Effect of Concentration, Temperature, and Base Changes on Retrovirus Dimerization

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Abstract:

RNA-RNA junctions between hairpin loops are a common feature of RNA retroviruses, accounting for their high mechanical stability. Dimerization of Moloney Murine Leukemia Virus (MMLV) is dependent on H3 stem loop interactions, which are an example of such a junction. H3 in MMLV dimerizes via a two base pair hairpin loop enhanced by two adjacent stacking bases. All atom, explicit solvent molecular dynamics pulling simulations were performed to examine the stability of the junction. Salt concentrations and temperatures were varied and unstacking times of the flanking bases and breaking times of the kissing bonds were calculated. Survival curves revealed that increasing salt concentration, decreasing temperature, decreasing force, and having two adenine stacking bases as opposed to one guanine and one adenine stacking bases stabilizes the flanking bases of the complex. Additionally, breaking of bonds is made faster by increased temperature, pulling force, and two adenine stacking bases over one guanine and one adenine stacking bases. Increased salt decreases the breaking rate, but only at low temperatures. Simulation results were compared with single molecule optical tweezers experiments.
Acknowledgements:

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Introduction

Single Molecule Optical Tweezers Experiments

Optical tweezers have traditionally been used to study DNA, but are increasingly used to study RNA folding patterns. Intermediates are detected in the folding process by performing pulling experiments and viewing a force-extension curve. A conformation change in such curves usually appears as a peak [1].

The typical optical tweezers setup (Figure 1) consists of a bead coated in RNA kept in an optical trap by dual lasers, which holds it stationary. A second bead, also coated in RNA, is put in a micropipette and brought close to the stationary bead. When the beads are close enough, a bonding interaction occurs. The beads are then pulled apart again and a force-extension graph is made as seen in Figure 2. The pulling of the two beads is done at the 3’ and 5’ ends of the RNA. As the two beads are pulled apart by moving the micropipette, a force is exerted on the bead in the optical trap. This force, as measured by the optical trap as the displacement from the center, along with the displacement of the second bead to the first as the extension are input into the force-extension curves [2]. For small strands of RNA, a tether is typically added to allow the RNA to stay on the bead. Additionally, handles can be used to allow for base pair interactions, or as below to allow for the RNA hairpin to form. Experiments, such as the one below can be repeated several times with the same two beads.

![Image of optical tweezers setup](image)

**Figure 1.** Optical tweezer setup of a hairpin loop [2]. Newer methods involve using an optical trap to apply the force while keeping the micropipette stationary.
The advantage of the optical tweezer setup is that it allows for the effects of mechanical forces, salt concentration changes, and temperature changes to be simultaneously measured during the folding/unfolding of a single RNA molecule. All of these factors alter the intrinsic rates of RNA folding reactions. The magnitude of the external force can be altered by changing the stiffness of the laser, which is done by increasing the intensity of the laser, increasing the power output [3].

However, optical tweezer experiments can often take many trials to get interpretable results. The concentration of RNA on each bead must be small to prevent multiple strands of RNA from binding. If there are multiple binding sites, the results of the pulling experiments are often skewed. This is because for these experiments to occur, only a single binding site is necessary. If there are more binding sites, the forces to pull apart the complex appear greater.

**Molecular Dynamics Simulations**

Molecular dynamics simulations start with a crystal or NMR structure found in the PDB database [4]. Water and ions are added to a box with the molecule, simulating ambient environmental conditions. The box has a specified length, large enough for the molecule to fit without causing periodic image interactions. Simulations use periodic images to mimic a macroscopic environment. Force fields are used to describe the atomic interactions, such as stacking and hydrogen bonding. These simulations operate using the concepts of Newton’s
equations of motion to determine how intermolecular forces result in time-evolution of atomic positions [5].

Simulations are used to compare to experimental results, to explain phenomena that may occur too fast or may be too small to see experimentally, and to test reactions before going through experiments. Traditionally, simulations have been used to study folding patterns of proteins, but increasingly are used to study RNA and DNA folding patterns and interactions. Molecular dynamics simulations include all of the atoms and allow for small scale changes to be measured. Other methods for simulating, namely course-grained models, do not include all the atoms and often measure larger scale changes. The advantages of such course-grained models are that simulations can be run on a much larger timescale without as much computational time being used and it can be used on large systems. However, these models may fail to explain what is impossible to see in experiment, as smaller scale changes involving single atoms. Thus, molecular dynamics, all-atom, explicit solvent simulations are generally used for explaining phenomena not observable experimentally [2].

Pulling experiments can be modeled in molecular dynamics. A pulling force is applied to the 3’ and 5’ ends of an RNA to model after the optical tweezers setup. Figure 3 shows an example of this pulling. Since all of the atoms are being simulated, these pulling experiments are run at much shorter timescales than seen experimentally, usually in the nanosecond (ns) time range. Therefore, forces for simulated pulling experiments need to be higher than those used experimentally [6].

![Figure 3](image)

**Figure 3.** Pulling simulation of a shortened MMLV chain. A pulling force is applied to the 3’ and 5’ ends of each chain. [6]
With molecular dynamics, multiple simulations are run with different initial velocities. This is required, because typically when a complex breaks in a simulation, it does not reform, in contrast to pulling experiments where a complex can be repeatedly folded and unfolded. The advantage of simulating experimental data over performing the experiment is that the phenomena of interest can be measured exactly by applying specific constraints into the simulation. However, there are often large errors associated with observing too few events, requiring multiple simulations to be performed before statistically significant trends can be seen.

**Moloney Murine Leukemia Virus (MMLV)**

Kissing complexes are strong RNA-RNA complexes that connect two hairpin loops with complementary RNA bases. This interaction is important to the folding of many large RNAs. The MMLV genome is an example of an H3 step loop which consists of two eighteen base hairpin loops, which form a kissing complex. The kissing interaction occurs at the 10 and 11 positions of each strand, bonding a cytosine to a guanine base. Two flanking adenine bases at the 9 positions of each strand supply the complex with additional mechanical stability. An image of this complex is shown in figure 4.

![Image of MMLV](image)

**Figure 4.** Structure of MMLV, an H3 2-base pair kissing loop. [6]

Optical Tweezer experiments on this complex had forces ranging from 13.5 to 30 pN. The “unkiss” rate constant is increased with force [7]. Unkissing is defined as the breaking of both of the bonds that make up the kissing complex. As figure 2 shows, at low forces, only one transition occurs, the “unkiss” of the complex. At higher forces, however, two or three transitions occur that involve the unraveling of the chains [1]. Additionally, at higher temperatures, the complex requires
less force to unkiss. With a guanine in place of one of the flanking adenine groups (GA), less force is required to unkiss, indicating that the flanking adenine somehow provides additional stability over a flanking guanine. At higher temperatures, however, no kissing occurs in the GA complex [8]. It has been shown through optical tweezer experiments that it takes the same amount of force to break MMLV kissing complex as to unfold an 11 base pair helix [9]. Through simulations this was found to be caused by the flexibility of the hairpin loop junction allowing for the base-pairs to reform [6].

Pulling simulations of MMLV used forces of 100 to 400 pN. Bases along the chain were restrained so they would not unravel at these high forces and only the unkissing event would be measured. A rearrangement was seen in all of the simulations, as shown in figure 3. This rearrangement involves a lengthening of the complex through this irreversible conformation change due to the pulling of the complex, as seen in figure 5 [6].

![Figure 5. Conformational change seen in all simulations during the first few steps [6]](image)

Molecular simulation studies of these complexes revealed that when the flanking bases were restrained to where they were no longer stacked, the complex broke much faster than with the bases stacked. This indicates that the stacking bases provide the complex with additional mechanical stability. Moreover, as predicted in experiment, lower force takes longer to break [6]. Guanine also stacks less favorably than adenine, because of the negative charge on the carbonyl group on the adjacent guanine bonding base. This causes guanine to stack unfavorably on the cytosine-guanine bond [1]. The proposed model of this unkissing event is shown below in figure 6.
Weakening this complex is important, because when retrovirus dimerization is weakened, the virus does not replicate. Therefore, this type of experiment can aid in the design of future antiretroviral drugs. MMLV mimics the HIV-1 complex of Mal and Lai type. These HIV-1 complexes have a greater number of kissing base pairs than MMLV, but it is believed that when these complexes are pulled, they resembles the structure of MMLV, with two flanking bases and two base pairs [10]. For this reason, MMLV can be studied to determine how to destabilize such kissing complexes. Additionally, simulating MMLV takes less computational time, since there are less kissing bonds to break.

The objective of this thesis is to compare pulling simulations to single molecule pulling experiments. Moreover, it was sought to determine the mechanisms for unkissing events caused by changes in environmental conditions. Also, to determine if simulation time can be saved by only viewing unstacking of the flanking bases. Finally, to determine if the current model of unkissing is followed for all simulations, regardless of the environmental conditions.
Materials and Methods:

Simulation Conditions:

All atom, explicit solvent molecular dynamics simulations were performed with Gromacs-4.6.5 [11]. The PDB code for the MMLV used is 1F5U [4]. The box size was 60 Å x 120 Å x 60 Å, filled with TIP 3P water, three coordinate water [12], and KCl. KCl was added in by concentration with 34 additional K+ ions added to neutralize the system. This made for 99 K+ and 65 Cl- ions for 250 mM and 294 K+ and 260 Cl- ions for 1 M. The amount of water was 13748 molecules for 250 mM and 13358 molecules for 1 M. The Amber 99- Chen-Garcia force field was used [13]. The base pairs in the stem loop were restrained using simple distance restraints of 250 to 1000 kJ/mol [11]. All simulations were performed at constant temperature and constant pressure.

A total of 100 simulations or more were performed for each simulation condition, with times ranging from 6 ns, for A, D, and G, and 18 ns, for LFA. The time step for these simulations was every 2 fs, with an output written every 2 ps. A pulling force was applied to the C3’ and O5’ atoms. One of the flanking adenine bases was changed from a guanine to an adenine by using the program X3-DNA [14]. Table 1 shows the simulation conditions studied. AA represents the MMLV complex with two adenine flanking bases, while GA represents the MMLV with one flanking adenine base changed to a guanine. This base change in figure 7.

Table 1. Simulation Conditions

<table>
<thead>
<tr>
<th>Label</th>
<th>System</th>
<th>Salt Concentration (M)</th>
<th>Temperature (K)</th>
<th>Pulling force (pN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA</td>
<td>AA</td>
<td>0.250</td>
<td>298</td>
<td>150</td>
</tr>
<tr>
<td>A</td>
<td>AA</td>
<td>0.250</td>
<td>298</td>
<td>200</td>
</tr>
<tr>
<td>B</td>
<td>AA</td>
<td>1.00</td>
<td>298</td>
<td>200</td>
</tr>
<tr>
<td>C</td>
<td>AA</td>
<td>1.00</td>
<td>310</td>
<td>200</td>
</tr>
<tr>
<td>D</td>
<td>AA</td>
<td>0.250</td>
<td>310</td>
<td>200</td>
</tr>
<tr>
<td>LFG</td>
<td>GA</td>
<td>0.250</td>
<td>298</td>
<td>150</td>
</tr>
<tr>
<td>G</td>
<td>GA</td>
<td>0.250</td>
<td>298</td>
<td>200</td>
</tr>
</tbody>
</table>
Figure 7. A) Unmodified MMLV, with two adenine flanking bases (AA). B) Modified MMLV, with a guanine and adenine flanking base (GA). Modified using X3DNA.

Data Analysis:

Data Analysis was completed using the g_dist function in Gromacs-4.6.5 [11]. Distances were found between the flanking base and the base it is stacked on, with the distances between C4 on the adenine or guanine base and C2 on the cytosine. If this distance was less than 1 nm, the adenine or guanine was considered to be stacked, and if over this threshold, the base is considered to be unstacked. The distances between a base pair of the kissing complex were found by taking the distance between H1 on guanine and N3 on cytosine. If this distance was less than 0.5 nm, the residues were considered to be broken. A python script was used to determine the unstacking and breaking times. After these times were recorded, likely death curves were made using Matlab [15]. A stochastic pure death model was used to determine rate constants of unstacking and breaking.

Stochastic Pure Death Model:

A stochastic pure death model was used to mu values for single exponential curves. In order for this model to work, it is assumed that when an event breaks or unstacks, it does not reform. Although this is not always true in experiments, a reformation is rare and the method is a
fair approximation. The equation of this model is written as in equation 1 below, where n is the number of events that survive at a certain time, \(n_0\) is the total number of survivors at time 0, t is the time, and \(\mu\) is the death rate [6].

\[
p_n(t|\mu) = \left(\frac{n_0}{n}\right)e^{-n\mu}(1 - e^{-\mu t})^{n_0-n}
\]  

(1)

This is a probability function. It works by recalculating the probability for each new time. In the analysis, events were censored based on pathway. For example, if an event went along pathway 1, it could not have gone along pathways 2 and 3, so it was not included as the total \(n_0\) of these pathways. If there are a certain number of events that still survive after the longest time, these are added into all three pathways \(n_0\), because these events have the possibility to go along any pathway, and it is unknown which pathway it follows unless the simulations were run longer. This probability function gets a new probability for each time. These peaks overlap and form a single peak, which is Gaussian in shape. An example of this peak is shown below in figure 8.

![Gaussian peak that comes about from multiple probabilities added together.](image)

**Figure 8.** Gaussian peak that comes about from multiple probabilities added together.

From the graph in figure 8, the x-value associated with the maximum height is taken. Error is calculated through the full width at half max function shown in equation 2. In this equation, \(\sigma\) represents the standard deviation. This equation can be used, because the graph in figure 8 is essentially normal [6].

\[
\text{FWHM} = 2\sqrt{2\ln 2} \sigma \approx 2.355 \sigma
\]  

(2)
To graph these mu values, equation 3 is used. This makes the smooth exponential likely death curves seen in the following graphs. The error lines were plotted using the same formula by adding and subtracting the σ value.

\[ y = 100e^{-(\mu \pm \sigma)t} \]  (3)
Results and Discussion:

Model of Unkissing Events:

Through analysis of the simulations, there appears to be three pathways that lead to an unkiss event. The first pathway, as shown in figure 6 is one in which unstacking occurs before breaking [6]. In this model, an unstack event causes a bond to break. The second pathway involves a break event followed by unstacking. A break event is the first event to occur which causes an unstack event in this model. In the third model, it is unclear which event happens first, if an unstack causes a break or a break causes an unstack. This model is followed when the break and unstack events occur in close succession of one another, which was defined as the time difference between break and unstack of 20 ps, simulation time, or less. Figure 9 shows these pathways. It is important to note that the conformational change in figure 5 is seen with all environmental conditions studied.

Figure 9. Pathways to unkiss event. Pathway 1 was proposed in figure 6 above.

Table 2 shows the percent of simulations in each pathway category. Refer to Table 1 for the meaning of the simulation labels. It is important to note that this table only includes events where an unstack or break occurred. As seen in the table, at higher salt concentrations, regardless of temperature, pathways two and three are followed 40% of the time, indicating that salt concentration has the greatest effect on the reaction pathway. An increase in salt concentration is more likely to follow pathway one, but follows pathways two and three more than with any other simulation condition studied. Salt appears to disrupt the cytosine-guanine bond that makes up the kissing complex, while stabilizing the stacking bases. The positively charged, K+ ions appears to disrupt these bonds by binding to the partial negatively charged carbonyl group on guanine, which
is important in the bonding with cytosine, causing the bond to break before unstacking or both events to occur simultaneously. Moreover, an increase in temperature at low salt concentration follows pathways two and three to a greater extent than with low salt concentration at room temperature. The GA system is least likely to follow pathways two and three, possibly due to the unstable stack with the cytosine-guanine bond. The flanking guanine appears to be unfavorably stacked and may disrupt the bond if it remains stacked, so it is more likely to follow pathway one to reduce this strain on the system [1]. Finally, force appears to have no effect on the reaction pathway. This indicates that the pulling force does not push the reaction pathway to two and three, but rather how the complex is being pulled from the 3’ and 5’ ends, or other extraneous factors may cause pathways two and three to be followed.

Table 2. Percent of Simulations following each pathway

<table>
<thead>
<tr>
<th>Simulation Label</th>
<th>Pathway One (%)</th>
<th>Pathway Two (%)</th>
<th>Pathway Three (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>78</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>B</td>
<td>60</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>C</td>
<td>60</td>
<td>28</td>
<td>12</td>
</tr>
<tr>
<td>D</td>
<td>70</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>LFA</td>
<td>78</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>G</td>
<td>94</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>LFG</td>
<td>91</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

Also from the table, it is clear that regardless of simulation condition, pathway one is followed most, then pathway two, and finally three, except with the lower force GA complex. For processes occurring biologically (i.e. very low force), the most important pathway is pathway one. However, the other pathways do partially affect the rates, so they are explored below.

Pathway One:

At higher salt concentrations of 1 M KCl, the unstacking rate constant is lower than that at 250 mM KCl. Salt concentration has the greatest effect on unstacking rate. Regardless of temperature, a higher salt concentration leads to a lower rate constant of unstacking. This suggests that the salt has a stabilizing effect on the flanking bases in AA complexes. Simulations that occur
at temperatures of 310 K unstack faster than those that occur at 298 K. Temperature may cause a slight melting in the complex, which makes the flanking bases more prone to unstack. As shown in figure 10, the stochastic death model follows the same trends for unstacking. The pause times, the time it takes for a complex to break after unstacking, do not follow the death model well. This is because after unstacking, the complex either breaks within several hundred picoseconds or takes nanoseconds to break as water comes in and breaks apart the bond. Due to the variation in pause times, a likely death curve does not uniformly decrease. However, the entire unkiss event can be fit to a stochastic death model, as shown by Alan Chen’s previous work [6]. The temperature effects follow the results of previous simulations and experiments.

![Figure 10](image)

**Figure 10.** Survival curves for temperature and salt concentration changes on the AA system. Smooth curves represent the stochastic death model, with the error shown as dashed lines. Experimental data is shown in the darker colors.

Adenine flanking bases are more stable than guanine stacking bases. For this reason, AA complexes take longer to unstack than GA complexes. Guanine less favorably stacks due to the unfavorable stacking interaction on C-G, where the carbonyl group on the base-paired guanine causes the flanking guanine to fly up, which minimizes the effect of this unstable stack [1].
Additionally, as the force decreases, the time it takes for the complex to unstack increases. This is shown in figure 11, and agrees with previous simulation and experimental results. As before, the pause times for these simulations do not follow the stochastic death model well due to few points and a large variation in pause times.

![Survival curves for base and force changes in pathway 1. These curves only include first unstacking times. As before, the smooth curves represent the results of stochastic death model, with the dashes representing the error. Non-smooth curves represent the simulation data.](image)

The unstacking of guanine does not appear to influence the pause time. In fact, guanine almost always unstacks before adenine in GA complexes, and the pause times are often far less in numbers, and are longer than expected. This may be due to the fact that guanine’s unfavorable stack negatively impacts the cytosine-guanine bond causing it to stabilize when the guanine
unstacks. Eventually, the bond will break as more water comes in between the bonds, and for this reason there are often larger pause times for these complexes. However, overall, unkissing happens much faster in GA complexes as seen in previous experiments [1].

**Pathways Two and Three:**

Pathways two and three generally follow the same trends in the likely death curves for breaking. Pathway two shows that high temperatures, regardless of salt concentration causes the complex to break faster. At higher salt concentration and higher temperature, the complex breaks slightly more slowly than at low salt concentration and high temperature. However, these two are almost indistinguishable. Higher salt concentration at low temperature is the slowest to break. Temperature appears to have the greatest effect on breaking with pathway two. Salt concentration is the next most important factor when it comes to breaking in pathway two.

![Figure 12. Pathway 2 breaking survival curves for salt and temperature changes.](image-url)
Pathway three follows the same trends as pathway two. At high temperature the two curves are almost indistinguishable due to the standard deviation values. This shows that regardless of pathway, two or three, breaking is essentially the same with temperature and salt concentration changes. Table 3, however, shows that the values of the rate constants differ for pathways two and three. High temperature may cause a slight melting to occur, so the bond is more likely to break.

Although pathways two and three are followed to a greater extent at high salt concentrations, it appears that at room temperature, the salt has a slight stabilizing effect. Eventually, however, the bonds of the kissing complex are disrupted by the K+ ions as opposed to the flanking bases being disrupted. Figure 13 shows the breaking/unstacking times for pathway three, changing salt concentration and temperature. Compared to pathway one, pathways two and three are more affected by temperatures and less affected by salt concentrations. Pathways two and three do not follow the stochastic death model as well as pathway one, possibly due to the small number of simulations that follow these pathways.

![Figure 13](image)  
**Figure 13.** Pathway 3 breaking survival curves with temperature and salt concentration effects.
There is a clear influence of pulling force on breaking rate for pathway two. When pulled at 200 pN, the GA and AA complexes break much faster than at 150 pN. These two curves are almost indistinguishable at high forces, because of the large standard deviation values. At low forces, GA breaks less often and slower than AA. A possible explanation for this event is that GA complex may be stabilized by the salt, as K+ neutralizes the carbonyl group on the stacked guanine. This allows for guanine to be stacked with more stability and prevent the complex from both breaking and unstacking. However, the small amount of break events for the GA complex and large standard deviation, which is within two standard deviations of the AA complex at low force, makes this assumption impossible to ascertain. Figure 14 shows this graph.

**Figure 14.** Pathway 2 survival curves for base and force changes.

Pathway three shows the same trends as that of pathway two. As seen, the only measurable difference is with force. At low forces, the complex takes longer to unstack/break. The GA and AA complexes are indistinguishable at both forces. This is due to the large standard deviation
values, which only separate the values by forces. Figure 15 shows the survival curves for pathway three with the influence of flanking base and force.

The same influence of force is seen with pathway one as with pathways two and three. However, guanine is always less favorably stacked, and unstacks more often than in AA complexes. This influence of force on breaking and unstacking rates follows experiment, and previous simulations.

![Pathway 3 survival curves for base and force changes.](image)

**Figure 15.** Pathway 3 survival curves for base and force changes.

**Analysis of Models:**

As shown in table 3 below, the first step of pathway one always occurs faster than the first steps in pathways 2 and 3. Additionally, except at low force for GA type MMLV, pathway 2 always occurs faster than pathway 3. This table shows the death rates that are graphed as the smooth single exponential curves in figures 10 to 15 Pathways 2 and 3 happen to a lesser extent than pathway 1,
but these pathways are still important for the overall mechanism for break events, and as shown in table 2, are affected by environmental conditions.

**Table 3.** Rate constants for the first step of each pathway for each simulation.

<table>
<thead>
<tr>
<th>Simulation</th>
<th>$\mu$ pathway 1 (ns$^{-1}$)</th>
<th>$\mu$ pathway 2 (ns$^{-1}$)</th>
<th>$\mu$ pathway 3 (ns$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.2640 ± 0.004714</td>
<td>0.0990 ± 0.007984</td>
<td>0.0630 ± 0.01321</td>
</tr>
<tr>
<td>B</td>
<td>0.0880 ± 0.002548</td>
<td>0.0620 ± 0.004119</td>
<td>0.0360 ± 0.003779</td>
</tr>
<tr>
<td>C</td>
<td>0.1750 ± 0.004204</td>
<td>0.1730 ± 0.008663</td>
<td>0.1710 ± 0.02000</td>
</tr>
<tr>
<td>D</td>
<td>0.4020 ± 0.006667</td>
<td>0.1960 ± 0.01028</td>
<td>0.1780 ± 0.02013</td>
</tr>
<tr>
<td>G</td>
<td>0.6356 ± 0.01023</td>
<td>0.1390 ± 0.04344</td>
<td>0.1200 ± 0.06468</td>
</tr>
<tr>
<td>LFA</td>
<td>0.1230 ± 0.002038</td>
<td>0.0480 ± 0.003355</td>
<td>0.0250 ± 0.005266</td>
</tr>
<tr>
<td>LFG</td>
<td>0.3220 ± 0.005606</td>
<td>0.0260 ± 0.009852</td>
<td>0.0320 ± 0.007899</td>
</tr>
</tbody>
</table>

Likely death models appear to work well for describing the first step of each pathway, but pause times are not described well with this model. This is because pause times are often few in numbers and do not linearly decrease. To prevent this, it may be necessary to run the simulations for larger time scales to get more pause times. Pause times from a break event that causes an unstack are often much smaller than those for an unstack event that causes a break. Assumptions cannot be made as to which step of each pathway is rate limiting, because the simulations were not run long enough to get data for the following steps. It happens that pathway 1 is the most energetically favorable pathway, as shown by the greater number of simulations that follow this pathway.
Conclusions:

MMLV unkissing is studied due to its importance for understanding RNA tertiary junctions and for its implications for antiretroviral drug design. Simulations were compared to results from optical tweezers experiments. It was determined that the unkissing can follow three pathways, but pathway one, which was previously proposed and consists of an unstack event causing a break event, is the pathway that is most followed. For this reason, the current pathway works well in describing experiments, but the other two pathways are still important. Pathways 2 and 3 cannot be ruled out as statistically insignificant, because at their greatest these two pathways account for 40% of the total pathways followed. The overall rate can change based on the pathway that is followed. Higher temperature simulations are more likely to follow pathways 2 and 3.

The simulations used were only run for short times, 6 to 18 ns. If these simulations were run longer, a total rate constant could be determined. However, if only the first step is viewed in each of the simulations, specifically those that follow pathway 1, the same trends are seen as those in experiments. It is therefore possible to use shorter simulations to determine the effects of environmental conditions on the complexes. By using the stochastic pure death model, rate constants for the first step can be determined. This model works best for pathway 1.

Temperature appears to disrupt bonding more than unstacking, where the unstacking rate is more affected by changes in salt concentration. The salt appears to have a stabilizing effect on the flanking bases, but a destabilizing effect on the bonds where it interacts with the carbonyl group, disrupting the bond. Lower pulling forces stabilize both the bonds and the flanking bases. Guanine in place of adenine as a flanking base appears to have little effect on bonding, but a large effect on stacking, which causes it to unstack faster than AA complexes. Guanine, as previously shown is not a stable flanking base for this complex.

Further projects with these types of simulations could be done at temperatures and salt concentrations in between the two used. This would aid in better determining the pathways and mechanisms for an unkiss event. Additionally, simulations of the GA type could be completed with differing salt concentrations and temperatures to see if these complexes match experiment. Finally, in order to determine if pause times can be adequately fit to the stochastic pure death model, the current simulations could be run longer to get more data points.
Citations:


