The assembly of DNA threading intercalation complexes quantified from nano-mechanical single-molecule measurements

by Ali A. Almaqwashi

B.S. in Physics, King Abdul-Aziz University
M.S. in Physics, University of Oregon and Oregon State University

A dissertation submitted to

The Faculty of
the College of Science of
Northeastern University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

December 03, 2015

Dissertation directed by

Mark C. Williams
Professor of Physics
Acknowledgements

I would like to thank my family for supporting my quest for knowledge, and uplifting my spirit, I would not have achieved anything without your love.

I would like to thank my adviser Prof. Mark Williams for believing in me, guiding my research, and elevating my experimental and theoretical skills. Thank you for your patience and kindness, I am proud to be one of your students.

I would like to thank the current and past Williams Lab members for their generous help, thoughtful discussions, and true friendship.

I would like to thank my research collaborators for their insights, and revision of my research work.

I would like to thank my dissertation committee members: Prof. Timothy Sage, Prof. Armen Stepanyants and Prof. Meni Wanunu for their effort in reviewing my work.

I would like to thank the professors who taught my graduate courses, I am in debt to their knowledge.

I would like to thank the physics department at Northeastern University, faculty and staff, for their assistance.

I would like to thank King Abdul-Aziz University for supporting my scholarship.

I am really thankful for all the people whom I have been blessed to meet and learn from.

Thank you for everything, I will always be grateful.
Abstract of Dissertation

There is a wide range of applications for DNA intercalation, a DNA-ligand assembly process in which small planar aromatic molecules are reversibly inserted between adjacent DNA base pairs. In particular, the recently developed dumbbell-shaped DNA threading intercalators, binuclear ruthenium complexes, represent the next order of structural complexity relative to simple intercalators, and can provide significant new insights into the molecular mechanisms that govern DNA-ligand intercalation. This model intercalating system involves passing the end of the dumbbell through broken base pairs before reaching the equilibrium intercalation state, which induces dynamic DNA structural distortion and exhibits equilibrium and kinetics properties desirable for DNA-targeting therapeutics. Single-molecule force spectroscopy using optical tweezers can provide precise measurements, enabling comprehensive investigation of diverse DNA assemblies. From efficiently controlled nanomechanical single-molecule measurements, force spectroscopy can determine the kinetics of DNA-ligand assembly and disassembly and the intercalation affinity as well as reveal the magnitude of equilibrium and dynamic structural deformations of the DNA-ligand complex. This work examines several structural modifications aimed toward optimizing DNA threading intercalation by binuclear ruthenium complexes. The results demonstrate robust DNA structural recognition of DNA threading intercalators. Essential molecular aspects of DNA-ligand intercalation are characterized, including the reshaping of the threading energy landscape due to the modifications of each structural subunit. The findings of this work will hopefully guide the rational design of intercalating molecular systems which are optimized for DNA-targeted synthetic drugs, optical probes, or DNA-integrated self-assembly processes.
# Table of Contents

Acknowledgements............................................................................................................. ii

Abstract of Dissertation...................................................................................................... iii

Table of Contents................................................................................................................ iv

Chapter 1: Introduction......................................................................................................... 1

1.1 DNA intercalation............................................................................................................. 2

1.2 Optical tweezers............................................................................................................. 5

1.3 Single-molecule force spectroscopy................................................................................ 7

Chapter 2: Conventional and unconventional Intercalators............................................... 11

2.1 Conventional DNA Intercalation..................................................................................... 12

2.2 Unconventional DNA Intercalation................................................................................ 18

Chapter 3: Single-transition DNA threading intercalation................................................ 21

3.1 DNA-ligand intercalative transition ................................................................................ 22

3.2 Threading mono-intercalator........................................................................................... 22

Chapter 4: Double-transition DNA threading intercalation................................................. 36

4.1 Fast and slow intercalative transitions ........................................................................... 37

4.2 Threading bis-intercalator.............................................................................................. 37

4.3 Converted threading mono-intercalator......................................................................... 52

Chapter 5: Steroselectivity of DNA threading intercalation................................................. 62

5.1 Chiral intercalators ....................................................................................................... 63

5.2 Steroselective threading mono-intercalator................................................................... 62

Chapter 6: Conclusions......................................................................................................... 77

6.1 Conclusions.................................................................................................................... 78
List of Publications.................................................................80
List of Presentations.............................................................81
References..............................................................................82
Chapter 1:

Introduction

1.1 DNA intercalation

1.2 Optical tweezers

1.3 Single-molecule force spectroscopy

Portions of this chapter are from:

Mechanisms of DNA intercalation probed by single-molecule force spectroscopy

Ali A. Almaqwashi, Thayaparan Paramanathan, and Mark C. Williams

(Submitted)
1.1 DNA INTERCALATION

The self-assembled structure of the deoxyribonucleic acid (DNA) molecule was revealed in 1953 (1-3), a double stranded helix where each stand is a polynucleotide constructed of nucleic acid bases attached to deoxyribo sugar-phosphates. Each deoxyribo sugar in the polynucleotide is bound to two phosphate groups with phosphodiester bonds at carbons 3’ and 5’. The double stranded helix is formed by pairing a purine base of adenine or guanine with a pyrimidine base of thymine or cytosine, respectively. The two strands are paired anti-parallel where the deoxyribo sugar-phosphate backbone runs from 3’ to 5’ for the first strand and from 5’ to 3’ for the second complementary strand, while the hydrophobic base pairs are transverse to the helical axis and protected by the double helix backbone (Fig.1.1.1). The DNA in its common B form has a helical twist of about 36° per base pair (bp), a separation of 0.34 nm between every two adjacent base pairs, a lateral width of 2 nm, and a net negative charge of -2 per bp.

Figure 1.1.1. The double stranded helix, the backbones are in black and silver for the first and second strand respectively, purine bases are orange and yellow for adenine and guanine, pyrimidine are purple and violet for thymine and cytosine. The hydrophobic bases are protected inside the double helix, and base pairs are formed by two or three hydrogen bonds between the purine and pyrimidine bases as shown with dashed black lines. (This B-DNA structure is from PDB file 1BNA by Drew et al (4), and visualized by VMD)

The unveiling of the molecular structure of DNA (1-3) was shortly followed by the prediction (5) and confirmation (6-8) of DNA intercalation, in which a planar aromatic moiety is noncovalently inserted between adjacent DNA base pairs (5-11), which stabilizes the double-stranded DNA helix (12, 13). The identification of intercalation as a DNA binding mode was first motivated by studying genetic transformation, potential mutagens, and to explain the DNA-acridine interaction mechanism (5, 14, 15), one of the early clinically used antitumor synthetic small molecule drugs (16). DNA intercalators, which
can be cationic or neutral, disrupt the continuity of the encoded genome, in contrast to ligands that bind DNA electrostatically, allosterically, and into the major or minor grooves (10, 17) (Fig. 1.1.2A). Single DNA intercalation events result in DNA helix unwinding, which can exceed 50% of the native twist (9, 18, 19), as well as helix elongation that almost doubles the natural base pair separation (5, 6, 8, 18, 20, 21) in order to accommodate the intercalating moiety (see Fig. 1.1.2B). While intercalated DNA helix unwinding compensates for the helix extension and maximizes the base-intercalator stacking, the DNA-ligand complex deformation maintains crucial aspects of the native DNA backbone structure, such as the nearly invariant distance between the adjacent phosphate groups (5, 9, 11, 18, 22). Such an invasive, yet reversible, DNA binding mode provides the basis of wide range of applications in which DNA molecules are targeted, probed, or integrated into assembly processes.

Figure 1.1.2. A) Different DNA binding modes, 1 is major groove binder, 2 is minor groove binder, 3 is electrostatic/allosteric binding, and 4 is intercalator. DNA backbone is in red and base pairs in grey. B) Diagrammatic Illustration of intercalated DNA helix elongation (left) and unwinding (right), the phosphate groups in red, Deoxy sugar in black, base pairs in grey, and intercalator in blue. It shows that the separation between the intercalated base pairs is nearly doubled, and more than half the native DNA helix twist is unwound to compensate for the elongation.

There is interdisciplinary interest in DNA intercalation, as evident in broad range of applications. For instance, X-ray crystal structures established that DNA intercalation is a common mode of action for naturally occurring antibiotic drugs such as Actinomycin D (ActD) and daunomycin as well as the antimalarial drug Cryptolepine (1, 7, 22-24). Employed in DNA-targeting antitumor therapeutics, DNA intercalators were found to act as topoisomerase II poisons, disrupt helicase activity, and cause ribosomal frame-shift mutations (11, 17, 25, 26). Consequently, both natural and synthetic DNA intercalating drugs are studied and rationally optimized for growing pharmaceutical interests (10, 11, 16, 17, 27-29).

Furthermore, the optical properties associated with “light-switch” synthetic intercalators upon intercalating DNA has become of great interest for visualized probe applications (29-38). In this regard, maintaining the native mechanical properties of stained DNA molecules is explored in ongoing research (21, 23, 39,
The reversibility of DNA intercalation also enables a controllable mechanism for integration of DNA in synthetic biology. For example, nano-films of DNA origami loaded with intercalating drugs are demonstrated as means of controlled release of anti-cancer agents for localized drug delivery (41). DNA intercalation was also recently utilized to dynamically modulate the parameters of a DNA-nanoparticle supper-lattice (42).

The molecular assembly of DNA intercalation can be classified according to the number of intercalating events per ligand, and the overall timescale to reach the equilibrium state as shown Fig. 1.1.3A. DNA intercalation can involve the insertion of one intercalating moiety (mono-intercalator), two intercalating moieties (bis-intercalator) or multi-intercalating moieties. Here each intercalation moiety elongates the natural DNA contour length by typical reported values of 0.2-0.4 nm (20, 21, 43-45). However, the timescale for reaching the final DNA-ligand intercalation state ranges over six orders of magnitude, depending on the structural deformation of the DNA-ligand complex that governs the molecular assembly. In particular, conventional intercalators that involve direct insertion of the intercalating have relatively fast association kinetics that can be from milliseconds such as for DNA mono-intercalation by ethidium (46), up to seconds as observed for the bis-intercalator YOYO (47). For unconventional intercalators, which involve a pre-intercalation DNA base pair threading (48, 49) or groove binding accommodation (43) of non-intercalating moieties, can exhibit extremely slow kinetics that range from minutes to days (43-45, 50, 51). Examples of intercalators from different classifications are shown in Fig. 1.1.3B.

Figure 1.1.3. A) DNA intercalators as classified by the number of intercalation event per ligand, and typical timescale to reach the equilibrium intercalative state, intercalator in blue, DNA backbone in red and base pairs in grey. M stands for mode, and T for threading. B) Examples for Intercalators from different classifications.
The DNA-intercalator molecular self-assembly process is investigated in bulk by different experimental means including X-ray diffraction (6), NMR (52), fluorescence spectroscopy (31), mass spectrometry (53), and linear and circular dichroism (54) spectroscopy. However, bulk studies of DNA intercalation can be constrained by several experimental and systematic challenges such as a limited range of detected concentration and uncontrolled non-intercalative molecular processes (10, 44). With its outstanding sensitivity and ability to distinguish other binding modes from intercalation, single-molecule force spectroscopy enables direct and real time measurement of the progressing dynamic assembly of single DNA-ligand complexes with as well as precise control of the experimental conditions (55-58). The unwinding and elongation of the DNA intercalation event provides mechanical signatures traceable by single molecule techniques such as atomic force microscopy (AFM) (59-61), magnetic tweezers (19, 62), and optical tweezers (20, 50, 63). The following section introduces optical tweezers, followed by a brief section outlining the use of single-molecule force spectroscopy in investigation DNA-ligand assemblies.

1.2 OPTICAL TWEEZERS

In 1986 Ashkin et al reported the first optical tweezing of dielectric particles with a refractive index $n'$ ranging from 1.5 to 3 and a radius ranging from 25 nm to 10 µm in water solution $(n=1.33)$ (64). The dielectric micro-and nano-particles of $n'$ larger than $n$ of the surrounding solution are attracted to the laser beam due to dipole forces. The particles are trapped in three-dimensions at the focus of the beam intensity gradient (64). The lateral trapping is due to the transverse intensity gradient of the Gaussian beam, and the axial trapping is due to focusing of the laser beam, which causes an intensity gradient maximized at the beam minimal waist centered in the focal plane.

Optical trapping for particles of diameter $d > 1$ µm can be explained by ray optics (65). As the light ray passes through the particle, the particle medium exerts piconewton force refracting the light ray, and therefore the particle experiences an equal and opposite forces exerted by the light ray. In lateral displacement from the beam (Fig1.2.1A1), more intense light ray is refracted by the particle side near the center of the beam relative to the intensity of the refracted light ray by the far side of the particle. This leads to a net transverse force directed toward the center of the laser beam (66). In the optical axis, the net force is directed toward the focal plane due to the focusing of the laser beam. Downstream of the focal plane where the particle is placed against the laser propagation (Fig.1.2.1A2), the net force by the refracted rays, which pulls the particle back to the focus, are required to overcome the net force exerted by the scattered light rays, which pushes the particle away on the optical axis. Accordingly, the particle is trapped in a potential well minimum at the center of the Gaussian beam (Fig.1.2.1B).

For single-beam optical tweezers, the laser beam is strongly focused to create a steeper intensity gradient along the optical axis to overcome the scattering forces and stabilize the optical trap.
Alternatively, two counter propagating beams in dual-beam optical tweezers neutralize the scattering forces with a relaxed intensity gradient, which is the optical tweezing setup used in this work. Once the particle is stably trapped at the laser beam focus, the trap restoring force for small displacements in each dimension can be considered as an elastic spring response, $F = k_x x$, where $k_x$ is called the trap stiffness along the x axis, and likewise $k_y$ and $k_z$ can be measured for the y and z axes (67). This equipartition approximation is valid for small displacements from the optical trap minimum. However, this approximated parabolic potential is less valid as the displacements grow bigger, where the trap potential is characterized instead by a Gaussian potential (68).
Figure 1.2.1: A) Optical trapping as explained by ray optics for beads with diameter \( d > 1 \mu m \), 1 is for lateral displacement from the laser beam, and 2 for displacements in the optical axis. B) The beads are trapped in a potential well with a minimum at the center of the Gaussian beam.

1.3 SINGLE-MOLECULE FORCE SPECTROSCOPY

In force spectroscopy experiments, a single DNA molecule is stretched and the force experienced by the molecule is measured as a function of its extension (56-58). These force-extension curves provide insights into the elastic properties of the nucleic acid molecule. Various reversible binding modes can be characterized by observing the changes in the DNA force-extension curves obtained in the presence of these reversibly binding molecules. Multiple force spectroscopy studies using optical tweezers (20, 50, 63) have measured the DNA binding properties of intercalators precisely. These techniques measure binding properties at single molecule level to characterize DNA intercalating drugs. In general the DNA-drug binding properties are obtained as a function of force and extrapolated to obtain these properties in the absence of force (20).

Typical optical tweezers experiments use polystyrene beads or cylinders coated with streptavidin to chemically attach a single DNA molecule that is labeled with biotin on the opposite strands (Fig. 1.3.1A). The bead attachment to the opposite strands of DNA allows the DNA to freely rotate about its axis. This torsionally unconstrained DNA configuration is commonly used to investigate the interactions of intercalators. The unconstrained DNA configuration relieves any torsional strain caused by the unwinding of the DNA double helix, so that only the change in DNA extension is measured. One of these beads is held by the optical trap while the other bead is held by a micropipette tip, glass plate or another optical trap that can be moved with the help of piezoelectric controls to provide stretching of DNA. When the DNA is stretched to a known extension, the bead in the trap will be displaced in proportion to the force exerted on the trapped bead by the DNA. The bead displacement causes the laser to deflect after passing through the bead. By calibrating the force as a function of laser displacement using known forces, the force exerted on the DNA is determined by measuring the laser displacement. In magnetic tweezers experiments a single DNA molecule is attached between the bottom glass plate of the flow cell and a magnetic bead. The magnetic bead is then moved with the help of moving magnet to stretch the DNA (Fig. 1.3.1B). In an atomic force microscopy (AFM) experiment one end of the DNA is attached to a substrate (such as mica) and the other end is attached to a tip of the AFM cantilever (Fig 1.3.1C).
Figure 1.3.1: A) dual-beam optical tweezers trapping a bead, while a second bead is held by micropipette tip, image is for the dual-beam optical tweezers setup used in this work. B) Stretching DNA with magnetic tweezers. C) Stretching DNA with AFM.
A DNA stretching curve (black data in Fig 1.3.2A) obtained with torsionally unconstrained DNA can be divided into four distinct regions (56-58). At low extensions (data shaded in violet) the DNA is stretched to its normal length with little change in the force. Since the force applied in this region is used to reduce the entropy of the DNA molecule, this region is known as the entropic stretching region. Further stretching beyond the normal contour length of DNA (0.34 nm/bp at F=30 pN) shows that the double helix behaves elastically (data shaded in blue), where a small extension results in a rapid force increase, which is known as the elastic region. Once the force reaches around F=65 pN, the force remains almost constant while the extension is almost doubled (data shaded in green) indicating a clear phase transition. Recent experiments have confirmed that the cooperative and progressive DNA elongation during this transition is due to force induced double helix unwinding and base pairs melting transitions, and may depend on the solution conditions (69-74). At the end of the overstretching transition, most of the double stranded DNA (dsDNA) is converted into single stranded DNA (ssDNA) but a few GC rich regions may hold the two strands together. Stretching beyond the transition exhibits again distinct elastic properties (data in red shade). Relaxing back slowly recovers the original elasticity of dsDNA indicating that this is a reversible processes, except in very low salt when melting is not reversible (69, 70, 72, 73).

The dsDNA can be explained as a homogeneous elastic rod with a smooth distribution of bending angles. The polymer model that describes the dsDNA is known as the worm-like chain model and effectively characterize the dsDNA length based on the end to end contour length ($L_{ds}$) and persistence length ($P_{ds}$). The force dependence of the dsDNA length at high force ($F$) is given by an approximate solution

$$L_{ds}(F) = L_{ds,c} \left( 1 - \frac{1}{2\sqrt{F \cdot P_{ds}/k_B T}} \cdot \frac{F}{S_{ds}} \right),$$

(1.3.1)

where $S_{ds}$ is a stretch modulus of backbone extensibility, $k_B$ is Boltzmann’s constant and $T$ is the temperature.

On the other hand the ssDNA is explained by the Freely Jointed Chain (FJC) model. FJC describes the one dimensional polymer as a collection of independent segments with a characteristic length (Kuhn length) connected via freely rotating hinges with varying bond angles (75). The length of the ssDNA ($L_{ss,c}$) at force $F$ can be characterized by the persistence length ($P_{ss}$) and the end-to-end or contour length ($L_{ss}$) of ssDNA

$$L_{ss}(F) = L_{ss,c} \left[ \coth \left( \frac{2P_{ss}F}{k_B T} \right) - \frac{k_B T}{2P_{ss}F} \right] \left[ 1 + \frac{F}{S_{ss}} \right],$$

(1.3.2)
where $S_{ss}$ is stretch modulus added to account for backbone extensibility of ssDNA. Fig 1.3.2B illustrates that the DNA stretching data fits the WLC model (blue solid curve) before reaching the force induced melting transition and fits the FJC model (red solid curve) beyond the melting transition. The DNA force-extension curves also provide information about the melting free energy associated with converting dsDNA to ssDNA. The area confined between the experimental data and the FJC polymer model (green striped area in Fig 1.3.2B) yields this melting free energy (69).

Figure 1.3.2: A) DNA stretching curve, the entropic stretching region, the first elastic region, the overstretching transition, and the second elastic region, respectively. B) Illustration of the free energy of the melting transition.
Chapter 2:
Conventional and unconventional intercalators

2.1 Conventional DNA Intercalation

2.2 Unconventional DNA Intercalation

Portions of this chapter are from:
Mechanisms of DNA intercalation probed by single-molecule force spectroscopy
Ali A. Almaqwashi, Thayaparan Paramanathan, and Mark C. Williams
(Submitted)
2.1 CONVENTIONAL DNA INTERCALATION

Studies of conventional DNA mono- and bis-intercalators in bulk showed that equilibrium binding is reached on a timescale ranging from milliseconds to seconds. This fast kinetics relative to the typical timescale of DNA stretching experiments (~10 to 100 seconds) provided the basis for examining DNA-ligand assembly in equilibrium for the first set of single-molecule observations of DNA interaction with conventional intercalators in 1996 using scanning force microscopy and optical tweezers. Coury et al. used scanning force microscopy to measure single DNA molecule lengthening upon binding to ethidium, daunomycin and 2,5-bis (4-amidinophenyl) furan (APF) (59). Cluzel et al. utilized intercalators to investigate the overstretching transition observed during single molecule DNA stretching with optical tweezers (76). A saturated concentration of ethidium (~25µM) was used to show that the overstretching transition disappears, in an attempt to explain the overstretching transition, which was then believed to be from B form DNA to S-DNA. In 2000, Krautbauer et al. used AFM to explain how force spectroscopy can be used to distinguish conventional intercalators from other binding modes like minor groove binding and cross-linking (77). Since then, measuring the extension of DNA upon binding to the intercalator has become a standard practice to quantify binding properties of the intercalator in force spectroscopy studies. The first complete characterization of conventional intercalators was proposed by Vladescu et al. in 2005 by analyzing the effects on DNA stretching curves in the presence of ethidium as a function of force and concentration (78). This study later lead to the novel quantitative method of measuring force-dependent binding properties using optical tweezers and extrapolating them to the zero-force values (20). In 2010, Lipfert et al. brought magnetic tweezers into studying intercalators. By examining torsionally constrained DNA, they characterized the double helix twist due to DNA unwinding upon binding to intercalators by measuring the rotation angles between the successive base pairs upon binding to ethidium (19).

Qualitative effects observed with conventional intercalators

In all force spectroscopy experiments DNA lengthening is observed upon intercalator binding to dsDNA. In addition, stabilization of dsDNA structure upon intercalation is represented in the force extension curves by an increase in the melting force in the presence of the intercalator (Fig 2.1.1A). Combining the above two effects as we progressively increase intercalator concentration, the melting transition plateau progressively become shorter and disappears (Fig 2.1.1B). Beyond some critical concentration the lengthening of dsDNA upon binding to intercalator makes dsDNA-drug complex indistinguishable from ssDNA (Fig 2.1.1B). In comparison to the classical thermodynamic example of liquid to gas phase transition at a constant temperature, a critical point is observed in the dsDNA to ssDNA phase transition at a particular ligand concentration. As we increase the intercalator concentration, at some critical concentration we won’t see a phase transition, and we cannot distinguish between the intercalator bound...
dsDNA and ssDNA, the same way we cannot distinguish between gas and liquid beyond the critical temperature. The phase diagram for ethidium (Fig 2.1.1C) can be mapped to show the similarity with the classical PVT phase diagrams of gaseous systems (78).

Figure 2.1.1: A) DNA elongation due to intercalation by ethidium, the DNA-ligand complex is stabilized which increases the melting transition. B) DNA transition plateau becomes shorter and vanishes at high concentration of ethidium. C) The phase diagram from DNA intercalation by ethidium.

Quantifying the DNA binding properties of intercalators

The binding of intercalators to DNA at a particular force can be quantified by the fractional lengthening observed. The fractional lengthening directly corresponds to the fractional ligand binding \( \Theta(F,C) \) associated with that concentration at a particular force, and is given by the ratio between the lengthening observed due to the binding of the intercalator at that concentration \( \Delta L_{eq}(F,C) \) compared to the lengthening observed at saturated concentration \( \Delta L_{eq}^{sat}(F) \):

\[
\Theta(F,C) = \frac{\Delta L_{eq}(F,C)}{\Delta L_{eq}^{sat}(F)} = \frac{L_{eq}(F,C) - L_{ds}(F)}{L_{eq}^{sat}(F) - L_{ds}(F)},
\]

(2.1.1)

Where \( L_{eq}(F,C) \) is the equilibrium extension observed with the drug-DNA complex at drug concentration \( C \) and at a particular force \( F \), \( L_{eq}^{sat}(F) \) is the extension of the drug-DNA complex at saturated drug concentration, and \( L_{ds}(F) \) is the extension of the dsDNA in the absence of drug. The experimentally obtained fractional binding measurements described above are fitted to a simple binding isotherm, an
approximate binding isotherm models, or the McGhee-von Hippel (MH) binding isotherm (21, 43, 78). The MH non-cooperative binding isotherm accounts for the effect of neighbor exclusion and has been used as a standard binding isotherm in single-molecule DNA intercalation studies. By fitting to the MH binding isotherm (as shown in Fig 2.1.2A for DNA intercalation by ethidium), we obtain the equilibrium binding constant \( K(F) \) (or alternatively the equilibrium dissociation constant \( K_d(F) = 1/K(F) \)) and the binding site size \( (n) \). The MH isotherm is described by:

\[
\Theta(K(F), n) = K(F) C \frac{n (1 - \Theta)^n}{\left(1 - \Theta + \frac{\Theta}{n}\right)^{n-1}},
\]

Although the binding constant obtained using this isotherm agreed well with bulk experiments, the binding site sizes of ethidium at high force appears to violate nearest neighbor exclusion expected for DNA intercalation (see Table 2.1.1). Due to the sugar puckering of the backbone that occurs with intercalation of ethidium, it was found to bind every other site and was reported to have a binding site size of 2 in the absence of applied force. This constraint seems to be relieved by high forces in single-molecule experiments. Stretching the backbone of DNA to nearly twice its length may enable the ethidium to bind to every available binding site, yielding \( n \) of less than 2. This method was also used to find the binding constants and the binding site sizes (Table 2.1.1) for ruthenium complexes \([Ru(phen)]^2+, [Ru(bby)_2dppz)]^2+\), \([Ru(phen)_2dppz)]^2+\) and \([Ru(phen)_2dppx)]^2+\) using with AFM and optical tweezers (79). Note that the fractional ligand binding per binding site \( \nu = \Theta/n \) is sometimes alternatively used in fitting the MH isotherm (20).

The determined binding constant \( K(F) \) and binding site size \( n \) are force-variant and further analysis is required to obtain the zero-force binding properties (Figure 2.1.2B). The binding constant at various forces for ethidium and ruthenium based intercalators \([Ru(phen)]^2+, [Ru(phen)_2dppz)]^2+\) showed a clear exponential dependence on force (20). Vladescu et al. demonstrated that the binding constant in the absence of the force \( K(F) \) can be determined from the exponential dependence on force in Eq.2.1.3

\[
K(F) = K(0) e^{F \Delta x_{eq}/k_B T},
\]

The term \( F \Delta x_{eq} \) in the exponential component is the shift in the zero-force free energy of DNA intercalation as the force facilitates the required double helix equilibrium elongation \( \Delta x_{eq} \) for each ligand to reach the intercalation equilibrium state. A complete analysis using this method to determine the zero force binding constant \( K(0) \) and the lengthening upon forming a DNA-ligand intercalation complex, \( \Delta x_{eq} \) for intercalators ethidium (20), \([Ru(phen)]^2+\) (20), \([Ru(phen)_2dppz)]^2+\) (20), and YO (80) are shown in Table1. The measurements of the zero force binding constant as a function of salt concentration showed a significant decrease in the binding affinity with increased salt concentration (21). A similar approach
was made with magnetic tweezers by Celedon et al. to quantify the torque dependence of ethidium binding constant \( (81) \). The torque or twisting of the DNA disfavors the binding of an Intercalator and it is given by

\[
K(F) = K(0)e^{\frac{T \Delta \mu}{k_BT}},
\]

\[2.1.4\]

Figure 2.1.2: A) McGhee-von Hippel (MH) binding isotherm fits (Eq 2.1.2) for the fractional DNA elongation as a function of ethidium concentration at each stretching force. B) Fitting the binding constant \( K(F) \) to the exponential dependence on force (Eq 2.1.3).

**Elastic properties of saturated DNA-intercalator complexes**

Fitting the force-extension curves obtained in the presence of intercalators to the worm like chain model yields the contour length, persistence length, and elastic modulus of the DNA-intercalator complex. All experimental measurements of the DNA contour length in the presence of the intercalators have supported the monotonic increase in contour length with intercalator concentration until it reaches the saturation value which is obtained from Eq. 2.1.5 \((20, 80, 82, 83)\). The contour length from the saturated DNA-intercalator complex \( L_{eq}^{sat}(F) \) and the equilibrium elongation per ligand \( \Delta x_{eq} \) provides a complementary estimation of the binding site size \( n \) (Eq. 2.1.6).
\[
L_{eq}^{sat}(F) = L_{eq,c}^{sat} \left(1 - \frac{1}{2\sqrt{F \cdot \frac{P}{k_BT}}} + \frac{F}{S}\right), \quad (2.1.5)
\]

\[
n = \frac{\Delta X_{eq}^{sat}}{\Delta L_{eq,c}^{sat}} = \frac{\Delta X_{eq}}{L_{eq,c}^{sat} - L_{ds,c}}, \quad (2.1.6)
\]

By combining Eq. 2.1.1 and Eq. 2.1.2 we obtain the DNA-intercalator complex extension as a function of the applied stretching force at each ligand concentration.

\[
L_{eq}(F) = L_{ds}(F) + \Theta(K(F), n) \cdot \Delta L_{eq}^{sat}(F), \quad (2.1.7)
\]

Then we substitute equations Eq. 2.1.3 and Eq. 2.1.6

\[
L_{eq}(F) = L_{ds}(F) + \Theta(K(0), n) \cdot \Delta L_{eq}^{sat}(F) \cdot e^{\left[n \Delta L_{eq,c}^{sat} \right] F / k_BT}, \quad (2.1.8)
\]

This alternative approach allows fitting the force-variant DNA equilibrium extension at each constant ligand concentration to estimate the zero-force binding constant and binding site size. The general trend for persistence length in the presence of intercalators was that \( P \) decreased compared to the dsDNA value (20, 80-83). Optical tweezers experiments showed the elastic modulus is reduced to almost one fourth of bare DNA upon binding to intercalators (20, 80) and magnetic tweezers experiment showed that the twist stiffness is also reduced to almost one third of the bare DNA twist stiffness (81). Some optical tweezers experiments at low force (<2 pN) showed that the persistence length increases upon binding to intercalators and increases with concentration until a critical value and then drops below the DNA persistence length to give the general trend (82).

**Studying the binding kinetics of conventional intercalators**

In regard to the binding kinetics, many conventional intercalators have rapid association rates that can be as fast as microseconds, which is challenging to measure in single molecule force spectroscopy experiments. However, recent experiments with optical tweezers have used this method to probe relatively slow conventional mono- and bis-intercalators (21). As the single-molecule techniques are steadily improving in time resolution, it is expected to explore the kinetics from elongation measurements of sub-second association timescale. Meanwhile, the kinetics investigations for unconventional intercalators are providing a general analysis framework, which can be in principle used for fast intercalating ligands.
Table 2.1.1: Quantitative results of the binding constant (K), the binding site size (n), and the equilibrium elongation per ligand ($\Delta x_{eq}$). (Atomic Force Microscopy (or scanning force microscopy), Magnetic Tweezers, Optical Tweezers).

<table>
<thead>
<tr>
<th>Intercalator</th>
<th>Binding Constant K ($10^6$ M$^{-1}$)</th>
<th>Binding Site Size n (base pairs)</th>
<th>Binding Equilibrium elongation $\Delta x_{eq}$ (nm/bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium bromide</td>
<td>0.036 ± 0.005&lt;sup&gt;a&lt;/sup&gt; 10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.01&lt;sup&gt;a&lt;/sup&gt; 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25 ± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.46 ± 0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.3 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.13 ± 0.04&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.9 ± 0.1&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.145&lt;sup&gt;g&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daunomycin</td>
<td>0.066 ± 0.024&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>AFP</td>
<td>2.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>[Ru(phen)$_3$]$_2^{2+}$</td>
<td>0.0088 ± 0.0003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.0 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.28 ± 0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.0016 ± 0.0002</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>[Ru(phen)$_2$dppz]$_2^{2+}$</td>
<td>0.15 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.2 ± 0.1 (10 pN)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.0 ± 0.5 (10 pN)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.90 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.29 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.38 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>[Ru(bpy)$_2$dppz]$_2^{2+}$</td>
<td>0.15 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3.2 ± 0.1 (10 pN)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.0 ± 0.5 (10 pN)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Oxazole Yellow (YO)</td>
<td>0.578 ± 0.080&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td>0.233 ± 0.013&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.29 ± 0.09&lt;sup&gt;i&lt;/sup&gt;</td>
<td>3.8 ± 1.0&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.31 ± 0.03&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Psoralen</td>
<td>0.088 ± 0.024&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1.43 ± 0.13&lt;sup&gt;h&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>SYTOX Orange (SxO)</td>
<td>2.4 ± 0.5&lt;sup&gt;i&lt;/sup&gt;</td>
<td>3.0 ± 0.4&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.30 ± 0.02&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>SYTOX Green (SxG)</td>
<td>14 ± 3&lt;sup&gt;i&lt;/sup&gt;</td>
<td>2.6 ± 0.6&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.27 ± 0.02&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>SGold (SbG)</td>
<td>7.8 ± 3.3&lt;sup&gt;i&lt;/sup&gt;</td>
<td>3.2 ± 0.5&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.30 ± 0.01&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
2.2 UNCONVENTIONAL DNA INTERCALATION

In experimental terms, unconventional intercalators often exhibit DNA-ligand kinetics slower than typical single DNA molecule stretching rates (40, 43-45, 50, 84). The slow association/dissociation kinetics results in considerable hysteresis between the force-extension (F-L) curves in a cycle of stretching the DNA molecule to a higher force and then releasing the DNA back to the initial extension (43, 44, 84). The irreversible F-L curves indicate non-equilibrium measurements and can be explained based on force-facilitated DNA intercalation. The observed hysteresis indicate that the DNA-intercalator complex extension is first underestimated during the stretching to higher forces due slower ligand associating than the pulling rate, then the extension is overestimated in the receding to lower forces due to slower dissociating for the ligand that intercalated DNA at higher forces (Fig 2.2.1A) (44). Paramanathan et al demonstrated for ActD that the DNA-ligand equilibrium elongation is indeed larger than the stretching extension and smaller than releasing extension (43). First, the DNA stretching proceeded until an assigned force, then a force-feedback maintained the applied force while tracing DNA elongation. Similar force-clamp experiments are done but initiated when the DNA-ActD complex extensions are relaxed from higher forces to the assigned lower force. The data showed that the DNA-intercalator elongation converged to similar equilibrium extension values (Fig. 2.2.1B). In principle, a DNA pulling rate slower than the intercalation kinetics would produces a reversible stretching-releasing cycle. In this regard, Kleimann et al, investigating DNA-Triostin A intercalation, showed that by slower the pulling rate reduces the hysteresis in the stretching-releasing cycle (84).

Figure 2.2.1: A) Stretch-release cycles of DNA-ActD complex show considerable hysteresis between the force-extension (F-L) indicating non-equilibrium measurements. B) Illustrating the slow kinetics of DNA unconventional intercalation, constant force measurements ensure reaching DNA equilibrium elongations.
The slow kinetics observed for unconventional DNA intercalators may arise from a required DNA structural accommodation of a non-intercalative moiety required in order to reach the intercalative equilibrium state. For instance, some naturally derived antibiotics such as ActD, Tristin A and Thiocoraline, which are all neural polypeptides, have been demonstrated by single-molecule force spectroscopy to unconventionally intercalate DNA (40, 43, 84). Crystal structures showed strong DNA unwinding in order to fit their linked/separate bicyclic peptide chains into the DNA groove before inserting the intercalating moieties (7, 85, 86). Further structural deformations such as DNA bending and base pairs flipping are also reported (43). Another category of unconventional DNA intercalators is composed of complexes that have intercalating moieties engulfed in between non-intercalating moieties. This molecular design requires that the non-intercalating moiety first thread through the DNA helix in order to bring the planar intercalating moiety in proximity of the base pairs (44). One of the early examples for such unusual molecular assembly was first reported for Nogalamycin, an anthracycline antibiotic, which has a dumbbell molecular structure composed of the intercalating chromophore in the middle between two bulky sugar substituents (87). Later, synthetic complexes of mono, bis, and multi-threading intercalators were demonstrated (48, 49, 51). In particular, DNA threading intercalation was recently characterized by single-molecule force spectroscopy for binuclear ruthenium complexes such as the mono-intercalator \( \Delta,\Delta-\text{[µ-bidppz(phen)]}_4\text{Ru}_2^{4+} \) (\( \Delta,\Delta-P \)), and the bis-intercalator \( \Delta,\Delta-\text{[µ-C4(cpdppz)]}_2(\text{phen})_4\text{Ru}_2^{4+} \) (\( \Delta,\Delta-Pc \)) (see Fig. 1.1.3B) (44, 45).

**Equilibrium elongation measurements**

In essence, equivalent equilibrium binding isotherm analysis is used for all DNA intercalators. However, the slow equilibrating DNA elongation of unconventional intercalators requires a revised experimental approach rather than typical single-molecule stretching. In order to apply the equilibrium analysis, each F-L data point is obtained at a given ligand concentration by conducting force clamp measurements on a single DNA molecule. In the typical single-molecule force clamp approach, the applied DNA stretching force \( (F) \) is maintained constant by force feedback control within a force resolution of \( \pm 1 \) pN, while the single DNA molecule elongates from the ligand-free DNA extension \( L_{ds}(F) \) until reaching an equilibrium extension \( L_{eq}(F,C) \), where the DNA-ligand complex extension becomes essentially time-invariant. For each applied force, measurements are obtained at higher concentrations until no additional elongation occurs, namely reaching saturated extension \( L_{sat}^{eq}(F) \) (as it will be illustrated in the following chapter).

Once the equilibrium intercalated DNA extension measurements are obtained, the equilibrium analysis proceeds in the same manner as it was established for the conventional intercalators. All equilibrium properties are similarly provided from fitting \( L_{sat}^{eq}(F) \) data points to the WLC model (Eq. 2.1.5) to determine the DNA-ligand complex elasticity, and fitting \( L_{eq}(F,C) \) for each applied force to a binding isotherm, such
as M-H model (Eq. 2.1.2) to yield the force-dependent affinity, then estimating the zero-force affinity and the equilibrium DNA deformation in the intercalative state from the exponential dependence on force (Eq. 2.1.3). Alternative to the force clamp approach detailed here, equilibrium F-L data points can be also acquired by force decay measurements. In this experimental approach, the tethered DNA molecule is stretched to an assigned extension and the applied tension is allowed to relax back by intercalated DNA elongation to an equilibrium force at a given ligand concentration (47). Furthermore, force-jump approach is also used, in which force-clamp is used to obtained at force F1 the equilibrium extension $L_{\text{eq}}(F1,C)$, then jumping to a higher force F2 determines $L_{\text{eq}}(F2,C)$. Note that in this last approach, only the first measured $L_{\text{eq}}(F1,C)$ starts from the free-DNA, a distinction that can be relevant when investigating the kinetics approach to equilibrium.
Chapter 3:
Single-transition DNA threading intercalation

3.1 DNA-ligand intercalative transition

3.2 Threading mono-intercalator

 Portions of this chapter are from:

Strong DNA deformation required for extremely slow DNA threading intercalation by a binuclear ruthenium complex

Ali A. Almaqwashi, Thayaparan Paramanathan, Per Lincoln, Ioulia Rouzina, Fredrik Westerlund and Mark C. Williams


Impact Factor: 9.11
3.1 DNA-LIGAND INTERCALATIVE TRANSITION

The time-dependent non-equilibrium elongation reveals significant characteristics of DNA intercalation complex assembly. The dynamic structural deformations and the governing energy landscape of DNA-ligand formation are derived from the force-dependent kinetics. In the force clamp experimental approach, most unconventional DNA intercalation by mono-intercalators show single-rate exponential dependence during DNA-ligand association. The single exponential time-dependence is indicative of a single molecular transition from the non-intercalative DNA state (NI) to the intercalated DNA state (I) shown in Eq. 3.1.1. Note that $k_{on}$ is the forward rate resulting from the product $k_a \cdot C$, where $k_a$ is the bimolecular association rate, and $k_{off}$ is the unimolecular reverse rate. At constant DNA stretching force and ligand concentration, the time dependent non-equilibrium DNA extension $L(t)$ is fitted by Eq. 3.1.2 yielding $L_{eq}(F,C)$, which is used in the equilibrium analysis, as well as the net relaxation rate $k_{total} = k_{on} + k_{off}$, which provides the kinetics analysis.

$$L(t) = L_{eq}(F,C) - \Delta L_{eq}(F,C) \cdot e^{-k_{total}t},$$

(3.1.2)

The kinetic analysis is useful only after determining at least two of the following quantities $K_{eq}(F)$, $k_{total}(F)$, and $k_{off}(F)$. The reverse intercalative rate $k_{off}(F)$ are found to be measureable for some single-transition DNA intercalation complexes by rinsing out the bound ligands, as long as the ligands dissociate from the NI state into the ligand-free solution significantly faster than $k_a(F)$. For the “wash off” experiments, a force clamp is applied while tracing the DNA-intercalator complex extension back to the ligand-free DNA extension. The traced extension at constant stretching force is fitted to a single exponential time-dependence, replacing $(L_{eq}-\Delta L_{eq}, k_{total})$ in Eq. 3.1.2 by $(L_{ds}+\Delta L_{eq}, k_{off})$. The single transition rate, consistently measured by single-molecule force spectroscopy for most DNA intercalators, is in contrast to multiple rates reported in bulk measurements. (31). This is possibly attributed in part to constraining DNA-DNA contacts in single-molecule experiment which prevents competitive non-intercalative molecular transitions such as DNA-ligand aggregation. Some bulk methods may also measure rates that involve external binding kinetics, and the ligand non-intercalative accommodation in the DNA grooves. In contrast, single-molecule measurements are specifically sensitive to DNA elongation due to intercalative binding.

3.2 THREADING MONO-INTERCALATOR

When synthesizing DNA-targeted drugs, high DNA binding affinity and slow DNA binding kinetics are considered essential aspects that enhance their therapeutic capability. (17, 56, 88-90). Higher affinities maximize the treatment effect with minimal dose exposure, lower association rates enable selective
binding molecules to scan DNA for a specific targeted sequence, and lower dissociation rates ensure
disruption of DNA transcription and duplication (11, 56, 88, 90-93). These desired properties are found in
ligands that intercalate DNA by threading (48, 50, 51, 94), an interaction that first requires non-
intercalating moieties of a ligand to pass between DNA base pairs before the intercalative binding occurs
(91, 92, 95, 96). The threading step is required in order that the ligand brings the intercalating moiety in
close proximity to the base pairs and reach the equilibrium state, resulting in a very slow binding process
(49, 91, 95, 97). This is in contrast to conventional intercalation, which occurs by simple insertion between
the DNA base pairs.

First synthesized more than a decade ago (31), the binuclear ruthenium complex Δ,Δ-[µ-bidppz-
(phen)]$_2$Ru$_2$]$^4^+$ (Δ,Δ-P) (Fig 3.2.1) exhibits extremely slow DNA intercalation kinetics, as determined by
luminescence and circular dichroism (CD) bulk experiments, due to the threading intercalation binding
mechanism (48, 91, 98). The equilibrium dissociation constant for the DNA-Δ,Δ-P complex and the
molecular mechanism governing the threading intercalation have not been quantitatively determined, due
to the limitations of traditional techniques for investigating ligands with extremely slow kinetics and high
binding affinities. In this study, the elongation is mechanically probed for each single DNA molecule at
constant applied force while the intercalation interaction approaches equilibrium (43, 50, 56). Optical
tweezers can resolve single DNA molecule intercalation elongation of a few nanometers while
maintaining an applied constant force with 1 pN resolution (43, 50, 99-102). From the single-molecule
measurements, presented here is a complete characterization of DNA threading intercalation by Δ,Δ-P.

Figure 3.2.1. Chemical structure of the binuclear ruthenium complex Δ,Δ-P (Δ,Δ-[µ-bidppz-(phen)]$_2$Ru$_2$]$^4^+$).

RESULTS
Equilibrium extension measurements at fixed force yield DNA-Δ,Δ-P titration curves and saturated
complex elasticity

Equilibrium DNA extension measurements were performed for forces ranging from 7 pN to 60 pN at
ligand concentrations from 5 nM to 150 nM, as shown in Fig. 3.2.2A along with the previously reported
measurements at 2 nM (50). Throughout the measurements, the force was maintained constant (±1 pN), which is illustrated in Fig. 3.2.2A for a 40 pN measurement at 5 nM of ΔΔ-P. At each constant force, the extension of the DNA-ΔΔ-P complex increased exponentially with time and converged to an equilibrium value, $L_{eq}(F,C)$, (per bp) (Fig. 3.2.2B). The time evolution of the DNA-ΔΔ-P complex extension at all forces is well fit by a single exponential with a net relaxation rate, $k_{total}(F,C)$ (see Eq. 3.1.2), supporting a single molecular transition for threading intercalation, at least under our force-facilitated conditions. This confirms previously reported results from optical tweezers experiments for λ-DNA, and it is in agreement with bulk CD measurements for AT-DNA (50, 98). However, previous luminescence measurements and CD measurements on mixed sequence DNA were fitted with multiple exponential equations, indicating multiple step behavior for ΔΔ-P binding. It is possible that such bulk observations, which were made under very low applied forces, reflect more subtle non-threading processes (98). Such non-threading processes may include pre-intercalation binding to dsDNA, ligand rearrangements due to DNA-DNA contacts, as well as post-intercalation DNA-ligand interactions that do not contribute to DNA elongation. In contrast, the rate that is found in single DNA molecule experiments is measured exclusively from the mechanical length change due to intercalation on the timescale of tens of minutes.
Figure 3.2.2: A) Average measurements of at least three DNA-ΔΔ-P complex equilibrium extensions for a range of constant forces between 7 and 60 pN at ligand concentrations of 2 to 150 nM as color coded, circles are measurements carried out in this study for concentrations 5 to 150 nM, squares are previously reported measurements at 2 nM, cyan crosses show non-equilibrium extensions for forces 5 to 50 pN obtained from averaging fast release curves (obtained with a release rate of 200 nm/s) after equilibrium extension measurements with constant force (60 pN) at 50 nM concentration. Dashed lines are fits to the DNA-ligand complex WLC model. B) The time-dependent extension as a function of time (open circles) for DNA in the presence of 5 nM Δ,Δ-P at several constant forces. The dashed lines are fits to the single exponential dependence. Experiments were conducted at 21 °C (10 mM Tris buffer, 100 mM NaCl, pH 8).

For each constant force we obtained extension measurements at increasing concentration until we reached the saturated extension, $L_{eq}^s(F)$. The equilibrium extensions per bp of the DNA-Δ,Δ-P complex, $L_{eq}(F,C)$, are presented in Fig. 3.2.2A for several force values between 10 and 60 pN. For each force the fractional equilibrium binding of ligand $\Theta(F,C)$, which ranges from zero to one, was determined by comparing $L_{eq}(F,C)$ to the saturated extension at the same force, $L_{eq}^s(F)$, (43, 56):

$$\Theta(F,C) = \frac{L_{eq}(F,C) - L_{ds}(F)}{L_{eq}^s(F) - L_{ds}(F)},$$

(3.2.1)

where $L_{ds}(F)$ is the DNA extension in the absence of ligand binding. Afterward, we use $\Theta(F,C)$ as a function of force at each fixed concentration to obtain the equilibrium force-extension curves $L_{eq}(F,C)$.
(and its reciprocal function \( F(L_{eq}, C) \)). It follows from Eq. 3.2.1 that the equilibrium force-extension \( L_{eq}(F, C) \) for each ligand concentration \( C \) can be calculated as a linear combination of \( L_{ds}(F) \) and \( L_{eq}^{sat}(F) \) weighted with the fraction of each component, governed by \( \Theta(F, C) \), according to the relationship:

\[
L_{eq}(F, C) = [1 - \Theta(F, C)] \cdot L_{ds}(F) + \Theta(F, C) \cdot L_{eq}^{sat}(F)
\]  

(3.2.2)

Fits to the experimental force-extension curves obtained by using Eq. 3.2.2 are shown as dashed lines in Figure 3.2.2A. The saturated DNA-\( \Delta,\Delta \)-P complex extensions \( L_{eq}^{sat}(F) \) are fitted to WLC (see Eq. 2.1.5) which yields an effective persistence length of \( \sim 2 \) nm. This is significantly lower than the persistence length of the saturated DNA- monomer \( \Delta \)-p complex of \( \sim 14 \) nm (20), which intercalates DNA without threading.

Our observation that the complete \( L_{eq}(F, C) \) curves calculated in this manner fit the equilibrium extensions obtained as a result of relaxation at each concentration supports the assumption that the intercalated DNA is indeed a mixture of the ligand-free and ligand-saturated DNA to a good approximation. It is important to note that, due to slow, force-dependent ligand binding, the equilibrium force-extension curve cannot be obtained directly, as this would require pulling on the timescale of many hours. To illustrate this point, a force-extension curve obtained after stretching the DNA to 60 pN at 50 nM, waiting at constant force until equilibrium is reached, and then releasing at a rate of 200 nm/s, shows very different elasticity due to the non-equilibrium nature of the stretching curve (cross symbols, Fig. 3.2.2A). In principle, a very slow release (over hours) of the DNA-\( \Delta,\Delta \)-P complex from its equilibrium extension at 60 pN would reflect the equilibrium extension fit obtained from Eq. 3.2.2.

**\( \Delta,\Delta \)-P-DNA binding affinity and the site size are strongly affected by force**

The measured equilibrium length of the DNA-\( \Delta,\Delta \)-P complex \( L_{eq}(F, C) \) is presented in Fig. 3A as a function of concentration. The McGhee-von Hippel binding isotherm (58, 79, 102-104)

\[
\Theta(K_d, n) = \frac{C}{K_d} \left( \frac{n \cdot (1 - \Theta)^n}{1 - \Theta + \frac{\Theta}{n}} \right)^{n-1}
\]  

(3.2.3)

is employed to fit these measurements by comparing the values obtained from Eq. 3.2.1 to the fit values obtained from Eq. 3.2.3, shown as dashed lines in Fig. 3.2.3A. There are two fitting parameters in Eq. 3.2.3, the equilibrium dissociation constant \( K_d \) and the binding site size \( n \), which are varied independently to fit the experimental \( L_{eq}(F, C) \) curves at each force. The binding curves could not be fit well to a single value of \( n \) for all curves. The fitted \( K_d(F) \) values decrease with force from 21±4 nM at 10 pN to 2.8±0.1 nM.
at 60 pN (see Fig. 3.2.3B). In addition, the fitted value of the binding site size of Δ,Δ-P, n(F), decreases from 3.7±0.2 at 10 pN to 1.7±0.1 at 60 pN (Fig. 3.2.3C). Analyzing the fitted $K_d(F)$ values in terms of the expected exponential force dependence for ligand binding processes that elongate DNA (20, 43, 56, 105)

$$K_d(F) = K_d(0)e^{-F\Delta x_{eq}/kT}$$

(3.2.4)
gives a zero-force equilibrium dissociation constant $K_d(0)$ of 44±2 nM. We also obtain a value of $\Delta x_{eq}=0.19\pm0.01$ nm, which represents the increase in DNA equilibrium extension due to one ligand binding event.

The measured force-dependent DNA-Δ,Δ-P complex elongation per bp observed at saturation, along with the fitted value of the binding site size $n(F)$, provide an independent estimate of $\Delta x_{eq}$ at each force

$$\Delta x_{eq} = n(F) \cdot L_{eq}^n(F).$$

(3.2.5)

Substituting the fitted values of $n(F)$ and $L_{eq}^n(F)$ into Eq. 3.2.5 we obtain the force-independent value of DNA elongation due to the single Δ,Δ-P intercalation event, $\Delta x_{eq} = 0.23\pm0.01$ nm, averaged over all measured forces. The result is in reasonable agreement with the value of the same parameter $\Delta x_{eq} = 0.19\pm0.01$ nm obtained by fitting $K_d(F)$ to Eq. 3.2.4. The decreasing binding site size shown in Figure 3.2.3C implies that higher forces promote additional ligand intercalation at saturation, such that at 60 pN almost every DNA stack can be intercalated. In contrast, at 10 pN only every 3.7 stacks are intercalated at saturation.
Figure 3.2.3. A) Measured equilibrium extensions as a function of concentration fitted to the McGhee-von Hippel binding isotherm, yielding the dissociation constant ($K_d$) and the binding site size ($n$) for a range of applied forces between 10 pN and 60 pN. The dashed lines are the fits at each constant force as color coded, closed triangles are measurements, filled squares are previously reported measurements at 2 nM and unfilled marks are obtained from the DNA-ligand complex WLC fit (Eq. 3.2.2). B) Measured equilibrium dissociation constant $K_d(F)$ (symbols) along with the fit to an exponential dependence on force (dashed line). The fit allows us to determine the zero force binding constant $K_d(0)= 44\pm2$ nM and the change in DNA length after equilibrium binding $\Delta x_{eq} = 0.19\pm0.01$ nm. C) The binding site size obtained from the McGhee-von Hippel binding isotherm ranging from $3.7\pm0.2$ at 10 pN to $1.7\pm0.1$ at 60 pN.
Kinetics analysis unveils pronounced base pair displacements required for binding

The kinetics of Δ,Δ-P binding to DNA can be followed as an increase in the DNA-ligand complex length over time, which fits well to a single exponential (see Fig 3.2.2B) with a net relaxation rate \( k_{\text{total}} = k_{\text{on}} + k_{\text{off}} \), where \( k_{\text{on}} \) and \( k_{\text{off}} \), are the bimolecular forward and the unimolecular reverse threading rates, respectively. As the DNA-Δ,Δ-P complex relaxation kinetics is determined at a fixed force, all rates pertain to that particular force. Because we also determined \( K_a(F) \), we can use this information to obtain the individual \( k_{\text{on}}(F) \) and \( k_{\text{off}}(F) \) rates at each constant force (43):

\[
k_{\text{on}}(F) = \frac{k_{\text{total}}(F)}{1 + K_a(F)/C}
\]

(3.2.6)

\[
k_{\text{off}}(F) = \frac{k_{\text{total}}(F)}{1 + C/K_a(F)}.
\]

(3.2.7)

Furthermore, \( k_{\text{on}}(F) \) and \( k_{\text{off}}(F) \) at each ligand concentration can be individually fitted to an exponential dependence on force, which provides a measurement of the zero-force rate, \( k_{\text{on/off}}(0) \), and the change in DNA length between the unbound and the forward transition state, \( x_{\text{on}} \), and the intercalated and the reverse transition state, \( x_{\text{off}} \), respectively.

\[
k_{\text{on/off}}(F) = k_{\text{on/off}}(0)e^{x_{\text{on/off}}F/kT}
\]

(3.2.8)

Fig. 3.2.4A presents the force-dependent kinetics analysis at 5 nM concentration. Interestingly, both \( k_{\text{on}}(F) \) and \( k_{\text{off}}(F) \) are strongly facilitated by force, due to the significant complex elongations required for both ligand association (\( x_{\text{on}} = 0.33\pm0.01 \) nm) and dissociation (\( x_{\text{off}} = 0.14\pm0.01 \) nm). Similar dynamic displacements are obtained from analyzing the kinetics at all other concentrations below saturated binding (data not shown). The difference between the measured complex elongations to the transition state in the forward and reverse directions provides another independent estimate of the equilibrium complex elongation upon a single Δ,Δ-P intercalation event (Fig. 3.2.5), \( \Delta x_{\text{eq}} = x_{\text{on}}- x_{\text{off}} = 0.19\pm0.01 \) nm, which agrees with the two other measurements of this parameter discussed above.

The forward rates \( k_{\text{on}}(F) \) for all measured forces show a linear dependence on ligand concentration, whereas the backward rates \( k_{\text{off}}(F) \) are independent of concentration, as expected for a bimolecular process. For example, the forward rate \( k_{\text{on}} \) at 30 pN ranges from \( \sim10^{-4} \text{ s}^{-1} \) (\( \tau \sim2 \) hours) at 2 nM to \( \sim10^{-2} \text{ s}^{-1} \) (\( \tau \sim2 \) mins) at 150 nM, as illustrated in Figure 3.2.4B. The slope from the linear concentration dependence at each measured force gives the association rate \( k_a(F) \), as shown in Figure 3.2.4C for all measured forces. By fitting the association rates to the exponential dependence on force, we obtain a
zero-force association rate of $k_{a(0)} \approx 10^4 \, M^{-1}s^{-1}$. This bimolecular association rate is about 5 orders of magnitude slower than the association rate limited by diffusion.
Figure 3.2.4. A) The on rates ($k_{on}$, green data points) and off rates ($k_{off}$, red data points) at a ligand concentration of 5 nM fitted to a single exponential dependence on force (dotted lines) to obtain the distances associated with the on and off transitions, $x_{on} = 0.33\pm0.01$ nm and $x_{off} = 0.14\pm0.01$ nm and $k_{on}(0) = 1.6\pm0.1\times10^{-4}$ and $k_{off}(0) = 1.4\pm0.1\times10^{-3}$. B) The on rates ($k_{on}$) and off rates ($k_{off}$) as a function of concentration at 30 pN force. The triangles are for concentrations 5-150 nM, squares are from analyzing previously reported kinetics at 2 nM, and the dotted lines are linear fits. C) The association rates $k_a(F)$ (data points) for forces from 20 pN to 60 pN fitted to Eq. 3.2.8 (dashed line), yielding the zero-force association rate $k_a(0) = 1.01\pm0.001\times10^4$ M$^{-1}$s$^{-1}$ and the length change required for ligand association $x_{on} = 0.31\pm0.01$ nm.
Figure 3.2.5. Illustration of the mechanism governing the slow kinetics of DNA threading intercalation by \( \Delta,\Delta-P \), where the separation between the base pairs is nearly doubled before each association/dissociation event while the DNA-\( \Delta,\Delta-P \) complex relaxes at an equilibrium separation of 0.53 nm per bp.

**DISCUSSION**

**Equilibrium intercalation properties of \( \Delta,\Delta-P \)**

DNA threading by \( \Delta,\Delta-P \) is dramatically facilitated by force, as indicated by the exponential decrease in the DNA-ligand equilibrium dissociation constant with applied stretching force (Fig. 3.2.3B). This \( \Delta,\Delta-P \) - intercalated DNA state is stabilized by force because each intercalation event results in a DNA elongation of \( \Delta x_{eq} = 0.19 \) nm. Also, the DNA stretching force appears to relieve significant steric constraints against \( \Delta,\Delta-P \) threading, leading to saturated \( \Delta,\Delta-P \)-DNA intercalation every 1.7 bp at 60 pN force (Fig. 3.2.3B), in contrast to the observed saturated intercalation of only every \( \sim 3.7 \) bp at low forces (10 pN).

Table 3.2.1. Comparison of the equilibrium intercalative parameters for mononuclear and binuclear ruthenium ligands.

<table>
<thead>
<tr>
<th>Property of intercalated complex</th>
<th>( \Delta-p ) ( \text{Ru(phen)}_2\text{dppz}^{2+} )</th>
<th>( \Delta,\Delta-P ) ( \text{\Delta,\Delta-[\mu \text{- bidppz} \cdot \text{(phen)}_4\text{Ru}_2]^{4+} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta x_{eq} ) (nm)</td>
<td>0.38 ± 0.02</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>( n(F=10\text{pN}) )</td>
<td>2.9 ± 0.1</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>( n(F=60\text{pN}) )</td>
<td>2.5 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>( K_d(F=0), ) ( (\mu\text{M}) )</td>
<td>1.1 ± 0.1</td>
<td>0.044 ± 0.002</td>
</tr>
<tr>
<td>Persistence length (nm)</td>
<td>14.3 ± 0.1</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Elastic modulus, (pN)</td>
<td>324 ± 17</td>
<td>598 ± 30</td>
</tr>
</tbody>
</table>

In Table 3.2.1, we compare the parameters describing equilibrium \( \Delta,\Delta-P \) binding with the analogous parameters for the corresponding mononuclear complex, \( \text{Ru(phen)}_2\text{dppz}^{2+} \) (\( \Delta-p \)), also measured by DNA stretching with optical tweezers (20). Only the equilibrium intercalation by these two related ligands can be compared, as the rapid intercalation of the \( \Delta-p \) molecules on our DNA stretching time scale \( \sim 1 \) min does not allow studies of its intercalation kinetics (20). At low forces (\( F=10 \) pN), the intercalative binding site size of \( \Delta,\Delta-P \) (3.7 bp) is somewhat larger than that of \( \Delta-p \) (2.9 bp), as could be expected from the larger size of the former complex. However, increasing force has a much more dramatic effect on the binding site size of \( \Delta,\Delta-P \), which decreases by 2 bp to 1.7 bp at 60 pN, relative to the binding site size of
Δ-p, which decreases by only 0.4 bp (Table 1). Surprisingly, the equilibrium DNA elongation upon monomer Δ-p intercalation Δx_{eq}=0.38 nm is almost twice as large as the elongation upon Δ,Δ-P intercalation, Δx_{eq}=0.2 nm. Also, the measured zero-force equilibrium dissociation constant of the Δ,Δ-P molecule K_{d}=44 nM is 25-fold smaller than for Δ-p, suggesting additional interactions that drive Δ,Δ-P intercalation, despite the similar dppz stacking moiety for these two ligands. Finally, the flexibility of the equilibrium ligand-saturated DNA complex for both monomer- and dimer- ligands is much higher than for the ligand-free double-stranded B-form DNA, as described by the corresponding persistence length of these complexes of 14 nm, 2 nm and 50 nm, respectively.

These results may be interpreted in terms of the recent crystal structure of Δ,Δ-P molecule intercalated into a double-stranded DNA 6-mer d(CGTACG) (49). According to this study, the Δ,Δ-P molecule traverses the DNA double helix and displaces an AT base pair from the DNA stack to position the two bulky Ru centers on the opposite groove sides of the double helix. These A and T bases that are displaced into the minor groove make extensive stacking contacts with the phen aromatic moieties of the Ru center located in the minor groove. The overall DNA–Δ,Δ-P binding mode was termed “insertion threading intercalation”, as opposed to non-displacive intercalation, which occurs for the mono-nuclear ruthenium compound Δ-p (106, 107). The difference between these two types of intercalation provides a potential explanation for the difference in DNA elongation after these binding events, as observed in our DNA stretching experiments. Indeed, the classical intercalation typical of the Δ-p ligand leads to duplex elongation by ~0.38 nm, which is similar to the length of a normal base pair stack ~0.34 nm, as for most of the other classic intercalators (20, 78, 79). In contrast, the inserted aromatic dppz moiety of Δ,Δ-P takes the place of the extruded base, thereby leading to a much smaller DNA elongation. The more severe duplex deformation caused by insertion threading intercalation is also consistent with the much smaller persistence length of the saturated Δ,Δ-P-DNA complex of 2 nm observed here. While this is much smaller than the B-form DNA persistence length of 50 nm, it still involves 4-6 intercalated DNA base pairs, in agreement with the relatively straight conformation of the final equilibrium bound state, in which the DNA duplex can be described as a distorted form of B-DNA and appears to be underwound (49).

**Kinetics of DNA intercalation by Δ,Δ-P**

The very slow forward and reverse threading kinetics is related to the major DNA duplex deformations required for either process. Our data suggests that the threading of Δ,Δ-P involves a rate-limiting step of DNA elongation by 0.33 nm per ligand, which might involve severe deformation including opening of one or two base pairs. Estimates of the enthalpy of this deformation obtained by studying the temperature dependence of Δ,Δ-P threading rate (94) are in the range of ΔH~ 22-40 kcal/mol. This can be compared to the enthalpy of a single bp fluctuational opening in the middle of the duplex, which was determined based on NMR studies to be ΔH_{1m}=10-26 kcal/mol (108). Therefore, the enthalpy of the threading
deformation is likely dominated by opening of one to two base pairs. A smaller, but still significant, DNA deformation of $x_{off}=0.14$ nm required for dissociation of $\Delta,\Delta$-P from the intercalated state, follows from our observation that the stretching force facilitates dissociation (Fig. 3.2.3B). As the forward process is promoted by force more strongly than the reverse process, the overall binding is exponentially facilitated by force, in agreement with the resulting overall equilibrium displacement $\Delta x_{eq} \approx 0.2$ nm $\approx x_{on} - x_{off}$. The observation that the force promotes insertion threading intercalation by $\Delta,\Delta$-P via DNA duplex destabilization is consistent with previous solution studies, which showed that $\Delta,\Delta$-P binding to DNA is strongly facilitated by negative super-helical stress in closed circular DNA (109), by the presence of AT-rich sequences (109, 110), by structural defects (111), and at higher temperatures (48, 94). Moreover, the propensity of $\Delta,\Delta$-P to specifically intercalate DNA quadruplex structures with both of its dppz moieties suggested by the recent crystal structure (49) opens the possibility for $\Delta,\Delta$-P to be able to recognize specific DNA structural motifs.

The force-facilitated $\Delta,\Delta$-P-dsDNA on rates in our experiments can be well-described by a single exponential dependence on time (Fig. 3.2.2B). This is in contrast to the complex threading intercalation kinetics observed in bulk solution experiments with mixed sequence DNA (98). We suggest that the simple kinetics is typical of the fastest intercalation event, which dominates the process under the action of applied force. Because we see strong saturation of DNA with $\Delta,\Delta$-P during this process, this likely constitutes the major intercalative pathway. The measured bimolecular $\Delta,\Delta$-P association rate $k_a(0) = 10^4$ M$^{-1}$s$^{-1}$ is about 5 orders of magnitude slower than the association rate limited by diffusion $k_{off} \sim 10^9$ M$^{-1}$s$^{-1}$ (112). The fact that the reaction appears bimolecular implies that the first $\Delta,\Delta$-P binding step, which is known to involve the fast non-intercalative DNA ligand binding of primarily electrostatic nature (48, 113), is very unstable at ligand concentrations typical of intercalative binding $K_d \sim 10$ nM, such that the ligand dissociates from this non-intercalated state much faster than it threads. The overall forward intercalative rate $k_a(0) = k_{off} e^{G_{thread}/k_BT}$ is a product of the non-intercalative bimolecular association rate $k_{off}$ and the probability of intercalation during non-specific binding, $e^{G_{thread}/k_BT}$, where $G_{thread} \sim k_BT \ln(k_{off} / k_a(0))$ such that $G_{thread} \approx 11.5 k_BT = 6.8$ kcal/mol is the free energy of DNA deformation involved in the rate limiting step of $\Delta,\Delta$-P threading. This free energy is consistent with the complete melting of 1-2 base pairs in the middle of the DNA duplex. While the stability of the single terminal duplex bp is only 2-4 $k_BT$, the creation of the helix/coil boundaries associated with local duplex disruption can easily introduce an additional 8-10 $k_BT$ (114). The free energy cost of threading deformation is much smaller than its enthalpy estimated from the temperature dependence of threading, discussed above. This result is not surprising, as the large enthalpy cost of threading deformation can be partially compensated by the associated entropy gain.

In vivo it is likely that the very slow threading of $\Delta,\Delta$-P into stable polymeric dsDNA would prevent this ligand from intercalating most chromosomal DNA if it reaches the nucleus. However, DNA undergoing
high rates of transcription and replication, such as may be found preferentially in cancer cells, may already be destabilized by RNA polymerase or other motor proteins, facilitating rapid initial binding to these locations, followed by very slow dissociation. Therefore, the slow dissociation kinetics combined with its high DNA binding affinity highlights the potential therapeutic use of ligands with binding properties similar to Δ,Δ-P (115, 116).
Chapter 4:
Double-transition DNA threading intercalation

4.1 Fast and slow intercalative transitions
4.2 Threading bis-intercalator
4.3 Converted threading mono-intercalator

Portions of this chapter are from:

A ruthenium dimer complex with a flexible linker slowly threads between DNA bases in two distinct steps

Meriem Bahira, Micah J. McCauley, Ali A. Almaqwashi, Per Lincoln, Fredrik Westerlund, Ioulia Rouzina, and Mark C. Williams


Impact Factor: 9.11
4.1 FAST AND SLOW INTERCALATIVE TRANSITIONS

In contrast to the single-transition DNA intercalation model, two distinctive fast and slow rates are observed by single-molecule studies for unconventional bis-intercalators, such as the polypeptide bis-intercalator Thiocoraline, and the threading bis-intercalator Δ,Δ-Pc (40, 45). The fast first transition is from the non-intercalative state NI to an intermediate intercalative state I‡, and the slow second transition is to the final intercalative state I. The first molecular transition has rapid forward $k_fC$ and reverse $k_f^{-1}$ rates, and the second transition involves a slow unimolecular threading intercalation step with forward $k_2$ and reverse $k_2^{-1}$ rates.

$$
\text{NI} \xrightarrow{k_f} \text{I}^\dagger \xrightarrow{k_2^{-1}} \text{I},
$$

(4.1.1)

Based on the assumption that the NI and I‡ states rapidly equilibrate before the second transition is initiated ($k_fC + k_f^{-1} \gg k_2 + k_2^{-1}$), we relate the measured fast and slow rates to the elementary association rates (Eq. 4.1.2), while $\rho$ pre-equilibrium factor determined from the first transition rates.

$$
k_f = k_f C + k_f^{-1}, \quad k_s = k_s^{-1} + k_s, \quad \rho = \frac{k_f C}{k_f^{-1}},
$$

(4.1.2)

$$
\Delta L(t) = \Delta L_{eq} - \Delta L_s \cdot e^{-k_s t} - \Delta L_s \cdot e^{-k_f t},
$$

(4.1.3)

The fast and slow rates are experimentally obtained from fitting the time-variant DNA elongation $\Delta L(t)$ to Eq.4.1.3, where the DNA elongations of the fast, slow and equilibrium are $\Delta L_f$, $\Delta L_s$ and $\Delta L_{eq}$ respectively. The elementary association rates $k_f$ and $k_s$, as well as the reverse rates $k_f^{-1}$ and $k_s^{-1}$, are determined by fits to the concentration dependence in (Eq. 4.1.2) for $k_f$ and $k_s$ values at each applied force.

4.2 DNA THREADING BIS-INTERCALATION

In this work we probe the threading bis-intercalation mechanism of Δ,Δ-Pc, which is a binuclear ruthenium complex based on the monomer Δ-p. It involves two Rudppz groups connected by a flexible four-carbon linker (Fig. 4.2.1A&B). The molecule is analogous to natural antibiotics that bis-intercalate DNA and achieve high affinity for DNA by linking two or more subunits of known DNA mono-intercalators to form poly-intercalating compounds (117, 118). Intercalation of these ligands was traditionally studied by optical spectroscopic approaches, based on the observations that the luminescence of the chiral aromatic groups of these molecules increases greatly upon their intercalation between DNA bases (119). However, the time scales for binding of threading intercalators often exceed tens of hours, and therefore the binding mechanisms cannot be adequately studied by these approaches.
The kinetics of the DNA length changes is followed upon Δ,Δ-Pc association at a fixed force. We find that the fast and the slow intercalation modes of Δ,Δ-Pc come from the first and the second intercalation events during single ligand DNA intercalation (Fig. 4.2.1C). By measuring the ligand concentration dependence of the fast and slow binding rates at several forces and fitting this dependence to a three state model, we show that the fast mode is a bimolecular intercalation of the first dppz moiety, in pre-equilibrium to the ~10-fold slower intercalation of the second dppz moiety which validates the assumption in the proceeding double-transition analysis.
Figure 4.2.1 Ru-phen-dppz motifs elongate DNA. A) The large aromatic dipyridophenazine ring of Ru(phen)$_2$dpdz$^{2+}$ (referred to as Rudppz, or simply p) intercalates into dsDNA. B) Two Rudppz connected by a flexible linker (the complex referred to as Δ,Δ-Pc). C) Cycles of force extension and release for DNA (black lines) and DNA in the presence of 5 nM of Δ,Δ-Pc (green lines). To elucidate the kinetics of Δ,Δ-Pc's interaction with DNA, a constant force was applied and released after different time points, resulting in a linear increase in extension with time. D) Concentration dependence of the extension of DNA in the presence of Δ,Δ-Pc. The extension increases with increasing concentrations of Δ,Δ-Pc, with the maximum extension occurring at 7 nM.
Pc, the force was fixed at 50 pN, 30 pN and 20 pN (20 pN is shown here in the grey box), while the increasing extension was recorded. D) \( \Delta,\Delta\)-Pc intercalation kinetics for increasing ligand concentrations of 1, 3, 5 and 7 nM (purple, blue, green and red) when held at a force of 20 pN. Fits (black) are to Eq. 4.1.3, and these results are included in Fig. 4.2.3 and Fig. 4.2.4.

RESULTS

Quantifying DNA-bis-\( \Delta,\Delta\)-P binding from force-extension curves

Presented in Fig. 5.2.1C are stretch and release curves for DNA alone, which shows a region of entropic elasticity up to 0.34 nm/bp B-form DNA contour length, followed by the overstretching transition at ~62 pN (in 100 mM Na\(^+\)). A solid green line shows the DNA extension curve in the presence of 5 nM \( \Delta,\Delta\)-Pc, which shows an increase in length relative to DNA at forces above 5 pN. When the extension curve reaches 20 pN, we initiate a force-feedback loop that increases the DNA length to keep the force constant as the DNA-\( \Delta,\Delta\)-Pc complex increases in length as more intercalators bind. Fig. 4.2.1D shows the extension vs. time at a constant force of 20 pN for different concentrations. The results suggest continuous binding on a time scale of hundreds of seconds. From the fits to the data, we obtain the intercalation rates as well as the equilibrium fractional binding for a given force and ligand concentration. Because of the slow ligand dissociation, the dotted return curve reflects the amount of ligand bound at 20 pN, which does not significantly change on the timescale of the release.

Equilibrium and dynamic intercalative properties from the kinetic rates

The solid black lines in Fig. 4.2.1D represent time-dependent fits to the DNA extension as the DNA is bound by \( \Delta,\Delta\)-Pc at a constant force of 20 pN. These curves do not fit to a single exponential dependence on time, indicating that the DNA-\( \Delta,\Delta\)-Pc binding process is multi-state. A typical elongation vs. time \( \Delta L(t) \) trace can be satisfactorily fitted to the two-exponential expression in Eq.4.1.3. Because the ligand does not dissociate on the time scales of these experiments (see Fig. 4.2.1C), a new DNA molecule is used for each new length relaxation experiment. The constant-force length relaxation is repeated at least three times for each ligand concentration and at three forces (20, 30, and 50 pN) to fully understand the \( \Delta,\Delta\)-Pc binding mechanism.

Presented in Fig. 4.2.2A are the fitted values of \( k_0 \) and \( k_s \) as a function of ligand concentration (\( C \)) for 20, 30, and 50 pN. The fits of these dependencies (see Eqs. 4.1.2) yield the elementary reaction rates \( k_1, k_{-1}, k_2 \) and \( k_{-2} \) as a function of force in Fig. 4.2.2B. The fits to the experimental \( k_1(C) \) and \( k_s(C) \) dependencies supports the two-step intercalation model that we used here.

Interestingly, both on and off processes for each intercalation step appear to be exponentially force-dependent and can be well-described by the relationships:

\[
k_{1,2}(F) = k_{1,2}^0 \cdot e^{F \chi_{1,2}/k_BT}
\]  

(4.2.1)
Here \( k_{1,2}^0 \) are the zero-force rates, and \( x_{1,2}^\dagger \) are the corresponding length changes either from the unbound to the transition state, or from the bound to the transition state, for each reaction step. The fitted values of \( k_{1,2}^0 \) and \( x_{1,2}^\dagger \) parameters for each of the two reaction steps are collected in Table 4.2.1. Importantly, the elementary rates satisfy the initial assumption that the faster first intercalation step being in pre-equilibrium to its slower conversion into the doubly-intercalated step at all forces. Thus, the zero-force off rate for the mono-intercalation step, \( k_{1}^0 = (6.8 \pm 0.4) \times 10^{-2} \) s\(^{-1}\) is ~10-fold faster than the conversion step into the bis-intercalated state, \( k_{2}^0 = (5.8 \pm 1.0) \times 10^{-3} \) s\(^{-1}\), which is then ~1.6-fold higher than the dissociation rate for the bis-intercalated \( \Delta,\Delta\)Pc, \( k_{2}^0 = (3.6 \pm 1.0) \times 10^{-3} \) s\(^{-1}\).

The stretching force facilitates the on rates for both the first and the second intercalation events with corresponding elongations of \( x_1^\dagger = 0.19 \pm 0.02 \) nm and \( x_2^\dagger = 0.08 \pm 0.01 \) nm. Interestingly, the reverse intercalation processes are slowed down by force, and according to Eq. 4.2.1 are associated with small negative elongations of \( x_1^\dagger = -0.06 \pm 0.01 \) nm and \( x_2^\dagger = -0.15 \pm 0.03 \) nm, implying that the DNA in the transition state is longer than the non-intercalated state, but slightly shorter than in the mono-intercalated state. In contrast, the second transition state is closer to the mono- than to the double-intercalated state. The physical meaning of these fitted kinetic parameter values and their relationship to the structure and intercalation mechanism of the \( \Delta,\Delta\)Pc/DNA complex are considered in the Discussion.

The elementary reaction rates \( k_1, k_{-1}, k_2 \) and \( k_{-2} \) obtained above can be used to calculate the equilibrium constants for each step and for the net reaction as follows:

\[
K_{d1} = \frac{k_{-1}}{k_1} = \frac{C}{K_1}, \quad K_2 = \frac{k_2}{k_{-2}} \quad \text{and} \quad K_d = \frac{K_{d1}}{K_2} = \frac{k_{-1}}{k_1} \cdot \frac{k_{-2}}{k_2} \tag{4.2.2}
\]

Here the \( K_{d1} \) and \( K_d \) are the dissociation constants for the first step and for the entire reaction, and \( K_2 \) is the equilibrium constant for the second step. Their values calculated according to Eqs. 4.2.2 are presented as a function of force in Fig. 4.2.2C. The force dependence for these equilibrium constants are well-fitted by the exponential expressions:

\[
K_j(F) = K_j^0 \cdot e^{Fx_j/kT} \tag{4.2.3}
\]

where \( K_j^0 \) is the zero-force value of the corresponding equilibrium binding parameter. The zero-force values \( K_{d1}^0 = 35 \pm 9 \) nM, \( K_2^0 = 1.8 \pm 0.6 \) and \( K_d^0 = 15 \pm 6 \) nM demonstrate a high affinity first intercalation event, followed by a strongly driven second intercalation step that makes the overall binding even stronger. The DNA length changes \( x_j \) from the exponential force dependence are in Table 4.2.1.
A

B

Elementary Rates

Force (pN)

Measured Rates (s⁻¹)

Ligand Concentration (nM)

k_{\text{fast}} \quad k_{\text{slow}}

50 pN
30 pN
20 pN
Figure 4.2.2 Kinetics of Δ,Δ-Pc binding to dsDNA. A) Measured fast and slow rates vs. concentration of Δ,Δ-Pc at three forces: 20, 30 and 50 pN (red, green and blue). Data points are rates $k_f$ and $k_s$ obtained by fitting the length vs. time for the Δ,Δ-Pc/DNA complex to Eq. 4.1.3. Lines are the results of fits to Eq. 4.1.2 (solid lines fits for $k_f$ and dotted lines fits for $k_s$) that determine the elementary rates of the two-step reaction. B) Fitted values of elementary rates of the two-step intercalation, giving the forward rates $k_1$ and $k_2$ (solid purple and orange symbols) and reverse rates $k_1^-$ and $k_2^-$ (open purple and orange symbols). Lines represent fits to Eq. 4.2.1, and give the force independent elementary rates and transition distances. Fitted parameters are shown in Table 4.2.1. C) Force dependent binding constants for each step $K_{d1}$ (cyan) and $K_2$ (gold) and for overall binding $K_d$ (magenta), determined from the elementary rates. Lines denote fits to Eq. 4.2.2, which give the force independent binding constants and equilibrium length changes, which are included in Table 4.2.1.

Also, according to the definition of $K_i(F)$ given by Eqs. 4.2.2 and expressions for the rates (Eq. 4.2.1), the equilibrium DNA length change upon intercalation of a single Δ,Δ-Pc molecule in the corresponding binding step (first, second) $x_i^0$ can be found either from fitting of $K_i(F)$ to Eq. 4.2.3, where $K_i(F)$ is calculated according to Eqs. 4.2.2 using the elementary reaction rates, or simply from the fitted elongations associated with each elementary reaction rate (see Table 4.2.1) as follows:

$$x_i = x_{+i} - x_{-i}$$  \hspace{1cm} (4.2.4)

The first mono-intercalation event leads to DNA-Δ,Δ-Pc complex elongation by $x_1 = 0.19\pm(-0.06) = 0.25$ nm, and the second intercalation event of the same Δ,Δ-Pc molecule leads to the additional complex...
elongation by \( x_2 = 0.08 \cdot (-0.15) = 0.23 \text{ nm} \). The total elongation of the DNA-\(\Delta,\Delta\)-Pc complex upon double-
intercalation of both dppz moieties of a single ligand molecule is: \( x = x_1 + x_2 = 0.25 + 0.23 = 0.48 \text{ nm} \).

Table 4.2.1. Zero-force kinetic and equilibrium parameters for \(\Delta,\Delta\)-Pc bis-intercalation of the dsDNA. The data and the analysis method for the “kinetic” and “equilibrium” approaches are discussed in the main text, and graphically presented in Fig. 4.2.2 and Fig. 4.2.4, respectively. Uncertainties determined as errors in the mean directly from each fitting step.

<table>
<thead>
<tr>
<th>Kinetic Approach</th>
<th>Equilibrium Approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_0^0, k_i^0 )</td>
<td>( x_i^0 ) (nm)</td>
</tr>
<tr>
<td>( K_d ) (nM)</td>
<td>15 ± 6</td>
</tr>
<tr>
<td>( K_{at} ) (nM)</td>
<td>35 ± 9</td>
</tr>
<tr>
<td>( K_2 ) (-)</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>( k_1 ) (x10^-3 nM^-1 s^-1)</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>( k_1 ) (x10^-3 s^-1)</td>
<td>68 ± 4</td>
</tr>
<tr>
<td>( k_2 ) (x10^-3 s^-1)</td>
<td>5.8 ± 1.0</td>
</tr>
<tr>
<td>( k_2 ) (x10^-3 s^-1)</td>
<td>3.6 ± 1.0</td>
</tr>
</tbody>
</table>

Table 4.2.2. Comparisons of polymer properties for dsDNA and ssDNA, including dsDNA in saturating
concentrations of Rudppz (p) and \(\Delta,\Delta\)-Pc. All parameters determined from fits to Eqn. 1 and results are
shown in Fig 2. aData from Wenner et al. (120), bData from Vladescu et al. (20), cFrom this work. dSingle-
stranded DNA (34 kbps length) in the same solution conditions as the dsDNA in this work.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>( L_{eq,c}^{tot} ) (nm/bp)</th>
<th>( P ) (nm)</th>
<th>( S ) (pN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsDNA(^a)</td>
<td>0.340 ± 0.001</td>
<td>47 ± 2</td>
<td>1270 ± 200</td>
</tr>
<tr>
<td>dsDNA + p(^b)</td>
<td>0.41 ± 0.01</td>
<td>14.3 ± 0.8</td>
<td>320 ± 17</td>
</tr>
<tr>
<td>dsDNA + (\Delta,\Delta)-Pc(^c)</td>
<td>0.49 ± 0.01</td>
<td>2.9 ± 0.8</td>
<td>800 ± 50</td>
</tr>
<tr>
<td>ssDNA(^d)</td>
<td>0.680 ± 0.002</td>
<td>1.2 ± 0.1</td>
<td>1220 ± 70</td>
</tr>
</tbody>
</table>
Saturated DNA-Δ,Δ-Pc measurements reveal DNA intercalation of both dppz moieties

Higher concentrations of Δ,Δ-Pc lead to faster ligand association, and to longer equilibrium lengths (Fig. 4.2.1D). The Δ,Δ-Pc saturated DNA stretching curve, $L_{eq,c}^{sat}$, is presented in Fig. 4.2.3. The five data points that form this curve were obtained as the final equilibrium extensions of DNA-Δ,Δ-Pc complex from relaxation process of 10 min performed at constant force in the presence of 20 nM ligand. Also shown in Fig. 4.2.3 for comparison are the ligand-free single-stranded (ss) and double-stranded (ds) DNA stretching curves, as well as the saturated Δ-p, all fitted to the extensible worm-like chain (WLC) model of polymer elasticity (121) in the form:

$$L_{eq}^{sat}(F) = L_{eq,c}^{sat} \left(1 - \frac{1}{2\sqrt{F^*P/k_BT}} + \frac{F}{S}\right),$$

(4.2.5)
Fitting parameters, $I_{eq,c}$, $A$ and $S$, for all four curves are collected in Table 4.2.2. The zero-force contour length of the saturated DNA-$\Delta,\Delta$-Pc complex $I_{eq,c}^{st}$ = 0.49 nm/bp, which yields an intercalative binding site of 3 bps (see Eq. 2.1.6). For comparison, the saturated zero-force DNA intercalation by $\Delta$-p (see pink $\Delta$-p-saturated curve in Fig. 4.2.3 and the corresponding fit parameters in Table 4.2.2) leads to monointercalation of every 4 bp at zero force. Thus, the compact bis-intercalation of every other stack by the $\Delta,\Delta$-Pc dimer leads to a much higher maximum intercalated density of this ligand that is similar to saturated intercalation by the classical intercalator ethidium (20, 78). In addition to a longer contour length, the saturated DNA-$\Delta,\Delta$-Pc complex has a much shorter persistence length ($P$~3 nm) than either B-form DNA (50 nm) or the saturated Rudppz-DNA complex (15 nm). This shorter persistence length of $\Delta,\Delta$-Pc-saturated DNA is consistent with frequent intercalation of this ligand, inducing additional random bends in DNA upon binding. Finally, the saturated DNA-$\Delta,\Delta$-Pc complex has an elastic modulus of ~800 pN, which is ~3-fold higher than the elastic modulus of the saturated $\Delta$-p intercalated DNA, but is 1.5-fold smaller than the elastic modulus of B-DNA (Table 4.2.2). This high resistance of $\Delta,\Delta$-Pc-saturated DNA to extension beyond its contour length is consistent with a “stapling” effect of $\Delta,\Delta$-Pc bis-intercalation, in which every two bp are “stapled” by a short stretched linker (122).

Fig. 4.2.4. A) Fits to Eq 4.2.7 for the equilibrium DNA elongations induced by $\Delta,\Delta$-Pc intercalation, triangles are data points and dashed lines are fits at each applied force, (purple: 50 pN, green: 30 pN, yellow: 20 pN). B) Fitting $K_d$ (magenta), determined from Eq 4.2.7 to the exponential force dependence, providing the force independent binding constants and equilibrium length changes, which are included in Table 4.2.1. Fits are dashed lines and $K_d$ values at each applied force are opened circles.
Equilibrium elongation measurements independently confirm the properties of the final intercalative state

In fitting the DNA-ΔΔ-Pc per base pair extension over time, $ΔL(t)$, to Eq. 4.1.3 we obtain not just the fast and the slow rates of this process, but also the equilibrium extension $ΔL_{eq}$, as a function of $C$ at each applied constant $F$. We can use $L_{eq}$ to determine the equilibrium characteristics for the final bis-intercalated state. Fits to $L_{eq}$ are presented in Fig. 4.2.4A.

\[
Θ(K_d(F), n) = \frac{C}{K_d(F)} \frac{n (1 - \Theta)^n}{(1 - \Theta + \Theta/n)^{n-1}},
\]

\[
L_{eq}(F) = L_{\infty}(F) + Θ(K_d(F), n) \cdot ΔL_{sat}(F),
\]

The fits provide $K_d(F)$ ranging from 2.4±0.6 nM at 20 pN to 0.3±0.07 nM at 50 pN. The force-variant $K_d(F)$ then fitted to the exponential force dependence in Eq. 4.2.3 (Fig. 4.2.4B) yielding the zero-force $K_d(0)$ of 12±2 nM. The magnitudes of DNA elongation for DNA-ΔΔ-Pc complex in the equilibrium bis-intercalative state $Δx_{eq}$ is smaller (0.3±0.4 nm) when determined from the equilibrium analysis, compared to the values obtained from kinetic analysis (0.44±0.4 nm) (Table 4.2.1). As our kinetic data (the fitted $ΔΔ$-Pc/DNA length relaxation rates) and the equilibrium data (the magnitudes of fast, slow and total equilibrium extensions, see Eq. 4.1.3) contain independent information, these two approaches are complementary. The good agreement between the results of these two approaches strongly supports the conclusions of this study. It is important to mention that our kinetic approach in this study is more reliable than the equilibrium one. This is because the rates are universal, in contrast to higher uncertainty in the estimated final equilibrium extensions within the time scale of the experimental measurements.

DISCUSSION

In this work we find that the saturated DNA-ΔΔ-Pc complex is 44% longer than B-form DNA, consistent with each of the two dppz moieties of ΔΔ-Pc being fully intercalated, extending the duplex by ~0.30 nm each at every other bp stack (Fig. 4.2.3 and Table 4.2.2). This result is consistent with previous measurements of the DNA-ΔΔ-Pc binding stoichiometry by optical methods (123), which show that the binding site size of ΔΔ-Pc is 4 bp per molecule, or 2 bp per dppz moiety, with similar stacking of each set of dppz aromatic rings with DNA. We also find that the saturated DNA-ΔΔ-Pc complex is ~16-fold more flexible than B-form DNA. Indeed, the persistence length of the saturated ΔΔ-Pc-DNA complex is ~3 nm and this length contains ~6 bp of saturated DNA-ΔΔ-Pc complex. For 3 bp binding site size of ΔΔ-Pc, the saturated DNA-ΔΔ-Pc complex behaves as a polymer with a free random bend at every other ligand binding site. Also, the saturated ΔΔ-Pc/DNA complex is ~1.5-fold more extensible than B-form DNA (elastic modulus of ~800 pN), but ~3-fold less extensible than the saturated mono-Rudppz
intercalated DNA (see Table 1), consistent with a fairly inextensible DNA defined by the “stapling” of every other adjacent bp by the 0.75 nm four-carbon linker, as previously suggested (122, 123).

The kinetics of DNA-$\Delta \Delta$-Pc binding can be minimally described as bi-exponential (Fig. 4.2.2), and is consistent with a two-step sequential intercalation of two dppz moieties of this ligand, in contrast to previously measured conventional bis-intercalators (47, 124, 125). Our measured force-dependencies for the four elementary reaction rates (Fig. 4.2.2B) of this process yield the zero-force values of all rates, and the elongation of the DNA-$\Delta \Delta$-Pc complex associated with each of these four processes, as summarized in Table 4.2.1. This information is also presented graphically in Fig. 4.2.5 for a zero-force free energy profile of the DNA-$\Delta \Delta$-Pc complex as a function of its elongation. The three free-energy minima on this diagram correspond to the non-intercalated, mono-intercalated, and bis-intercalated states. At $\Delta \Delta$-Pc concentration of $C = K_d = 15 \text{nM}$, the free energy of the non-intercalated and bis-intercalated states are the same, and are taken here as a zero free energy reference state. At higher $C = K_{dT} = 35 \text{nM}$ the free energies of the non-intercalated state and of the mono-intercalated states are the same, and equal to $k_B T \ln(K_d) = k_B T \ln(35/15) = 0.85 k_B T$ relative to the reference state.

Only the non-intercalated state free energy is affected by $C$, as illustrated in Fig. 4.2.5 with its two free energy values 0 and 0.85 $k_B T$, corresponding to 15 nM and 35 nM of $\Delta \Delta$-Pc, respectively. The free energy barriers between the local free energy minima are defined by the elementary rate constants, but can only be estimated up to the unknown constant $k_0 = k_B T \ln(k_0)$, as $\Delta G^i = k_B T \ln(k_0/k^i_0)$, where $k^i_0$ are the zero-force rates of the on and off processes for the first or the second step (i.e. $i = +/ - 1$ or +/-2), and $k_0$ is the unknown attempt rate. The transition free energies shown in Fig. 5 were calculated for $k_0 = 1 \text{s}^{-1}$. The much higher second barrier $\Delta G^2 = - k_B T \ln(3.6 \times 10^{-3}) = 5.6 k_B T$ reflects the much slower second intercalation transition compared to the first one with $\Delta G^1 (C = 15 \text{nM}) = k_B T \ln(15 \times 1.8 \times 10^{-3}) = 3.6 k_B T$.

The extensions of the complex at each free energy minimum and at the transition states are derived from the force dependence of all elementary reactions rates (summarized in Table 4.2.1), and are shown in Fig. 5 relative to non-intercalated complex length. It is notable that the positions of both transition states are highly asymmetric. Thus, during the first intercalation only the on- process is associated with significant complex lengthening by $x^i_1 = 0.19 \text{ nm}$, leading to the unstable intercalated transition state, which requires only minor additional elongation by $-x^i_1 = 0.06 \text{ nm}$ to become a stable mono-intercalated state. The reciprocal off process for the first dppz intercalation thus does not involve major duplex deformation, and is rather fast, with a timescale of $1/k_1^0 \sim 15 \text{s}$. This off process for the mono-intercalated state is clearly not rate-limited by dppz unstacking, which by itself is known to only take $\sim 1 \text{s}$ (122, 123), and would be associated with significant complex shortening. Instead, as the C4 linker is located at the intercalating edge of dppz moiety, we hypothesize that it is the passing of C4 linker through the DNA
duplex that rate-limits both on and off processes during the first intercalation step. This hypothesis is consistent with the dissociation timescale for the intercalated mono-Rudppz with the attached C4 (Δ,Δ-Pc without second Ru complex) being ~15 s (122, 123). The fact that the on rate for the first intercalation step in DNA-Δ,Δ-Pc binding, \( Ck_1 \), is bi-molecular implies that there is an additional fast and unstable non-intercalative binding mode of Δ,Δ-Pc to DNA that is in pre-equilibrium to the next slower intercalation. Furthermore, the observation that most of the complex elongation that occurs during mono-intercalation is associated with the association process implies that dppz intercalation occurs in rapid pre-equilibrium to the slower process stabilizing this mono-intercalated step. This is an example of a multi-step process for which the kinetics of its faster less stable steps cannot be distinguished, and the net on-rate is modified by the equilibrium constants of these prior steps in rapid pre-equilibrium to the rate-limiting step. This initial process can be characterized by a single high transition barrier.

Once the mono-intercalated state is stabilized, the DNA-Δ,Δ-Pc complex is further driven towards bis-intercalation due to \( K_2 = k^0_{2i}/k^0_{2c} = 1.8 > 1 \), resulting in additional lowering of the complex free energy by \( k_B T \ln(K_2) = 0.59 \ k_B T \) after the second intercalation event. This second intercalation event is much slower, \( 1/k^0_{2} \sim 170 \ s \), and is not rate-limited by intercalation itself, as it is accompanied by a minor complex elongation of just \( x^i_{2e} = 0.08 \ nm \). This slow process must involve a conformational adjustment in the C4 linker, positioning the second dppz moiety in a state optimal for intercalation. This C4 conformational change is by itself highly unfavorable, leading to the second transition barrier, which is unfavorable relative to the mono-intercalated state by \( 5.63 - 0.85 = 4.8 \ k_B T \). It is then stabilized by a fast second intercalation event leading to significant complex extension by \( x^f_{2e} = 0.15 \ nm \). The reciprocal slow \( 1/k^0_{2} = 280 \ s \) off process for the second intercalation step has the intercalation itself in fast pre-equilibrium to the subsequent slow conformational change in C4, which rate-limits both on and off processes for the second intercalation. The net DNA elongation upon bis-intercalation of the single Δ,Δ-Pc molecule is 0.48 nm, of which \( x^i_{1e} = x^i_{2e} = 0.25 \ nm \) comes from the first, and \( x^f_{2e} = 0.23 \ nm \) comes from the second intercalated dppz moiety. This result is semi-quantitatively consistent with the conclusion from the WLC analysis of the saturated Δ,Δ-Pc/DNA intercalated complex (see Fig. 4.2.3 and Table 4.2.2), suggesting that the double-intercalation of Δ,Δ-Pc leads to DNA extension per ligand of 0.60 nm, or 0.3 nm per dppz intercalation event.
Fig. 4.2.5. Zero-force free energy profile of the DNA/Δ,Δ-Pc complex vs. elongation. The three free-energy minima on this diagram correspond to the non-intercalated, mono-intercalated, and bis-intercalated states illustrated in the figure. At the Δ,Δ-Pc concentration of \( C = K_d = 15 \) nM, the free energy of the non-intercalated and bis-intercalated states are the same and are taken here as the zero free energy reference state. At higher \( C = K_{d1} = 35 \) nM the free energies of the non-intercalated and the mono-intercalated states are the same, and equal to \( k_B T \ln(K_d) = k_B T \ln(35/15) = 0.85 \ k_B T \). Only the non-intercalated state free energy is affected by \( C \), as illustrated by the two lines (solid line for 15 nM and dashed line for 35 nM). The free energy barriers \( \Delta G_{1,2}^\ddagger \) between the local free energy minima were calculated, as discussed in the main text, and all extensions are taken from Table 4.2.1.
We can now compare our results on DNA-$\Delta,\Delta$-Pc bis-intercalation to the results of the previous solution studies on $\Delta,\Delta$-Pc and related ligands. Previous studies presented models of the doubly-intercalated DNA-$\Delta,\Delta$-Pc complex obtained by free-energy minimization of the complex using molecular dynamic simulations (122, 123). This model was based on information obtained in the optical study (122), suggesting that the $\Delta,\Delta$-Pc intercalates B-form DNA with both dppz moieties, each in a similar way, without interaction between the intercalated dppz moieties, and with a binding sites size of 4 bp per $\Delta,\Delta$-Pc ligand. In addition, energy minimization of the DNA-$\Delta,\Delta$-Pc complex suggested that both dppz moieties intercalate from the minor groove side of B DNA and are separated by one non-intercalated base stack. This conformation allows for the optimum stacking of each dppz moiety, and the additional interactions of the Ruphen side groups with the DNA minor groove, and is also consistent with the $\sim$0.75 nm length of the stretched C4 linker "stapling" two bp stacks on the opposite side of the duplex. This equilibrium state model is fully consistent with our DNA-$\Delta,\Delta$-Pc stretching results summarized above. In addition, we show that the two dppz moieties intercalate sequentially, one after another, with the second step rate-limited by the slow passage of the C4 linker through the base pairs to allow the two dppz moieties to intercalate in the same orientation and separated by only one base stack.

The case of DNA-$\Delta,\Delta$-Pc bis-intercalation characterized in here can be contrasted with the mono-intercalation of the $\Delta,\Delta$-P. The $\Delta,\Delta$-P ligand is different from the $\Delta,\Delta$-Pc ligand studied here only by the absence of the C4 linker, as the two Rudppz groups are instead connected by a single covalent bond. The $\Delta,\Delta$-P intercalates DNA with only one dppz moiety in a single step with a bi-molecular on rate of $10^5$ nM$^{-1}$s$^{-1}$, which is 100-fold slower than the first dppz intercalation event for $\Delta,\Delta$-Pc. Also, the off rate $k_{\text{off}}$ $\sim$1/(700 s) is $\sim$50-fold slower than the off rate for the first intercalation event of $\Delta,\Delta$-Pc. Importantly, both on and off processes for $\Delta,\Delta$-P intercalation are associated with very large DNA elongations of 0.33 nm and 0.14 nm, respectively. In other words, both on and off processes are strongly facilitated by DNA stretching force. This is in contrast to both $\Delta,\Delta$-Pc intercalation events, for which the on rates are facilitated by force but off rates are inhibited by force (see Table 4.2.1 and Fig. 4.2.5). Interestingly, the net DNA elongation upon equilibrium dppz intercalation of $\Delta,\Delta$-P is $\sim$0.2 nm, which is similar to the DNA elongation associated with the first dppz intercalation $\sim$0.25 nm of $\Delta,\Delta$-Pc. Thus, it is mostly the $\sim$100-fold slower kinetics for the $\Delta,\Delta$-P that distinguishes the mono-intercalation events for these two binuclear ruthenium ligands. The very slow kinetics of $\Delta,\Delta$-P intercalation is clearly rate-limited by the slow “threading” of its bulky out of plane Ru-phen groups through the duplex required for both on and off processes. This is in stark contrast to the case of the both $\Delta,\Delta$-Pc-dppz intercalation events, for which the intercalation events themselves are fast, while the conformational changes in the C4 linker occurring in between the two intercalation events, and stabilizing both of them, are rate-limiting, but are not associated with major duplex lengthening. In the latter case of $\Delta,\Delta$-Pc intercalation, only one of the rates (on or off) for each of the two intercalation steps is associated with significant DNA elongation, while the reverse process leads to only minor additional elongations of the complex. In the case of bulkier “threading”
intercalation, both on and off processes require major duplex elongations, associated with strong local duplex destabilization. The latter process leads to the ~100-fold slower kinetics of Δ,Δ-P intercalation relative to the mono-intercalation of Δ,Δ-Pc. At the same time, the apparent slow off rate for the Δ,Δ-Pc molecule is defined by the slow conformational changes in this molecule as it binds, with duplex shortening associated with reverse intercalation of the second dppz in fast pre-equilibrium. As a result, the Δ,Δ-Pc molecule combines overall fast association kinetics, \( k_+^0 \sim 1/(15 \text{ s}) \) at \( K_d=35 \text{ nM} \), with slow off kinetics \( k_-^0 \sim 1/(280 \text{ s}) \) and strong binding, \( K_d \sim 15 \text{ nM} \), a combination of qualities suitable for an anticancer drug.

Finally, it is instructive to compare DNA-Δ,Δ-Pc intercalation with the intercalation of the single mono-Rudppz molecule (20). The mono-intercalation of Rudppz is at least 10-fold faster than even the fastest first step of Δ,Δ-Pc intercalation \([k > 1/(15 \text{ s})]\). However, the Rudppz mono intercalation affinity, with \( K_d=1100 \text{ nM} \), is much weaker than either mono- or bis-intercalation of Δ,Δ-Pc with \( K_d \sim 35\text{nM} \) and \( 15 \text{ nM} \), respectively. The much stronger binding of Δ,Δ-Pc is probably due to the additional non-intercalative electrostatic interactions of Δ,Δ-Pc \((4+)\) with DNA, relative to Rudppz \((2+)\) as well as the additional interactions between the Δ,Δ-Pc phen side groups in the minor groove of DNA. Our ability to characterize such diverse scenarios of DNA intercalation by closely related Ru-based ligands illustrates the power of the single molecule DNA stretching approaches to characterize the intercalation mechanisms of slow ligands in unprecedented quantitative details. The method developed here can be also applied to other types of ligands and biomolecules that may increase DNA length upon binding in multiple steps.

This study suggests specific mechanisms of DNA interaction that maximize molecular characteristics that are desirable for anti-cancer drugs. In particular, its two-step binding mechanism gives Δ,Δ-Pc \((K_d(0)=15 \text{ nM})\) a higher binding affinity than the Δ,Δ-P molecule \((K_d(0)=44 \text{ nM})\), which contains the same moieties configured to allow only one-step binding. In contrast, Actinomycin D has on and off rates that are a factor of ten lower than those of Δ,Δ-Pc \((43)\), but also has a one-step binding mechanism with an overall binding affinity \((K_d(0)=1.2 \mu\text{M})\) that is much lower than that of Δ,Δ-Pc. These results suggest that it may be useful to target the development of multi-step DNA binding ligands similar to Δ,Δ-Pc to optimize DNA binding affinity, but with the slower off rates characteristic of the one-step ligands such as Δ,Δ-P and Actinomycin D. Unlike Δ,Δ-Pc, both of these ligands bind DNA dynamically through a lock mechanism, in which DNA length must increase to allow ligand dissociation, to limit their off rates. Hence it may be desirable to combine two-step binding with a lock mechanism.

4.3 CONVERTED THREADING MONO-INTERCALATOR

The two intercalative steps of the threading bis-intercalator Δ,Δ-Pc are linked, as quantified above, to two intercalative events by two intercalating moieties. Here we introduce a new mechanism of DNA intercalation, where a single intercalating moiety is converted from a fast assembling semi-conventional
intercalative state to a slow assembling final unconventional intercalative state. This intercalative conversion is revealed for the rotationally flexible binuclear ruthenium complex Δ,Δ-[μ-bipb(phen)_4Ru_2]^{4+} (Δ,Δ-Pi) shown in Fig.4.3.1A (92, 126). Considering the structure of Δ,Δ-Pi, note that that it has the same bulky side group as Δ,Δ-P at each end of the dumbbell (Fig.4.3.1B), but the bridging moiety is different. While the two Ru(phen)_2dppz^{2+} monomers of Δ,Δ-P are linked by single semi-rigid bond, the two Ru(phen)_2ip^{2+} in Δ,Δ-Pi are linked by two bonds through a single aromatic ring.

In particular, previous reports showed that Δ,Δ-Pi strongly condenses DNA, using effective ligand concentration of ~1 uM (92). The strong DNA condensation poses a challenge in bulk experiments, such that even the DNA-ligand binding mode could not be resolved. We examined the kinetics of Δ,Δ-Pi interactions with single λ-DNA molecules as a function of constant applied forces 10 to 50 pN and ligand concentration of 0.15 to 40 nM. Fig. 4.3.2A shows force clamp measurements in which DNA-ligand intercalation is monitored over tens of minutes, starting from the free DNA extension until the DNA-ligand complex reaches equilibrium. The DNA elongation measurements illustrate two distinct phases during association; rapid intercalation that is analogous to classic intercalation, followed by very slow intercalation that approaches equilibrium with a rate that is comparable to that observed for other threading intercalators. We Then measured Δ,Δ-Pi dissociation after rinsing the binding ligands from the surrounding solution (Fig. 4.3.2B), and observed that the DNA-ligand complex extension decreases to the DNA-only extension over a timescale longer than the association process. Interestingly, the dissociation measurements fit well to a single rate that is slower than the dissociation rate estimated for Δ,Δ-P.
Fig. 4.3.2: A) The time-dependent equilibrium extensions of the DNA-ΔΔ-Pi complex at ΔΔ-Pi concentration of 5 nM and constant forces 10 pN to 50 pN as color coded, dots are data, and solid lines are fits to Eq. 4.1.3. B) The time-dependent extensions of the DNA-ΔΔ-Pi complex dissociation fitted with single exponential rate giving a direct measurement of $k_2$ at for each applied force as color coded.
Fig. 4.3.3: A&B) Demonstrating the three-state model by stopping the association after 0.72 min, 1.44 min and 15 min from the beginning of (I*) intermediate intercalated state formation. By rinsing the ligand out of solution, there is a fast drop in the extension that decreases as the DNA-ligand complex reaches the equilibrium final intercalated (I). While dissociation of (I*) takes less than 0.5 min, the dissociation of DNA-ligand complex when fully converted to the final state (I) is fitted with single exponential and it takes over 10 min.

Furthermore, the dissociation is investigated after several intercalative relaxation times from the initial fast intercalation phase (Fig. 4.3.3). The measurements demonstrate that there is fast dissociation rate has extension amplitudes that are inversely proportional to the intercalative relaxation time. The fast dissociation time scale is within ~10 s, which is in the range of dissociation rates for conventional DNA intercalators. For a relaxation time of tens of min, the extension amplitude of the fast dissociation rate is diminished. This is consistent with a time-dependent conversion from conventional/semi-conventional intercalation to a threading intercalation state. After the conversion is completed (Fig. 4.3.3, dark blue measurement after 15 min), the slow dissociation rate corresponds to the step that limits the reverse rate of the unthreading transition. Once ligands dissociate from the final intercalative state, individual rapid dissociation events to the non-intercalative state take less time than the resolution that the measurements can detect.
Figure 4.3.3: Kinetics of Δ,Δ-Pi binding to DNA. A) Examples at forces 20 pN and 50 pN for the measured $k_f$ and $k_s$ rates (as color coded) fitted to the concentration dependence from Eqs. 4.1.2, the measured $k_2$ show no concentration dependence. B) Fitted values of elementary rates $k_1$, $k_{-1}$, $k_2$ and $k_{-2}$ (as color coded) to Eq. 4.2.1, values from the fits are provided in Table 4.3.1. C) Force dependent binding constants $K_d$, $K_{1d}$ and $K_2$ (as color coded) determined from the elementary rates, fits are to Eq. 4.2.2,
which give the force independent binding constants and equilibrium length changes, which are included in Table 4.3.1.

RESULTS

From the double-transition approach that we outlined in sections 4.1 and 4.2, the obtained fast and slow association rates are analyzed, in addition to experimentally determining the reverse rate of the second transition. Fig. 4.3.3A shows fits from Eqs 4.1.2 to the measured $k_f(C)$ and $k_s(C)$ at constant forces, while $k_{-2}$ measurements show no concentration dependence as expected for a unimolecular dissociation rate. The determined elementary rates $k_1$, $k_{-1}$, $k_2$ and $k_{-2}$ are then fitted to an exponential dependence on force as shown in Fig. 4.3.3B. Similarly, the equilibrium constants $K_{d1}$, $K_{2}$ and $K_{d}$ for the first and second transition, as well as the final DNA-ligand intercalative state, are fitted exponentially in force as shown in Fig. 4.3.3C. The zero-force rates, equilibrium constants, and their related DNA length changes are provided in Table 4.3.1.

The saturated DNA-$\Delta\Delta$-$\Pi$ extensions, $L_{\text{sat eq,c}}$, are also fitted to the WLC model in Fig. 4.3.4A to obtain the equilibrium elastic properties of DNA-$\Delta\Delta$-$\Pi$, which are provided by Table 4.3.2. The obtained elastic properties are comparative to values previously found for threading intercalators. Fig.4.3.4A also shows the aggregation which only observed at high concentrations when holding DNA at very low initial extensions comparable to the bulk experimental conditions. After the first DNA stretches in the presence of ligand in solution, the elongation due to the fast intercalation is observed, but the slow assembling final state is not reached even after several consecutive stretching and releasing cycles. Furthermore, we used the M-H model (Eqs. 4.2.6 and 4.2.7) to fit the equilibrium extensions $L_{\text{eq}}$ that we obtained from the constant force measurements. The fits are shown in Fig. 4.3.4B, yielding the equilibrium constant $K_d$ at each applied force. Fig. 4.3.4C shows that the equilibrium approach confirms the equilibrium dissociation constants obtained from the kinetic measurements, and independently providing a consistent zero-force equilibrium dissociation constant $K_d(0)$ of 10 nM, and an equilibrium DNA elongation per ligand $\Delta x_{\text{eq}}$ of 0.27 nm (see Table 4.3.1).
A

40 nM

B

Concentrations (nM)

Extension (nm/bp)

50 pN
30 pN
20 pN
10 pN
Figure 4.3.4: A) As color coded: WLC fit of the equilibrium extension show persistence length of ~10 nm consistent with values from previous threading intercalators (see Table 4.3.2). Showing the DNA-ΔΔ-Δ-Pi aggregations when introducing the ligand in solution while DNA molecule is held at low extension comparable to the bulk conditions, the 1st stretches of three different DNA molecules show significant aggregation as shown with different colors in the dashed circle. The second stretches (not shown) have much less aggregation, and successive stretches show DNA elongation due to fast phase intercalation, the average of the 6th stretch show that the DNA molecules are far from the equilibrium extensions obtained by force clamp measurements. B) Measured equilibrium extensions for forces 10-50 pN and concentrations 0.15-40 nM, along with fits to the McGhee-von Hippel binding isotherm, yielding the dissociation constant $K_d(F)$ ranging from 0.42 nM at 50 pN to 5.47 nM at 10 pN, and the binding site size ($n$) has no significant variation and fixed at 2.8 that optimizes the fits of all forces. C) Fitting dissociation $K_d(F)$ from the equilibrium approach (dashed line) to Eq. 4.2.2, which closely agrees with the kinetic approach (dotted line), values from the fits are provided in Table 4.3.1.
Table 4.2.1. Zero-force kinetic and equilibrium parameters for Δ,Δ-Pi double-transition intercalation of the DNA, shows values from the kinetic and equilibrium approaches.

<table>
<thead>
<tr>
<th></th>
<th>Kinetic Approach</th>
<th>Equilibrium Approach</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ (nM)</td>
<td>$K_{eq}$</td>
</tr>
<tr>
<td></td>
<td>$K_{sat}$ (nM)</td>
<td></td>
</tr>
<tr>
<td>$K_2$ (-)</td>
<td>$4.7 \pm 0.7$</td>
<td>$0.16 \pm 0.02$</td>
</tr>
<tr>
<td>$k_1$ ($\times 10^{-3}$ nM$^{-1}$s$^{-1}$)</td>
<td>$0.4 \pm 0.03$</td>
<td>$0.23 \pm 0.02$</td>
</tr>
<tr>
<td>$k_{-1}$ ($\times 10^{-3}$ s$^{-1}$)</td>
<td>$28 \pm 2$</td>
<td>$0.07 \pm 0.01$</td>
</tr>
<tr>
<td>$k_2$ ($\times 10^{-3}$ s$^{-1}$)</td>
<td>$9 \pm 1$</td>
<td>$0.12 \pm 0.01$</td>
</tr>
<tr>
<td>$k_{-2}$ ($\times 10^{-3}$ s$^{-1}$)</td>
<td>$1.5 \pm 0.2$</td>
<td>$0.0 \pm 0.00$</td>
</tr>
<tr>
<td></td>
<td>$x_0^0$ (nm)</td>
<td>$x_{eq}^0$ (nm)</td>
</tr>
</tbody>
</table>

Table 4.2.2. Polymer properties for dsDNA in saturating concentration of Δ,Δ-Pi. \textsuperscript{a}Data from Wenner et al. (120), \textsuperscript{b}From this work.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$L_{eq,c}^{tot}$ (nm/bp)</th>
<th>$P$ (nm)</th>
<th>$S$ (pN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsDNA\textsuperscript{a}</td>
<td>$0.340 \pm 0.001$</td>
<td>$47 \pm 2$</td>
<td>$1270 \pm 200$</td>
</tr>
<tr>
<td>dsDNA + Δ,Δ-Pi\textsuperscript{b}</td>
<td>$0.42 \pm 0.003$</td>
<td>$10 \pm 2$</td>
<td>$414 \pm 40$</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The equilibrium affinity of Δ,Δ-Pi for is found to be \approx 5-fold higher than the threading mono-intercalator Δ,Δ-P. The quantified dynamic DNA deformations show that the double-transition intercalative mechanism has also a moderate molecular lock in which equilibrium DNA deformation in the final state is less than the dynamic DNA deformation that is required for DNA-ligand assembly. While the overall DNA elongation in the forward transitions is

$$x_{en} = x_{en}^f = x_{eq} = 0.35,$$

the DNA-Δ,Δ-P complex is relaxed to

$$\Delta x_{eq} = 0.27 \text{ nm}$$

in the final equilibrium state. These findings confirm our prediction in the Δ,Δ-Pc study that
combining the two-step intercalation with the lock mechanism would result in higher DNA intercalation affinity. The first intercalative step has an equilibrium constant of ~80 nM which is close to the ~100 nM equilibrium constant reported in bulk for the conventional DNA intercalation by one Ru(phen)$_2$ip$_2^{2+}$ monomer attached to the aromatic moiety (Fig.4.3.1A) (127).

This supports a model for DNA-Δ,Δ-Pi assembly that involves initial partial semi-conventional intercalation by the aromatic moiety, leading to unwinding of the double helix, which makes the threading transition an order of magnitude energetically more favourable than the threading of the same side moiety in Δ,Δ-P. Following the threading transition, DNA-Δ,Δ-Pi complex reaches an equilibrium state in which DNA intercalation by the aromatic ring is maximized. Note that the rotational flexibility of Δ,Δ-Pi may facilitate initial non-intercalative minor groove binding, the transition from the intermediate state to the final state, and the accommodation of both ends of the dumbbell in the minor and major grooves. This outlined intercalation mechanism indicates that the intercalating moiety switches between two adjacent intercalative sites. Thus, the intercalating moiety is partially inserted between two initial base pairs, the following threading transition occurs between other adjacent base pairs. As the threading transition progresses, the intercalating moiety is spontaneously released from the initial intercalative site into the final intercalative site. These novel findings for Δ,Δ-Pi not only overcome the limitation of bulk measurements in resolving the binding mode of the DNA-Δ,Δ-Pi interaction, but also present a convincing illustration of single-molecule studies providing important insights in the rational design of DNA-targeting ligands.
Chapter 5:
Steroselectivity of DNA threading intercalation

5.1 Chiral intercalators

5.2 Steroselective threading mono-intercalator

Portions of this chapter are from:

Dissecting the dynamic pathways of stereoselective DNA threading intercalation

Ali A. Almaqwashi, Johanna Andersson, Per Lincoln, Ioulia Rouzina, Fredrik Westerlund and Mark C. Williams

(In preparation)
5.1 CHIRAL INTERCALATORS

Many intercalators have a right- or left-handed chiral structure, for which the molecule and its mirror image cannot be superimposed. In synthesizing DNA-targeting therapeutics, the optimization of chiral intercalators may provide significant drug dose reduction to achieve more efficient treatment. Currently used polypeptide natural antibiotics, such as Actinomycin D, have a very complex structure with multiple chiral subunits. Yet only simple intercalators with a single chiral subunit have been previously fully characterized. The dumbbell-shaped DNA threading intercalators, such as binuclear ruthenium complexes, represent the next order of complexity relative to simple intercalators and can provide significant new insights into the molecular mechanisms that govern DNA-ligand intercalation. This study represents the first direct and quantitative measurement of the effect of stereoselectivity on the static and dynamic structural DNA deformation upon intercalation by a ligand with more than one chiral subunit.

5.2 SETEROSELECTIVE THREADING MONO-INTERCALATOR

Rational design of unconventional intercalators requires optimizing not only the equilibrium state, but also the dynamic intercalative transition. While crystal structures, when available, may reveal an equilibrium DNA-ligand conformation, the structural dynamics of the threading pathways remain obscure. Altering even fine structural characteristics may change the threading dynamics and result in a reshaping of the energy landscape. For example, some studies on chiral ligands reported higher affinity for the right handed (Δ) enantiomer of DNA intercalators than the left-handed (Λ) enantiomer when intercalating a right-handed DNA molecule (106, 128, 129). This stereo-selectivity was particularly confirmed by crystal structures for the light-switching mononuclear ruthenium complex Ru(phen)_2dppz^2+ (106). The equilibrium conformations showed that intercalation by the dipyridophenazine (dppz) moiety is associated with preferential minor groove accommodation for the phenanthroline (phen) moieties in the Δ configuration (106).

The stereoselectivity of unconventional DNA intercalators, such as threading intercalators, is far less understood. Bulk studies reported significant stereoselectivity for the binuclear ruthenium complex [µ-dppzip(phen)_4Ru_2]^{4+} that diverge from the dominant preference for the Δ configuration (90). This asymmetric complex, referred to hereafter as (Piz) and shown in Fig. 5.2.1A, consists of two different monomers linked by a single bond. It is a shorter analogue of the parent semirigid binuclear ruthenium complex [µ-bidppz(phen)_4Ru_2]^{4+}, referred to as (P) (see section 3.2). Previous fluorescence experiments indicated that for Piz the intercalating subunit is the dppz moiety and the distal subunit is the ip moiety. Andersson and Lincoln examined threading intercalation of AT-DNA segments by Piz with all four possible configuration sets of the chiral bulky phen moieties (Fig. 5.2.1A) attached to the intercalating and distal subunits, respectively (dppz,ip); (Δ,Λ), (Δ,Δ), (Λ,Λ) and (Λ,Δ) (90). Although they were unable to quantify the ligand binding affinities, both fluorescence and circular dichroism results showed robust preference for (Δ,Λ) configurations (Fig. 5.2.1B) relative to (Λ,Λ) and (Δ,Δ) respectively, while the
threading intercalation fraction was almost negligible for $(\Lambda, \Delta)$ (90). Surprisingly, for longer mixed sequence DNA, such as calf thymus (ct)-DNA, bulk experiments could not detect threading intercalation by Piz (90, 92).

Here, we probe the threading intercalation of Piz by mechanically tracing over time single $\lambda$-DNA molecules elongation due to threading intercalation by Piz with all four possible Piz stereoisomers (or alternatively Piz variants) at several constant applied forces (43, 50). The elongation is then traced back to the free-DNA extension as Piz dissociates over time from the equilibrium intercalated state. The applied forces of 10-50 pN enable us to study the threading intercalation process without the DNA aggregation that may be facilitated by DNA-DNA contacts in bulk experiments.
Figure 5.2.1. A) Left column: chemical structure of the binuclear ruthenium complex [µ-dppzip- (phen)₄Ru₂]⁴⁺(Piz), in violet: Ru(phen)₂dppz, in cyan: Ru(phen)₂zip. In the second row are the chemical structures of the dipyridophenazine (dppz), imidazophenanthroline (ip), and phenanthroline (phen) moieties respectively. Right column: Right-handed (Δ) and left-handed (Λ) chirality of the phen moieties around the ruthenium ion. B) Previously published model of Δ,Λ-Piz thread-intercalated into a oligonucleotide duplex, the major groove is to the left, dppz-part: violet, ip-part: cyan. Left is side view, right is top view (only the surrounding base-pairs shown for clarity) (90).

RESULTS

Kinetics measurements yield intercalation affinity and DNA structural deformations

For all Piz variants, the threading intercalation extensions were obtained in real time for applied forces of 10-50 pN. First, the DNA-Piz complex elongates at ligand concentration C of 5 nM until reaching equilibrium, then the complex dissociates in ligand-free buffer to recover the free DNA extension as shown for (Δ,Δ) in Fig. 5.2.2A (other stereoisomers shown in Fig. 5.2.2B). The time evolution of the extension traces is fit to Eq. 5.2.1, describing a single DNA molecule extension trajectory (43). The single exponential dependence on time provides the net relaxation rate $k_{\text{total}}(F)$ from the association trace, and the unimolecular reverse rate $k_{\text{off}}(F)$ from the dissociation trace:

$$L(t) = L_i \mp \Delta L_{\text{eq}} \cdot e^{-k_i t}, \quad (5.2.1)$$

Where $L_i$, the second term sign, and $k_i$ are ($L_{\text{eq}}, -\Delta L_{\text{eq}}, k_{\text{total}}$) for association of the complex, and ($L_{\text{ds}}, +\Delta L_{\text{eq}}, k_{\text{off}}$) for dissociation of the complex, $L_{\text{eq}}$ is the equilibrium relaxed extensions for the elongated DNA-Piz complex, $L_{\text{ds}}$ for the recovered free DNA at each stretching force, and $\Delta L_{\text{eq}}$ is the equilibrium change in length due to ligand binding.
Figure 5.2.2. A) The time-dependent DNA extension as a function of time for constant applied forces, as color coded, in the presence of 5 nM Δ,Δ-Piz (left) and in ligand-free buffer (right). The dashed lines are fits to the single exponential dependence in Eq. 5.2.1 in the association and the dissociation forms. B) The time-dependent extension as a function of time for DNA constant applied forces, as color coded, in the presence of 5 nM concentration of each Piz variant [(Δ,Δ), (Δ,Λ), (Λ,Δ), respectively] (first row) and in ligand-free buffer (second row). The dashed lines are fits to the single exponential dependence in Eq. 5.2.1 in the association and the dissociation forms. Experiments were conducted at 21 °C (10 mM Tris buffer, 100 mM NaCl, pH 8).

Direct measurements of $k_{\text{total}}$ and $k_{\text{off}}$ enable determination of the equilibrium dissociation constant in a kinetic approach for each experimental cycle as the following:

$$K_a(F) = C \cdot \frac{k_{\text{off}}(F)}{k_{\text{total}}(F) - k_{\text{off}}(F)}, \quad (5.2.2)$$

Note that $k_{\text{total}}(F) = k_{\text{on}}(F) + k_{\text{off}}(F)$, and $k_{\text{on}}$ is the forward rate resulting from the product $k_s(F) \cdot C$, and $k_d$ is the bimolecular association rate. The obtained intercalative rates $k_{\text{on}}(F)$ and $k_{\text{off}}(F)$, as well as the dissociation constant $K_d(F)$, are fitted to the exponential force-dependence in Eq. 5.2.3, which is a characteristic of molecular assembly processes that change DNA length:

$$\kappa_i(F) = \kappa_i(0) \cdot e^{-FX_i/k_dT}, \quad (5.2.3)$$

Here $\kappa_i$ is $K_d$, $k_{\text{on}}$, or $k_{\text{off}}$ for $X_i$ corresponding to $\Delta x_{\text{eq}}$, $-x_{\text{on}}$ and $-x_{\text{off}}$, respectively, where $\Delta x_{\text{eq}}$ is the equilibrium DNA elongation per ligand, $x_{\text{on}}$ is the change in length during DNA-ligand association, and $x_{\text{off}}$
is the change in length required for DNA-ligand dissociation from the intercalated state. The analysis reveals robust stereoselectivity in the DNA-Piz threading intercalation process, where the intercalative properties are optimal for (Δ,Λ), minimized for (Λ,Δ), and in between for (Δ,Δ) and (Λ,Λ) (see Table 1). The zero-force equilibrium dissociation constant $K_d(0)$ ranges from 27±3 nM for (Δ,Λ) to 622±55 nM for (Λ,Δ), $\Delta x_{eq}$ from 0.30±0.02 nm to 0.48±0.03 nm, $x_{on}$ almost doubles from 0.25±0.02 nm to 0.46±0.03 nm, and $k_{off}(0)$ decreases by an order of magnitude from $2.8 \times 10^5$ M$^{-1}$s$^{-1}$ to $2.0 \times 10^4$ M$^{-1}$s$^{-1}$. This slowing of the association rate correlates with increasing DNA deformation during the threading process. In contrast, the dissociation (or unthreading) rates of all Piz variants are about similar, $k_{off}(0)\sim10^2$ s$^{-1}$, and rather fast compared to the unthreading rate of Δ,Δ-P (Table 5.3.1). Interestingly, the measured dissociation rates for λ-DNA-Piz are in close agreement with values reported by bulk experiments for the unthreading of AT-DNA-Piz, which range from $\sim10^2$ s$^{-1}$ to $2 \times 10^3$ s$^{-1}$ (90). It is also notable that Piz dissociation rates are coupled with limited DNA structural dynamics, exhibiting weaker force dependence that ranges from inhibition for (Δ,Λ) by $x_{eff}=0.09\pm0.01$ nm to a nominal facilitation for (Δ,Δ) with $x_{eff}=0.01\pm0.01$ nm.
Figure 3. A) The forward rates ($k_{\text{on}}$, green data points) and reverse rates ($k_{\text{off}}$, red data points) at a ligand concentration of 5 nM fitted to the exponential force dependence (dotted lines) for each Piz variant to obtain DNA deformation lengths for the on and off processes, $x_{\text{on}}$ and $x_{\text{off}}$ and the zero-force forward and reverse rates $k_{\text{on}}(0)$ and $k_{\text{off}}(0)$, (see table 5.2.1. B) The equilibrium dissociation constants $K_d(F)$ obtained from Eq. 5.2.2 along with the fits to the exponential dependence on force of Equation 5.2.3 (dotted lines) for all Piz variants as color coded. The fits determine the zero force binding constant $K_d(0)$ and DNA deformation length in the equilibrium state $\Delta x_{\text{eq}}$, which are provided in table 5.3.1. C) Average equilibrium elongation measurements of at least three DNA molecules obtained at constant forces of 10-50 pN at 5 nM (triangle data points) and the saturating concentrations (circle data points) of each Piz variants as colour coded, saturating concentrations, $(\Delta,\Lambda):100$ nM, $(\Delta,\Delta):150$ nM, $(\Lambda,\Lambda):400$ nM, $(\Lambda,\Delta):1.5$ µM. Dashed lines are fits to the DNA-Piz complex WLC model in Eq. 5.2.4, and dotted lines are fits to Eq. 5.2.8.

Equilibrium measurements of saturated elongations provide DNA-Piz polymer properties

For constant forces of 10-50 pN we obtained the saturated equilibrium extensions $L_{eq}^{\text{sat}}(F)$ measured at concentrations of Piz beyond which the single DNA molecule does not further elongate. The concentration independent measurements of $L_{eq}^{\text{sat}}(F)$ allow us to construct the equilibrium stretching curve of the DNA-Piz polymer from WLC model:

$$L_{eq}^{\text{sat}}(F) = L_{eq,c}^{\text{sat}} \left(1 - \frac{1}{2 \sqrt{F \cdot P / k_B T}} + \frac{F}{S} \right).$$  (5.2.4)
The fits to Eq. 5.2.4 (dashed lines), along with experimental measurements (circle data points), are shown in Fig. 5.2.3C. The DNA-Piz complex contour lengths \( L_{eq,c} \) obtained from Eq. 5.2.4, the free DNA contour length \( L_{ds,c} \), and \( \Delta x_{eq} \) obtained from Eq. 5.2.3 provide an estimate of the intercalative site size per bound ligand \( n \),

\[
 n = \frac{\Delta x_{eq}}{\Delta L_{eq,c}} = \frac{\Delta x_{eq}}{L^{sat}_{eq,c} - L^{ds}_{eq,c}}. \tag{5.2.5}
\]

The elastic properties and \( n \) are provided in Table 5.2.2. Consistently with the stronger DNA threading intercalation by \((\Delta,\Lambda)\) relative to \((\Lambda,\Delta)\), the saturated DNA molecule with \((\Delta,\Lambda)\) exhibits a significantly smaller persistence length \( P=6\text{nm} \) relative to \( P=21\text{nm} \) for \((\Lambda,\Delta)\), and the intercalative site size of \( n=2.7 \) for \((\Delta,\Lambda)\) compared to \( n=5 \) for \((\Lambda,\Delta)\).

From the saturated elongation measurements, we obtain the intercalative fraction bound for each Piz variant as function of concentration and force:

\[
 \Theta(F,C) = \frac{\Delta L_{eq}(F,C)}{\Delta L^{sat}_{eq}(F)} = \frac{L_{eq}(F,C) - L_{ds}(F)}{L^{sat}_{eq}(F) - L^{ds}(F)}, \tag{5.2.6}
\]

The intercalative fraction bound is also provided by the McGhee-von Hippel binding isotherm as a function of \( K_d(F) \) and \( n \):

\[
 \Theta(K_d(F),n) = \frac{C}{K_d(F)} \left( \frac{n (1-\Theta)^n}{\left(1 - \Theta + \frac{\Theta}{n}\right)^{n-1}} \right), \tag{5.2.7}
\]

As previously outlined (in section 2.1), equations 5.2.3, 5.2.6 and 5.2.7 gives:

\[
 L_{eq}(F) = L_{ds}(F) + \Theta(K_d(0),n) \cdot \Delta L^{sat}_{eq}(F) \cdot e^{\left[ n \Delta L^{sat}_{eq,c} \right] F/k_BT}. \tag{5.2.8}
\]

This allows us to obtain the equilibrium elongation as a function of force at fixed concentration, fitting only to \( K_d(0) \) and \( n \). The fits for \( L_{eq}(F) \) at \( C=5\text{nM} \) (dotted lines fits and triangle data points) for all Piz variants are shown in Fig. 5.2.3C, providing values of \( K_d(0) \) and \( n \) in good agreement with the kinetics analysis (see Table 5.2.3).

Table 5.2.1. Comparison of the equilibrium dissociation constants, the DNA deformation lengths, and the kinetic rates for the intercalative molecular assembly of Piz variants as well as \( \Delta,\Delta-P \).
Table 5.2.2. Comparison of the equilibrium intercalation parameters for Piz variants

<table>
<thead>
<tr>
<th>Piz (dppz,ip)</th>
<th>(Δ,Λ)</th>
<th>(Δ,Δ)</th>
<th>(Λ,Λ)</th>
<th>(Λ,Δ)</th>
<th>Δ,Δ-P (bi-dppz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_d(0) (nM)</td>
<td>27±3</td>
<td>44±4</td>
<td>135±11</td>
<td>622±55</td>
<td>44±2</td>
</tr>
<tr>
<td>Δx_eq (nm)</td>
<td>0.30±0.02</td>
<td>0.31±0.02</td>
<td>0.39±0.02</td>
<td>0.48±0.02</td>
<td>0.19±0.01</td>
</tr>
<tr>
<td>x_on (nm)</td>
<td>0.25±0.01</td>
<td>0.33±0.02</td>
<td>0.35±0.02</td>
<td>0.46±0.02</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>x_off (nm)</td>
<td>-0.09±0.01</td>
<td>0.01±0.01</td>
<td>-0.04±0.01</td>
<td>-0.02±0.01</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>k_d(0) (M⁻¹s⁻¹)</td>
<td>28(±4)x10⁴</td>
<td>14(±1.6)x10⁴</td>
<td>6.4(±0.6)x10⁴</td>
<td>2.0(±0.2)x10⁴</td>
<td>1.0(±0.01)x10⁴</td>
</tr>
<tr>
<td>k_off(0) (s⁻¹)</td>
<td>13(±2)x10⁻³</td>
<td>5.4(±0.4)x10⁻³</td>
<td>8.3(±0.5)x10⁻³</td>
<td>9.8(±1)x10⁻³</td>
<td>1.4(±0.1)x10⁻³</td>
</tr>
</tbody>
</table>

Table 5.2.3: Comparison of the zero-force equilibrium dissociation constant, DNA equilibrium deformation lengths, and the intercalative site size for all Piz variants obtained by the kinetics results, and from equilibrium elongation fits (Equation 5.2.8).
The intercalation bimolecular association rate of Δ,Δ-Piz as determined by titration measurements

For comparison with the parent threading intercalator Δ,Δ-P, and to validate the preceding kinetic and equilibrium approaches in characterizing the binding process, we also obtained $K_d(F)$ for Δ,Δ-Piz from equilibrium titration measurements. The DNA equilibrium elongations and the net relaxation rates at ligand concentrations ranging from 1 nM to 150 nM were measured at constant forces of 10-50 pN. The intercalation fractional binding at each force and concentration $\Theta(F,C)$ is experimentally given by Eq. 5.2.6, and then fitted to the McGhee-von Hippel binding isotherm. The intercalation equilibrium elongations and the corresponding fits are shown for each force in Fig. 5.2.4A. For comparison with the analysis from the kinetic approach, $K_d(F)$ obtained by Eq. 5.2.7 are plotted in Fig. 5.2.4A and fit to the exponential force dependence of Equation 5.2.3. From the measured net relaxation rates and the determined equilibrium dissociation constants we obtain $k_{on}(F)$ and $k_{off}(F)$, as shown in Eq. 5.2.9 and Eq. 5.2.10 respectively (43):

$$k_{on}(F) = k_{total}(F) \cdot \frac{1}{1 + K_d(F)/C}$$  \hspace{1cm} (5.2.9)

$$k_{off}(F) = k_{total}(F) \cdot \frac{1}{1 + C/K_d(F)}$$  \hspace{1cm} (5.2.10)

Furthermore, $k_{on}(F)$ and $k_{off}(F)$ at each ligand concentration are fit to Eq. 5.2.3, producing comparable force dependence. The rates at each force are plotted as function of concentration, illustrating the expected linear dependence on concentration for $k_{on}(F)$ as well as the independence of concentration for $k_{off}(F)$.
The slopes of \( k_{\text{on}}(F) \) as a function of concentration provide the values of \( k_{\text{a}}(F) \), which increase by the applied constant force, as shown in Fig. 5.2.4C. Fitting \( k_{\text{a}}(F) \) exponentially in force using Equation 5.2.3, as shown in Fig. 5.2.4D, we obtain a zero-force bimolecular association rate \( k_{\text{a}}(0) \) of \( 1.4\pm0.1\times10^5 \) M\(^{-1}\)s\(^{-1}\) for \( \Delta,\Delta\)-Piz, confirming an order of magnitude higher association rate than the reported \( k_{\text{a}}(0) \) of \( \sim10^4 \) M\(^{-1}\)s\(^{-1}\) for \( \Delta,\Delta\)-P. The zero-force unimolecular reverse rate \( k_{\text{off}}(0) \) of \( 6\times10^{-3} \) s\(^{-1}\) is also about six-fold higher for \( \Delta,\Delta\)-Piz relative to \( \Delta,\Delta\)-P.

Figure 4. A) Measured average equilibrium elongations of at least three DNA molecules with threading intercalation by \( \Delta,\Delta\)-Piz as a function of concentration, ranging from 1-150 nM. Dashed lines are fits to the McGhee-von Hippel binding isotherm of Equation 5.2.7 for a range of constant applied forces (10-50 pN) as color coded, yielding. B) Fitting the equilibrium dissociation constant \( K_{\text{d}}(F) \) for DNA threading intercalation by \( \Delta,\Delta\)-Piz, obtained from the kinetic rates in Eq. 5.3.2 (filled circles data points, dotted fit lines) and from the equilibrium elongations in Eq. 5.2.7 (open circles data points, dashed fit lines). Fits are to exponential force dependence in Eq. 5.2.3. C) The forward rates \( (k_{\text{on}}) \) as a function of concentration at constant applied forces 10-50 pN as color coded. The dotted lines are fits to the linear dependence on concentration. D) The association rates \( k_{\text{a}}(F) \) (data points) for forces 10-50 pN obtained from the slopes of the linear concentration dependence in Figure 5.2.4C. The dashed line is the exponential dependence on force from fitted to Eq. 5.2.3.
DISCUSSION

Diverse DNA structural deformations lead to stereoselective threading intercalation

Fig. 3.2.5A illustrates both the DNA intercalation mechanism and the corresponding ligand-DNA lengths changes for all Piz variants on a relative scale. First, it is notable that the equilibrium deformations become larger in the order (Δ,Λ), (Δ,Δ), (Λ,Λ), and (Λ,Δ), while the on rate decreases (see table 5.2.1). Interestingly, the off rates are about the same for all, as they are not accompanied by significant duplex deformation. Second, we consider the three Piz variants (Δ,Λ), (Δ,Δ) and (Λ,Λ) to further compare the impact on structural deformations due to changing the chirality of each subunit of the asymmetric Piz complex. By preserving the right-handed chirality associated with the intercalating Ru(phen)₂dppz subunit in (Δ,Λ) and (Δ,Δ), we notice that Δxeq is constant and relatively small. However, the dynamic structural deformation xon is increased by switching chirality associated with the distal Ru(phen)₂ip subunit from Λ to Δ. In contrast, switching the chirality associated with the intercalating Ru(phen)₂dppz subunit from right-handed to left-handed between (Δ,Δ) and (Λ,Δ) impacts both Δxeq and xon. This indicates that, while the intercalating moiety has the ultimate influence on the equilibrium structural deformation due to threading intercalation, the non-intercalating subunit of the threading intercalator can reshape the energy landscape by modulating the threading transition and consequently the equilibrium itself. This novel and systematic dissecting of the DNA deformations appears to suggest that strong steric clashes occur with the ip-adjacent phen groups, leading to significant DNA duplex deformation, which slows down the threading transition relative to the most favorable stereoisomer (Δ,Λ).
Figure 5.2.5. A) Illustration of the equilibrium and dynamic DNA deformation lengths in the threading intercalation for each Piz variant. The displacement from the natural separation between the base pairs is scaled relative a maximum displacement of 0.5 nm. B) Comparing the free energy landscape of $\Delta,\Delta$-P and $\Delta,\Delta$-Piz relative to a reference non-intercalative state free energy $\Delta G_0$. It illustrates the effect of replacing bi-dppz with dppz-ip leading to steric stress that prevents DNA helix deformation from relaxing to less elongation in the equilibrium state, solid lines for $\Delta,\Delta$-P and dotted-lines for $\Delta,\Delta$-Piz, black arrows indicate the shifts in equilibrium elongation and the threading free energy barrier heights. $\Delta G$ values determined at a concentration of both ligands equal their $K_d(0)=44$ nM.

The relaxed DNA deformation values in the intercalation equilibrium state $\Delta x_{eq}$ of ~0.30 nm found in this study for $(\Lambda,\Lambda)$ and $(\Delta,\Delta)$ are in the range of elongations previously reported for mono-intercalators (20, 21, 43). The DNA extension associated with intercalation of merely a planar aromatic ring system, such as in the case of the simple fast intercalator ethidium, is somewhat smaller, with $\Delta x_{eq}$ of 0.25 nm (20). On the other hand, the intercalation of the monomeric ruthenium complex $\Delta$-P reportedly leads to a significantly larger DNA extension of $\Delta x_{eq} = 0.38$ nm, similar to the 0.39 nm observed here for $(\Lambda,\Lambda)$ (20). However, it seems unlikely that the intercalation of the dppz moiety alone would lead to the very high $\Delta x_{eq}$ value of 0.48 nm observed for $(\Lambda,\Delta)$. It is, therefore, reasonable to suggest that the larger duplex extension of the monomer $\Delta$-p as well as the Piz variants $(\Lambda,\Lambda)$ and $(\Lambda,\Delta)$ is associated not only with the intercalation of the dppz group, but also with additional duplex extension and unwinding associated with fitting the bulky phen side groups into both grooves of the duplex. This effect is expected to be more sensitive to the spatial arrangement of the side groups, including their chirality, rather than to the nature of the intercalating moiety itself.

The $(\Lambda,\Delta)$ spatial arrangement shows extreme equilibrium and dynamic deformations for a mono-intercalator, but less than that observed for most bis-intercalators (21). While $(\Lambda,\Delta)$ was not found to intercalate either ct-DNA or AT-DNA in bulk experiments (90, 92), it could be that $(\Lambda,\Delta)$ weakly threads into bubbles of melted DNA base pairs, as we found a relatively larger intercalative site size for $(\Lambda,\Delta)$ and very slow association rate (see Table 5.2.1 and Table 5.2.2). Strong helix unwinding may lead to unorthodox intercalative orientation of the DNA-ligand complex, which may interfere with optical detection of intercalation in bulk studies. Considering DNA intercalation affinity, $(\Lambda,\Delta)$ has $K_d(0) \sim 0.6$ µM, a very weak affinity compared to threading intercalation by the parent binuclear ruthenium complex $\Delta,\Delta$-P ($K_d(0) \sim 50$ nM) (44), and it is merely 2-fold higher affinity than the conventional mono-intercalator $\Delta$-P ($K_d(0) \sim 1$ µM) (20). In terms of the intercalated DNA equilibrium elasticity, $(\Lambda,\Delta)$ also has a surprisingly large persistence length, in contrast to all other measured values for threading intercalators. Despite the weak effect of $(\Lambda,\Delta)$ on DNA persistence length, both $(\Delta,\Lambda)$ and $(\Lambda,\Delta)$ uniquely share a stronger interaction with the DNA backbone, evident by significant lowering of the DNA elastic modulus relative to that observed for $(\Delta,\Delta)$ and $(\Lambda,\Lambda)$. Only $(\Lambda,\Lambda)$ appears to have optimized stereoselective access to both interact with
DNA backbone (reducing elastic modulus) and interact with DNA base pairs (reducing persistence length).

**Optimizing the properties of chiral intercalators for DNA-targeting therapeutics**

The preference for DNA intercalation by right-handed chiral ligands was previously reported for crystal structures of the ruthenium complex monomer $\Delta$-Ru(phen)$_3$dppz$^{2+}$ ($\Delta$-p) and its enantiomer $\Lambda$-p (106). The studies showed that for both $\Delta$-p and $\Lambda$-p, the base pairs are intercalated by the dppz subunit from the minor groove, but with more favorable accommodation of the bulky phen moieties for $\Delta$-p relative to $\Lambda$-p. Recently, a crystal structure revealed threading DNA intercalation by the binuclear ruthenium complex $\Delta,\Delta$-P in an equilibrium state was reported (49). This crystal structure shows that, similar to $\Delta$-p, dppz is inserted from the minor groove. The preferred right-handed chirality of the phen side group adjacent to the intercalating dppz moiety observed in this study is exactly the same, as for the original studies on $\Delta$-p and $\Lambda$-p mono-Ru intercalators. However, the chirality of the phen side group adjacent to the distal ip moiety appears to be important for the threading rate and the equilibrium intercalation, and exhibits oppositely the left-handed preference. The significant effect of the ip-adjacent side group on the equilibrium and kinetic properties of Piz can be explained by the fact that the ip moiety is smaller than dppz. This may cause more severe steric clashes in the DNA major groove, than in the case of the symmetric binuclear ruthenium complex $\Delta,\Delta$-P with the larger bidppz moiety in place of dppzip for Piz. There is no available study of any other binuclear threading intercalators in which hetero-chirality is explored to the extent measured here. It is possible that the observed ($\Delta,\Lambda$) preference is limited to Piz, as a characteristic of the substituted bridging moiety dppzip. However, the hetero-chirality could be a global preference at least for binuclear ruthenium complexes with similar bulky phen sidechains. This would make, for example, $\Delta,\Lambda$-P a rational optimization of $\Delta,\Delta$-P in terms of maximizing affinity and minimizing structural deformations. In this regard, the hetero-chirality of $\Delta,\Lambda$-P could allow minimization of the angle between the two subunits bridging the opposite sidechains, providing an optimum intercalating plane.

It is important to note that replacing the bidppz bridging moiety in $\Delta,\Delta$-P by dppzip, does not seem to impact the affinity of DNA threading intercalation by $\Delta,\Delta$-Piz, but rather leads to larger equilibrium elongation by $\sim$0.1 nm, and faster threading kinetics relative to $\Delta,\Delta$-P. Regarding the altered kinetics, the structural modification is found to reduce the threading energy barrier by a few $k_B T$. The difference in free energy barrier is $\sim k_B T \cdot \ln(k_{a,2}(0) / k_a(0))$, where $k_{a,1}(0)$ is $k_a(0)$ for $\Delta,\Delta$-P, and $k_{a,2}(0)$ is for $\Delta,\Delta$-Piz, which is $\sim 2.6 k_B T$. At C=44 nM, which is $K_d(0)$ for both ligands, the non-intercalative state and the intercalative state are at an equal reference free energy level determined by a reference attempt rate $k_0$. By combining the energy barrier difference with the determined equilibrium and dynamic DNA deformations, we obtain an approximate threading free energy landscapes for both $\Delta,\Delta$-P and $\Delta,\Delta$-Piz (Fig. 5B). Although the DNA length changes during association are similar for $\Delta,\Delta$-Piz and $\Delta,\Delta$-P, the DNA deformation during
dissociation is different. While Δ,Δ-Piz has a nominal DNA deformation during dissociation, the molecular
lock mechanism in Δ,Δ-P involves strong complex relaxation to much smaller equilibrium extension.
Therefore, the reverse process of Δ,Δ-P dissociation also requires DNA stretching, which leads to much
higher transition free energy barrier (Fig. 5.2.5B), and slower Δ,Δ-P kinetics in its both on and off
processes (44). The elongation required for dissociation that slows the reverse rate is an important
property shared by both the binuclear ruthenium complex Δ,Δ-P and the naturally occurring antibiotic and
anticancer agent ActD (43, 44). More importantly, the ability of both DNA-Δ,Δ-P and DNA-ActD
intercalation complexes to relax back to a smaller deformation in the equilibrium state relative to the
transition state is indicative of better accommodation of their side groups. This may also reflect attractive
interactions in DNA grooves that involve partial reannealing of a flipped out base pair, as reported in
some crystal structures (49, 130).

The consideration of hetero-chirality for DNA-targeting chiral anticancer agents may potentially enhance
their therapeutic properties. In fact, such molecular optimization is scarce in nature, where either right-
headed chirality or left-handed chirality is dominant. Examples to such a rarity are found in polypeptide
natural antibiotics such as the unconventional intercalator ActD. The subunits of ActD involves hetero-
chirality structural elements of both right-handed D-amino acid residues and left-handed L-amino acid in
contrast to only L-amino acids residues found in proteins (131, 132). This structural complexity indicates
that therapeutic activity may require robust optimization to precisely match the targeted DNA chiral
structure. Our study illustrates the significance of refining the chirality of each structural subunit of DNA
threading intercalators. The systematic measurements presented here highlight the potential of sterically
modulating DNA equilibrium and dynamic deformations, reshaping the entire energy binding landscape,
and optimizing both the binding affinity and kinetics for therapeutics applications.
Chapter 6:
Conclusions

6.1 Conclusions

 Portions of this chapter are from:

Mechanisms of DNA intercalation probed by single-molecule force spectroscopy

Ali A. Almqwashi, Thayaparan Paramanathan, and Mark C. Williams

(Submitted)
6.1 CONCLUSIONS

As highlighted here, unconventional DNA intercalation can provide a combination of both higher affinity and slow binding kinetics, which is desired for DNA-based applications. In particular, we found that the threading mono-intercalator Δ,Δ-P has ~20-fold higher affinity than its monomer Δ-p, and at least three order of magnitude slower kinetics. When the semi-rigid bond connecting the two Δ-p in Δ,Δ-P is replaced with a flexible longer linker in Δ,Δ-Pc, the affinity of the threading bis-intercalator is further increased by ~5-fold relative to Δ,Δ-P (45), bringing the equilibrium dissociation constant to 15 nM, one of the highest affinity measurement reported for a DNA intercalator. The effective dissociation rate Δ,Δ-Pc is extremely slow, in fact no significant dissociation is observed in single-molecule wash off experiment. As the dynamic DNA deformation of Δ,Δ-Pc does not show the lock mechanism of Δ,Δ-P, we predicted that combining the two-step intercalation in Δ,Δ-Pc to the locking assembly of Δ,Δ-P may result in further higher DNA-ligand intercalation affinity (45). Indeed, this is what we found for the converted threading mono-intercalator Δ,Δ-Pi, with an equilibrium dissociation constant of 10 nM.

Furthermore, bulk studies have demonstrated that switching the chirality of intercalators may result in virtual termination of their ability to intercalate DNA (85, 90, 92). We examined robust stereoselectivity that is reported for the threading mono-intercalator Piz. In the absence of a crystal structure exploring the stereo-selectivity of static equilibrium conformations, this work revealed for the first time both the equilibrium and dynamic elongations of the DNA-Piz complex for all four stereoisomers (Δ,Λ), (Δ,Δ), (Λ,Λ) and (Λ,Δ). Our findings establish that there is strong coupling between DNA-ligand intercalation affinity for (Δ,Λ) and its minimal static and dynamic DNA deformations. By demonstrating the impact of chirality in optimizing the transition pathways of DNA-ligand assembly, these results should help guide the rational design of unconventional DNA-targeted intercalators.

However, it is important to note that optimizing the affinity and kinetics of intercalators for in vivo applications requires in addition considering the cellular uptake efficiency. Although the monomer Δ-p was recently demonstrated to have in vivo antibiotic activity in treating bacterial infection of the soil nematode (84), the binuclear ruthenium complexes Δ,Δ-P and Δ,Δ-Pc had less definite cellular uptake in vitro (36). In the absence of an effective drug delivery scheme, the high DNA binding affinity of these unconventional intercalators is insufficient to qualify them as DNA-targeting drug candidates. One of the solutions to cellular uptake deficiency is increasing the molecule hydrophobicity to enhance passive diffusion through the cellular membrane. This can be achieved by functionalizing ligands with lipid-like attachment such as alkyl chains (29). Another binuclear ruthenium complex that shares some of the Δ,Δ-P structural characteristics, namely [(Ru(phen)2)2(tpphz)]^{4+}, has proven more successful in penetrating membrane of breast cancer cells, even without hydrophobic attachments (29). The binding mode of this light-switching ligand is surprisingly yet to be resolved, and bulk studies were not conclusive whether it intercalates DNA or binds to the minor groove (34, 133).
Nevertheless, the variability in cellular uptake for seemingly related ligands (133) highlights possible advanced molecular recognition that can be sensitive to fine structural alteration. This confirms that more single-molecule studies are needed to reveal the governing assembly mechanisms, which may help guide and optimize rational design of a new generation of antibiotic and anti-cancer drugs. The urgent quest for synthesizing new therapeutic small molecules and optimizing currently used agents are fueled by a fierce race to surpass the molecular evolution of drug-resistance. Studies showed that combining a mix of drugs that have different binding modes to DNA, such as intercalation and cross linking, lower the chance to cancer reoccurrence (17, 84). Aside from the important application of DNA intercalators, these small molecules serve as basic models that may improve our capacity to examine and understand much bigger and more complicated biological systems. We hope that the outlined single-molecule nanomechanical measurements and analysis may help advance the interest in revealing both the equilibrium and dynamic characteristics of diverse DNA assemblies.
List of Publications

Strong DNA deformation required for extremely slow DNA threading intercalation by a binuclear ruthenium complex
Ali A. Almaqwashi, Thayaparan Paramanathan, Per Lincoln, Ioulia Rouzina, Fredrik Westerlund and Mark C. Williams


A ruthenium dimer complex with a flexible linker slowly threads between DNA bases in two distinct steps
Meriem Bahira, Micah J. McCauley, Ali A. Almaqwashi, Per Lincoln, Fredrik Westerlund, Ioulia Rouzina, and Mark C. Williams


Mechanisms of DNA intercalation probed by single-molecule force spectroscopy
Ali A. Almaqwashi, Thayaparan Paramanathan, and Mark C. Williams
(Submitted)

Dissecting the dynamic pathways of stereoselective DNA threading intercalation
Ali A. Almaqwashi, Johanna Andersson, Per Lincoln, Ioulia Rouzina, Fredrik Westerlund and Mark C. Williams
(In preparation)

Resolving DNA threading conversion by rotationally flexible mono-intercalator
Ali A. Almaqwashi, Johanna Andersson, Per Lincoln, Ioulia Rouzina, Fredrik Westerlund and Mark C. Williams
(In preparation)
List of Presentations

Kinetics of DNA threading intercalation by a rigid ruthenium complex dimer
(Biophysical Society 58th, 2014, San Francisco, Poster presentation)

Resolving the DNA binding mode of a rotationally flexible binuclear ruthenium complex
(Biophysical Society 59th, 2015, Baltimore, Poster presentation)

Quantifying the molecular mechanism for highly stereo-selective DNA threading intercalation
(American Physical Society March Meeting, 2015, San Antonio, Oral presentation)
References


