circles) does not match the stretching curve (blue solid points) may occur, depending upon solution conditions. DNA stretching and relaxation cycles exhibit similar force-extension curves on the timescale of the experiment in typical solution conditions, indicating that the process is reversible.

The wormlike chain model fit (purple solid curve) to dsDNA and the freely jointed chain model fit (green solid curve) to ssDNA shown in the diagram clearly illustrate that the overstretching transition at 65pN is a force induced melting transition, in which dsDNA is converted to ssDNA. Although there have been 2 models describing this transition, this issue will be discussed in detail in the next chapter. The area between the dsDNA and ssDNA curves (blue shaded area) reveals the free energy of equilibrium melting (ΔG) when the transition is reversible.
A thermodynamic model quantitatively describes the overstretching transition at 65 pN in terms of force-induced melting. The force exerted on the dsDNA molecule does work to increase the length of the DNA, converting dsDNA to ssDNA and disrupting both base pairing and base stacking interactions. In this model, the second transition at the end of the melting plateau is a non-equilibrium process involving the remaining base pairs of dsDNA which must break for strand separation. Force-induced melting is analogous to thermal melting, and the model predicts that solution conditions that influence thermal melting, such as salt, pH, and temperature, also affect the force-induced melting transition. DNA stretching experiments quantitatively confirmed these predictions, and recent modeling studies also support a force-induced melting model. Furthermore, experiments demonstrated that solution conditions and DNA binding ligands known to inhibit DNA reannealing induce strong hysteresis in the force-extension curves, providing additional evidence for melting of the DNA strands.

In an alternate model of the overstretching plateau, B-form duplex DNA lengthens in response to the applied force, undergoing a structural transition to a new form of DNA referred to as “S-DNA”. This form of DNA is predicted to preserve base pairing but not base stacking, a distinction based upon the observation that strand separation occurs at high forces. An early modeling study predicted a transition to this form of DNA at a significantly larger force than experiments observed. Recent studies use the proposed existence of S-DNA as a means to generate new parameters to fit stretching curves and other experimental results.
However, it is not clear that additional fitting parameters are needed to explain DNA stretching experiments. In addition, these models do not make predictions that can be tested with other experiments, making it difficult to test proposed S-DNA models \cite{31,60-61}. Magnetic tweezers experiments with both strands of a dsDNA molecule tethered to beads did not observe the transition at 65 pN, but instead measured a transition at 110 pN over a similar extension attributed to a combination of S-DNA and P-DNA, a form of melted DNA which is overwound \cite{62}. Although this particular transition of torsionally constrained DNA is consistent with force-induced melting, it was suggested that some features of DNA stretching curves were incompatible with force-induced melting \cite{63-64} and that the structure of DNA in the overstretching transition remains unclear \cite{65-66}.

It is essential to establish the nature of the overstretching transition in order to use single molecule force spectroscopy techniques to characterize DNA binding. Recent experiments use glyoxal, intercalating dyes, and single-stranded binding proteins (SSBs) to establish that this conformational transition involves base pair disruption, and therefore DNA overstretching is force-induced melting of dsDNA into ssDNA.

Glyoxal (C$_2$H$_2$O$_2$) is a small molecule which binds irreversibly to exposed guanine bases of DNA with slow kinetics \cite{67}. The modified guanine bases have three rings instead of two, introducing steric constraints that hinder base pair reannealing \cite{68}. λ-DNA molecules were held at fixed extensions for ~30 min in the presence of glyoxal, which is the timescale required for DNA binding \cite{67}. The DNA stretching curve exhibits a decrease in melting force and strong hysteresis (Figure 2.3.1), indicating that guanine bases exposed to solution are subject to glyoxal modification and subsequently prevent DNA reannealing \cite{67}. Therefore extension into the
overstretching plateau exposes ssDNA bases to solution, reflecting force-induced melting of the dsDNA molecule.

Figure 2.3.1: Force-Extension curve of single DNA molecule in presence of glyoxal
Shows lowering of melting force and huge hysteresis

As the DNA molecule is held at larger fixed extensions, the corresponding relaxation curves exhibit additional hysteresis (Figure 2.3.2). These results demonstrate that glyoxal binding increases as the DNA molecules are held further into the overstretching plateau, despite constant solution conditions. This indicates that greater extensions into the stretching transition result in exposure of additional bases, and the relaxation curves in the presence of glyoxal are a combination of dsDNA and ssDNA. The experimental data fits well to a linear combination of
the FJC and WLC polymer models (fits shown in Figure 2.3.2), where the measured contour length $b$ is a function of the ssDNA fraction $\gamma_{ss}$:

$$
\begin{equation}
(2.3.1)
\end{equation}
$$

and $b_{ds}$ and $b_{ss}$ are force-dependent DNA extensions from the WLC (Equation 1.5.3) and the FJC model (Equation 1.5.4), respectively. The fractional extension along the transition plateau agrees well with the fraction of glyoxal-stabilized ssDNA obtained from fits to Equation 2.3.1, which provides structural evidence that DNA overstretching is indeed the force-induced melting of dsDNA into ssDNA.

Figure 2.3.2: Force-Extension curve of single DNA molecule in presence of glyoxal
Shows increased hysteresis as we progress along the melting transition
The significant presence of ssDNA exposed to glyoxal modification in the overstretching transition is unlikely to arise from nicks in the DNA backbone \textsuperscript{69}, and further experiments with small molecules and SSBs confirm the force-induced melting model. Recent single-molecule studies have directly visualized the nature of the structural transition in a combination of optical tweezers and fluorescence imaging techniques \textsuperscript{70}. A DNA molecule stretched to a fixed extension in the absence of ligand is briefly transferred into the presence of YOYO, a fluorescent dye which intercalates into the paired bases of dsDNA \textsuperscript{13}. Subsequent imaging reveals only regions of dsDNA, to which the intercalator can bind \textsuperscript{70}. The fraction of dsDNA present at each fixed extension corresponds directly to fractional extension along transition plateau, illustrating a structural conversion from dsDNA into a form of DNA to which YOYO is unable to bind \textsuperscript{70}.

Experiments with fluorescent dye-labeled SSBs demonstrate that the form to which dsDNA is converted upon overstretching is ssDNA. Human mitochondrial SSB (mtSSB) binds and wraps relaxed ssDNA \textsuperscript{71}, but does not affect the overstretching transition or bind ssDNA which is under tension greater than \textasciitilde40 pN. When DNA is extended into the stretching transition and briefly placed in the presence of mtSSB, the images show fluorescent spots at both ends of the DNA molecule, indicating the presence of protein-wrapped ssDNA \textsuperscript{70}. These spots increase in brightness and move toward the center of the molecule as a function of extension, illustrating the relative increase of ssDNA with progressive movement into the stretching transition. This method also visualizes nicks in the DNA backbone, since the mtSSB wraps the relaxed ssDNA in the middle of the molecule. Molecules without nicks do not exhibit these binding events, and mtSSB fluorescence is confined to the ends of the DNA. Two-color fluorescent measurements
with both YOYO and mtSSB confirm that mtSSB-wrapped ssDNA forms at an interface with YOYO-labeled dsDNA.

In contrast with mtSSB, the SSB Replication Protein A (RPA) binds ssDNA under tension of at least 70 pN. RPA is able to bind both ssDNA under tension and relaxed ssDNA without wrapping it. Two-color fluorescence measurements with eGFP-labeled RPA and bis-intercalator POPO-3 show three fluorescent regions. The dsDNA segment has two bright spots of relaxed ssDNA on either side, followed by two ssDNA strands extending out to their respective attachment sites on each bead. Application of flow perpendicular to the axis of the molecule stretches out the relaxed ssDNA, clearly illustrating both strands of ssDNA created upon dsDNA overstretching.

Similar experiments with a DNA molecule attached to beads on both strands reveal the torsionally-constrained transition at 110 pN, with sites of POPO-3-labeled dsDNA and RPA-labeled ssDNA throughout the molecule. The negative correlation of dsDNA and ssDNA areas on the same DNA molecule implies spatial separation of melted regions, with no evidence to support an interpretation of separate S-DNA and P-DNA phases. The data also indicate that short regions of dsDNA remain when DNA is stretched to forces beyond the overstretching transition (in the second transition at the end of the overstretching plateau, near contour length of ssDNA). Thus, complete separation of the strands require application of unexpectedly high forces, but most of the DNA has been melted by force during overstretching. Although this pulling-rate dependent transition is not well-described, it exists even in the presence of ssDNA binding ligands, which is unexpected in the S-DNA model.
The results of these single-molecule fluorescence imaging experiments are consistent with formation of ssDNA during both structural transitions, an observation which is incompatible with the prediction of unexposed individual bases of the S-DNA model. Thus the overstretching transition is a force-induced melting transition, in which the applied force does work to melt dsDNA into ssDNA. Therefore DNA stretching experiments involve melting of the two strands. This result can be used as a basis for investigation of the biophysical mechanisms of DNA-ligand interactions with single molecule force spectroscopy techniques.
2.4 Viewing Drug-DNA Interactions with Optical Tweezers

Most of the drugs/potential drugs interact with DNA through reversible binding modes. Optical tweezers is a useful tool to distinguish different binding modes. The main features that distinguish different binding modes qualitatively are the melting force, the cooperativity of the transition, and the hysteresis observed during relaxation. All of the dsDNA binding modes increase the melting force\textsuperscript{26,75-78}, as expected thermodynamically. Intercalators show a force-dependent lengthening upon binding to dsDNA which increases with drug concentration and saturates at some concentration (Figure 2.4.1a). Also after a critical drug concentration the melting transition vanishes, indicating a critical point (Figure 2.4.1b) beyond which there is no distinction between ssDNA and dsDNA\textsuperscript{75}.

![Figure 2.4.1: (a) Force-extension curve in the presence of intercalator ethidium (b) Phase diagram and critical point of ethidium](image-url)
Major and minor groove binders have similar effects on DNA stretching curves, but major groove binders decrease the cooperativity of the melting transition\textsuperscript{11}. The ssDNA binding modes show lowering of the melting force\textsuperscript{48-50,53,67,77,79-81} and induce hysteresis between the stretching and relaxation. Significant hysteresis is also observed in the presence of bis-intercalators and threading intercalators, reflecting slow dissociation kinetics\textsuperscript{82-84}.

Although several studies discussed qualitative discrimination between intercalation, minor and major groove binding\textsuperscript{9-11}, the first quantitative approach was with low force (\(F \leq 15\text{pN}\)) extension measurements obtained using optical tweezers\textsuperscript{85}, followed by fits of the data to the WLC model (Equation 1.5.3) in the near-full extension limit\textsuperscript{86}. Several more recent studies use the WLC in both the and low high force limits to obtain the contour length \(B_{ds}\) and persistence length \(P_{ds}\) of different small molecules (Figure 2.4.2).

![Figure 2.4.2: Fractional increase in contour length (right) and persistence length (left) of the DNA-ligand complex for different drugs that exhibit a variety of binding modes.](image-url)
The contour length obtained from fits to the WLC model in both low (blue bars) and high (brown bars) force limits provide the same insights. Minor groove binders (drug names shaded in yellow) and major groove binders (drug names shaded in purple) do not change the contour length, while intercalators (drug names shaded in light blue) and the bis-intercalator YOYO exhibits an increase in contour length for DNA-ligand complexes. Concentration-dependent studies on intercalators and bis-intercalators show that the contour length increases with the increase in concentration until saturation (green bars).

Measurements of minor groove binders at low force (less than 15 pN) generally show an increase in DNA persistence length, whereas a measurement of minor groove binder Distamycin-A at higher force showed an increase in DNA persistence length. Major groove binders cause a decrease in persistence length. Concentration-dependent studies of intercalators show that the persistence length of the DNA-intercalator complex initially increases with concentration, dropping to the value of dsDNA, and then dropping even lower at very high or saturated concentrations.

In addition the binding constant and binding site size of the drugs can be estimated using the McGhee and von Hippel isotherm. The site exclusion binding isotherm of McGhee and von Hippel relates the occupancy $\gamma$ to the drug concentration $c$, for a drug with an equilibrium association binding constant $K$ and binding site size $n$.

$$\gamma = Kn c \frac{(1-\gamma)^n}{\left(1-\gamma + \frac{\gamma}{n}\right)^{n-1}}$$ (2.4.1)
The force-dependent fractional occupancy of the DNA lattice \( \nu \) is defined in terms of the binding site size \( n \):

\[
\nu = \frac{\gamma}{n} \tag{2.4.2}
\]

Thus ligand binding may be described in terms of fractional occupancy \( \gamma \), which runs from 0 to 1, or fractional occupancy per base pair \( \nu \), which accounts for binding site size and therefore runs from 0 to \( 1/n \). The fractional occupancy per base pair for intercalators, \( \nu_{\text{int}} \), at a given force \( F \) is:

\[
\nu_{\text{int}} = \frac{b_{\text{ds}}(F,c) - b_{\text{ds}}(F,0)}{B_{\text{ds}}} \tag{2.4.3}
\]

where \( b_{\text{ds}}(F,c) \) is the extension in the presence of the intercalator at concentration \( c \), \( b_{\text{ds}}(F,0) \) is the extension in the absence of intercalator, and \( B_{\text{ds}} \) is the DNA contour length in the absence of intercalator. This factional binding per base pair \( \nu \) is fit to the McGhee von Hippel isotherm (Equations 2.4.1 and 2.4.2 combined) to obtain the equilibrium association constant \( K_F \) and binding site size \( n_F \) at a given force \( F \) (Figure 2.4.3).
Single molecule studies propose a method of quantifying force-dependent intercalation (Figure 2.4.4) which extrapolates the results to zero force, characterizing ligand binding to relaxed DNA \(^7\). This method is now widely used to analyze intercalation of small molecules. The force \(F\) applied to stretch the DNA molecule reduces the free energy of intercalation \(\Delta G_0\) by \(F\Delta x\), where \(\Delta x\) is the dsDNA elongation upon a single intercalation event, leading to an exponential force dependence of the binding constant \(^7\):

\[
K = K_0 \exp \left( \frac{F\Delta x}{k_B T} \right)
\]

(2.4.4)

where \(K_0\) is the binding constant in the absence of the force, \(k_B\) is the Boltzmann constant and \(T\) is the absolute temperature. Figure 2.4.5 presents \(K_0\), \(\Delta x\), and effective binding site size \(n_0\) for ethidium \(^7\), \(\text{Ru(phen)}_2\text{dppz}^{2+}\) \(^7\), \(\text{Ru(phen)}_3^{2+}\) \(^7\) and \(\text{YO}\) \(^8\) obtained using this method.
These results show that single molecule force spectroscopy is a useful method for quantitatively characterizing the thermodynamics and kinetics of drug interactions with DNA.

Figure 2.4.5: Binding parameters for various intercalators at zero force

These results show that single molecule force spectroscopy is a useful method for quantitatively characterizing the thermodynamics and kinetics of drug interactions with DNA.
Chapter 3: DNA Threading of Binuclear Ruthenium Complexes

3.1 Ruthenium Complexes

3.2 Mechanically Manipulating the DNA Threading Rate

3.3 Quantifying the Threading of the Binuclear Ruthenium Complexes

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Mechanically Manipulating the DNA Threading Intercalation Rate
Thayaparan Paramanathan, Fredrik Westerlund, Micah J. McCauley, Ioulia Rouzina, Per Lincoln and Mark C. Williams


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3.1 Ruthenium Complexes

As we have discussed in the previous sections, studies of the interactions of small molecules with DNA are essential for designing and developing better probes for diagnosis and drugs for cancer therapy. Since [Ru(phen)$_3$]$_2^{2+}$ (phen=1,10 phenanthroline) (Figure 3.1.1) was introduced in 1984$^{89}$, ruthenium complexes have played an important role in the design of DNA binding small molecules. In 1995 scientists from United Kingdom, Germany, Netherlands, Italy and Czech Republic started a project called “COST” to discuss the activity in the field of ruthenium anti-tumor compounds. Synthesis of new ruthenium compounds and testing of their pharmacological activity and DNA interactions were main goals of the project$^{90-92}$.

Recent studies succeeded in introducing carriers to target the metal compounds to specific places in the organism. The ruthenium metal complexes are inserted into transferrin, the protein that is used to transfer the iron$^{91,93-94}$. Transferrin acts like a Trojan horse and delivers ruthenium to the tumors, which actually requires excess iron. Recent tests have shown that our ruthenium compounds are much more effective than the clinically used therapeutic drugs, in particular in intestinal tumors and they are already entered clinical trials$^{95}$.

One goal in the construction of molecules that target the reproduction of tumor cells is that the developed complexes should have high affinity to DNA as well as slow dissociation from DNA$^{96}$. 
Several modifications have been made to ruthenium complexes to improve their DNA binding properties. For example, the replacement of one of the phen moieties with dppz, (dppz=dipyrido[3,2-a:2’,3’-c]phenazine) (Figure 3.1.1) increases the binding constant by three orders of magnitude\textsuperscript{76,97}.

The binding kinetics are significantly altered by covalently linking two \([\text{Ru(phen)}_2\text{dppz}]^{2+}\) molecules to form binuclear ruthenium complexes (Figure 3.1.2), one such example being the semi-rigid \(\Delta,\Delta\) - \([\mu\text{-bidppz(phen)}_4\text{Ru}_2]^{4+}\) [bidppz=11,11’ – bi(dipyrido[3,2-a: 2’3’-c]phenazinyl)]\textsuperscript{14}, which shall here be denoted as \(\Delta\Delta\text{-P}\) (\(\Delta\) denoting the ruthenium coordination to be right-handed). This dumbbell shaped binuclear ruthenium complex has been shown to have extremely slow DNA binding kinetics.
Initially the complex was believed to interact with DNA exclusively by binding to the grooves, but it was later shown that the dominant binding mode was one of threaded intercalation (Figure 3.1.3), in which the bridging bidppz ligand is sandwiched between adjacent DNA base pairs\textsuperscript{98}.

To reach this final state the propeller-like bulky end of this molecule must thread through the DNA base pairs, which transiently requires the opening of base pairs, i.e. local DNA melting, to an unknown extent. In bulk experiments this process relies on rare thermal fluctuations to open up and allow threading, which results in an extremely long time to reach the final threaded state\textsuperscript{98-100}. Once the complex is bound by threading intercalation, it exhibits very slow dissociation\textsuperscript{100-101} and hence it is an excellent model compound for therapeutic applications.

Figure 3.1.3: ΔΔ-P (red) threaded through DNA
3.2 Mechanically Manipulating the DNA Threading Intercalation Rate

In the current studies, we use optical tweezers (discussed in Chapter 2.1) to stretch single lambda DNA molecules in the presence and absence of the binuclear threading intercalator ΔΔ-P. We have also previously shown (Chapter 2.3) that DNA intercalation can be quantified with high precision and sensitivity by measuring the increase in DNA contour length with intercalator concentration. In the case of simple intercalators, ligand association and dissociation occurs rapidly on the time scale of a DNA stretching experiment, such that equilibrium ligand-DNA association constants could be obtained as a function of force, and the equilibrium constant increased exponentially with applied force.78

In contrast, the binuclear ruthenium complex ΔΔ-P, which has to thread between DNA strands to reach its intercalated form, exhibits very slow association and dissociation kinetics, such that the increase in DNA contour length exhibited in DNA force-extension curves (Figure 3.2.1) do not represent the equilibrium DNA-intercalator complex. This slow binding is consistent with bulk experiments, which showed that many hours were required for DNA threading to be observed at room temperature and low ionic strength98. It was also noted that elevated temperature makes the kinetics little faster in the bulk experiments, which is analogous to applying force in the stretching experiments.
As expected we see the effects of intercalation right away during the first stretch (purple solid curve in Figure 3.2.1a) after adding the drug in our stretching experiments, which is in the order of minutes. The consecutive second, third and fourth stretches (blue green and orange solid curves in Figure 3.2.1a) don’t overlay the first stretching curve suggesting that the DNA-drug complex is not in equilibrium.

Figures 3.2.1: DNA stretching curves in the presence of 0.1 nM ΔΔ-P (a) First four stretching shows non equilibrium (b) 4-8th stretches indicates that we are closer to equilibrium. Experiments were performed at 20 °C in 10mM TRIS buffer (100mM Na+, pH 8).

Fifth to eight stretches (Figure 3.2.1b) shows that they are closer to equilibrium. Although consecutive stretching curves overlap still there is hysteresis between the stretching and relaxation curves which may suggest that they are still not in equilibrium.
In order to obtain equilibrium force-extension curves for DNA in the presence of $\Delta\Delta$-P, we decided to stretch the DNA rapidly to a particular force and stay there maintain constant force until it reaches equilibrium, which is on the order of hours at room temperature. The extension measurements taken as function of time at constant force allow us to directly measure the rate of DNA threading intercalation as a function of force.

Figure 3.2.2: Constant force extension measurements with 2 nM $\Delta\Delta$-P at different constant forces (open circles) and their corresponding single exponential fits (solid lines). Experiments were performed at 20 °C in 10 mM TRIS buffer (100 mM NaCl, pH 8).

The constant force extension data (open circles in Figure 3.2.2) taken at five different forces, 28 pN (red) 44 pN (orange), 48 pN (green), 54pN (blue) and 59 pN (purple) at 2 nM $\Delta\Delta$-P concentration fit well to a single exponential dependence (solid curves) with time.
The equilibrium extensions obtained from the exponential fittings of the constant force extension measurements is plotted (red data points in Figure 3.2.3) next to the equilibrium DNA force-extension curve (solid black curve in Figure 3.2.3) in the absence of intercalator. The arrows in the figure indicate the extension change with time and clearly illustrates that the final equilibrium extension increases significantly with force at a constant intercalator concentration.

The slow rate of $\Delta\Delta-P$ /DNA intercalation in combination with its exponential dependence on applied force suggests that the ligand threading rate is limited by the very small probability of melting of $n$ DNA base pairs, which is required before threading can occur.

Figure 3.2.3: Force extension measurements of single DNA molecule in the presence of 2nM binuclear complex $\Delta\Delta$-P (red circles) and in the absence of complex (black). The red line is a fit to guide the eye. The broken arrows indicate the lengthening of the DNA. Experiments was performed at 20 °C in 10mM TRIS buffer (100mM Na$^+$, pH 8).
$$k = k_c e^{-n\Delta G/k_BT}$$  \hspace{1cm} (3.2.1)

where \(\Delta G\) is the free energy associated with the melting of single base pair, \(k_B\) is the Boltzmann’s constant and \(T\) is the absolute temperature.

The applied force favors the more extended melted state of DNA, thereby decreasing the melting free energy by \(Fn\Delta x\), and increasing the probability of melting by the factor \(e^{Fn\Delta x/k_BT}\). Here \(F\) is the applied force and \(\Delta x\) is the difference in length per base pair between ssDNA and dsDNA at that force, which is constant over the force range studied, and \(n\) is the number of transiently melted base pairs required for ligand threading.

$$k = k_c e^{-n(\Delta G_0-F\Delta x)/k_BT}$$  \hspace{1cm} (3.2.2)

where \(\Delta G_0\) is the free energy associated with the melting of single base pair. Therefore, the threading time constants obtained from the fits in Figure 3.2.2 are expected to decrease exponentially with applied force as:

$$\tau = \tau_c e^{n\Delta G_0/k_BT} \cdot e^{-Fn\Delta x/k_BT}$$  \hspace{1cm} (3.2.3)

$$\tau = \tau_0 \cdot e^{-Fn\Delta x/k_BT}$$  \hspace{1cm} (3.2.4)

The result from fitting our measured force-dependent time constant to the predicted exponential behavior is shown as a solid line in Figure 3.2.4. The data fit well to this dependence, and the resulting change in DNA length upon melting required for the threading is
0.239 ± 0.025 nm. Therefore, assuming an average Δx value of 0.22 nm for DNA in the linear approximation region,48,77 we have determined that only 1.08 ± 0.11 bp must be melted by thermal fluctuations in order for the binuclear ruthenium complex to thread between the bases and intercalate into the DNA. If we extrapolate our results to zero force, we obtain a reaction time constant of 120 ± 30 min.

![Figure 3.2.4: Time constants for threading of the binuclear complex ΔΔ-P into DNA. Green data points are experimental and solid red line is fit to an exponential dependence on force.](image)

Previous measurements of threading intercalation kinetics for ΔΔ-P intercalating into polydisperse calf thymus DNA, obtained using spectroscopic methods in the temperature range
of 40 °C to 60 °C, have shown a two phase process, with the first phase at least 6 times faster than the second.\textsuperscript{98,100} The observation of two rates in those experiments may be due to processes not detected by DNA stretching, such as ligand rearrangement after binding or conformational changes in the DNA. In Appendix B, we show that measurements of the enthalpy of the rate-limiting threading step from previous high temperature spectroscopic measurements are consistent with the enthalpy of one base pair opening in the middle of the DNA duplex.

To conclude, we have demonstrated that the DNA threading rate into a single DNA molecule can be directly measured at constant force using optical tweezers. Our results show that the known dependence of DNA destabilization upon force can be used to predict the dependence of the threading rate on force. Because we manipulate the measurable physical parameters of length and force, we are able to directly determine the physical length change responsible for lowering the reaction barrier. From this data, we find that only one base pair must be melted in order for threading intercalation of $\Delta\Delta$-P to occur. This result is consistent with the minimum steric requirements for threading based on structural considerations.
3.3 Quantifying the Threading of Binuclear Ruthenium Complexes

Equilibrium force-extension curves obtained at different concentrations are shown in Figure 3.3.1. These curves were the ones reached equilibrium after several stretches. Each data point shown in the curve is average of more than three different stretching curves which were believed to be closer to equilibrium. Comparing these results with one obtained with constant force measurements discussed in the previous section, it is clear that only near the melting transition force are the extensions at equilibrium.

Figure 3.3.1: Force extension measurements of single DNA molecule in the presence of different concentration ΔΔ-P (colored) and in the absence of complex (black). Experiments were performed at 20 °C in 10mM Hepes buffer (100mM Na⁺, pH 8).
Using similar methods to those used to quantify intercalators\textsuperscript{78} the fractional elongation at 60 pN force was estimated at different concentrations using equation:

$$\gamma_F(c) = \frac{b_{ds}(F,C) - b_{ds}(F,0)}{b_{ds}(F,0)}$$

where $b_{ds}(F,C)$ is the extension in the presence of the intercalator at concentration $C$ and $b_{ds}(F,0)$ is the extension in the absence of intercalator. These fractional elongations were fitted to McGhee and von Hippel isotherms\textsuperscript{87-88} (Figure 3.3.2) discussed in chapter 2.4 by equation 2.4.1.

$$\gamma = Kn_c \left( \frac{1 - \gamma^n}{1 - \frac{\gamma}{n}} \right)$$

![Figure 3.3.2: McGhee and von Hippel fitting at 60 pN for $\Delta\Delta$-P (red solid) and the error range (green solid)](image)

$n = 4.9 \pm 0.5$ bp

$K = (5.1 \pm 2.0) \times 10^9 \text{ M}^{-1}$
The fitting yields the binding constant $K = (5.1 \pm 2.0) \times 10^9 \text{ M}^{-1}$ and the binding site size $n = 4.9 \pm 0.5 \text{ bp}$ for $\Delta\Delta\text{P}$ at 60 pN. Using the force dependence of binding constant (equation 2.4.4) established for intercalators in chapter 4.3:

$$K = K_0 \exp \left( \frac{F\Delta x}{k_BT} \right)$$

combining it with the value obtained for the binding constant at 60 pN and the $\Delta x = 0.38 \text{ nm/bp}$ dppz moiety intercalation $^{78}$, we can estimated the binding constant in the absence of force $K_0 = (2.1 \pm 0.6) \times 10^7 \text{ M}^{-1}$. Binuclear ruthenium complex has two orders of magnitude higher binding affinity compared to the binding affinity of other intercalators at zero force $^{78}$ and it is three orders of magnitude higher at the force range of melting (~60 pN).

Considering the force dependence of the binding site size for other intercalators, $^{78}$ it is likely that the zero force binding site size is greater than 4.9 bp. Further comparing it to the binding site size of the mononuclear ruthenium complex Ru(phen)$_2$dpdz$^{2+}$ which is 2.55 bp at 60 pN and 3 bp at zero force $^{78}$ we can imagine the binuclear complex running from 4.9 bp to around 6 bp. Based on these results we can explain a binding model for binuclear ruthenium complex comparing it with mononuclear complex.

In this model the propeller-like phen rings are illustrated by the orange shaded circular discs and the dppz moiety is illustrated as brown triangular flat portions. For simplicity the DNA is shown as a straight ladder (black). Each figure contains the side view of the DNA so that it is easy to visualize the occupation of the number of base pairs. According to the previous studies with mononuclear ruthenium complexes that have phen side chains it can be induced that each complex takes 3 bp space between successive intercalation. If we assume the saturated binding
you can imagine consecutive mononuclear complexes binding from the opposite side of the DNA ladder as shown in the Figure 3.3.3 (left). This model suggests that the propeller itself is taking somewhat little less than 6 bp. Now if we consider the case when these complexes are bridged together to form the binuclear complex binding from the opposite site of the ladder is hindered by the phen rings on that side of ladder after threading as shown in the Figure 3.3.3 (right). This explains the binding site size we obtain which is close to 6 bp and almost double the binding site size of the mononuclear complex.

**Figure 3.3.3:** Model comparing the binding of mononuclear ruthenium complex (left) and binuclear ruthenium complex ΔΔ-P (right) both figures shows the side view of DNA so that it is easy to understand the model.
Chapter 4: Resolving the ActinomycinD Binding Controversy

4.1 Actinomycin D

4.2 DNA Stretching Exhibits Multiple Binding Modes

4.3 Direct Measurement of ActD Binding Kinetics

4.4 Converging to Single Binding Mode
4.1 Actinomycin D (ActD)

Actinomycin D (ActD) is one of the most studied biologically active small molecules due to its therapeutic activity as an antibiotic and an anticancer agent in current clinical use, as well as its ability to inhibit HIV-1 reverse transcription. As discussed in chapter 1.3 reversible small molecule-DNA interactions are divided into three modes: intercalation between double stranded DNA (dsDNA) base pairs, binding to single stranded DNA (ssDNA), and binding in the dsDNA grooves. Surprisingly, the mode of ActD-DNA interactions remains a controversial issue, with many workers characterizing ActD binding primarily as intercalation and the others characterizing its binding to ssDNA as the primary and stronger binding mode. It was also shown that when co-stacking between the bases of the opposite strands is impossible, ActD can still intercalate between the bases of the same single DNA strand, a hybrid binding mode termed hemi-intercalation. However, despite numerous studies of ActD’s effect on DNA, major questions regarding the nature of its DNA interactions and biological role remain unclear.

In the present study we directly measure the kinetics of ActD binding to a single polymeric DNA molecule in both ssDNA and dsDNA forms. We find that ActD binds to a distorted form of dsDNA that is close in length to stretched ssDNA, but it can approach this state from either the dsDNA or ssDNA form, with differing kinetics. In addition to reconciling previous seemingly contradictory reports, our results show that destabilization of dsDNA by an applied stretching force leads to enhanced ActD binding, supporting a model in which the biologically active mode of ActD binding is to pre-melted dsDNA, as found in transcription.
bubbles. Since DNA destabilization occurs naturally at transcription bubbles, they are likely a favorable target for rapid ActD binding and subsequent inhibition of RNA synthesis\textsuperscript{104,114-115}.

ActD is an electrostatically neutral small molecule (Figure 4.1.1) that contains a planar tricyclic phenoxazone ring and two cyclic pentapeptide side chains. During intercalation ActD inserts its chromophore phenoxazone ring preferentially between the two G bases in the subsequent base pairs in the opposite strands\textsuperscript{96} and fits the pentapeptide side chain in the minor groove\textsuperscript{23,96}. The side chains were found to interact with Guanine bases by hydrogen bonding\textsuperscript{24}, a finding that was consistent with NMR data\textsuperscript{106}.

When the crystal structure\textsuperscript{25,107-108,116} of intercalated ActD (Figure 4.1.2) became available, it was noticed that in contrast to other intercalators, ActD induces strong deformations around the site of its intercalation, including strong duplex bending. Later studies report that the ActD can be intercalated at non-GpC sites\textsuperscript{117-118}. Recent NMR\textsuperscript{119} and x-ray crystallography\textsuperscript{116} studies indicate that ActD can even bind to duplexes with base pair mismatches. Furthermore, it was shown that when the co-stacking between the bases of the opposite strands is impossible, the ActD chromophore ring can still “hemi-intercalate” between the bases of the same single DNA strand,\textsuperscript{111-112} with strong preference for G-A residues. The authors of the earlier studies tend to argue that ActD interferes with DNA transcription and replication by preferentially binding to
and stabilizing duplex DNA in front of the replication fork, as expected for a standard intercalator. However, studies showing that ActD primarily blocked RNA synthesis led to the proposal that the biologically significant ActD binding activity consists of binding to pre-melted double stranded DNA\textsuperscript{113}, such as that found at transcription bubbles.

![Figure 4.1.2: (a) Crystal structure of ActD (cyan and green) intercalated through dsDNA (red) (b) same structure with pentapeptide side chain color coded blue (c), (d) and (e) three views of space filled form of the structure rotated about vertical axis Color code: pentapeptide (blue), phenoxazone ring (cyan and green), DNA (red) and water (white) Figures obtained from the pdb file IMNV by Hou et. al. (2002) Nucleic Acids Res. 30: 4910-4917](image)

ActD binding to specific sites on ssDNA\textsuperscript{18-19,21,109-110} was more recently examined and such binding was associated with ActD’s inhibitory effect on DNA transcription\textsuperscript{105}. However, such inhibition of \textit{in vitro} ssDNA transcription required ActD concentrations $\geq 30\ \mu\text{M}$\textsuperscript{105}, which is at least an order of magnitude higher than needed for \textit{in vivo} anti-cancer activity. Furthermore, it was found that ActD interferes with minus strand DNA strand transfer during HIV-1 reverse transcription by the suppression of the TAR RNA/DNA annealing at ActD concentrations $\sim 1-4\ \mu\text{M}$\textsuperscript{15-17}. 
Due to the great diversity of the findings from these studies, the physical mechanism of ActD’s anti-cancer activity remains elusive. One hypothesis that may explain all of the previous studies is that ActD is able to stabilize imperfect or even non-complementary hairpin DNA as well or better than perfect nucleic acid duplexes. To test this hypothesis, we use optical tweezers to systematically analyze the effect of Act D on the stretching behavior of single DNA molecules in solution. As DNA is stretched, dsDNA is destabilized until a force-induced melting transition occurs, such that DNA is converted from dsDNA to ssDNA as the extension is increased\textsuperscript{26,46}. In the presence of a DNA binding ligand, this method tests the ligand’s thermodynamic preference for and kinetic approach to binding a range of DNA forms, from stable dsDNA to fully denatured ssDNA\textsuperscript{26}. 

Selman Waksman (1888-1973) was awarded Nobel Prize in Physiology and Medicine for his discovery of streptomycin, which was the first antibiotic used clinically. Many think that this was the first antibiotic. But he isolated Actinomycin in 1940, 3 years before streptomycin. In a article title “Man of the soil” Time magazine have sited that “He still keeps in his littered desk samples of the first antibiotic he isolated, in 1940, called Actinomycin” 
4.2 DNA Stretching Exhibits Multiple Binding Modes

At low (nM) ActD concentrations (Figure 4.2.1), the first DNA stretching curve after adding Act D (red solid curve) overlaps the stretching curve for dsDNA in the absence of ActD (black solid curve) until the melting plateau is reached, at which point the melting transition shows a small slope and the relaxation shows hysteresis, in which the relaxation curve (red broken curve) does not match the stretching curve. The subsequent second (green solid curve) and third (blue solid curve) stretching curves of the DNA-ActD complex are shifted to the right with respect to the naked DNA stretching curve and the first stretching curve. These data show that DNA has to be melted to bind ActD at low concentrations and short times, while the hysteresis shows that DNA destabilized by force is rapidly bound by ActD. In addition, ActD binds only to ssDNA created by force-induced melting at low concentrations on the short (~1 minute) time scales of these experiments.

Figure 4.2.1: DNA Stretching in the presence of 50 nM ActD
In contrast, at higher (µM) concentrations we are able to observe the effect of ActD on the first stretch, and consecutive stretching-relaxation curves always overlap (Figure 4.2.2a). The concentration profile for stretching-relaxation cycles (Figure 4.2.2b) shows force-dependent lengthening prior the melting plateau, similar to that observed for standard intercalators\textsuperscript{26,78}. Some aspects of the stretching curves resemble those for intercalation, such as the shape change with concentration from convex to concave. Other characteristics, such as a progressive decrease in the force at the beginning of the melting plateau and the presence of hysteresis between the stretching and relaxation curves, are consistent with ssDNA binding. The observed hysteresis indicates slow ActD-DNA binding kinetics, as previously, with time scales of up to ~1000 s.\textsuperscript{96}

\textbf{Figure 4.2.2: (a) DNA Stretching in the presence of 0.75 µM ActD (b) High concentration (~µM) profile of ActD}
4.3 Direct Measurement of ActD Binding Kinetics

To test the binding kinetics, we stretched the molecule rapidly to a certain force while adding ActD and held it at constant force until the lengthening due to ActD binding reached equilibrium. Measurements at several constant forces (Figure 4.3.1a) indicate a clear force-dependent lengthening upon ActD binding, suggesting intercalation even at low concentrations for longer time scales (~nM). Once the equilibrium was reached ActD was washed away with the flow of buffer to measure the change in the length of the complex as ActD dissociates (Figure 4.3.1b).

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**Figure 4.3.1:** (a) Constant force extension measurements while adding 500nM ActD (b) Constant force extension measurements while washing the drug with buffer flow
Further fitting of the extensions observed while adding the drug \(x(t)\) to an exponential time dependence (solid curves Figure 4.3.1a) yields the total rate \((k_{on}+k_{off})\) of binding and the equilibrium extension \((x_{eq})\)

\[
x(t, F) = x_{eq}(F) + (x_{eq}(F) - x_{0}(F))\exp[-(k_{on}(F) + k_{off}(F))t]
\]

(4.3.1)

From the equilibrium extension \((x_{eq}(F))\) observed at a particular force \((F)\) the fractional binding of ActD to DNA at that force \((\theta(F))\) can be determined using the drug saturated extension at that force \((x_{sat}(F))\) and the dsDNA extension at that force \((x_{ds}(F))\).

\[
\theta(F) = \frac{x_{eq}(F) - x_{ds}(F)}{x_{sat}(F) - x_{ds}(F)}
\]

(4.3.2)

Using a simple binding isotherm the equilibrium dissociation constant at that force \((K_d(F))\) can be obtained for a particular concentration.

\[
K_d(F) = c \frac{1 - \theta(F)}{\theta(F)}
\]

(4.3.3)

The exponential force dependence of the dissociation constant \((K_d(F))\) can then be extrapolated to determine the equilibrium dissociation constant in the absence of force \((K_d(0))\).

\[
K_d(F) = K_d(0) \exp \left(-\frac{F\Delta x}{k_BT}\right)
\]

(4.3.4)
Extrapolation of the force dependence of ActD binding (Figure 4.3.2) yields a dissociation constant of $0.52 \pm 0.06 \, \mu M$ in the absence of force after averaging out the values obtained at different concentrations. This value falls in the proper range of the values obtained from different studies which range from $1\,\mu M$ for dsDNA intercalation$^{96}$ and the strongest $0.01\,\mu M$ for certain sequences of DNA oligomers$^{110}$.

Assuming a bimolecular relationship, which was verified in direct measurements (Figure 4.3.3), the on rate $k_{on}(F)$ (green points in Figure 4.3.4) and off rate $k_{off}(F)$ (red points in Figure 4.3.4) at that force can be separated

$$K_d(F) = c \frac{k_{off}(F)}{k_{on}(F)} \quad (4.3.5)$$

The off rates can also be obtained directly from the washing off
experiments described above (Figure 3.3.1b). The directly measured off rates (blue points in Figure 4.3.4) agree very well with the ones estimated from the above relationship. The on rate \( k_{on}(F) \) at a particular concentration strongly grows with the applied force \( F \). Fitting this to a simple exponential (green line in Figure 3.3.4):

\[
    k_{on}(F) = k_{on}(0) \exp\left( \frac{Fx_{on}}{k_B T} \right)
\]

yields the on rate in the absence of force \( k_{on}(0) = (3.3 \pm 0.3) \times 10^{-4} \text{ s}^{-1} \) and the DNA elongation associated with the rate limiting step of a single intercalation event \( x_{on} = 0.33 \pm 0.01 \text{ nm} \).

Figure 4.3.4: Force dependent on rates (green) and off rates (red) calculated from the exponential fittings while adding the drug using bimolecular rates of dissociation constant compared with the off rates directly measured from washing experiments (blue)
To verify the effect of flow in the above results we also did experiments in the absence of flow at 50 nM ActD, in which the flow cell that contains the DNA was first filled with ActD and then the constant force extension measurements were performed (Figure 3.4.5a).

The on rate obtained with 50 nM ActD (Figure 4.3.5b) in the absence of force $k_{on}(0) = (6.8 \pm 1.9) \times 10^{-5} \text{ s}^{-1}$, which is an order of magnitude smaller to the one obtained with the flow at 10 times higher concentration (500 nM) as expected. This demonstrates that the low flow rate used does not affect the rates we measured.
4.4 Converging to Single Binding Mode

To determine the type of ActD binding mode, after filling the flow cell with 50nM ActD we stretched the DNA rapidly (red solid curve in Figure 4.4.1) to reach 30pN and held it at constant force (orange curve in Figure 4.4.1) until it reached equilibrium and then relaxed (red broken curve in Figure 4.4.1). Immediately we stretched (purple solid curve in Figure 4.4.1) the same molecule throughout the melting transition (forming two parallel melted DNA strands) then relaxed (purple broken curve in Figure 4.4.1) to the same force (30pN) and held it at constant force (green curve in Figure 4.4.1) to reach equilibrium and finally relaxed back (blue broken curve in Figure 4.4.1) to the initial point.

Figure 4.3.5: Converging to the same point at equilibrium either from dsDNA intercalated state or ssDNA bound state
It is clear that both binding modes either from the dsDNA form while stretching or from the two largely melted ssDNA strands while relaxing tend to reach the same point at equilibrium. The above result shows that there is only a single dominant mode of binding for ActD at equilibrium. In addition, the equilibrium point is much closer to the two melted ssDNA strands, suggesting that DNA melting facilitates the binding of ActD.

Our quantitative picture of force-facilitated ActD binding to DNA allows us to propose the physical mechanism of this process. We observed that the ActD-DNA complex forms on average a single type of distorted intercalated complex. We also showed that binding from melted DNA is rapid while binding from the dsDNA form was extremely slow ($\sim 10^{-5}$ s$^{-1}$). Taken together, we have demonstrated that, while ActD is an intercalator, its preferred and likely biologically relevant binding mode is one that is approached from a state of two melted parallel strands. This observation provides a simple biophysical explanation for its selective inhibition of RNA synthesis$^{104,114-115}$ and inhibition of the reverse transcription of HIV-1$^{15-17,105}$.
Chapter 5: Conclusions and Discussions
Conclusions and Discussion

We have used optical tweezers to study complex DNA binding kinetics by stretching single DNA molecules in the presence of small molecules. In our studies we have quantified the binding of a potential drug, the binuclear ruthenium complex ΔΔ-P and a drug in practice, Actinomycin D (ActD). Both studies revealed potential new information about the thermodynamics and kinetics of complex and multiple binding modes of these drugs.

The study of a binuclear ruthenium complex has shown that the single molecule force spectroscopy can be used as an effective technique to study slow binding processes such as those of threading intercalators. By mechanically manipulating the drug-DNA complex we have shown that only one base pair must be melted to thread this dumbbell-shaped molecule through the DNA bases. The threading observed on the order of minutes in the presence of force agrees with bulk experiments upon extrapolation to zero force, which suggest the zero force time constant is on the order of hours. The binding affinity of the binuclear ruthenium complex is 3 orders of magnitude higher than that of typical mononuclear complexes at high forces (~10^9 M^-1 and 10^6 M^-1 respectively) and two orders of magnitude higher in the absence of force (10^7 M^-1 and 10^5 M^-1 respectively). By estimating the binding site size using McGhee and von Hippel fits and comparing the results with those of the mononuclear complex, we proposed a model for the organization of a saturated lattice of bound intercalators.

The most challenging project was to resolve the controversy of ActD binding, which has persisted for more than half a century since its discovery. We explored different methods to
quantify the binding of ActD to DNA and developed unique method to measure the on and off rates directly while flowing the drug in and washing away the drug. By doing control experiments we have shown that in these optical tweezers experiments the small flow rate used doesn’t affect the kinetics of the molecular binding events. The slow binding kinetics of ActD suggests that ActD binds the destabilized dsDNA which has a length closer to the stretched ssDNA. This final state can be reached either by binding to dsDNA or by binding to two melted ssDNA strands. This is consistent with the model which predicts ActD binding to pre-melted DNA, as found in transcription bubbles. This leads to the conclusion that the likely biologically significant binding mode of ActD occurs at transcription bubbles, thereby inhibiting RNA synthesis.

In our studies we have extended the use of optical tweezers to study complex binding processes like threading intercalation and multiple mode binding processes like in the case of ActD. We have introduced a method to study these slow processes by stretching the DNA and holding it at constant force to reach equilibrium, which directly measures the on rates of these complex binding modes. Also we have shown that relatively slow off rates can be measured directly by employing the constant force extension measurement technique while washing the drug with buffer flow. In addition any force-dependent measurements obtained with optical tweezers can be appropriately extrapolated to zero force to predict the binding parameters in the absence of force. Therefore optical tweezers can be used as a valuable tool in exploring drug – DNA interactions to provide new insights to design potential drugs for challenging disease like cancer and AIDS.
List of Publications

Mechanically Manipulating the DNA Threading Intercalation Rate
Thayaparan Paramanathan, Fredrik Westerlund, Micah J. McCauley, Ioulia Rouzina, Per Lincoln and Mark C. Williams
*Journal of the American Chemical Society (Communication)* **130**: 3752 -3753 (2008)

Biophysical Characterization of DNA Binding from Single Molecule Force Measurements
Kathy Chaurasiya, Thayaparan Paramanathan, Micah J. McCauley, Mark C. Williams
*Physics of Life Reviews 7*: (2010)  (*In Press*)

ActinomycinD Binding Controversy Resolved by Single Molecule Kinetics (*In preparation*)

Equilibrium Binding Affinity and Saturated Binding of Binuclear Ruthenium Complexes (*In preparation*)
Thayaparan Paramanathan, Fredrik Westerlund, Micah J. McCauley, Ioulia Rouzina, Per Lincoln and Mark C. Williams

Studying DNA Interactions with Small Molecules Using Optical Tweezers (*Book chapter in preparation*)
Thayaparan Paramanathan, Micah J. McCauley, Mark C. Williams
List of Presentations

Oral Presentations

**Threading of Binuclear Ruthenium Complex through DNA Bases**
- APS March Meeting (March 17, 2009)
  (Awarded DCP Travel Award to Present at the Meeting)

**Exploring Threaded Intercalation Using Optical Tweezers**
- APS March Meeting (March 8, 2007)
  (Awarded DBP Travel Award to Present at the Meeting)

Poster Presentations as Lead Presenter

**Binding Mode of ActinomycinD Reveals the Basis of Its Potential HIV-1 and Cancer Activity**
- 54th Annual Biophysical Society Meeting (February 20-24, 2010)

**Quantifying Multiple DNA Binding Modes of ActinomycinD using Optical Tweezers**
- 16th Conversation - Albany Meeting (June 16-20, 2009)

**Drug-DNA Interactions: View with Optical Tweezers**
- 2009 EXPO, Northeastern University (March 26, 2009) (Awarded Best Presentation of the EXPO in Physical & Life Science Category)

**Distinguishing Dual DNA Binding Modes of ActinomycinD using Optical Tweezers**
- 53rd Annual Biophysical Society Meeting and 17th International Biophysics Congress (February 28-March 3, 2009)

**Kinetics of Binuclear Ruthenium Complex Threading Through DNA Bases**
- 52nd Annual Biophysical Society Meeting and 16th International Biophysics Congress (February 3-6, 2008)

**Threading Through DNA Bases: View with Optical Tweezers**
- 234th ACS National Meeting (August 19-23, 2007)
Appendix A: The Gradient Force on Dielectric Particles

The Lorentz force on a single charge in an electromagnetic field is given by:

\[ \vec{F} = q \left( \vec{E} + \frac{d\vec{x}}{dt} \times \vec{B} \right) \]

When small dielectric particles are trapped by a considerably smaller wavelength of light compared to the size of the particle, the particle can be treated as a point dipole which has two charges of opposite sign separated by an infinitesimal distance \( \vec{d} = \vec{x}_1 - \vec{x}_2 \). The force on this dipole can be written as:

\[ \vec{F} = q \left( \vec{E}_1(x_1, y, z) - \vec{E}_2(x_2, y, z) + \frac{d(\vec{x}_1 - \vec{x}_2)}{dt} \times \vec{B} \right) \]

\[ \vec{F} = q \left( \vec{E}_1(x_1, y, z) - \left( \vec{E}_1(x_1, y, z) - \left( (\vec{x}_1 - \vec{x}_2) \cdot \nabla \right) \vec{E} \right) + \frac{d(\vec{x}_1 - \vec{x}_2)}{dt} \times \vec{B} \right) \]

\[ \vec{F} = q \left( (\vec{x}_1 - \vec{x}_2) \cdot \nabla \vec{E} + \frac{d(\vec{x}_1 - \vec{x}_2)}{dt} \times \vec{B} \right) \]

\[ \vec{F} = (p \cdot \nabla) \vec{E} + \frac{dp}{dt} \times \vec{B} \]

where \( \vec{p} = q\vec{d} = q(\vec{x}_1 - \vec{x}_2) \) is the polarization of the dipole.

If we assume that the dielectric particle is linear, that is \( \vec{p} = \alpha \vec{E} \), then

\[ \vec{F} = \alpha \left( (\vec{E} \cdot \nabla) \vec{E} + \frac{d\vec{E}}{dt} \times \vec{B} \right) \]
But from vector analysis \( (\vec{E}.\nabla)\vec{E} = \frac{1}{2} \nabla E^2 - \vec{E} \times (\nabla \times \vec{E}) \) and

From Maxwells’ equations \( \nabla \times \vec{E} = -\frac{d\vec{B}}{dt} \)

\[
\vec{F} = \alpha \left( \frac{1}{2} \nabla E^2 + \vec{E} \times \frac{d\vec{B}}{dt} + \frac{d\vec{E}}{dt} \times \vec{B} \right)
\]

\[
\vec{F} = \alpha \left( \frac{1}{2} \nabla E^2 + \frac{d}{dt} (\vec{E} \times \vec{B}) \right)
\]

The term \( \vec{E} \times \vec{B} \) is related to the power passing through the surface, and if the power of the laser remains constant over the sampling the time derivative turns out to be zero and the second term vanishes. Therefore the gradient force on the dielectric particle can be written as:

\[
\vec{F} = -\frac{1}{2} \alpha \nabla E^2
\]
Appendix-B: Spectroscopic Results of Bimolecular Ru-complex

In this section, we will compare with spectroscopic measurements our conclusion that only one DNA base pair must be melted for DNA threading by the binuclear ruthenium complex \( \Delta,\Delta-[\mu\text{-}\text{bidppz}(\text{phen})_4\text{Ru}_2]^{4+} (\Delta\Delta\text{-P}) \). While previous measurements were obtained at high temperatures, the temperature dependence of the DNA threading rates at high temperatures can also be used to obtain information on the barrier to DNA threading.

In previous experiments, the rate of DNA threading by \( \Delta\Delta\text{-P} \) was determined spectroscopically over a temperature range from 45 to 60 °C. The temperature-dependent threading rate was then used to determine the threading enthalpy, \( \Delta H^\ddagger \), using a Van’t Hoff analysis:

\[
\Delta H^\ddagger = -R \cdot \frac{d \ln(k_{\text{thread}})}{d(1/T)}, \tag{A1}
\]

where \( k_{\text{thread}} = 1/\tau_{\text{thread}} \) is the threading rate, or the inverse of the observed time constant for DNA threading by this compound. From this previous work, barrier energies of 94 kJ/mol=22 kcal/mol, obtained in 100 mM Na\(^+\) solution\(^9\) and 167 kJ/mol=40 kcal/mol, obtained in 150 mM Na\(^+\) solution have been determined\(^\text{10}\).

To test the conclusion from our DNA stretching experiments that only a single base pair must be melted for threading to occur, the threading enthalpies obtained from previous measurements can be directly compared to the enthalpy for melting a single DNA base pair in the middle of the DNA molecule. The enthalpy for melting a single DNA base pair in the middle
of a DNA duplex has been determined from proton exchange Nuclear Magnetic Resonance experiments over a temperature range from 10 °C to 35 °C. The authors found that the equilibrium base pair opening enthalpy varied over the range of 10 kcal/mol to 26 kcal/mol, depending on the sequence context, with an average value of approximately 20 kcal/mol. Based on the uncertainty in the range of bulk DNA threading measurements, the threading enthalpy measured spectroscopically for ΔΔ-P at high temperatures are consistent with the enthalpies for single base pair opening as measured by NMR.

We can also compare the value of our zero force threading time τ₀ = 120±30 min measured in this work at 20 °C and the threading time measured at higher temperatures in the bulk spectroscopic experiments. The latter work contains three measurements of the DNA threading time constant at 50 °C, which average to τ = 30±20 min. The large variation of the latter quantity is due to variation in the DNA sequence, ligand concentration and method of measurement. If we assume that the temperature dependence of the threading rate is dominated by the temperature dependence of single base-pair opening, which has an average entropy change of ΔS=30 cal/mol/K, as determined by NMR at 37 °C, we estimate that the threading rate is expected to decrease by ~4-5- fold when the temperature is reduced from 50 °C to 20 °C. Therefore, the zero force DNA stretching and high temperature spectroscopic measurements agree within uncertainty.

In summary, high temperature fits of spectroscopic DNA threading rate measurements can be fitted to Eq. (1) to obtain the DNA threading barrier enthalpy. By comparing the measured barrier enthalpy at high temperatures to proton exchange NMR experiments, we find
that the spectroscopic measurements of DNA threading by ΔΔ-P are fully consistent with the accompanying result that the rate is determined by the requirement that a single base pair must open for DNA threading to occur. In addition, extrapolation of the zero force DNA threading time constant measured by DNA stretching to 50 °C yields good agreement with the spectroscopic measurements of the DNA threading time constant at high temperatures.
References


