Sense Dependent DNA Dynamics in a Membrane Channel

Hasina Visram
Rowland Institute of Science at Harvard
Harvard REU Program 2003

Abstract

The importance of single stranded DNA orientation with respect to its 5'and 3' ends on its travel trough the α-Hemolysin membrane channel was examined in this study through a series of feedback-controlled experiments and DNA hairpins that restricted entry to the pore via either the 5' end or 3' end. By systematically varying either the amount of time the hairpins were driven into the channel or the time they were allowed to diffuse out of the channel it was shown that when entering the pore from the cis terminal and traveling towards the trans terminal under the influence of an electric field, poly-adenine tails entering via their 5' ends travel approximately three times slower than their 3' entering counter parts. However, no distinction between the behavior of hairpins traveling from trans to cis in the pore could be detected regardless of whether an electric field was present or not. It is suggested that the negative free phosphate group on the 5' end of the poly-adenine tail is strongly repelled by the negative charges at the trans terminal of the channel when traveling towards the trans terminal and thus the hairpins entering via their 5' ends travel slower than the 3' entering hairpins that do not have a strong negative charge. In the reverse direction, however, the influence of the repulsion is not detectable due to the ends not being in the electric field of the negative trans terminal.

Introduction

Membrane channels are in integral part a great number of biological systems ranging from neural transmission to viral infections and thus the study of such proteins can shed insight on various biological areas. Often, membrane channels are used to transport RNA and occasionally single and double stranded DNA between a cell nucleus and the surrounding cytoplasm. Given the asymmetry of a single stranded DNA chain, single stranded DNA can enter the α-Hemolysin pore with one of two senses. Specifically, it may enter with either the 3' or the 5' end leading.

Due to differences in shape and charge of the 3' and 5' end of single stranded DNA, it is reasonable to question whether the sense of single stranded DNA influences its behavior in a membrane channel. The study presented here employed DNA hairpins to restrict the entry of DNA into the α-Hemolysin pore in one sense in order to investigate the effects of orientation on the dynamics of DNA in a membrane channel.

Methods

In this study of DNA dynamics in a membrane channel, a Teflon cell containing two closed chambers connected via a tube was used as shown in figure 1.

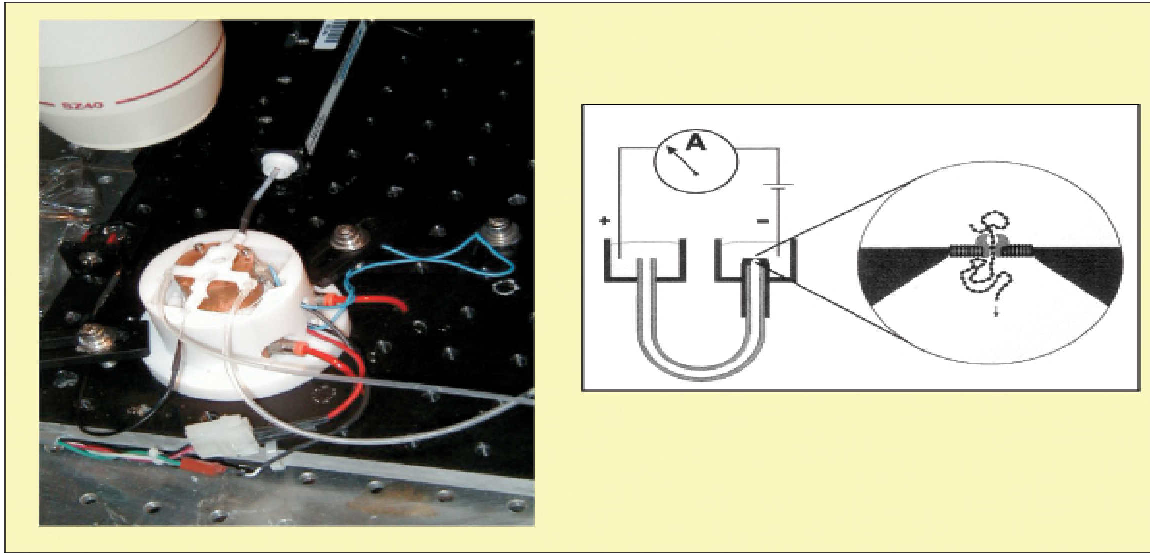

Figure 1: Photograph of the Teflon cell employed in this study with a schematic of cell setup.

The opening of the tube located in the trans chamber of the cell was approximately 2mm in diameter while the opening of the tube located in the cis chamber was approximately 15 um in diameter. During the experiment, both chambers and the connecting tube were filled with a 1.0 M KCl/ 100 mM Tris-Cl buffer that conducted a current between the two cell chambers under the influence of an electric field.

In the preparation stages of the experiment, a phospholipid bilayer, or membrane, is formed over the smaller cis opening of the connecting tube effectively hindering the passage of current between the two chambers of the Teflon cell. At this point, a single α-Hemolysin pore is incorporated into the membrane by adding a miniscule amount of the protein to the cis cell chamber. Once the pore is incorporated, DNA is added to the cis chamber of the cell. Upon the application of an electric field between the chambers, the DNA is made to enter the pore. The channel of the α-Hemolysin pore, due to its size, can only accommodate single stranded DNA and thus the double stranded loop of the DNA hairpins cannot enter the pore's channel. The presence of DNA in the pore is detected by the absence of current passing through the pore due to the DNA blocking the passage of ions.

The experimental method used for feedback-controlled measurements was adapted from previous studies conducted on single stranded DNA chains. Briefly, a constant voltage of 120 mV was applied across the membrane to force the DNA to enter the pore. The entry of single stranded DNA into the channel causes the current passing through the pore to drop to less than 10% of the open pore current, and thus its entry is easily detected. At the time of DNA entry, the voltage across the membrane was maintained at 120 mV for a time, T_{Drive}, in order to drive the DNA further into the channel. Then for a period of time, T_{Off}, the voltage across the membrane is set to zero to allow the DNA to passively diffuse out of the pore. At the end of the T_{Off} period, the voltage across the membrane was set to 40 mV for a time, T_{Probe}, to probe for the

presence of the DNA in the pore. If the DNA had remained in the pore beyond the T_{Off}, then no current could pass through the pore in the presence of the probe voltage. However, if the DNA had diffused out of the pore during T_{Off}, then current could pass though the pore during T_{Probe} and be detected. If a molecule had been in the pore at the onset of T_{Probe} but had left the pore during its duration, a sharp rise in the current from 0pA to approximately 25 pA could be detected at the time at which the DNA had left the pore. In summary then, once a single stranded segment of DNA enters the pore channel, it is driven into the pore for a time T_{Drive}, and then allowed to diffuse for a time T_{Off}, after which its presence in the pore is monitored for a time, $T_{Probe.}$ Any molecules that escaped the pore during T_{Drive}, were not used in data analysis.

The feedback-controlled method described above could be altered to allow the detailed study of hairpin dynamics in the pore under the influence of an electric field to be conducted. In this case, the voltage during T_{Drive} was maintained at 120 mV while the duration of T_{Drive} was sufficiently long enough to allow for the double stranded loop of the hairpin to reach the top of the pore channel; its lowest point. Then, if the duration of T_{Off} is 0μs and the duration of T_{Probe} is long enough to witness the transition from no currant passage through the pore to detectable current pass indicating the leaving of the hairpin from the pore, the time it takes for the hairpin to dislodge from the pore under the electric field created by the probe voltage can be found.

Description of Hairpins

The two similar DNA hairpins used in this study consisted of a single stranded poly Adenine region composed of 50 adenine base pairs joined to a 10 base pair loop displayed in figure 2.

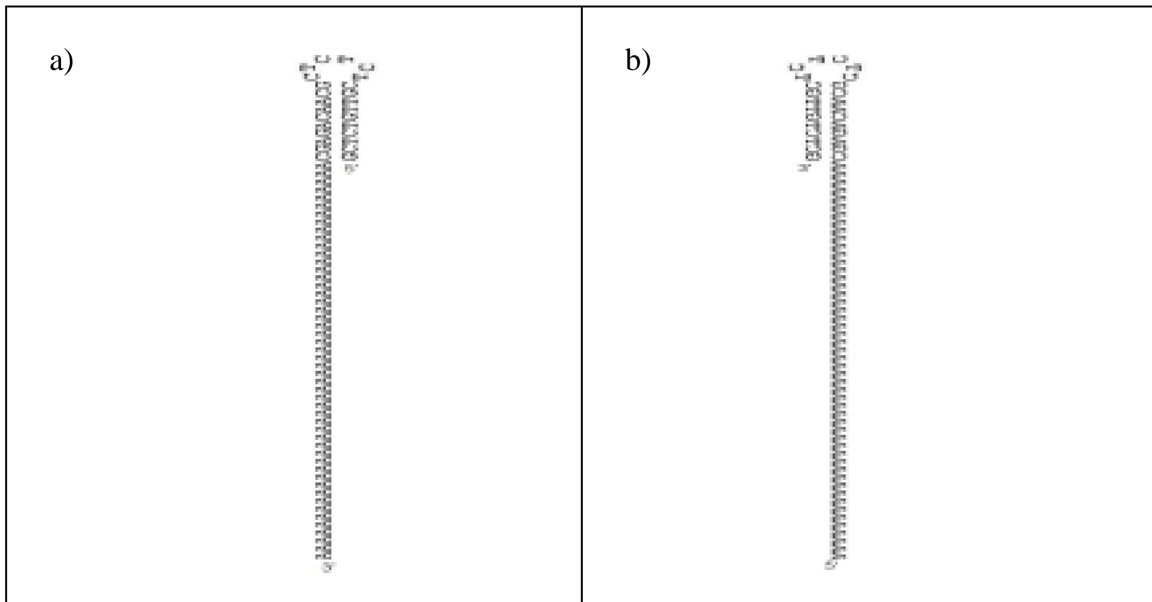

Figure 2: Schematic representation of the two hairpins employed in this study. The molecule in a) enters the membrane pore via its 3' end while the molecule in b) enters via its 5' end.

In one hairpin, the single stranded tail is found at the 3’ end while the single stranded region in the other hairpin is situated at the 5’ end of the molecule. Thus one hairpin is forced to enter the pore via its 3’ end and the other via its 5’ end.

Results

Diffusion Experiments with Varying Driving Times and Constant Off Times

Using the feedback-controlled protocol described above, a set of experiments was conducted where the driving time was varying while the time where the hairpins were allowed to diffuse was kept constant. By changing T_{Drive} it was possible to alter the position of the hairpin in the pore at the onset of T_{Off}. In the case of the hairpins studied here, T_{Off} was set to 300 μs. For each value of T_{Drive} selected, the probability of the hairpin escaping from the pore through diffusion was calculated directly as the fraction of molecules that were not found in the pore after the 300 μs T_{Off} time as detected by the probe voltage. Figure 3 displays this probability of escape as a function of driving time for both hairpins studied.

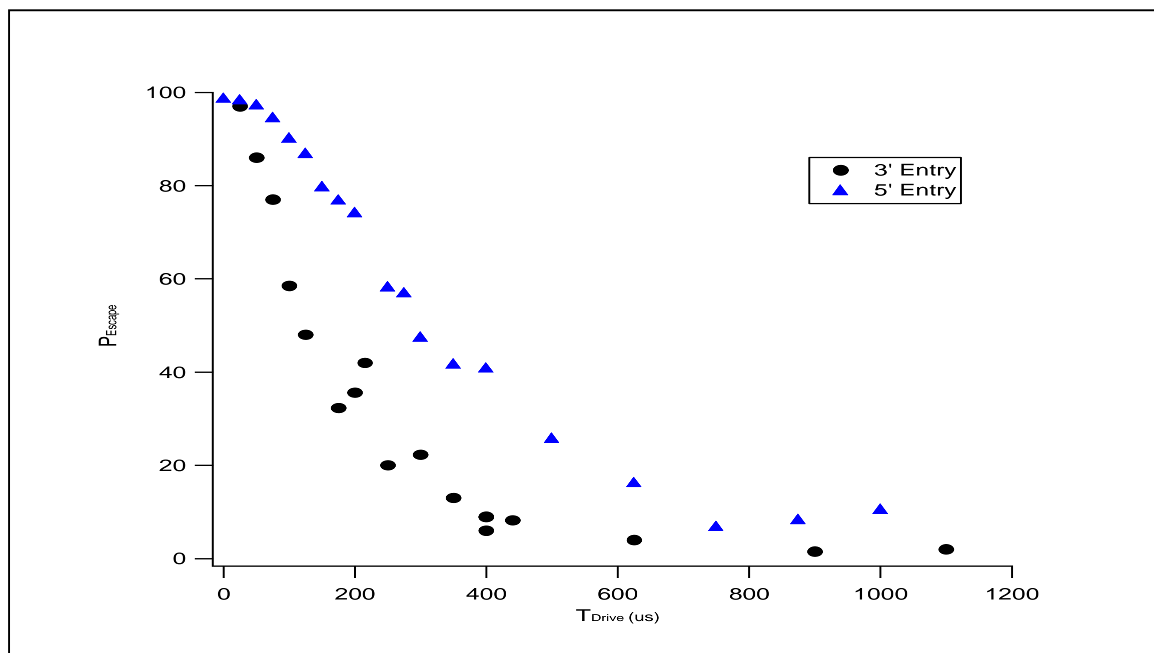

Figure 3: The probability of DNA escaping from the pore within a T_{Off} time of 300 μs as a function of T_{Drive}.

At nearly any giving driving time, the probability of escape for a hairpin entering the pore via its 3’ end was significantly less than for a hairpin entering via its 5’ end. Additionally, the minimum probability of escape for a hairpin entering via its 3’ end is less than that for a hairpin entering via its 5’ end. An explantion of the shape of the curves in figure 3 is presented in the discussion below.

Diffusion Experiments with Varying Off Times and Constant Driving Times

A set of feedback-controlled experiments where the driving time remains constant and the off time varies can provide insight to the diffusion of DNA through the α-Hemolysin pore over a time period. For the hairpins that entered the pore by their 3' end, five such sets of experiments were performed with constant driving times of 25µs, 35µs, 100µs, 300µs, and 750µs with the results displayed in figure 4.

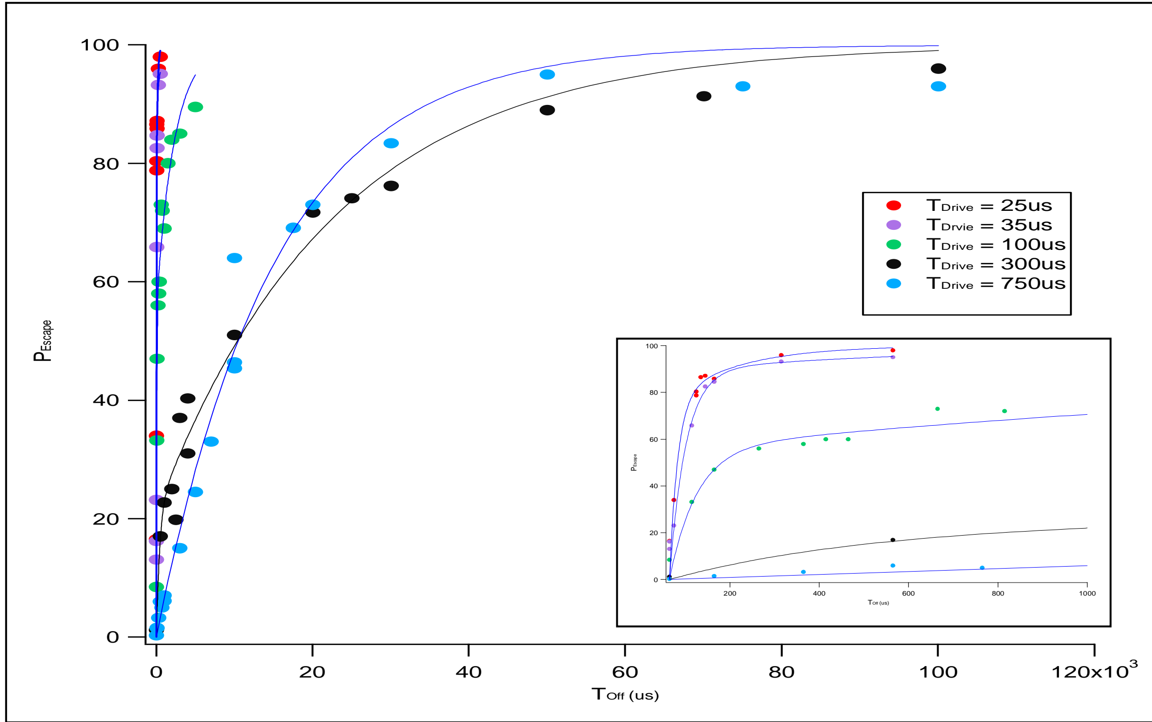

Figure 4: Constant T_{Drive} scans with varying T_{Off} times for DNA hairpins entering via their 3' end. Inset: A detailed view of results where T_{Off} varied between 0µs and 1000µs. Each of the curves was fit to a sum of two exponentials whose results can be found in table 1.

Each curve displayed in figure 4 was fit to the sum of two exponentials in equation [1].

$$P_{Escape} = 100 - [a*\exp(-(T_{Off}-65)/T_{Fast}) + (100-a)*\exp(-(T_{Off}-65)/T_{Slow})] \qquad [1]$$

where a is the relative weighting of the two time scales, T_{Fast} is the fast time scale constant and T_{Slow} is the slow time scale constant. The results of these fits are listed in table 1.

Table 1: Fit Results for data presented in figure 4 describing diffusion of hairpins entering via their 3' end.

T_{Drive}	a	T_{Fast}	T_{Slow}
25µs	77%	18µs	156µs
35µs	89%	36µs	590µs
100µs	56%	61µs	2313µs
300us	21%	422µs	22750µs
750µs	0%	----	13060µs

As with the hairpins that entered the pore with their 3' end, sets of experiments with a constant T_{Drive} and varying T_{Off} were conducted for hairpins that entered the pore with their 5' end first. For these molecules, T_{Drive} was set at 100µs, 300µs, and 750µs while T_{Off} was varied. The results of these experiments are displayed in figure 5.

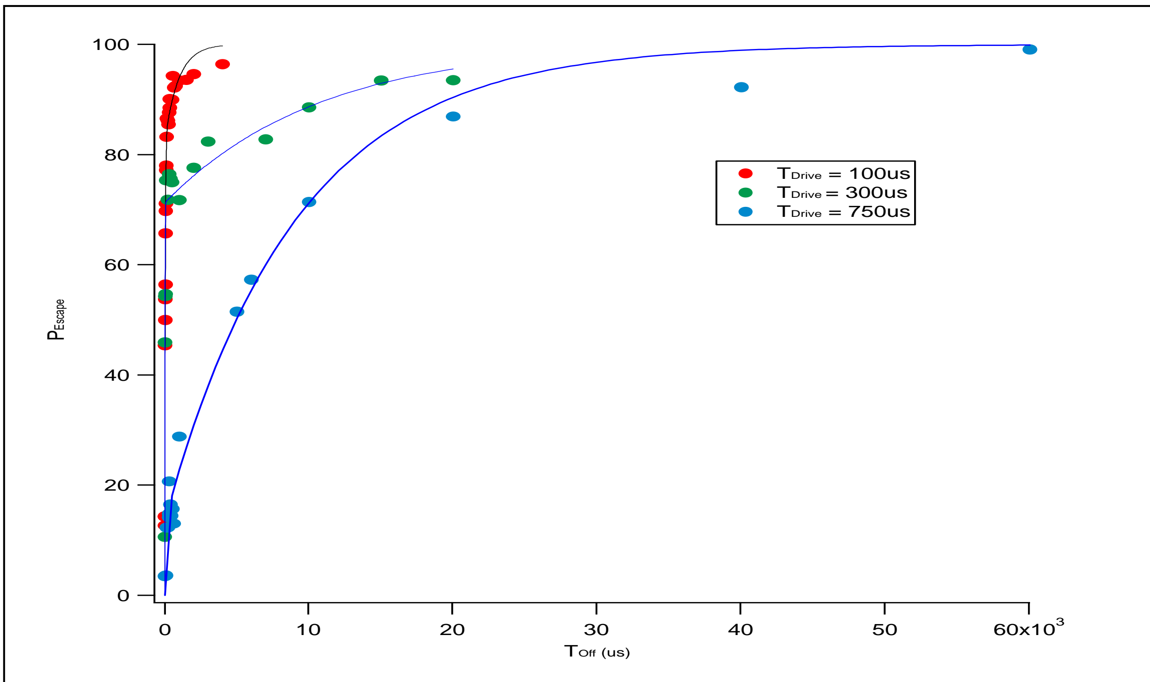

Figure 5: Constant T_{Drive} scans with varying T_{Off} times for DNA hairpins entering via their 5' end. Each of the curves was fit to a sum of two exponentials whose results can be found in table 2.

Each of the curves displayed in figure 5 was fit to the sum of two exponentials in equation [1] with the results listed in table 2.

Table 2: Fit Results for data presented in figure 5 describing diffusion of hairpins entering via their 5' end.

T_{Drive}	a	T_{Fast}	T_{Slow}
100µs	82%	32µs	941µs
300us	71%	14µs	10715µs
750µs	14%	145µs	9123µs

The presence of fast and slow timescales for the diffusion curves in the above experiments could represent the diffusion of the hairpin loop from the α-Hemolysin vestibule and the diffusion of the single stranded tail from the channel. This possibility is further explored in the discussion.

DNA Dynamics Under Varying Electric Fields

As described earlier, feedback-controlled mechanisms were employed to study detailed hairpin dynamics under an electric field. Based on results in figure 3, a T_{Drive} time of 750µs was selected as a time where the greatest number of hairpins would be at the furthest point in the pore. The voltage across the membrane during T_{Probe} was set to –40 mV for one set of experiments and –80 mV for 20 ms. In the analysis stage of the study, the presence of the hairpin the pore was checked for at increasing times. At each point in time, the probability that the hairpin escaped the pore was calculated as the fraction of molecules that were no longer in the pore at that time. Figure 6 displays the probablility of escape as a function of time over which a voltage was applied across the membrane.

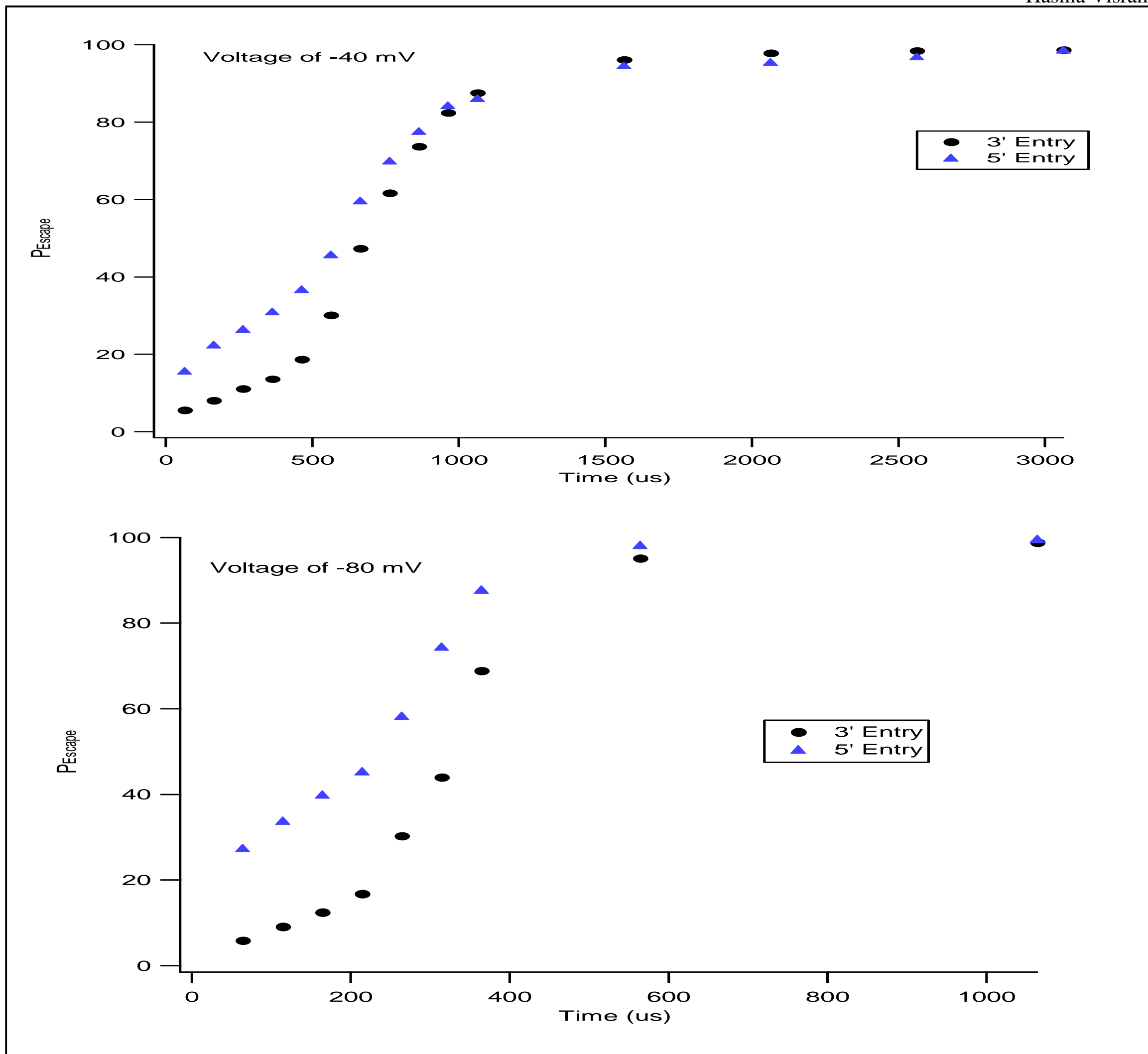

Figure 6: The driving of hairpins in the trans to cis direction in the pore through the use of an electric filed with either –40 mV or –80 mV applied across the membrane.

As the time that the negative voltage is applied across the membrane increases the probability that the hairpin will escape the pore increases as well. There are three distinct regions of the traces in figure 6 that demonstrate that there are three processes that take place. A explanation of the shape of the curve and the processes driving it is offered in the below discussion.

Discussion

Using the feedback controlled protocol described previously with a varying T_{Drive} and a constant T_{Off}, as shown in figure 3, allowed for differences in between the behaviors of the two hairpins to be detected. The results showed the probability of the hairpin to be located in the pore's channel 300 µs after the voltage across the membrane was set to 0mV. From this information the position of the DNA hairpin in the α-Hemolysin pore after 300µs diffusion time to be inferred in relation to driving time. From figure 3, however, it is not possible to determine whether the difference in position of the

two hairpins is due to a difference in behavior while under the influence of the electric field, a difference in hairpin diffusion in the pore, or both.

Three distinct regions in the traces in figure 3 can be attributed to three distinct pore environments the hairpin can be located in as illustrated in figure 7.

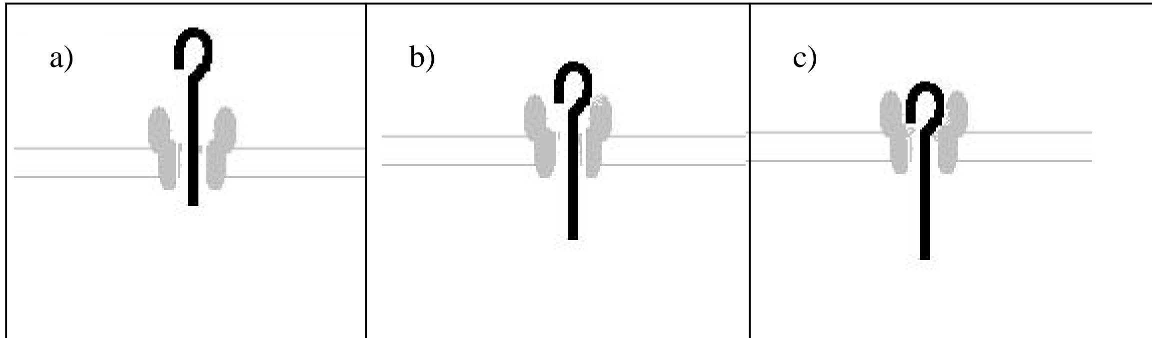

Figure 7: a) When T_{Drive} is short, only the single stranded region of the hairpins come in contact with the pore and interactions between the vestibule and double stranded loop do not occur. b) When T_{Drive} is of medium duration, the single stranded portions of the hairpin is located in the pore's channel while minimal interactions between the pore vestibule and hairpin loop can occur due to their proximity in space. c) When T_{Drive} is long, the single stranded portion of the hairpin is located in the channel of the pore and maximal interactions between the pore's vestibule and hairpin's loop can occur because the loop is located in the vestibule.

When T_{Drive} is short, only the single stranded region of the hairpins come in contact with the pore and interactions between the vestibule and double stranded loop do not occur because due to the distance between them. However, as T_{Drive} is increased, the hairpins travel further through the pore bringing the hairpin's loop and pore's vestibule to come closer and allowing interactions between the two to occur. As more of the loop and vestibule come into contact, the strength of loop vestibule interactions increases until T_{Drive} is long enough to drive the single stranded region of the hairpin to its junction with the loop such that the loop is in the pore's vestibule.

In the case where only the relatively weak interactions of the single stranded region and the pore's channel are interacting, the probability of escape for the molecule within the 300μs T_{Off} time is relatively high and the rate of the probability of escape decrease with respect to driving time is relatively low. Once the hairpins loop and pore's vestibules are allowed to interact, the probability of the hairpin escaping decreases at a significantly than when the loop and vestibule cannot interact. Finally, once the loop is located within the vestibule, the probability of the hairpin escaping from the pore is extremely low and relatively constant.

While it is evident that the hairpins behave differently while in the α-Hemolysin pore, it is difficult to discern whether the hairpins behave differently while under the influence of the an electric field, without the presence of a field, or in both conditions by only examining figure 3. However, results from experiments where T_{Off} was increased for a particular T_{Drive} can shed insight into this matter.

Plotting the results where T_{Drive} was set to 25µs and 35µs for the hairpins entering via their 3' end with the results where T_{Drive} was set to 100µs for hairpins that enter the pore via their 5' ends illustrates that these three curves are similar. Likewise the results of the experiment where T_{Drive} was set to 100µs for molecules entering the pore with their 3' end resembled the results for the experiment where T_{Drive} was set to 300µs for molecules that enter the pore with their 5' end when plotted together. Such combined traces are displayed in figure 8.

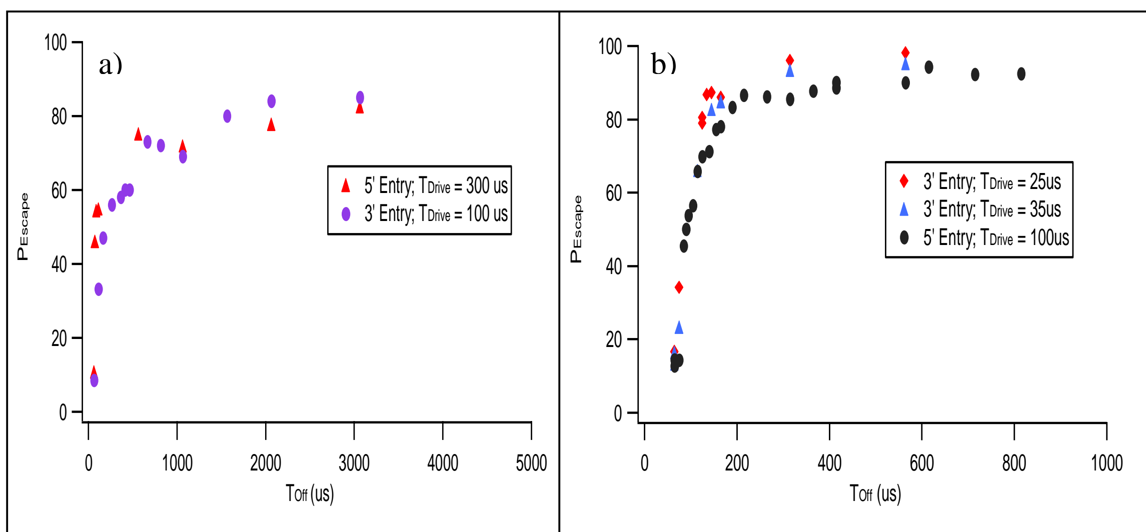

Figure 8: Combined traces of experiments were T_{Drive} was kept constant and T_{Off} was varied illustrating that for different T_{Drive} times, the results are similar between different hairpins.

The similarity in the traces show above would indicate that the molecules compared started to diffuse from the same position in the pore and moved out of the pore in a similar fashion. That is, a hairpin entering with it's 3' end that is driven into the pore for 100µs reaches the same position as a hairpin entering with its 5' end that is driven for 300µs. Similarly, a hairpin entering with it's 3' end that is driven for 25µs to 35µs reaches the same position as a hairpin entering with its 5' end that is driven for 100µs. These results indicate that the orientation of the DNA entering the pore influences how the molecules enter through the pore, but do not influence how they diffuse out of the pore. However, it is impossible, from the traces above, to determine whether the difference in orientation influence is due to the presence of the electric field, the direction of DNA travel (i.e. cis to trans), or a combination of both. To address this issue, the traces in figure 6 where the hairpins were driven out of the pore in the trrans to cis direction must be examined.

The plots in figure 6 clearly contain three different regions starting from low driving time to high driving time. First, there is a small region with a relatively small slope that would correspond to the loop of the hairpin being driven out of the vestibule. Next, there is region with a large slope corresponding to the single stranded region of the hairpin being driven out. Lastly, there is a region with constant slope relating to a region

of saturation where the entire hairpin has been pushed out of the pore. Based on evidence discussed above, driving both hairpins for 750μs may not render them in the same position in the pore, thus the starting position for the molecules entering with their 3' end first may not be the same as the position for the molecules entering via their 5' end. However, both molecules are in the same position at the onset of the second region of the plots in figure 6, which is the position where the hairpin's loop has just left the pore and only the remaining single stranded tail remains in the pore. Thus, by scaling the plots to start at a probability of escape of zero once the loop has left the vestibule, regions on the plots that probe the same process, specifically driving the single stranded region towards the cis side of the pore, can be compared.

The appropriate transformation required to scale the plots is as follows:

$$P_{Escape}^* = (P_{Escape} - \text{Min } P_{Escape})/(\text{Max}P_{Escape} - \text{Min } P_{Escape}) \quad [2]$$

where P_{Escape}^* is the scaled probability of escape, $\text{Min}P_{Escape}$ is the minimum probability of escape found at the point where the single stranded region has just begun to be driven out and $\text{Max}P_{Escape}$ is the maximum probability of escape at the point of saturation. Figure 9 displays the scaled P_{Escape}^*.

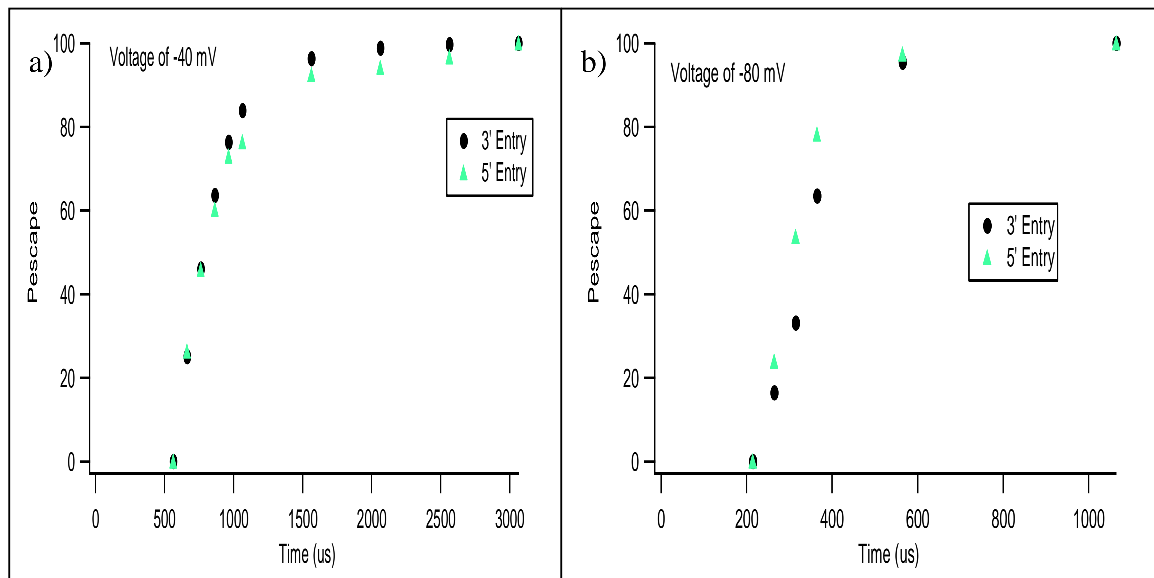

Figure 9: Scaled probabilities of escape for driving hairpins out of the α-Hemolysin pore towards the cis side examining only the diving of the single stranded region.

Allowing for a small offset between the curves compared in figure 9 a) and b) due to a time offset at which the hairpin loop has left the vestibule, it is clear that orientation of the hairpin in terms of 5' and 3' entry does not influence the driving of the single stranded region of the hairpin out of the pore.

Combining the interpretation of all results then, the DNA hairpins, at least in the single stranded regions, behave differently when moving from cis to trans in the pore (i.e. from the vestibule towards the channel) but behave similarly when moving trans to

cis in the channel and that the presence of an electric field is not a necessary factor in observing this phenomenon.

A plausible explanation for this observation comes from a brief analysis of the charge map of the α-Hemolysin pore. It is know that charges are dispersed throughout the pore but that a high concentration of negative charge is found at the trans terminal of the pore. Additionally, for purines, including the adenine bases composing the hairpin's entire single stranded region, the negative phosphate group is located at the 5' end a DNA chain. The 3' end of a chain of single stranded adenine does not have as strong of a negative charge as the 5' end. As the adenine chain enters the pore via the cis terminal, and in driven toward the negatively charged trans terminal of the pore it enters the electric field of the trans terminal. Chains entering via their 5' terminal face greater repulsion from the trans terminal than do chains entering via their 3' end due to the negatively charged 3' end. Thus as the molecules travel towards the trans terminal of the pore, their orientation affects their behavior. However, as molecules are traveling towards the cis terminal of the pore and are no longer within the electric field of the trans terminal's negative charge, the orientation of the molecule with respect to 5' and 3' is no longer an influence on their behavior.

A follow up set of experiments to support such a theory would include the study of hairpins with a cytosine tail. Pyrimidines, including cytosine, contain the free phosphate group at their 3' terminal and not at their 5' terminal. If the above theory is correct, then experiments conducted identically to those in this study would show identical results for processes where the DNA is moving from trans to cis in the pore but reversed results for processes where the molecules are moving from cis to trans with respect to 3' and 5' orientation.

Conclusion

The dynamics of DNA interactions with the α-Hemolysin pore with respect to its 3'/5' sense were effectively probed through a series of feedback-controlled experiments. Combining insight gained from experiments where DNA was allowed to diffuse without the influence of an electric field with those where it was constantly under the influence of a field revealed that while traveling from the cis terminal of the pore towards the trans terminal, 3'/5' orientation is a consideration in the molecules' behavior. Additionally, it was demonstrated that when traveling in the reverse direction, that is from trans terminal to cis terminal, 3'/5' orientation does not impact the molecules' behavior whether it is under the influence on an electric field or not.

Future experiments in this area should address the structure and charge map of the α-Hemolysin pore containing DNA in both orientations to identify key causes of the distinct behavior of the different orientations while molecules are moving cis to trans. Additionally, experiments similar to those studied here with pyrimidine base chains could shed insight to the importance of the phosphate group found at the end of single stranded DNA chains.