The Thermodynamic Characterization of the DNA Four-Way Junction Melting Process

by

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Abstract

The DNA four-way junction (4WJ) is an essential structural intermediate in the process of homologous recombination. Initial experiments have potentially elucidated the intermediates of the 4WJ melting process (Savage, 2017) and have led to the development of a “pseudo-duplex” model of 4WJ melting. In this thesis, we report the use of multiple spectroscopic techniques to test the validity of this new model and to characterize the global thermodynamic parameters of the melting process.

Firstly, the thermodynamic parameters of the 4WJ melting process were characterized using DNA concentration-dependent absorbance spectroscopy. For a more nuanced understanding of the process, fluorescent nucleoside analogs 2-aminopurine (2AP) and 4-amino-6-methyl-8-(2-deoxy-beta-d-ribofuranosyl)-7(8H)-pteridone (6MAP) were incorporated into J3, providing single-base observation of melting behavior.

Through our comparison of 2AP and 6MAP reporting in analogous base positions, we concluded that the pseudo-duplex melting behavior previously reported with 6MAP (Savage, 2017) was unique to the junction, and not to the 6MAP probe itself. Additionally, solvent exposure of the analogs was tested through titration with a known fluorescence quencher molecule. These experiments indicated that all probes are similarly exposed to the solvent, suggesting that the fluorescence reported by each probe was not skewed by differing base orientations in duplex.

Using data from these experiments, differential scanning calorimetry scans, and coarse-grained simulations, we have outlined a reaction coordinate diagram for
the pseudo-duplex model of junction melting. This schematic yields both a qualitative and quantitative characterization of the pseudo-duplex melting model of the DNA four-way junction.
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Chapter 1
Introduction

1.1 Theories of Homologous Recombination

The scientific understanding of heredity and the field of genetic research has exploded during the past century. Experiments related to the generational transfer of information have led to an understanding of the genetic code and the mechanisms behind genetic variability. Researchers are constantly advancing our understanding of the processes that make every individual within a species unique.

Our genetic information is encoded within our cells in the form of deoxyribonucleic acid (DNA). DNA is structurally stable, and its sequence of bases is what encodes the entire blueprint for humanity. Upon fertilization and the creation of a new individual, information from the genes of each parent are exchanged to create a unique genetic code in the daughter cell. This process is called meiotic homologous recombination. In this work, we will focus on the DNA four-way junction (4WJ), a structural DNA intermediate that forms during the recombination process.

The concept of the 4WJ was first theorized in 1964, when molecular biologist Robin Holliday proposed a mechanism for genetic recombination in fungi. Holliday’s theory of meiotic recombination was the first to highlight an intermediate formed upon the separation of both DNA parent duplexes and subsequent annealing of homologous strands (Holliday, 1964). The intermediate referenced here is the 4WJ, a structure composed of two DNA duplexes that form four branched arms. Because of Holliday’s mechanistic proposal, the 4WJ goes by another name: the Holliday junction.
**Figure 1.1.1:** Schematic for the initial steps of the Holliday model of genetic recombination. Parent duplexes (1) separate into single strands (2), which crossover each other to form a 4WJ (3) (Holliday, 1964)

During the two decades following Holliday’s proposal, the dynamics of the 4WJ during genetic recombination were uncovered. Using a molecular model, Sigal & Alberts were the first to demonstrate that the Holliday junction is a biologically plausible intermediate. The model demonstrated the capability for isomerization of the structure during recombination (Sigal & Alberts, 1972).

In 1975, Meselson & Radding built upon the work of Sigal & Alberts, proposing their own model for recombination. In this model, the recombination intermediate undergoes an isomerization to produce two possible outcomes upon cleavage: non-recombinant and recombinant DNA.
Figure 1.2.1: Isomerization of the four-way junction during homologous recombination (Sigal & Alberts, 1972), (Meselson & Radding, 1975)

Potter and Dressler used electron microscopy to produce the first experimental observation of the 4WJ in 1976. The group observed self-replicating colicin plasmid DNA molecules and located apparent dimer and multimer ring structures that were candidates for the presence of recombination intermediates. To test that these structures were not simply folded monomer rings, the researchers treated their samples with the restriction enzyme EcoRI. EcoRI would site-specifically cleave a monomer ring only once, but would be expected to cleave a dimer undergoing recombination twice. EcoRI cleavage of a dimer resulted in the following image.
The researchers were able to determine that the area of covalent linkage was an area of homology because the DNA duplexes were split into two pairs of equal length arms when cleaved with the restriction enzyme. Thus, Potter & Dressler became the first to successfully identify the Holliday junction, the recombination intermediate that had been speculated upon for years prior.

Direct observation of the 4WJ prompted further research into recombination. In 1983, almost twenty years after Holliday’s work, Szostak, Orr-Weaver, & Rothstein proposed the double-strand break repair (DSBR) model for genetic recombination. Today, this model is the most well-known and widely accepted.
The DSBR process is named for its first step, a double strand break in one of the homologous duplexes. The breaks are widened by endonucleases to create 3’ overhangs, one of which acts as the invading strand into the other homologous duplex. This invasion forms a Holliday junction and D-loop structure. The D-loop is enlarged as the 3’ end of the invading strand is lengthened by repair synthesis, which uses the homologous region of the other parent duplex as a template. The ends of the single strands anneal, forming a second Holliday junction, while the gap from the second strand break is repaired. The junctions can be resolved in one of two ways; cleavage of the inner two strands or of the outer two strands.

If both junctions are cleaved in the same way, the resulting duplexes are in the non-crossover configuration. If one is cleaved at the inner strands while the other is cleaved at the outer strands, the result will be duplexes in the crossover configuration.
Physiologically, most duplexes are formed in the crossover configuration. The model cites the junction isomerization process as a possible driving force behind site-specific cleavage to yield recombinant DNA (Szostak, Orr-Weaver, & Rothstein, 1983).

As research surrounding the subject progressed, it became increasingly clear that the DNA 4WJ was necessary for genetic recombination, and therefore was necessary to maintain genetic variability within our species. Given this significance, elucidation of the structure and dynamics of the 4WJ became the focus of several biochemical studies.

1.2 4WJ Structure and Dynamics

Though it had been identified as a vital intermediate to recombination, the structural characteristics of the four-way junction remained mysterious until the late 1980s. Theories of recombination identified the need for a process called branch migration to allow the exchange of genetic material between homologous duplexes. Throughout branch migration, the branch point, or the center of the junction is displaced along the sequence. Experimental observation of branch migration in 4WJ began with the Thompson group in 1976. The results of their work displayed that the center of the junction shifts at approximately 6,000 base pairs per second at 37°C (Thompson, Camien, & Warner, 1976). However, in the presence of magnesium, this rate is approximately 1,000 times slower (Panyutin & Hsieh, 1994). Further research showed that the thermodynamics of branch migration are sequence-dependent,
creating a rugged energy landscape for a multistep branch migration (McKinney et al., 2005).

Physiological, mobile junctions present a particularly difficult problem for experimental study. In solution, without the aid of proteins, the hybridization of two individual duplexes is more energetically favorable than the hybridization of the 4WJ. Thus, in order to study the junction, oligonucleotides must be designed in such a way that promotes the formation of the 4WJ. These sequences are carefully constructed through the minimization of duplex symmetry and equilibrium calculations based on known thermodynamic parameters of DNA (Kallenbach, Ma, & Seeman, 1983). The development of the synthetic, immobile junction opened the door for research into the structure and dynamics of the 4WJ.

In 1987, Diekmann & Lilley demonstrated that the addition of salt to a pseudo-junction, formed from an inverted repeat sequence, yielded faster electrophoretic migration. They proposed that this result indicated an ion-binding
event at the center of the junction that allows for a more streamlined movement through a gel matrix (Diekmann & Lilley, 1987).

Then, Duckett et al. used restriction enzyme digests to produce the first in-depth study of junction structure.

![Diagram of DNA junctions](image)

**Figure 1.2.2**: 80 base pair junction (A) used by Duckett, et al. with specific restriction enzyme cleavage sites on each strands (B) (Duckett et al., 1988)

In the case of an open, tetrahedral junction, six equally shaped DNA fragments would be produced upon pairwise restriction enzyme cleavage. However, the electrophoretic results showed three different species.

Because of the ion-dependent change in structure demonstrated by both Diekmann & Lilley and Duckett et al., the Lilley group observed the structure of synthetic junctions in various metal ion solutions. Their results showed that the presence of cations folded the junction into a stacked form, where one duplex arm stacked coaxially on the other. The group proposed that metal ions allow for the
adoption of the stacked geometry due to cation stabilization of the negative repulsion created when the two phosphate backbones move into close proximity to one another (Duckett, Murchie, & Lilley, 1990). The different isomers of the junction observed through these studies lent experimental validity to Sigal & Alberts’ and Meselson & Radding’s theories of junction isomerization during recombination, as the synthetic junction adopted the physiologically relevant conformers.

Coaxial stacking in the presence of salt has implications for branch migration. Because branch migration is significantly slower at high salt, it is likely that the structure of the junction plays a role in the capability for spontaneous migration (Panyutin & Hsieh, 1994). Single molecule studies of the 4WJ have demonstrated that the unstacked form of the junction is a prerequisite for branch migration (Lushnikov et al., 2003), (McKinney et al., 2005).

After the different 4WJ conformers were isolated, further studies sought to understand the structural differences between them. Fluorescence energy transfer experiments revealed that the stacked form of the junction is antiparallel, and adopts one of two possible conformers (Murchie et al., 1989).
Figure 1.2.3: Right-handed, antiparallel, stacked structure of the 4WJ (top) and possible conformers of the junction (bottom: middle junction is open and left and right junction are stacked) as reported by fluorescence resonance energy transfer (Murchie et al., 1989)

The preferred conformer is dependent only on identity of the two base pairs in the center of the junction (Duckett et al., 1988), (von Kitzing, Lilley, & Diekmann, 1990). There is a “helical twist” at the center of the junction that prevents steric contact of the exchanging strands (von Kitzing, Lilley, & Diekmann, 1990). In 1999, a 4WJ was crystallized, revealing high-resolution structural information about the recombination intermediate. The 4WJ contained two GA mismatches in each duplex because no 4WJ with canonical base pairing had been successfully crystallized. Consequently, these mismatches helped facilitate crystallization of the structure with minimal disturbance to its stability (Ortiz-Lombardía et al., 1999).
The junction displays two-fold symmetry in the stacked-X structure, the angle between stacked arms is 40°, and central bases pairs display sequence specific hydrogen bonding (Ortiz-Lombardía et al., 1999). At the core of the junction, sterically driven interactions and favorability of hydrogen bonding determine the adoption of one conformer over the other.

![Schematic of a 4WJ with hydrogen bonding patterns illustrated as dotted lines, and phosphate groups as orange circles (left) and crystal structure (right) (Ortiz-Lombardía et al., 1999)](image)

**Figure 1.2.4:** Schematic of a 4WJ with hydrogen bonding patterns illustrated as dotted lines, and phosphate groups as orange circles (left) and crystal structure (right) (Ortiz-Lombardía et al., 1999)

Perhaps the most notable feature derived from the crystal structure is the presence of phosphate-base hydrogen bonding between the two central base pairs of each exchanging strand. This bonding pattern has major implications for the isomerization of the junction, as it implies the breakage of hydrogen bonds to fully isomerize. Thus, the junction must first open before the alternative isomer can be adopted. This is also consistent with the crossover isomerization process theorized years prior (Sigal & Alberts, 1972), (Meselson & Radding, 1975).
Since its initial proposal, a wealth of knowledge concerning the structure of the 4WJ has been uncovered. Further research has begun to explore the kinetics of the processes the junction is involved in, as well as the thermodynamic stability of the structure.

1.3 Coarse-Grained Simulations

The basis for the experimental research conducted in this work is a computational simulation of the 4WJ melting process, which predicted stepwise melting. Recent advancements in computational modeling have allowed for the development of a simulated DNA system, called the 3-site-per-nucleotide (3SPN) model. The 3SPN model is an alternative to all-atom DNA simulations, which require significant computational power to properly replicate experimental structure and dynamics. The 3SPN model is a coarse-grained system that reduces DNA bases into their basic components: sugar, phosphate, and a nucleotide base. The validity of this model system has been displayed through agreement with experimentally obtained hybridization rates and structural properties (Hinckley et al., 2013).

Initial experiments have indicated that the 3SPN.2 model (a refined version of 3SPN) properly replicates the isomeric preferences of the 4WJ, as well as ion-dependent melting behavior (Wang, et al., 2016). Further investigation of 4WJ melting using the 3SPN.2 model indicated an unexpected structural melting pattern: one arm of the 4WJ destabilized while the torsionally strained center of the junction remained intact. Initial solution experiments using fluorescent nucleoside analogs
have supported this melting model (Savage, 2017), but questions regarding the thermodynamics of the process still remain. To achieve further characterization of the 4WJ melting process, we will employ multiple spectroscopic methods through this work. Ultimately, we hope to validate the predictions of the 3SPN.2 model through the use of several biophysical approaches.

1.4 Thermostability of DNA and the 4WJ

1.4.1 Melting Behaviors of Nucleic Acids

DNA structure has evolved optimal stability in order to protect and maintain the genetic code. The stability of native DNA draws from many factors, two of the most important being base stacking and base pairing. The energetic contribution of both stacking and pairing interactions depend on the sequence identity of the DNA strands. In base pairing, the two canonical Watson-Crick base pairs experience different hydrogen bonding motifs. GC base pairs form three interstrand hydrogen bonds, while AT base pairs form only two.
Because of these different bonding patterns, the thermodynamic stability of duplexes of the same length varies with the base content of each strand. A GC-rich oligomer is likely to be more thermodynamically stable than an AT-rich oligomer as more heat energy is required to break the additional hydrogen bonds between the GC pairs. Different bases also stack differently with their neighboring base pairs to promote favorable hybridization.

Quantitatively, then, the thermodynamic contribution of each base pair is based on its own identity, as well as the identity of its neighboring base pairs (SantaLucia Jr. & Hicks, 2004). Thermostability can be estimated by calculating the energetic contribution of each base pair and its nearest neighbor through both base pairing and base stacking. Predictions of thermostability are important in interpreting and understanding the melting behavior of different DNA structures.
1.4.2 4WJ Melting

This thesis will focus on the melting process of the DNA four-way junction. Through the study of junction melting, we will not only gain insight into the stepwise intermediates in the process, but also about the relative thermodynamic stability of the junction. The relative stability of the junction at the center and along the arms has implications regarding the process of branch migration.

Because of the torsional strain in the center of the 4WJ, it is reasonable to hypothesize that this region would be less stable than the duplex arms. However, recent experiments have provided data that refutes this concept. Spectroscopic and calorimetric studies conducted on intramolecular 4WJs, which stack similarly to intermolecular 4WJs, indicate that the melting process occurs in stages (Carr & Marky, 2017).

![Figure 1.4.2: Schematic for intermolecular 4WJ melting (Carr & Marky, 2017)](image)

The stages of the melting process, as displayed in the above figure, are based only on the stabilities of the individual arms. It is unclear whether this observed
melting pattern occurs in all intramolecular 4WJs, and how the pattern may vary with intermolecular junctions.

In addition to the results of Carr & Marky, recent experiments from the Mukerji lab and Starr lab at Wesleyan University have led to the proposal of a new melting model for intermolecular 4WJ based on coaxial stacking. This model suggests that the junction melts according to its “pseudo-duplex” components (Savage, 2017). The term pseudo-duplex has been introduced to describe the nearly continuous duplexes formed by the coaxially stacked arms of the junction.

![Diagram of the stacked conformation of the four-way junction in terms of its pseudo-duplexes](image)

**Figure 1.4.3:** Diagram of the stacked conformation of the four-way junction in terms of its pseudo-duplexes

The model proposes that junction melting is non-cooperative and sequence-dependent. The pseudo-duplex with a lower GC base-pair content is destabilized at a lower temperature relative to the other pseudo-duplex, leading to transition states in
the equilibrium reaction. Much of this work will focus on supporting and enhancing the pseudo-duplex melting model.

![Diagram of pseudo-duplex melting model](image)

**Figure 1.4.4:** Schematic of the pseudo-duplex model of DNA four-way junction melting

The melting process of the junction is understood as an equilibrium reaction, meaning that all intermediates are present in solution at different concentrations depending on temperature. The work of Marky & Breslauer outlines the calculations for the thermodynamic parameters of equilibrium data obtained from a melting curve of any molecularity. These parameters can be applied to the four-way junction, which forms from four non-self-complementary and non-identical strands.

$$K = \frac{[Junction]}{[Single\ strands]} = \frac{[A_1A_2A_3A_4]}{[A_1][A_2][A_3][A_4]} = \frac{\alpha C_T}{[1 - \alpha]C_T^4} = \frac{\alpha}{(C_T^4)^3(1 - \alpha)^4}$$

**Equation 1.4.1:** Definition of equilibrium constant (K) for the formation of a tetramer complex from four non-self-complementary single strands, where A represents DNA single strands, C_T represents total DNA concentration, and \( \alpha \) represents the fraction of DNA strands in 4WJ form (Marky & Breslauer, 1987)
In the analysis of melting curves, the fraction of strands in the native complex \( (\alpha) \) is plotted against temperature. To calculate \( \alpha \), the baselines of raw melting curve are fit with a line, and the deviations of points in the melting transition from each baseline are measured (Marky & Breslauer, 1987). These deviations are used to calculate the amount of native 4WJ in the sample at a given temperature.

**Figure 1.4.5:** Sample normalization of absorbance data

Because \( \alpha \) is representative of the fraction of DNA strands in junction form, information regarding the populations in solution may be understood from the above plot. At \( \alpha = 1 \), the solution is entirely 4WJ, at \( \alpha = 0.5 \), approximately half of the strands in solution are in junction, and at \( \alpha = 0 \), all strands are in the single-stranded state. The melting temperature (\( T_M \)) of the junction is defined at \( \alpha = 0.5 \). This analysis assumes a fully cooperative, two-state melting transition, which, although inconsistent to our proposed pseudo-duplex model, is useful in obtaining melting temperatures. Additionally, we can interpret the shape of the melting curve in terms of cooperativity. A fully cooperative process implies an all or none transition, with no
intermediates. A sigmoidal melting curve indicates a fully cooperative process (Chan, Bromberg, & Dill, 1995), while deviations from sigmoidality may indicate the presence of a transition state. Thus, in our analyses, we will consider both the shape of the melting curves and the relative trends reported by nucleoside analogs.

The approximation of $K_{eq}$ for the 4WJ melting process allows for the use of the Van’t Hoff equation to gather the thermodynamic parameters of the process. The Van’t Hoff equation utilizes the temperature dependence of the equilibrium constant of a reaction to determine the changes in enthalpy and entropy during the process. The equation was derived from two thermodynamic descriptions of Gibbs free energy, and can be arranged in a linear form (Kuriyan, Konforti, & Wemmer 2012).

\[
\Delta G = \Delta H - T\Delta S
\]

**Equation 1.4.2**: Thermodynamic description of Gibbs free energy

\[
\Delta G = -RT\ln(K_{eq})
\]

**Equation 1.4.3**: Dependence of Gibbs free energy on the equilibrium constant

\[
\ln K_{eq} = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}
\]

**Equation 1.4.4**: Linear form of the Van’t Hoff Equation

In this thesis, thermodynamic melting curves will be analyzed using the Van’t Hoff equation and by generating a plot of $\ln(K_{eq})$ vs. $1/T$. $\Delta H$ of the melting process will be calculated from the slope of the linear fit of this plot and $\Delta S$ will be calculated from the intercept.
1.5 A Spectroscopic Approach to 4WJ Melting

1.5.1 Absorbance Spectroscopy

Nucleic acids like DNA have characteristic absorbance patterns that arise mostly from their purine and pyrimidine bases. Temperature-dependent UV absorbance spectroscopy can reveal information about the melting transition of duplex DNA to single-stranded DNA.

The single-stranded product of a duplex melting transition yields a higher absorbance value at 260 nm than its parent duplex. This phenomenon, referred to as a hyperchromic shift, allows for the spectroscopic observation of nucleic acid melting. The hyperchromic shift observed upon melting results from a rearrangement of the transition dipoles in the molecule. An electronic transition dipole, which is directly proportional to absorbance intensity, is representative of the magnitude and direction of the electronic transition to the first excited state. The transition dipole of one nucleic acid base induces dipoles in neighboring bases. When bases are stacked and paired, as in duplex DNA, the direction of induced dipoles opposes that of the transition dipoles, weakening the overall magnitude of the electronic transition, and consequently reducing the absorbance of the molecule. When bases are randomly oriented in single-stranded conformation, the opposition of induced dipoles is decreased due to a lack of conformational restraint (Bloomfield, Crothers, & Tinoco, 2000).
Thus, it is possible to obtain a nucleic acid melting curve from temperature-dependent UV absorbance data, as absorbance increases when melting occurs. The melting curve may then be analyzed using the method demonstrated in Figure 1.4.5.

1.5.2 Fluorescence Spectroscopy

In addition to absorbance spectroscopy, we will use fluorescence spectroscopy to probe the 4WJ melting process. Fluorescence is a measurable, quantum mechanical phenomenon related to the structural properties of a molecule. Unmodified nucleic acids have little fluorescence capability, but the insertion of molecules called fluorescent nucleoside analogs allows for measurable fluorescence at the position of
the analog. In contrast to the global perspective gained through absorbance spectroscopy, the use of fluorescent nucleoside analogs provides a local view of the melting of different positions along the four-way junction.

Nucleoside analogs are molecules that mimic the structure of DNA bases, but contain specific modifications that lead to an increased fluorescence quantum yield. Upon base pairing and stacking, the fluorescence of nucleoside analogs is quenched, or reduced, due to collisional interactions with complementary base pairs. Thus, as hybridized DNA melts into its single-stranded components, the fluorescence of the analog increases (Rist & Marino, 2002). This structurally dependent fluorescent behavior allows for meaningful reporting on native state changes in nucleic acids. In this work, we will use two adenine analogs, 2-aminopurine (2AP) and 4-amino-6-methyl-8-(2-deoxy-beta-d-ribofuranosyl)-7(8H)-pteridone (6MAP) to investigate 4WJ melting.

6MAP is a pteridine-based analog, consisting of conjugated pyrimidine and pyrazine rings. The probe has been previously demonstrated to report meaningful nuances in four-way junction melting behavior (Savage, 2017). One of the major benefits of 6MAP as a molecular probe is that it does not induce significant instability in global nucleic acid structure, despite its large shape in comparison to adenine. The probe has been shown to decrease the melting temperature of oligonucleotides by only 2-4 °C (Hawkins et al., 2001), (Hawkins, 2001). Through this thesis, we will continue to evaluate the effectiveness and fluorescent properties of 6MAP.
Like other nucleoside analogs, fluorescence quenching upon base pairing enables the utility of 6MAP as an effective probe of nucleic acid behavior (Hawkins, 2001). Because of its recent characterization, there is little quantum mechanical data regarding the mechanism of 6MAP fluorescence. In a general sense, it is possible that the extension of the conjugated system by the introduction of a six-membered aromatic ring allows for a more stable excited state, thereby increasing the likelihood of a radiative, fluorescent decay pathway.

The second fluorescent probe we will use to investigate 4WJ melting is 2AP. 2AP is a well-characterized analog, used often to probe the thermostability of oligonucleotides.
The probe, an adenine isomer, can be substituted for any nucleotide base, but only forms a stable Watson-Crick base pair with thymine. 2AP hydrogen bonds with other nucleotide bases, but in a manner that disrupts the typical stability of stacked duplexes (Jones & Neely, 2015).

![Figure 1.5.4: Watson-Crick base-pairing of 2AP to thymine (Jones & Neely, 2015)](image)

Generally, 2AP is known to depress the melting temperature of small duplexes (heptamers to decamers) by 2-9°C (Eritja et al., 1986), (Nordlund et al., 1989), implying a decreased stability of the duplex. However, there is evidence to suggest that thermodynamic instability induced by the probe varies with the nucleotide base it is substituted for. Data produced by Law et al. show that in an 11-mer duplex where 2AP is substituted for adenine and base pairs with thymine, there is virtually no effect on the melting temperature or stability of the duplex (Law et al. 1996). Another study conducted on 13-mer duplexes suggests that the degree of instability caused by 2AP insertion may be dependent on more than just the substituted base, as a 3.5°C depression in melting temperature was observed (Dallmann et al. 2010). It is possible
that the size of the duplex or the site of probe insertion may also play a role in the
effect on global stability.

Since 2AP is so structurally similar to adenine, which has a notably low
fluorescent capability, the measurable fluorescence yield of 2AP is a curious
deviation that highlights the structural basis of fluorescence. Before exploring the
quantum mechanical basis of 2AP fluorescence, it is important to understand
molecular orbital theory in relation to radiative emission. Of relevance to the
following analyses are the n, π, and π* orbitals. Respectively, these orbitals are non-
bonding, bonding, and anti-bonding orbitals. In aromatic compounds, the π and π*
orbitals are delocalized above and below the plane of the n orbital.

![Schematic of delocalization of π (gray) and π* (white) orbitals around a benzene ring (Soderberg, 2016)](image)

**Figure 1.5.5:** Schematic of delocalization of π (gray) and π* (white) orbitals around a benzene ring (Soderberg, 2016)

Upon excitement, an electron in a conjugated system likely follows one of two
pathways: n → π* or π → π*. The out-of-plane n → π* transition creates a less stable
excited state than the planar π → π* transition. Consequently, π* → n relaxation is
significantly less radiative than π* → π relaxation, since slow, fluorescent emission
can occur more readily from a stable excited state (Guilbault, 1990). Principles of fluorescent emission will be discussed further in Chapter 2.5.1.

Theoretical quantum mechanical studies have speculated upon the reasoning behind the increased fluorescence of 2AP compared to adenine. Several possible mechanisms regarding molecular orbitals have been proposed to explain the difference between the fluorescent properties of the two isomers.

One computational analysis of the properties of both molecules suggested that the bond lengths of the six-membered ring are altered due to the changed position of the amino group. This structural difference affects the accessibility of the out-of-plane \( n \rightarrow \pi^* \) transition, allowing for a fast, nonradiative decay pathway in adenine but not in 2AP (Broo, 1998).

Also important in the consideration of fluorescence of these two molecules is the proximity effect, which accounts for the potential of interstate crossing upon excitation. While both 2AP and adenine experience an initial \( \pi \rightarrow \pi^* \) electronic transition, Gaussian calculations indicate the \( n \rightarrow \pi^* \) transition state shifts below the \( \pi \rightarrow \pi^* \) in adenine, but the same effect does not occur in 2AP (Menucci et al., 2001). Thus, vibrational relaxation occurs from the \( \pi^* \rightarrow n \) state in adenine after crossing from the \( \pi \rightarrow \pi^* \) state, while fluorescent relaxation occurs from the \( \pi^* \rightarrow \pi \) state in 2AP.

It is worth noting that with the advancement of quantum chemical techniques, the aforementioned literature has been accused of oversimplification of the decay mechanisms. A more recent quantum chemical study (Serrano-Andres et al., 2006)
has further explored the photophysical basis behind 2AP fluorescence using new concepts in modern photochemistry.

In this work, 6MAP and 2AP will be placed in the same positions in the 4WJ. The reporting of the two analogs will be used to verify that each reported behavior is independent of the specific probe. Consistent melting trends reported between the two probes will indicate that the melting patterns exhibited by each are specific to the 4WJ.

1.5.3 Fluorescence Quenching

In addition to the use of multiple fluorescent nucleoside analogs, we will also employ fluorescence quenching methods to explore the local environments of the probes. In this work, we will expose the fluorescently-labeled 4WJs to iodide to observe the degree of exposure to the solvent in each probe location.

Fluorescence quenching occurs when the fluorescence of a molecule is decreased by one of many possible processes. There are two main types of quenching that can occur: static and collisional. Collisional quenching occurs upon collisions between the quencher molecule and the excited state of the fluorophore, resulting in nonfluorescent decay to the ground state. Static quenching occurs upon the formation of a complex between the fluorophore and quencher, preventing a radiative decay pathway (Lakowicz, 2006).
Figure 1.5.6: Decay pathways and effect on Stern-Volmer plots of collisional and static quenching (Lakowicz, 2006)

In the case of many collisional quenching processes, including collisions with heavy atoms like iodide, the radiative decay pathway is prevented by electronic intersystem crossing from the singlet excited state to the triplet state. State crossing occurs upon disruption of electronic spin-coupling, causing the excited electron in the triplet state to spin parallel to the electron in the ground state. This means that an electronic spin-flip is required to return to the ground state. Because of the required spin-flip, relaxation from the triplet state to the singlet ground state occurs on a much slower timescale than fluorescence, making it difficult to observe (Roberts & Caserio, 1977). The mechanism behind state crossing during fluorescence quenching with heavy atoms is proposed as a disruption in the spin-coupling of π electrons by the electric field of the nucleus of the heavy atom quencher (Kasha, 1952). Thus, the quenching ability of halogen atoms is directly related to atomic number, as the strength of the electric field of the nucleus increases with increased charge.
Figure 1.5.7: Jablonski diagram indicating intersystem crossing upon collisional quenching with heavy atoms (Kasha, 1952)

Experimentally, we expect the fluorescence of the nucleoside analogs to be quenched differently depending on the relative exposure to solvent and consequently, heavy atoms such as iodide in solution. If a probe in one location in the junction is differentially base-stacked because of unfavorable interactions with neighboring base pairs, quenching will proceed at a more rapid rate, as the probe is more solvent exposed. Differing degrees of solvent exposure in each probe position may skew fluorescence reporting. For example, if a probe in the H-pseudo-duplex is base stacked differently than a probe in the X-pseudo-duplex, the relative trends in fluorescence may be a product of local probe environment rather than of probe position.
1.6 Project Goals

As mentioned throughout this introduction, the major purpose of this project is to elucidate the thermodynamics of the DNA four-way junction melting process. We also hope to further understand and characterize the use of different biophysical tools for the study of nucleic acids and the 4WJ in particular. Finally, we will further test the experimental validity of the 3SPN.2 model for DNA. We hope that our results will help to improve the model for future use in nucleic acid systems.

The implications of characterizing 4WJ thermostability are wide-ranging. First, there exists an entire class of recombination proteins that bind the 4WJ (West, 2003), some of which are yet to be completely understood. The pseudo-duplex model of 4WJ melting may help to explain the binding mechanics, domains, and recognition sequences of some of these proteins. As previously mentioned, the relative stability of the center of the 4WJ has implications in understanding the process of branch migration, where the branch point of the junction shifts in accordance with sequence homology. Finally, understanding the different fluorescence patterns of individual nucleoside analogs, like 2AP and 6MAP, will improve their characterization and make them more reliable tools for future research.
Chapter 2
Theory and Methods

For the following methods, buffer solutions were prepared as follows:

10X TBE was prepared with 890 mM Tris Base, 890 mM boric acid, and 20 mM EDTA in water. The pH was adjusted to 8.3. Solution was stored at 4°C. 10X DNA annealing buffer was prepared with 3M NaCl, 100 mM Tris Base, and 10 mM EDTA in water. Oligonucleotide loading buffer for purification was prepared as ten parts formamide to one part 10X TBE.

2.1 Oligonucleotide Purification

2.1.1 Oligonucleotide Purification Theory

Errors in oligonucleotide synthesis lead to truncated products in the oligonucleotide sample, meaning that DNA strands are present that are shorter than the desired sequence (Hecker & Rill, 1988). To remove these impurities, the purchased samples are purified through denaturing polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis (PAGE) separates molecules by size based on their mobility through a gel matrix. Urea, the denaturant in the gel, frees the single-stranded oligonucleotides of secondary structure. Removal of secondary structure ensures that the gel separates the molecules by sequence length and not by shape. A constant voltage is applied to the system, allowing the negatively charged
DNA molecules to move towards the cathode at the bottom of the gel. Smaller molecules have increased mobility in the porous gel, and therefore move further down the matrix than larger molecules in the same sample.

Once the oligonucleotides move through the gel, they can be visualized using a method called UV shadowing. UV shadowing is a technique in which the gel is quickly illuminated under a UV lamp on a TLC plate. The UV radiation passes through the gel itself but is absorbed by the DNA bands on the gel, creating a shadow where the bands occur (Hassur & Whitlock Jr., 1974). The largest and slowest moving DNA band can then be isolated and removed from the rest of the gel. However, this method can also lead to photo-induced chemical damage of DNA (Kladwang, Hum, & Das, 2012), so UV radiation must be carefully regulated and the bands must be visualized quickly to preserve DNA structure.

The oligonucleotide is removed from the gel matrix through electroelution. The gel fragments are placed in running buffer, and voltage is applied so that the DNA is eluted out of the neutral gel matrix towards the cathode of the system. A filter that blocks particles larger than DNA is placed in front of the cathode so gel fragments and other impurities are not collected. A filter that does not allow DNA to flow through it is placed next to the first filter to create a collection well (Lopez-Gomollon, & Nicolas, 2013).

The DNA is concentrated after collection using ethanol precipitation. Ethanol is added to the collected sample, and the increased polarity of the solution forces precipitation of the DNA. The ethanol supernatant is removed, and the DNA is resolubilized as a final, purified sample (Lopez-Gomollon, & Nicolas, 2013).
2.1.2 Oligonucleotide Purification Methods

Oligonucleotides were purchased from Integrated DNA Technologies. Single strands were purified using denaturing PAGE. For denaturing gel production, a solution of 200 µL 10% APS solution, 25.2 g urea, 6.0 mL 10X TBE, 22.0 mL water, and 31.6 mL 38:2 acrylamide/bis-acrylamide was used. Before polymerization, the urea in solution was dissolved at 50°C on a hot plate and the solution was degassed for 15 minutes through a vacuum source.

Gel plates were assembled according to the figure below. Plates were sealed with Vaseline and clipped together to prevent gel leakage. Extra Vaseline was removed with a bent syringe.

![Set up of denaturing polyacrylamide gel plates](image)

**Figure 2.1.1:** Set up of denaturing polyacrylamide gel plates (Front plate = 20 cm x 20 cm plate, back plate = 20 cm x 22 cm, blue spacer = 0.2 cm)

Before polymerization of the entire gel solution, an acrylamide plug was made to prevent gel leakage from the bottom of the plate. The plug was made from 3.0 mL
of the gel solution and 10.0 µL tetramethylethlenediamine (TEMED). Immediately upon addition of TEMED, the plug was poured between gel plates and was left to fully polymerize for 30 minutes.

After the plug completely polymerized, 50 µL of TEMED was added to the remaining gel solution. The sample was quickly poured between gel plates and a comb was inserted into the top of the gel. The comb was clipped to prevent slipping, and the gel was left to polymerize for one hour.

Once fully polymerized, the blue seal and comb were removed and the gel was transferred and clipped to the electrophoretic apparatus. The wells at the top and bottom of the apparatus were filled with 1X TBE. Air bubbles that formed from the addition of TBE near the bottom of the gel were removed with a bent syringe. The gel was pre-run at 400 V for one hour.

Oligonucleotide samples were prepared as one part oligonucleotide solution and one part loading buffer. The oligonucleotide concentration of the sample was 150 nM. The marker was prepared as one part water and one part loading buffer, with approximately 0.5 mg of dye. Two markers were used, one with coomassie dye and one with bromophenol blue. The final volume of the marker samples was 20 µL.

The oligonucleotide samples were heated at 90°C for three minutes, centrifuged at 6,000 rpm for five seconds, and placed on ice for two minutes. The marker and oligonucleotide samples were then pipetted into respective wells. The gel was run at 400 V for approximately two hours, or when the marker reached the acrylamide plug. The gel was run at room temperature (approximately 25°C).
The plates were removed and the gel was transferred to a saran wrapped TLC plate. The oligonucleotide bands were visualized with a UV lamp at low times of exposure. The largest and slowest moving band was removed from the gel with a razor, cut into small pieces, and saved at 4°C in 1X TBE for electroelution of the oligonucleotide.

Electroelution of the oligonucleotides from the gel was performed either immediately after gel electrophoresis or the following day. The electroelution tank was filled with 1X TBE. The electroelution chamber was filled with the oligonucleotide-containing gel fragments and 1X TBE. Whatman Elutrap BT1 and BT2 membranes were inserted into one side of the chamber to form a collection well for the eluted oligonucleotides. The other side contained only a BT1 membrane to prevent the backflow of DNA into the main tank. The apparatus was assembled so that a continuous flow of TBE would move through the chamber when voltage was applied. The positive electrode was connected to the side of the tank with the oligonucleotide collection well and the voltage was set to 100V. Sample was collected from the well after the first hour of applied voltage and then samples were collected every two hours. A total of five collections were made. To collect the samples, the current direction was reversed for one minute to position the DNA in the middle of the collection well. The sample was then gathered using a gel-tipped pipette as not to puncture the filters.

The eluted oligonucleotides were exchanged with water five times and were concentrated in a Sartorius Vivaspin 3,000 MWCO filtration unit in a centrifuge at 5,000 rpm until the final volume reached 200 µL. The flow through was saved and
stored at 4°C. The DNA concentration of the flow through was determined by monitoring the absorbance at 260 nm using a ThermoFisher Scientific NanoDrop One. If the absorbance intensity at 260 nm was below .05, the flow through was discarded. Otherwise, the sample was re-concentrated and added to the previously concentrated oligonucleotide sample.

For complete purification, the sample was precipitated with ethanol three times. The oligonucleotide sample was defrosted and 450 µL of 100% cold ethanol and 20 µL of 3 M NaCl were added. The sample was frozen for three hours, defrosted, and spun at 13,000 rpm for 30 minutes. The supernatant was removed and the DNA pellet was resuspended in 200 µL of water. After three rounds of precipitation, the oligonucleotide was dialyzed overnight in a ThermoFisher Scientific Slide-A-Lyzer Mini dialysis unit (3,500 MWCO) in 1L water. The oligonucleotide was finally stored at -20°C in water. The DNA concentration of the final sample was determined by monitoring the absorbance at 260 nm on a NanoDrop One.
2.2 DNA Annealing

2.2.1 DNA Annealing Theory

The theory of DNA annealing is heavily based in thermodynamic principle. Proper annealing, or hybridization, of DNA single strands is dependent on the availability of energy to overcome the entropy penalty of shifting from multiple strands in solution to one secondary structure. Thus, a hybridization reaction is enthalpically driven, as the enthalpy change of the reaction is favorable to due base pairing and stacking interactions that occur upon duplex formation.

However, forming single-stranded secondary structure, such as hairpins, competes with the bimolecular process of DNA annealing (SantaLucia Jr., & Hicks). The energy needed to force single strands into the extended conformation is supplied by an increase in temperature. Once secondary structure is broken at high heat, slow cooling of the DNA allows for formation of the more enthalpically stable structure, which, in this case, is the 4WJ.

2.2.2 DNA Annealing Methods

Samples were prepared at 300 nm NaCl, 10mM Tris (pH 8.0), and 1 mM EDTA. Select samples were annealed at 200 mM NaCl, 6.67 mM Tris (pH 8.0), and 0.67 mM EDTA to maintain a 200 mM salt concentration during high DNA concentration absorbance melting. Samples contained equimolar amounts of purified J3 single-strands. All fluorescently labeled junctions were annealed at 5 µM DNA. J3
was annealed at 10 µM DNA, to facilitate high DNA concentration absorbance melting experiments. Samples were placed in a 68°C water bath for six hours and slowly cooled to room temperature (approximately 25°C) in the bath for sixteen hours. 4WJ annealing was verified with native gel electrophoresis, which will be described in the next section of this chapter.

The 4WJ J3 was annealed. Single-stranded sequences are listed below. Nomenclature of the strands is consistent with that of the Duckett group, with each strand named in accordance with the restriction enzyme used to target it (Duckett et al, 1988). The base position of the probe within the single-stranded sequence is indicated by the number in the strand name.

<table>
<thead>
<tr>
<th>Strand Name</th>
<th>DNA Sequence (5' -&gt; 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>JB</td>
<td>CCTCCGTCTAGCAAGGGGCTGCTACCGGAAGGG</td>
</tr>
<tr>
<td>JH</td>
<td>CCCTCCCGGTAGCAGCCTGAGCCTGGTTGAAGGG</td>
</tr>
<tr>
<td>JR</td>
<td>CTTCAACCACCCTCAACTCAACTGCAGTCTGG</td>
</tr>
<tr>
<td>JX</td>
<td>CCAGACTGCAGTTGAGTCTGTAGGACGGAGG</td>
</tr>
<tr>
<td>J3R7-6MAP/2AP</td>
<td>CTTTCAFCCACCCTCAACTCAACTGCAGTCTGG</td>
</tr>
<tr>
<td>J3R18-6MAP</td>
<td>CTTCAACCACCCTCAACTCAACTGCAGTCTGG</td>
</tr>
<tr>
<td>J3R22-6MAP</td>
<td>CTTTCAACCACCCTCAACTCAACTGCAGTCTGG</td>
</tr>
<tr>
<td>J3R28-6MAP/2AP</td>
<td>CTTTCAACCACCCTCAACTCAACTGCAGTCTGG</td>
</tr>
</tbody>
</table>

Table 2.2.1: Oligonucleotide sequences for each arm of the 4WJ. F indicates position of fluorophore

Because J3 was annealed at 300 mM NaCl and fluorescence experiments were performed at 200 mM NaCl, we expect the stacked form of the junction to predominate in solution (Duckett, Murchie, & Lilley, 1990). The stacked conformer of the junction can exist as one of two isomers, referred to as IsoI and IsoII based on
which strands are exchanging and which are continuous (Grainger et al., 1998).
Previous research has demonstrated that J3 favors the stacked IsoII conformer, where H and X strands are continuous, and B and R are exchanging (Joo et al., 2004).

![Figure 2.2.1: Schematic of 4WJ J3 in its open (left) and stacked (right) form. Red circles indicate position of nucleoside analogs, all of which replace adenine bases.](image-url)
2.3 Native Polyacrylamide Gel Electrophoresis

2.3.1 Native Polyacrylamide Gel Electrophoresis Theory

A native polyacrylamide gel is used to verify DNA annealing. In contrast to a denaturing gel, a native gel preserves the secondary structure of DNA as it runs through the gel. Preservation of structure is necessary to visualize the amount of 4WJ and single strands in the sample. Other than the absence of a denaturant, the theory behind native PAGE is the same as denaturing PAGE: samples are separated based on size and shape.

2.3.2 Native Polyacrylamide Gel Electrophoresis Methods

A native 6.5% polyacrylamide gel was used to assess the efficacy of 4WJ annealing. The gel solution was produced with 19.21 mL water, 3.9 mL 29:1 acrylamide/bis-acrylamide, 0.6 mL 10X TBE, 120 uL 10% APS solution. 1.5 mm gel plates were assembled on the apparatus so that the gel solution could be poured in between them without leakage, allowing for efficient polymerization of the gel within the plates. 30 µL of TEMED was added to the gel solution, and the solution was quickly poured between gel plates. A comb was inserted at the top of the gel and the gel was left to polymerize for thirty minutes. Once fully polymerized, the gel was moved to the running apparatus, and the tank was filled with 1X TBE. The comb was removed, and the gel was pre-run at 100 V for thirty minutes at 4°C.
4WJ samples were prepared at 10 nm DNA, with 2 µL Ficoll and 1 µL 10X DNA annealing buffer. The sample volume was brought to 20 µL with water. The ladder was prepared with 8 µL 1X gel loading dye, 2 µL Ficoll, 0.3 µL pbr322 HAE III digest, and 9.7 µL water.

Samples were loaded onto the gel. The gel was run at 100 V at 4°C for approximately one hour, or when the marker reached the bottom of the gel. The gel was removed from the apparatus and stained with 10 µL SYBR Green in water for 15 minutes. Following staining, the gel was removed from solution and imaged used a SynGene GBOX at 1-2s exposure times. The gel image was analyzed with ImageQuant software, which determines the relative sample populations using the thickness and intensity of the bands on the gel. The sample was considered well-annealed if it contained less than 10% of single-stranded DNA. Otherwise, the sample was re-annealed and tested again on another native gel.

2.4 Ultraviolet (UV) Absorbance Spectroscopy

2.4.1 Ultraviolet (UV) Absorbance Spectroscopy Theory

In certain biomolecules, the energy of ultraviolet and visible light allows for electronic transitions to take place upon irradiation. Quantum theory states that these transitions are quantized, and that a molecule will only absorb light at a wavelength that matches the energy difference between the ground and the excited state. The energy required to undergo the transition to the molecular excited state is dependent
on the structural characteristics of a molecule. Thus, the absorbance properties of certain molecules are unique, and can serve to provide characterization of a sample (Tinoco Jr. et al., 2013).

Absorbance is measured as the energy required to promote an electron to an excited state. In absorbance spectroscopy, the commonly measured transitions are $n \rightarrow \pi^*$ transitions and $\pi \rightarrow \pi^*$ transitions. $n \rightarrow \pi^*$ transitions are symmetry “forbidden” due to a non-planar electronic transition. These low energy transitions lead to weak absorbance peaks. $\pi \rightarrow \pi^*$ transitions, which are more likely to occur in conjugated systems, are high energy, planar, and lead to strong absorbance peaks.

![Molecular orbital diagram for a carbonyl group, displaying $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions (Boyer, 2011)](image)

**Figure 2.4.1:** Molecular orbital diagram for a carbonyl group, displaying $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions (Boyer, 2011)

The nucleotide bases of DNA are conjugated ring structures that absorb strongly in the UV. Due to differences in conjugated $\pi$ systems between each nucleic acid base, AT and GC base pairs absorb at the same wavelengths, but with different intensities. While the exact maximum absorbance wavelength of a DNA strand is sequence-dependent because of the difference in base pair properties, DNA absorbance is typically monitored at 260 nm (Puglisi & Tinoco Jr., 1989).
Figure 2.4.2: Extinction coefficient as a function of wavelength for AT and GC base pairs (Puglisi & Tinoco Jr., 1989)

Beyond understanding the absorbance properties of a molecule, it is also important to understand how the absorbance of a molecule can be measured through spectroscopic methods. Absorbance of light at a certain wavelength by a molecule is quantized as the negative log of transmittance, where transmittance is the light that passes through the sample divided by the overall light intensity used by the spectrophotometer.

\[
A = -\log \left( \frac{I}{I_0} \right) = \varepsilon cl
\]

**Equation 2.4.1:** Defining absorbance through the Beer-Lambert law (Cantor & Schimmel, 1980) where \( I \) = intensity of light that passes through the sample, \( I_0 \) = intensity of incident light, \( \varepsilon \) = extinction coefficient, \( c \) = concentration, and \( l \) = path length of the measurement
The extinction coefficient ($\varepsilon$) is a measure of how strongly a molecule absorbs light at a given wavelength. It may be calculated for a molecule by monitoring the absorbance over a range of concentrations, since the Beer-Lambert law of absorbance states that concentration and absorbance are linearly related.

It is important to consider the consequence of the logarithmic definition of absorbance. The absorbance value that is obtained by the spectrometer must be between 0.1 – 1.0 if it is to be considered reliable. Values below 0.1 indicate a sample that is too dilute to yield trustworthy absorbance values, while values above 1.0 indicate that a sample is too concentrated to allow for a proper measurement of transmitted light.

### 2.4.2 UV Absorbance Spectroscopy Methods

UV absorbance melts were conducted on J3 at various DNA concentrations. All samples were prepared to a total volume of 35 µL with a 200 mM NaCl, 6.67 mM Tris (pH 8.0), and 0.67 mM EDTA buffer. Samples were covered with Parafilm “M” laboratory film and bubbled with helium for four minutes prior to collection of absorbance measurements to homogenize the environment of the solution. The samples were transferred to Beckman T_m cells and sealed with Teflon tape surrounding the cap to create a tight seal around the opening of the cell.

UV absorbance melts were also conducted on fluorescently labeled junctions. The procedure was conducted as described above, with two notable exceptions.
Sample volume was larger, at 350 µL, and DNA concentration was held constant at 1.18 µM.

A Beckman DU 650 spectrophotometer was used to monitor melting. The sample chamber was purged with nitrogen to prevent condensation and sample oxygenation. For concentration-dependent absorbance melts, the path length of the Tm cells was 1 mm to allow for absorbance values in the linear range at high DNA concentrations. For melts of fluorescently-labeled junctions, cells with a 1 cm path length were used. Absorbance was monitored at 260 nm and at 280 nm. DNA was melted from 30°C to 90°C with collection in 1°C increments. The ramp rate for the experiment was 1°C/minute.

2.5 Temperature-Dependent Fluorescence Spectroscopy

2.5.1 Temperature-Dependent Fluorescence Spectroscopy Theory

![Jablonski diagram](image)

**Figure 2.5.1:** Jablonski diagram (Lakowicz, 2006)

The figure above, referred to as a Jablonski diagram after physicist Alexander Jablonski, is a simplified view of the possible electronic transition pathways upon
excitation and relaxation. Upon absorption of light, an electron is excited to a higher energy state, called the excited state. A molecule is fluorescent if its electronic relaxation from the first singlet excited state to the singlet ground state occurs slowly through radiative emission (Lakowicz, 2006). In this case, energy is released as a photon, which can be monitored through spectroscopic techniques.

Fluorescence, or radiative decay, is only one of several relaxation pathways. Nonradiative decay pathways include internal conversion of energy and intersystem crossing to the triplet state, from where phosphorescence can occur (Guilbault, 1990). The prominent electronic decay pathway of a molecule, and therefore its capacity to fluoresce, is structurally dependent.

For the greatest likelihood of fluorescence, an electronic transition must be relatively low energy and the electron must not be excited to an orbital heavily involved in intermolecular bonding. There are also certain functional groups that can quench fluorescence (Guilbault, 1990). Typical fluorophores that fit these structural requirements are molecules with extended conjugated $\pi$ systems, such as aromatic compounds. The symmetry allowed $\pi \rightarrow \pi^*$ transition in a conjugated system is low energy, and provides a stable excited state to allow for relatively slow relaxation through the fluorescent release of a photon.

The fluorescence of a particular molecule is measured through quantum yield, which is the ratio of photons emitted to those absorbed (Lakowicz, 2006)

$$Q = \frac{\Gamma}{\Gamma + k_{nr}}$$

**Equation 2.5.1:** Fluorescence quantum yield, where $\Gamma$ = rate of radiative emission and $k_{nr}$ = rate of nonradiative emission (Lakowicz, 2006)
Though nucleic acid bases retain some of the properties of commonly fluorescent molecules, they have extremely short-lived excited states, developed to protect the genetic code against photo-induced damage (Jones & Neely, 2015). The fleeting existence of the nucleic acid excited state prevents fluorescent relaxation, and instead promotes decay through rapid internal conversion of energy. Thus, to monitor structural changes of DNA through fluorescence, a fluorophore must either be attached to the end of the structure or inserted into the sequence.

2.5.2 Temperature-Dependent Fluorescence Spectroscopy Methods

After 4WJ annealing was confirmed with a native gel, samples of fluorescently labeled junctions were prepared at a concentration of 200 nM DNA, 200 mM NaCl, 200 mM NaCl, 6.67 mM Tris (pH 8.0), and 0.67 mM EDTA. Samples were bubbled with helium for four minutes prior to fluorescence measurements to prevent collisional quenching of the sample by molecular oxygen in solution. A Fluoromax-4 spectrofluorometer was used to measure fluorescence.

For 6MAP-labeled junctions, samples were excited at 333 nm and emission was monitored at 420 nm. For 2AP-labeled junctions, samples were excited at 307 nm and emission was monitored at 385 nm. Background emission was monitored by excitation at 320 nm and emission at 600 nm. DNA was melted from 30°C to 90°C with collection in 1°C increments. Two minutes of equilibration time was allowed between collections with a 0.2°C tolerance range.
2.6 Fluorescence Quenching Titration Theory

2.6.1 Fluorescence Quenching Titration Theory

Collisional quenching of fluorescence is quantified through the Stern-Volmer equation.

\[
\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q]
\]

**Equation 2.6.1:** Stern-Volmer equation for collisional quenching, where \(F_0\) = initial fluorescence, \(F\) = fluorescence at quencher concentration \([Q]\), \(\tau_0\) = lifetime of the fluorophore in the absence of quencher, \(k_q\) = bimolecular quenching constant, and \(K_{SV}\) = Stern-Volmer quenching constant, representative of the degree of fluorescence quenching (Lakowicz, 2006)

Commonly, the Stern-Volmer equation is plotted as \(F_0/F\) vs. \([Q]\), where the slope of the linear plot is equal to \(K_{SV}\). It is possible to learn more about the nature of the quenching mechanism from the shape of this plot. As static quenching is based on the proximity of quencher and fluorophore upon excitation, both static quenching and collisional quenching will increase as a function of quencher concentration. Thus, the presence of both quenching mechanisms means that a Stern-Volmer plot will deviate from linearity as an upward curve (Laws & Contino, 1992).

Alternatively, the Stern-Volmer plot may deviate from linearity as an upward curve when there are multiple populations of a molecule with a fluorophore in solution. There exists the possibility that a fraction of the fluorophores is accessible to the solvent, while the other fraction is inaccessible (Lakowicz, 2006). Fractional
accessibility leads to a plateau in the Stern-Volmer plot, because the entirety of the exposed fraction eventually becomes quenched while there is no change in the buried fraction. The typical Stern-Volmer plot can then be modified to fit the curve and to understand the properties of the accessible fraction of fluorophores.

\[
F = \frac{F_{0a}}{1 + K_a [Q]} + F_{0b}
\]

**Equation 2.6.2:** Modified Stern-Volmer equation, where \(F_{0a}\) = fluorescence of the accessible fraction in the absence of quencher (e.g. \([Q]=0\)), \(F_{0b}\) = fluorescence of the inaccessible fraction in the absence of quencher, and \(K_a\) = the Stern-Volmer quenching constant of the accessible fraction (Lakowicz, 2006)

In a modified Stern-Volmer plot, \(F\) is plotted against \([Q]\) and fit with the above equation. The fit parameters \(F_{0a}\), \(F_{0b}\), and \(K_a\) can then be derived (Augustyn, 2002).

### 2.6.2 Fluorescence Quenching Titration Methods

Fluorescently-labeled junction samples were prepared at 100 nM DNA in 200 mM KCl, 6.67 mM Tris, and 0.67 mM EDTA. Samples were bubbled with helium for four minutes prior to fluorescence measurements. Potassium iodide (KI), a known quencher of fluorescence, was titrated into the sample until \([KI] \approx 150\) mM, while maintaining the ionic potassium concentration at 200 mM and the DNA concentration at 100 nm. Using a Fluoromax-4 spectrofluorometer, fluorescence was recorded after each addition from 350 nm – 500 nm for 6MAP-labeled junctions and from 345 nm - 500 nm for 2AP-labeled junctions. 6MAP-labeled junctions were excited at 333 nm
and 2AP-labeled junctions were excited at 307 nm. Eight total aliquots of KI were titrated into the sample solutions.
Chapter 3
Results

3.1 Experimental Summary

Figure 3.1.1: From left to right: Open structure of J3, stacked isoII conformer of J3, and stacked-X view of J3 isoII. Generated using 3DNA software (Lu & Olson, 2003) and (Karymov et al., 2008)

This work will discuss four sets of experiments. The first is DNA concentration-dependent absorbance melting, with which we have characterized thermodynamic parameters of the J3 melting process. The second set is base-specific fluorescence melting with the fluorescent nucleoside analogs 2AP and 6MAP, incorporated at 7th and 28th base position of the exchanging R-strand. These substrates will be referred to as J3R7 and J3R28, respectively. These experiments probe the junction melting process at different sites. The third set is fluorescence quenching titrations with J3R7 and J3R28. The fourth and final set of experiments is fluorescence melting with 6MAP probes in the 18th, 22nd, and 28th position of the R-strand. These substrates will be referred to as J3R18, J3R22, and J3R28, respectively.
3.2 DNA Concentration-Dependent Absorbance Melting Results

The unlabeled 4WJ J3 was melted over a range of DNA concentrations from 1.29 µM to 7.82 µM. Using these trials, we intend to derive well-characterized values for the thermodynamic parameters of the 4WJ melting process. We also intend to demonstrate that the melting of J3 to four single strands is, in fact, multimolecular. The molecularity of the melting reaction can be determined by the change in $T_M$ as higher DNA concentrations are approached. If the $T_M$ increases with an increase in DNA concentration, the reaction is said to be intermolecular, while the $T_M$ will not vary with DNA concentration for an intramolecular reaction (Puglisi & Tinoco, 1989). The following experiments demonstrate that the transition observed is intermolecular, and not the product of an intramolecular side reaction, such as hairpin or intramolecular junction melting.

![Graph showing absorbance melting curves](image)

**Figure 3.2.1:** Raw absorbance melting curves of J3 at varying DNA concentrations (Figure legend represents DNA concentration)
Raw absorbance curves were analyzed using the method outlined in Chapter 1 to obtain a plot of $\alpha$, or fraction of junction as a function of temperature at each concentration. DNA concentration was determined by applying the Beer-Lambert law to the last point on the melting curve (at 89-90°C). For the following analyses, we have averaged the results at 6.56 µM and 6.61 µM, indicated by a trial at 6.58 µM.

![Absorbance curve](image)

**Figure 3.2.2:** Normalized absorbance melting curves for J3 at varying DNA concentrations (Figure legend represents DNA concentration)

Normalization of the melting curves allowed for determination of $K_{eq}$ at each temperature, calculated using Equation 1.4.1. Note that Equation 1.4.1 gives $K_{eq}$ for junction formation. Since 4WJ melting is an equilibrium reaction, we will take the inverse of $K_{eq}$ of formation as $K_{eq}$ of melting. Once the equilibrium constants for all points in the melting transition were determined, we constructed Van’t Hoff plots for each transition in accordance with Equation 1.4.4.
Figure 3.2.3: Van’t Hoff plots for J3 melting at varying DNA concentrations (Figure legend represents DNA concentration)

Each linear fit had an R-squared value above 0.98. Thermodynamic parameters of the melting transition at each DNA concentration were obtained from the slope and intercept of the linear fit. Error in these parameters were calculated as shown below using the error obtained from the linear fit of the Van’t Hoff plot and the experimental error between the two trials at 6.58 µM.

\[ \sqrt{(Experimental\ error)^2 + (Fit\ error)^2} \]

Equation 3.2.1: \(\Delta H\) and \(\Delta S\) error calculation

Change in enthalpy upon 4WJ melting was positive, as heat energy is required to break the interstrand hydrogen bonds. Change in entropy was also positive, as the multiplicity of states in solution increases when the singular junction melts into its four component strands.
<table>
<thead>
<tr>
<th>DNA Concentration (µM)</th>
<th>ΔH (± 46.8 kcal/mol)</th>
<th>ΔS (± .14 kcal/mol K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.29</td>
<td>479.5</td>
<td>1.32</td>
</tr>
<tr>
<td>1.54</td>
<td>425.5</td>
<td>1.16</td>
</tr>
<tr>
<td>2.24</td>
<td>390.4</td>
<td>1.06</td>
</tr>
<tr>
<td>3.40</td>
<td>454.8</td>
<td>1.25</td>
</tr>
<tr>
<td>4.46</td>
<td>424.8</td>
<td>1.16</td>
</tr>
<tr>
<td>5.46</td>
<td>366.4</td>
<td>0.99</td>
</tr>
<tr>
<td>6.58</td>
<td>415.9</td>
<td>1.13</td>
</tr>
<tr>
<td>7.02</td>
<td>400.8</td>
<td>1.09</td>
</tr>
<tr>
<td>7.82</td>
<td>399.4</td>
<td>1.09</td>
</tr>
</tbody>
</table>

**Table 3.2.1**: Thermodynamic parameters obtained from Van’t Hoff plots

For additional analysis, we compared melting temperatures between trials. To calculate $T_M$, the melting curves were fit with a sigmoid function using software Origin 9.1. All curve fits had R-squared values above 0.98. $T_M$ was recorded at the temperature where the sigmoidal fit equation equaled 0.5.

**Figure 3.2.4**: Melting temperatures of the 4WJ at varying DNA concentrations. Error in $T_M$ was calculated using Equation 3.2.1, with experimental error in $T_M$ between the two trials at 6.58 µM and the error in the Boltzmann fit at 6.58 µM.
$T_M$ increased with increasing concentration, indicating the occurrence of an intermolecular reaction. It is possible that the high concentration of sample at 7.02 µM and 7.82 µM led to some experimental issues in recording absorbance as absorbance values approached 1.0.

We proceeded with this data by utilizing the relationship between melting temperature and DNA concentration, as described by Puglisi and Tinoco Jr., to gain a holistic characterization of the thermodynamic parameters of the melting process. Equation 3.2.2 relates enthalpy, entropy, DNA concentration, and melting temperature for a tetramolecular melting process. This equation is derived using the $K_{eq}$ for 4WJ melting and the Van’t Hoff equation. In the following derivation, $C_T$ is representative of the total concentration of DNA strands.

\[
K_{eq} = \frac{\left(\frac{C_T}{4}\right)^3 (1 - \alpha)^4}{\alpha}
\]

At the $T_M$, $\alpha = 0.5$. $K_{eq}$ becomes:

\[
K_{eq} = \frac{\left(\frac{C_T}{4}\right)^3 (.5)^4}{.5} = \frac{C_T^3}{512}
\]

The natural log of this equilibrium constant, which will be utilized in the following steps, is:

\[
\ln(K_{eq}) = 3 \ln(C_T) - 3 \ln 8
\]
By combining the Van’t Hoff equation at the $T_M$ and the natural log of the above $K_{eq}$, we arrive at:

$$\ln(K_{eq}) = \frac{1}{T_M} \left( -\frac{\Delta H}{R} \right) + \left( \frac{\Delta S}{R} \right) = 3 \ln(C_T) - 3 \ln 8$$

Rearrangement of the equation above brings us to Equation 3.2.2, which allows for direct analysis of the relationship between $T_M$ and $C_T$. It also allows us to extract values for enthalpy and entropy from the respective slope and intercept of the linear fit of $\ln(C_T)$ vs. $1/T_M$.

$$\frac{1}{T_M} = -\frac{3R}{\Delta H} \ln(C_T) + \left( \frac{\Delta S - 3R \ln(8)}{\Delta H} \right)$$

**Equation 3.2.2:** Relation of total DNA concentration and thermodynamic parameters $\Delta H$ and $\Delta S$ for a non-self-complementary, tetramolecular process (Puglisi & Tinoco Jr., 1989)
Figure 3.2.5: Plot of ln(Cₜ) vs. 1/Tₘ for nine absorbance melt trials at varying Cₜ

ΔH and ΔS were calculated from the linear fit of this plot. The values are within range of most of the parameters obtained through the analysis of individual trials, further indicating that the concentration-dependent analysis provides well-characterized values for enthalpy and entropy of the melting process. Error in ΔH and ΔS were calculated using the error in the slope and intercept from the linear fit. Error in ΔG was calculated using the sum of the percent errors in ΔH and ΔS.

<table>
<thead>
<tr>
<th>ΔH (kcal/mol)</th>
<th>ΔS (kcal/mol K)</th>
<th>ΔG₅ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>363 ± 70</td>
<td>.98 ± .22</td>
<td>90 ± 38</td>
</tr>
</tbody>
</table>

Table 3.2.2: Thermodynamic parameters obtained from linear fit of ln(Cₜ) vs. 1/Tₘ
3.3 J3R7 and J3R28 2AP/6MAP Fluorescence Melting

The probe 2AP was introduced into the same loci as 6MAP, with one probe on each pseudo-duplex, to test the initial fluorescence melting results with 6MAP that led to the development of the pseudo-duplex melting model (Savage, 2017) as described in Chapter 1.4.2. Fluorescence melting experiments were performed on J3R7 2AP, J3R28 2AP, J3R7 6MAP, and J3R8 6MAP.

**Figure 3.3.1:** Stacked-X structure of J3, colored by pseudo-duplex. H-pseudo-duplex/J3R7 probe = black, X-pseudo-duplex/J3R28 probe = red. The probes are shown as space filling spheres at their respective location on the exchanging R-strand.

The 2AP and 6MAP probes were incorporated at the same positions.

The labeled junctions were first melted while monitoring absorbance at 260 nm to determine if the probes had altered global stability of the junction. A drastic change in global stability or structure would prevent observation of native 4WJ melting.
Figure 3.3.2: Raw absorbance melting curves for 6MAP-labeled junctions, 2AP-labeled junctions, and unlabeled junction J3

Raw absorbance curves were normalized to compare the melting of labeled and unlabeled junctions.
Figure 3.3.3: Normalized absorbance melting curves (top) and melting temperatures (bottom) for 2AP labeled J3R7 and J3R28 with reference to unlabeled J3. Error calculated as error in Boltzmann fit of $\alpha$ average.

2AP-labeled junctions did not undergo a change in stability, which is consistent with previous results for adenine substitutions in large oligomers (Law et al. 1996). Interestingly, the 2AP-labeled junctions were slightly more thermodynamically stable than unlabeled junction.
Figure 3.3.4: Normalized absorbance melting curves (top) and melting temperatures (bottom) for 6MAP labeled J3R7 and J3R28 with reference to unlabeled J3. Error calculated as in Figure 3.3.3.

As demonstrated previously (Savage, 2017), the insertion of 6MAP into J3 decreases its melting temperature by approximately 1°C. This slight instability does not seem to be significant enough to affect the overall structure of the junction, but nonetheless will be considered in the analysis of fluorescence data. After global stability of all labeled junctions was assessed, fluorescence melts were performed.
The raw fluorescence of the probes showed promising melting transitions. It is likely that a tear in the cuvette cap allowed for evaporation of J3R28 2AP at higher temperatures, leading to the second increase in fluorescence. The normalization process used for absorbance curves was then applied to normalize fluorescence curves.
Figure 3.3.6: Normalized fluorescence melting transitions for J3R7 and J3R28 with 2AP (top left) and 6MAP labels (top right). Bottom figure displays relative melting behaviors of 2AP and 6MAP probes, as well as probe location in the stacked-X structure.

The relative melting curves display destabilization at the J3R28 position at a lower temperature than at the J3R7 position, indicating X-pseudo-duplex destabilization at a lower temperature than H-pseudo-duplex destabilization. To
understand the cooperativity of each melting transition, fluorescence melting curves were fit with a sigmoidal function.

![Melting curve graphs](image)

**Figure 3.3.7:** Normalized melting curves for all four fluorescently-labeled junctions and corresponding Boltzmann fits (blue)

The baselines of the 6MAP melting curves were not optimal for sigmoidal fitting, making them difficult to analyze in terms of cooperativity. We can attribute the poor baselines of the 6MAP curves to the large slope of the baseline in the raw fluorescence data. While fitting our baselines during data normalization, we encountered a problem where the sloping baselines forced an intersection of our baseline fits, causing large fluctuations in $\alpha$ at low temperatures. Thus, it is difficult to obtain clean normalized baselines for these probes. However, the deviation of the
2AP fluorescence melting curves from sigmoidality, especially in comparison to absorbance melting curves, likely indicates some element of non-cooperativity in local melting transitions.

Finally, the thermodynamics of the melting process of all four labeled junctions were analyzed with Van’t Hoff plots. Linear fits for all plots had an R-squared value above 0.97.

Figure 3.3.8: Van’t Hoff plots for 2AP and 6MAP-labeled J3R7 and J3R28
Table 3.3.1: Thermodynamic parameters of 2AP and 6MAP-labeled J3R7 and J3R28 melting process. Error in ΔH and ΔS were determined from fit error and trial error. Error in ΔG was determined using percent errors in ΔH and ΔS.

<table>
<thead>
<tr>
<th></th>
<th>ΔH (kcal/mol)</th>
<th>ΔS (kcal/mol K)</th>
<th>ΔG (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J3R7 2AP</td>
<td>338 ± 28</td>
<td>0.895 ± 0.08</td>
<td>84 ± 14</td>
</tr>
<tr>
<td>J3R28 2AP</td>
<td>301 ± 28</td>
<td>0.786 ± 0.08</td>
<td>81 ± 16</td>
</tr>
<tr>
<td>J3R7 6MAP</td>
<td>252 ± 21</td>
<td>0.647 ± 0.06</td>
<td>72 ± 13</td>
</tr>
<tr>
<td>J3R28 6MAP</td>
<td>198 ± 19</td>
<td>0.487 ± 0.05</td>
<td>63 ± 13</td>
</tr>
</tbody>
</table>

Reporting from both probes indicated that the change in enthalpy, entropy, and free energy (at 5°C) were smaller for J3R28 than for J3R7. This result suggests that less energy is needed to destabilize the junction at the R28 position (X-pseudo-duplex) than at the R7 position (H-pseudo-duplex). Thus, both sets of probes support the pseudo-duplex model of 4WJ melting, which predicts that the X-pseudo-duplex is less stable than the H-pseudo-duplex and melts at a lower temperature in the melting process.

3.4 J3R7 and J3R28 Fluorescence Quenching

While incorporating 2AP into the 4WJ in the same position at 6MAP may corroborate 6MAP reporting, there still remains the question of solvent exposure of each of these probes. It is possible that the probes could not be properly incorporated into the junction due to difference in sequence or structure at either of the two positions. Improper incorporation may lead to solvent exposure and unreliable
fluorescence reporting. Thus, a fluorescence quenching titration was performed for four labeled junctions: J3R7 2AP, J3R28 2AP, J3R7 6MAP, and J3R28 6MAP.

**Figure 3.4.1:** Emission scans for fluorescently labeled junctions. 2AP-labeled junctions were excited at 307 nm and 6MAP-labeled junctions were excited at 333 nm. Figure legend indicates [KI]

Fluorescence intensities were extracted at 420 nm for 6MAP-labeled junctions and at 385 nm for 2AP-labeled junctions. These values were analyzed as a function of quencher concentration using Stern-Volmer plots.
The upward curvature of the Stern-Volmer plots suggest that some population of probes in all four positions is solvent exposed. Because the 6MAP data displays a somewhat linear relationship, we fit $F_0/F$ vs. [KI] with the linear Stern-Volmer equation (Equation 2.6.1) and $F$ vs. [KI] with a modified Stern-Volmer curve fit (Equation 2.6.2). In both cases, the $R^2$ value was greater for the curve fit, indicating a
closer adherence to the modified Stern-Volmer equation. Thus, we proceeded to analyze fractional accessibility by constructing modified Stern-Volmer plots, or Lehrer plots. $R^2$ values for all curve fits were greater than 0.95.

**Figure 3.4.3:** Lehrer plots and fits for fluorescently labeled junctions
We determined $F_0a$, the initial fluorescence intensity of exposed probes, and $K_a$, the quenching constant of the accessible fraction, from the curve fit. By dividing $F_0a/F_0$, we obtain $F_a$, the fraction of probes exposed to the solvent.

<table>
<thead>
<tr>
<th></th>
<th>J3R7 6MAP</th>
<th>J3R28 6MAP</th>
<th>J3R7 2AP</th>
<th>J3R28 2AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_a$</td>
<td>.86 ± .04</td>
<td>.77 ± .06</td>
<td>.66 ± .08</td>
<td>.71 ± .07</td>
</tr>
<tr>
<td>$K_a$</td>
<td>30.3 ± 5.1</td>
<td>23.5 ± 6.3</td>
<td>20.2 ± 7.5</td>
<td>20.6 ± 6.3</td>
</tr>
</tbody>
</table>

**Table 3.4.1:** Fluorescence quenching parameters for fluorescently labeled junctions. Error calculated from trial error and error in curve fit.

6MAP and 2AP probes displayed slightly different degrees of solvent exposure. This discrepancy is likely due to the differences in base stacking between the two analogs. More significantly, these results indicate that the probes in both pseudo-duplexes have similar local environments, as the J3R7 and J3R28 positions for both probes yield quenching parameters within error of one another.

3.5 J3R18, J3R22, and J3R28 6MAP Fluorescence Melting

Previous fluorescence results demonstrated that the two pseudo-duplexes of the 4WJ had differing thermostabilities, which was consistent with the melting behavior predicted by the 3SPN.2 model (Savage, 2017). We seek to improve upon the pseudo-duplex model and test other melting predictions made by the 3SPN.2 simulations.
Firstly, it is important to note in the above figure that the 3SPN.2 model predicts the center of the junction to be a point of instability, which deviates from the experimentally obtained data outlined within this section. This discrepancy will be discussed further in Chapter 4 of this work. More significantly, the above figure indicates that the 3SPN.2 model predicts that both pseudo-duplexes show decreased stability at the end of each arm. Because the X-pseudo-duplex melts at an earlier temperature than the H-pseudo-duplex, the following experiment was designed to investigate the initial melting transition of the junction.

6MAP probes were incorporated along the JR arm of J3. Probes in the R18, R22, and R28 positions provide a more nuanced understanding of the pseudo-duplex melting model. With this placement, we have observed step-wise melting in the JR/JX arm, occurring from the end of the arm toward the center of the junction.
Figure 3.5.2: Stacked-X structure of J3, colored by pseudo-duplex. H-pseudo-duplex = black, X-pseudo-duplex = red. 6MAP probe positions J3R18 (purple), J3R22 (green), and J3R28 (red) are shown as spheres.

While previous experiments with J3R28 had already been performed, the following analyses use newly-collected fluorescence data for J3R28 so as to preserve experimental conditions between all three labeled junctions. As with all fluorescently labeled junctions, we first performed absorbance melts to observe changes in global stability.

Figure 3.5.3: Raw absorbance melting curves for J3R18, J3R22, and J3R28 6MAP-labeled J3
**Figure 3.5.4:** Normalized absorbance melting curves for J3R18, J3R22, and J3R28 6MAP-labeled J3 with unlabeled J3 for reference

Melting temperatures of each junction were extracted with a sigmoidal fit of the melting curves.

**Figure 3.5.5:** Melting temperatures of J3R18, J3R22, and J3R28 6MAP-labeled J3 with unlabeled J3 for reference
As consistent with previous thermodynamic results for 6MAP-labeled J3R7 and J3R28, J3R18 and J3R22 were slightly less stable than J3. Interestingly, the introduction of a fluorescent probe at the center of the junction did not additionally decrease the stability of the overall structure. We proceeded with fluorescence melting experiments.

![Figure 3.5.6: Raw fluorescence melting curves for 6MAP-labeled J3R18, J3R22, and J3R28. Red circle indicates consistent increase in fluorescence around 43°C for J3R22.](image)

The melting transitions of the three junctions occurred at higher temperatures than the previously observed transitions in other labeled junctions. We believe that this discrepancy was likely caused by a difference in buffer solution between these trials and previous experiments. This increased melting temperature should not affect relative trends in thermostability, but will be considered in our evaluation of thermodynamic parameters of each junction melting process.
It is immediately apparent that fluorescence of J3R22 is quenched relative to J3R18 and J3R28. Additionally, there was a consistent increase in fluorescence at approximately 43°C for J3R22, potentially indicating an element of secondary structure in either the single-stranded or junction form. To investigate this possibility, we conducted fluorescence melts on fluorescently labeled single strands R18, R22, and R28.

![Figure 3.5.7: Single stranded fluorescence melts for R18, R22, and R28](image)

Both R18 and R28 decreased in fluorescence due to an increased internal conversion of energy as temperature increased. R22 however, increased in fluorescence and also displayed the same noted increase at 43°C. Thus, the transition observed at 43°C in our raw fluorescence results can be attributed to a small population of single-stranded R22 in the sample, and not to a structural rearrangement of J3. We therefore proceeded with analysis of our fluorescence data.
Figure 3.5.8: Normalized fluorescence melting curves for J3R18, J3R22, and J3R28 6MAP-labeled J3, with inset displaying probe location within the stacked-X J3 structure

We encountered the same problem of intersecting linear fits of 6MAP baselines as described in section 3.3. The x-axis of the $\alpha$ plot above is cropped so as to display the more linear section of the native baseline. The trend displayed by these melting curves indicates that the R28 position experiences a reduction in stacking at an earlier temperature than R18 and R22, and that the R18 and R22 positions ultimately melt cooperatively. Additionally, the coincidence of the melting curves of J3R18 and J3R28 at the start of the melting transition may be indicative of a pre-melting transition at the center of the junction. Melting trends and the shape of melting curves will be discussed further in Chapter 4.

These fluorescence melting curves fit to a sigmoidal function more closely than previous fluorescence trials with J3R7 and J3R28, but still deviate from
sigmoidality at the beginning and end of the melting transition. These results indicate a higher element of cooperativity than was observed in J3R7 and previous J3R28 melts.

**Figure 3.5.9:** Normalized fluorescence melting curves for 6MAP-labeled J3R18, J3R22, and J3R28 and their sigmoidal fits (blue)

Finally, the thermodynamics of J3R18, J3R22, and J3R28 melting processes were analyzed using Van’t Hoff plots. All linear fits had an R-squared value above 0.96.
**Figure 3.5.10:** Van’t Hoff plots for 6MAP-labeled J3R18, J3R22, and J3R28

<table>
<thead>
<tr>
<th></th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$ (kcal/mol K)</th>
<th>$G_5$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>J3R18</strong></td>
<td>368 ± 86</td>
<td>0.97 ± .25</td>
<td>96 ± 47</td>
</tr>
<tr>
<td><strong>J3R22</strong></td>
<td>627 ± 63</td>
<td>1.74 ± .18</td>
<td>144 ± 31</td>
</tr>
<tr>
<td><strong>J3R28</strong></td>
<td>536 ± 76</td>
<td>1.47 ± .22</td>
<td>127 ± 39</td>
</tr>
</tbody>
</table>

**Table 3.5.1:** Thermodynamic parameters of 6MAP-labeled J3R18, J3R22, and J3R28 melting processes. Error in $\Delta H$ and $\Delta S$ determined from fit error and trial error. Error in $\Delta G$ determined using percent errors in $\Delta H$ and $\Delta S$.

Initial results are mostly consistent with predictions of the 3SPN.2 model. J3R22 stability is increased relative to J3R28 and J3R18. J3R18, the center of the junction, is less thermodynamically stable than the end of the duplex, J3R28. However, it is also important to consider the shapes of the melting curves in terms of stability. The melting curve for J3R18 indicates that complete destabilization does not occur until J3R22 melts, suggesting an element of stability not captured in the predictions of the 3SPN.2 model. The implications of these results will be discussed in the following chapter.
Chapter 4
Discussion

4.1 An Overall Characterization of the J3 Melting Process

We have characterized the changes in enthalpy, entropy, and free energy for the melting process of the DNA four-way junction J3 using UV-absorbance spectroscopy. While the global perspective of absorbance spectroscopy cannot directly support the pseudo-duplex melting model, this characterization provides values for the global thermodynamics of the process and indicates that J3, as expected, is a relatively stable DNA secondary and tertiary structure. Going forward, we will use the thermodynamic values obtained from our absorbance experiments in comparison to those obtained from calorimetric experiments. We will combine this thermodynamic characterization with the structural information gathered from fluorescence experiments to fully describe the J3 melting process.

4.2 Revisiting the Pseudo-Duplex Melting Model

4.2.1 Fluorescence Spectroscopy Data and Pseudo-Duplex Melting

We also monitored the 4WJ melting process from a more nuanced perspective, gaining single base resolution through the use of fluorescent nucleoside analogs. Our evidence suggests that these analogs are useful tools in understanding the structural dynamics of the DNA four-way junction. By investigating the reliability
of the inserted probes through solvent exposure experiments and the usage of both 2AP and 6MAP probes, we have successfully determined that the reported melting behaviors are characteristic of the 4WJ.

Additional experiments in this work have served to refine the proposed pseudo-duplex melting model. Previous work has shown that the entire X-pseudo-duplex, which has a lower GC content than the H-pseudo-duplex, destabilizes at a lower temperature than the H-pseudo-duplex (Savage, 2017).

\[
\begin{array}{|c|c|c|}
\hline
\text{Strand} & T_M (+/- 1.4 \degree C) & GC Content (%) \\
\hline
JB & 71.5 & 67.6 \\
JH & 71.9 & 67.6 \\
JR & 67.1 & 55.9 \\
JX & 67.4 & 58.8 \\
\hline
\end{array}
\]

**Table 4.2.1:** Estimated $T_M$s and GC contents of J3 single-strands from Integrated DNA Technologies. JB, JH, and JR compose the H-pseudo-duplex and JB, JR, and JX compose the X-pseudo-duplex.

The stepwise, pseudo-duplex melting pattern suggested that the non-cooperativity of the melting process is based on differences in sequence between the pseudo-duplexes. However, initial results did not report on the relative stability of an individual duplex arm.
Figure 4.2.1: Sequence labeled J3 in terms of pseudo-duplexes (Black = H-pseudo-duplex and red = X-pseudo-duplex)

Incorporation of 6MAP probes into the exchanging strand of the X-pseudo-duplex in this work has displayed that destabilization begins at the end of the JR/JX arm and continues down the helix as temperature increases. A caveat to the stepwise process is a pre-melting transition in the center of the junction as indicated by the fluorescence reporting of J3R18.

The fluorescence melting curve of J3R18 showed initial destabilization at the same temperature as J3R28, but did not show complete melting until J3R22 melted. This may be interpreted to indicate a “breathing” type behavior, where the stability in the center of the junction fluctuates before total melting. The cooperativity of the melting transition can be further assessed through the derivative of the melting curve.
A more negative minimum of the derivative curve indicates a more cooperative process, as a more intense peak indicates a steeper slope to the melting curve. Because fully cooperative processes occur over a smaller temperature range than non-cooperative processes, the slope of the melting transition is steeper if the melting transition is more cooperative. We may also infer an element of non-cooperativity from a derivative curve by the presence of a shoulder. This means that there is a change in the rate of melting during the transition, which would not occur during a cooperative melting process. In other words, cooperativity in the melting transition is indicated by a smooth, symmetrical first derivative curve.

**Figure 4.2.2:** First derivative curves of 6MAP-labeled J3R18, J3R22, and J3R28. Bottom right figure shows normalized overlay of three derivative curves.
Clear elements of non-cooperativity are displayed by the asymmetrical derivative curves of J3R18 and J3R28, while the melting transition of J3R22 occurs relatively cooperatively. Thus, these curves indicate that J3R18 and J3R28 undergo pre-melting transitions at a lower temperature than the melting of the global structure, while J3R22 melts along with the global structure.

Because the minimum of J3R18 is smaller in magnitude than that of J3R22 and J3R28, it is likely that J3R18 exhibits more non-cooperative melting than J3R28. We may interpret this result to support the idea that opening of the center of the junction occurs early in the melting transition, but complete destabilization does not occur until the entire junction melts apart. The non-cooperativity exhibited by J3R28 manifests itself differently than J3R18. The derivative curve of J3R28 has a similar minimum to J3R22, but displays a shoulder at a temperature around 63°C. The shape of this curve indicates that the JR arm melts at position 28 before the entire structure destabilizes. Thus, this result supports the prediction that melting occurs from the end of the duplex arm towards the center of the junction.

4.2.2 Applications to the 3SPN.2 Model

The stepwise melting process of the JR/JX arm is consistent with the 3SPN.2 model prediction for relative stability of the duplex arm. J3R18 and J3R28 were predicted to be less thermodynamically stable than J3R22, and therefore begin to melt at an earlier temperature.
There exists one discrepancy between experimental and simulated results. The center of the junction was predicted to be the most significant point of instability in the global structure by the 3SPN.2 model. Experimental results with a fluorescently-labeled center (J3R18) did not entirely support this prediction. While the center of the junction did undergo an initial destabilization at an earlier temperature than the global structure, it did not completely melt until the entire structure did as well. We believe that this discrepancy may be due to implicit ion modeling in the 3SPN.2 simulation. Rather than including explicit ions in the melting model, there is a uniform charge applied to the system. Implicit ion modeling thus differs from solution experiments, where ions bind the center of the junction to provide increased stability (Litke et al., 2016). We propose that the lack of these explicit ions in the simulation leads the 3SPN.2 model to predict decreased stability at the center of the junction.

With the exception of the thermostability at the center of the junction, the melting behavior observed through fluorescence spectroscopy experiments has been mostly consistent with the 4WJ melting behavior predicted by the 3SPN.2 model. We believe that this work will help to improve future 3SPN.2 simulations, especially in ion modeling, and will open the door for more experiments using this method.

4.2.3 Differential Scanning Calorimetry Data for J3 Melting

In addition to our own spectroscopic results, we have also obtained differential scanning calorimetry (DSC) data for J3 from the Marky group at the University of Nebraska. DSC is a technique that measures the difference in heat
required to raise the temperature of sample cell and a reference cell, giving the change in heat capacity between the two cells as the temperature is increased. The degree of cooperativity of a melting transition can be determined from these scans using the ratio of Van’t Hoff to calorimetric enthalpies ($\Delta H_{VH}/\Delta H_{cal}$). A ratio greater than one indicates a cooperative process (Spink, 2008).

Figure 4.2.3: DSC scan for junction J3 (black) and its curve fits (red). DNA concentration = 9.89 µM

<table>
<thead>
<tr>
<th>$\Delta H_{VH}$ (kcal/mol)</th>
<th>$\Delta H_{cal}$ (kcal/mol)</th>
<th>$\Delta H_{VH}/\Delta H_{cal}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>363 ± 70</td>
<td>473 ± 26</td>
<td>.78 ± .19</td>
</tr>
</tbody>
</table>

Table 4.2.2: Enthalpy data for J3. $\Delta H_{VH}$ obtained from absorbance experiments and $\Delta H_{cal}$ obtained from DSC experiments

Firstly, asymmetry in the melting curve indicates non-cooperativity in the melting process, as $\Delta C_p$ changes at an inconstant rate (Raudino, Sarpietro, & Pannuzzo, 2013). The analytical fit of the melting data to two curves suggests the
presence of an intermediate state and a more complex melting process. Additionally, \( \Delta H_{\text{VH}} / \Delta H_{\text{cal}} \) is less than one, indicating the presence of an intermediate.

Because our fluorescence results show stepwise melting of the X-pseudo-duplex at an earlier temperature than the melting of the H-pseudo-duplex, we have ascribed the intermediate state to the destabilization of the X-pseudo-duplex. While we propose that the two pseudo-duplexes destabilize at differing temperatures, we suggest that the first intermediate corresponds to the destabilized X-pseudo-duplex structure because we expect the initial destabilization to promote the destabilization of the rest of the junction. In other words, the H-pseudo-duplex becomes unstable at a higher temperature than the X-pseudo-duplex, but this second destabilization does not yield an isolatable intermediate state.

We have obtained the thermodynamic parameters for each of the two transitions from the Marky group. It is important to consider the difference in values obtained from absorbance spectroscopy and differential scanning calorimetry. Experimental differences, such as slight differences in buffer solutions, may lead to the discrepancy in \( \Delta H_{\text{VH}} \) and \( \Delta S \) between methods.

<table>
<thead>
<tr>
<th>( \Delta H_{\text{cal}} ) (kcal/mol)</th>
<th>( \Delta S_1 ) (kcal/mol K)</th>
<th>( \Delta G_1 ) (kcal/mol)</th>
<th>( T_M (1) ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>303 ± 2.8</td>
<td>.888 ± .053</td>
<td>58 ± 4</td>
<td>68.1 ± .05</td>
</tr>
</tbody>
</table>

**Table 4.2.3:** Parameters obtained from first curve fit of DSC data, representative of the X-pseudo-duplex destabilization process (\( H_{\text{cal}} = \) Enthalpy obtained from calorimetry scan)
Table 4.2.4: Parameters obtained from second curve fit of DSC data, representative of the subsequent melting process into four single-strands

<table>
<thead>
<tr>
<th>Method</th>
<th>$\Delta H_{\text{Total}}$ (kcal/mol)</th>
<th>$\Delta S_{\text{Total}}$ (kcal/mol K)</th>
<th>$G_{\text{Total}}$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSC</td>
<td>447 ± 5.1</td>
<td>1.31 ± .076</td>
<td>89 ± 6</td>
</tr>
<tr>
<td>Absorbance</td>
<td>363 ± 70</td>
<td>1.01 ± .231</td>
<td>90 ± 38</td>
</tr>
</tbody>
</table>

Table 4.2.5: Parameters obtained from DSC and absorbance data for the 4WJ melting process

Using this additional data describing the thermodynamics of the transition state, we were able to construct a free energy diagram for the melting process of the 4WJ.

Figure 4.2.4: Reaction coordinate diagram for the melting of the DNA four-way junction
ΔG₁, the change in free energy upon X-pseudo-duplex destabilization, and ΔG₂, the change in free energy upon melting into four single strands from the intermediate state, were derived from DSC experiments. ΔG_{Total} was calculated using both DSC and absorbance melting data. Our free energy diagram was constructed both with structural information from fluorescence experiments and with thermodynamic values obtained from absorbance spectroscopy and differential scanning calorimetry experiments. As such, this reaction coordinate yields a structural and quantitative scheme of the pseudo-duplex melting model of the DNA four-way junction.

4.3 Thermodynamic Predictions for J3 Melting

To compare the thermodynamics of each melting transition to predicted values, we used a database compiled by SantaLucia Jr. & Hicks (Santalucia Jr. & Hicks, 2004) to calculate the expected enthalpies and entropies of J3 and of each pseudo-duplex. We treated each pseudo-duplex as an individual duplex and used the base stacking parameters set by the database to calculate the thermodynamics of the melting of each duplex. As such, these predictions are limited in that they lack a parameter to describe the effect of the branch point on relative the stability of the duplexes.
<table>
<thead>
<tr>
<th></th>
<th>$\Delta H$</th>
<th>$\Delta S$</th>
<th>$\Delta G_{37}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH</td>
<td>276</td>
<td>.75</td>
<td>44</td>
</tr>
<tr>
<td>JX</td>
<td>272</td>
<td>.75</td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td>547</td>
<td>1.5</td>
<td>84</td>
</tr>
</tbody>
</table>

Table 4.3.1: Predicted values (SantaLucia Jr. & Hicks, 2004) for pseudo-duplex melting based only on stacking parameters (top) and obtained values for each melting transition (bottom). $\Delta G_{37}$ was calculated from our data using Equation 1.4.2.

Because we did not account for the instability induced by the center of the junction in our predictions, the estimation of the thermodynamic parameters for the overall melting process are higher than those obtained. However, even without adding the energy penalty induced by the strain at the center of the junction, we see that the first melting transition has a higher enthalpy and entropy than what would be expected for melting of the X-pseudo-duplex. This discrepancy indicates that there is more occurring in the first transition than the melting of the X-pseudo-duplex ends. Additional structural changes that lead to this unexpectedly high-energy transition likely include a disruption in coaxial stacking of the pseudo-duplex arms brought about by the pre-melting transition in the center of the junction.

We also observe that the energy associated with the second transition in the melting process is lower than what was predicted for melting of the H-pseudo-duplex. We believe that this difference may be explained by increased destabilization of the global structure induced by X-pseudo-duplex destabilization. Thus, while our
predictions of thermostability do not contain an explicit parameter for the effect of the branch point, they serve to elucidate more information about the melting model. Through these estimations of individual duplex stability, we see that the melting process cannot be reduced to the sequential melting of two duplexes.

4.4 Implications of Pseudo-Duplex Melting

Knowledge of the process of branch migration can help us to further explain our results. Reciprocally, our new understanding of 4WJ thermostability can be considered within the greater context of homologous recombination. It is important to recognize that regardless of the melting trends observed, a thermodynamic characterization of the relative stability of the 4WJ allows for further speculation into the process of branch migration.

The pre-melting transition we observe in the center of the junction is consistent with reported mechanisms of branch migration (Thompson, Camien, & Warner, 1976), (Parsons et al., 1995). Opening of the center of the 4WJ is necessary for migration to occur. Thus, it is likely that our fluorescence experiments report an opening of the 4WJ without complete melting of the structure, as this process occurs physiologically. Opening of the junction is also necessary for isomerization during recombination (Sigal & Alberts, 1972), (Meselson & Radding, 1975).
4.5 Considerations in Experimental Procedures

While we addressed some concerns about the reporting of fluorescent nucleoside analogs in this work, there still remains some questions about the 4WJ that may be addressed with future experiments.

Firstly, our results should be considered with reference to the recent results of Carr and Marky, which demonstrate that 4WJ melting occurs through a stepwise process. However, the fluorescence data from this research indicates that stepwise melting does not occur as a function of the stacked pseudo-duplexes (see Figure 1.4.2), but rather occurs as a function of individual arm stability. Because the Marky group studied an intramolecular junction, this result leads us to an interesting question: how does intramolecular junction melting differ from intermolecular junction melting? Also, to what degree does coaxial stacking play a role in stepwise melting? These questions may be investigated through the incorporation of nucleoside analogs into intramolecular junctions.

We also seek to understand the applicability of our model to other intermolecular 4WJs. While we hypothesize that we will observe the same sequence-dependent melting behavior in other 4WJs because the structure of the branch point remains the same, future directions for this project include investigation of the melting behavior of other 4WJs. We will elaborate upon this future work in the next section of this chapter.

Additionally, it has been previously demonstrated that J3 preferably adopts the stacked IsoII conformer relative to IsoI by a ratio of 77.4% to 22.6% (Joo et al., 2004).
Regardless of the local environment of the probes in each conformer, we do not expect the presence of the IsoI conformer to significantly affect our fluorescence results because of the low IsoI population relative to IsoII. Additionally, previous experiments with 6MAP in J3 (Savage, 2017) have shown pseudo-duplex melting behavior when probes were distributed between pseudo-duplexes regardless of isomeric conformation. The stacking energies calculated for the B-pseudo-duplex and R-pseudo-duplex of the IsoI conformer are similar to the values calculated for the H-pseudo-duplex and X-pseudo-duplex of the IsoII conformer, respectively. Thus, while the IsoI conformer may have a minimal impact on our results from trial to trial, we do not expect its presence to affect the relative trends observed in our data.
4.6 Future Directions

Future directions for this research include investigating 4WJ melting through circular dichroism (CD) spectroscopy. The degree of nucleic acid base stacking can be measured through a peak around 250 nm and base pairing can be measured around 280 nm (Johnson, 1996). Using a two-state model, each of these absorbance peaks can be analyzed in terms of fraction of native junction in the sample. This means that $K_{eq}$ at each temperature can be derived, leading the same modes of analysis as other spectroscopic methods. We hope that this method of spectroscopy is sensitive enough to detect changes in the rates of base unstacking and unpairing, thereby allowing for the observation of a non-cooperative melting process.

We also plan to investigate the melting of 4WJs other than J3, including mobile junctions, using fluorescence spectroscopy. Investigating mobile junctions, which occur physiologically, will provide us with more insight into how the pseudo-duplex melting model can be implicated in homologous recombination.

4.7 Conclusion

Understanding the melting process of the DNA four-way junction helps to elucidate information about the relative thermodynamic stability of the structure. The implications of this research extend into the theories of homologous recombination. The increased stability of the branch point of the junction relative to its arms may
provide insight into branch migration and thus the entirety of the recombination process.

It is a novel observation that the junction melts in accordance with its component duplexes, as the structurally anomalous center of the junction is thought to be a point of weak stability when compared to the canonical stability of the DNA duplex. The findings presented in this work thus provide the basis for future study into DNA thermostability and four-way junction dynamics.
References


