Published in final edited form as:

Mikrochim Acta. 2016 March ; 183(3): 973–979. doi:10.1007/s00604-015-1516-6.

α -Hemolysin nanopore studies reveal strong interactions between biogenic polyamines and DNA hairpins

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Abstract

The α -hemolysin (α -HL) nanopore analyzes DNA as it is electrophoretically driven through the pore. The respective current vs. time (i - t) traces depends on the DNA sequence, its secondary structures, or on the physical conditions of the analysis. The current study describes analysis of a DNA hairpin with a 5'-extension with the α -HL nanopore in the presence of the polyamines spermine (Spm), spermidine (Spd), and putrescine (Put). These studies identified a new i - t trace characteristic of the DNA-polyamine complex. Voltage-dependent studies determined that the hairpin-Spm complex formed with excess Spm was not unzipped and translocated through the pore even when the voltage was increased to 180 mV. The DNA hairpin sample was titrated with Spm, Spd, or Put that showed a dose-dependent response in the characteristic event patterns for hairpins bound to Spm or Spd, but not for Put. Plots of the event types vs. count were used to calculate binding constants for the Spm or Spd hairpin interactions under these conditions. The titration also demonstrated that the event rate decreased ~ 10 -fold when the Spm or Spd concentration was increased from 0 to 4 mM. These observations impose practical limitations on the ability to use Spm or Spd for DNA studies with the α -HL nanopore.

Graphical abstract

Hairpin DNAs bearing long single-stranded tail was studied with spermine via the α -hemolysin nanopore that generated three characteristic current signatures.

Keywords

α -Hemolysin; DNA Hairpin; Spermine; Spermidine; Putrescine

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Supporting Information: T_m data, PAGE data, sample i - t traces, and event duration histograms are available in the supporting information.

Introduction

The biogenic polyamines (Fig. 1) putrescine (Put), spermidine (Spd), and spermine (Spm) are linear aliphatic amines present in almost all living organisms at millimolar concentrations [1, 2]. These polycationic amines are essential for normal cell growth, gene regulation, and proliferation due to their ability to interact with negatively charged molecules such as DNA, RNA, and proteins [3]. While their levels decline with age, increased levels of polyamines have been observed in cancer cells [4]. Knocking out the genes encoding key enzymes for the biosynthesis of polyamines in mice is lethal, indicating their vital role in mammals [4, 5]. The cardinal function of polyamines has attracted much research interest, especially in the exploration of their interactions with the polyanionic nucleic acids.

More than half a century ago, Spd and Spm were found to stabilize calf thymus DNA by increasing the melting temperature (T_m) by ~ 10 °C [6]. Subsequent studies were conducted to probe the interactions between polyamines and nucleic acids by a variety of spectroscopic and calorimetric analytical methods [7–12]. From these studies, interactions between DNA and Put, Spd, or Spm can occur in the major and minor grooves of duplex DNA; the leading mode of molecular interaction between these compounds is electrostatic; a minor secondary mode of interaction is hydrophobic in nature; polyamines increase the thermal stability of DNA; there exists a degree of sequence dependence in the preference of polyamine binding to duplex DNA; and at high concentrations of polyamines DNA begins to aggregate. Moreover, the binding constants between polyamines and DNA provide a trend in which Spm, with the greatest positive charge, has the highest binding constant, and Put, with the smallest positive charge, has the lowest binding constant [7, 8, 10]. These studies provide a foundation to interpret new experimental results pertaining to DNA polyamine interactions.

Investigation of the interactions between polyamines and DNA by single-molecule detection techniques, such as protein nanopores, can provide insights not observed by bulk measurements. Emerging as one of the most utilized protein nanopores, α -hemolysin (α -HL) provides a rapid and inexpensive platform for detecting single molecules in solution [13–16]. The α -HL nanopore is composed of two parts, a wide vestibule with an opening of ~ 3.0 nm on the *cis* side leading to a narrow β barrel exiting on the *trans* side that has a constriction zone of about 1.4 nm separating these two regions (Fig. 2) [17]. Application of a voltage across the pore causes a flow of K^+ and Cl^- electrolytes that establish an open channel current I_o [18]. Charged molecules, such as DNA or RNA, are electrophoretically driven into the nanopore and generate a blocking current I [18, 19]. The size-selective properties of the α -HL nanopore allow translocation of single-strand DNA (ssDNA) and prevent free translocation of DNA duplexes, hairpins, or G-quadruplexes that must unfold or denature before translocation [20–28]. Previous studies demonstrated that blunt ended hairpins and fishhook hairpins (with one tail) can enter the vestibule, unzip and translocate through α -HL nanopore, producing characteristic current vs. time events [20, 25, 29–31]. Changes in stem sequence and length, as well as the loop size, lead to changes in the blocking current and/or event duration [20, 25, 30–32]. Nanopores have also been used to detect and quantify binding interactions between antibiotic or anticancer drugs with nucleic acids [33, 34]. These studies validate the α -HL nanopore as a sensitive detector of DNA-

secondary structures. The present work utilized the nanopore to study the binding of polyamines with DNA hairpins while monitoring the event duration and blocking current levels. For these studies, a fishhook hairpin with a 6 base pair (bp) stem and a 5'-GTTA-3' tetraloop was chosen to study Spm, Spd, and Put interactions with DNA via the α -HL nanopore.

Experimental

Materials

All chemicals used in these studies were obtained from commercial sources and used without further purification. Stock solutions of the polyamines were prepared at 0.1 M concentration in the buffered electrolyte solution used in the nanopore analysis (1.00 M KCl, 10 mM phosphate buffer, pH 7.4).

Oligodeoxynucleotide (ODN) preparation

The ODNs were synthesized from commercially available phosphoramidites (Glen Research, Sterling, VA, www.glenresearch.com) by the DNA-Peptide Core Facility at the University of Utah, followed by purification and characterization by previously described literature protocols [25]. After purification, all hairpins were annealed in a 1 mL solution by mixing 10 μ M DNA with nanopore buffer (1.00 M KCl and 10 mM phosphate buffer, pH 7.4) and incubating in a 90 °C water bath for 5 min followed by flash cooling on ice. The annealed samples were stored at -20 °C prior to their study.

Ion current recording and data analysis

The glass nanopore membrane (GNM) was constructed with a nanometer sized orifice ($r = 800$ nm) as previously described [35]. Insertion of a single, wild-type α -HL protein in a lipid bilayer spanning the orifice of the GNM composed of the phospholipid 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) was achieved as previously described [36]. The apparatus for conducting the ion current recordings was a customized, low-noise amplifier and data acquisition system constructed by Electronic BioScience (San Diego, CA, <http://electronicbio.com>). All experiments were performed at 22 ± 1 °C. The buffered electrolyte solution was composed of 1.00 M KCl, 10 mM phosphate buffer, pH 7.4. In each experiment, the concentration of hairpin ODN was 10 μ M while the concentrations of the polyamines were titrated from 0 to 4 mM. Data were collected with a 100-kHz low-pass filter at a 500-kHz acquisition rate. Events were extracted using QUB 1.5.0.31 and fitted using Origin 9.1. The density plots were constructed using custom software from Electronic BioSciences (San Diego, CA, <http://electronicbio.com>). Note: The addition of polyamines to the nanopore analysis chamber during the experiments irreversibly damaged the GNM surface such that they would no longer supported a lipid bilayer requiring a new GNM for each experiment.

Results and Discussion

Effect of Spm on the event profile for the fishhook hairpin studied

The hairpin 5'-ATCCTAGTTATAGGAT-3' (underlined region represents the tetraloop) was chosen for this work because of its established structure, high stability, and short length [37]. Thermal melting and native polyacrylamide gel electrophoresis (PAGE) control experiments confirmed the formation of the hairpin structure under the nanopore conditions studied (Fig. S1). For the α -HL nanopore experiments, a 5'-poly-dT₄₀ tail was attached to the hairpin for increasing the capture efficiency for this structure by facilitating tail-first entry. Analysis of this hairpin by the α -HL nanopore with a 100 mV (*trans* vs. *cis*) bias without a polyamine present provided two event types (Fig. 3A), consistent with our previous observations [25]. The first event type gave a deep blockage (I_1) to the current. Blockage current values are reported as a percentage of the I_o value (i.e., % I/I_o). The deep blockage value for the Type 1 events was % $I_1/I_o = 15 \pm 2\%$. The event times for these events were plotted to give a Gaussian population that had a mean event time of 0.28 ± 0.03 ms (Fig. S2). Furthermore, as the voltage was increased to 120 mV, the mean event time for the Type 1 events decreased supporting unzipping and translocation of the hairpin through the β -barrel of α -HL (Fig. S2). The second type of event observed (Type 2) provided a % $I_2/I_o = 45 \pm 5\%$ at an intermediate current level. The Type 2 event duration time plots gave a broad distribution; furthermore, as the voltage was increased the duration time increased (Fig. S2). These observations for the Type 2 events are consistent with loop entry of the hairpin into the vestibule of α -HL that cannot unzip and translocate through the pore, as we previously described (Fig. 3B) [25].

Next, a nanopore study was conducted with the same hairpin after incubation with 1 mM Spm for 5 min. Heat plots from this analysis provided three populations labeled Type 1, 2, and 3 (Fig. 3A). The Type 1 and 2 populations gave similar blocking currents and time distributions as observed for the same hairpin analyzed without Spm (Fig. 3A). These results support Type 1 events as tail entry into α -HL without Spm bound that unzip and translocate through the nanopore, and Type 2 events as loop entry of the hairpin without Spm bound (Fig. 3B). The new event type observed, Type 3, gave a % $I_3/I_o = 24 \pm 1\%$ that was a deep level blockage similar to Type 1 events but the event time was much longer. Interestingly, as the voltage was increased the Type 3 event time also increased, as did Type 2 events, an observation that supports the inability of these events to unzip and translocate through the α -HL nanopore (Fig. S3). On the basis of these results, we propose the Type 3 events occur when the hairpin is captured with a bound Spm (Fig. 3B). The nearly +4 charge on Spm (Fig. 1) neutralizes the hairpin leading to a current increase (+9%) observed while the complex was captured by the pore. Furthermore, the stabilization effect of Spm prevents the hairpin from unzipping and translocating through the pore, as will be discussed more below. Knowledge of how the events change with the addition of Spm allowed two additional studies to be conducted: (1) Investigation of Put and Spd to determine if they give the same profiles as observed with Spm at 1 mM concentration, and (2) a titration study with Spm, Spd, or Put to determine if a dose-dependent response in the events can be detected.

Hairpin unzipping with the presence of Spd and Put

Studies were conducted with the 5'-tail hairpin and 1 mM Spd or Put to determine if the event populations were similar to those observed with 1 mM Spm. When Spd was studied, nearly identical event types were observed as seen with Spm (Fig. 4A and B). Moreover, the blocking currents and event durations for the Type 3 events were similar. Specifically, for Spd $\% I_3/I_0 = 23 \pm 1\%$ while Spm gave $\% I_3/I_0 = 24 \pm 1\%$ (Fig. 4A and B), and both events gave broad time distributions. For Spd and Spm the net positive charge at pH 7.4 is +3 and +3.8, respectively (Fig. 1), which based on the current results does not change the blocking current recorded when the hairpin polyamine complex resides in the vestibule of α -HL. Both polyamines have previously been demonstrated to interact with duplex DNA similarly and have similar binding constants [10]. Therefore, the present observation that Spm and Spd yield nearly identical profiles when bound to the hairpin analyzed with the α -HL nanopore was not surprising.

In contrast to the Spm and Spd results, when 1 mM Put was added with the hairpin and analyzed, Type 3 events were not observed (Fig. 4C). This observation indicates that Put does not interact with the hairpin under these conditions or the interactions are highly dynamic leading to loss of the Type 3 events. The net charge of +2 on Put (Fig. 1) does lead to interactions with DNA; however, they are much weaker than observed for Spd and Spm [8]. Therefore, the observation that 1 mM Put had no effect may result from two possible scenarios. (1) The DNA interactions with Put are too weak to be detected, or (2) the interactions are too dynamic to be observed when the complex was captured. The current results do not allow a conclusion to be made. A similar observation was observed when a single-stranded control (poly-dC₈₇) was studied with 4 mM Spm, in which no change in the event profile was observed. This observation supports long-lived and specific interactions between the amine and hairpin are required to observe the Type 3 events.

Hairpin unzipping profiles observe when titrated with Spm, Spd, or Put

Unzipping experiments with the 5'-tail hairpin were conducted in the presence of different concentrations of Spm, Spd, or Put. In each experiment, the hairpin was added to the *cis* side of the protein nanopore first followed by data collection for ~30 min. Next, polyamine was added and mixed in the analysis chamber (i.e., *cis* side) in aliquots to achieve an increase of amine by 1 mM, followed by data collection for another 30 min. These experiments were conducted at 0, 1, 2, 3, and 4 mM concentration for the three polyamines. For each addition of Spm into the analysis chamber, the distribution of event types changed as a function of the Spm concentration. When 0 mM Spm was present, only Type 1 and 2 events were observed (Fig. 4A). Next, as the Spm concentration increased, the fractional amount of Type 1 and 2 events (i.e., hairpins without bound Spm) decreased and the Type 3 events increased (i.e., hairpins with bound Spm; Fig. 4A). Furthermore, these changes in event type distribution show a dose dependence that further supports Type 3 events as those that occur when the hairpin and Spm are interacting when captured in the vestibule of α -HL (Fig. 3B). Plots of fractional abundance of events that occur without Spm (Type 1 + Type 2) and with Spm (Type 3) gave dose-response trends (Fig. 5C). From these plots, the dissociation constant (K_D) can be extracted from the halfway point through the transition (i.e., the concentration of polyamine that yields polyamine bound and unbound signals in a 1:1 ratio).

In the current experiment, K_D for the Spm hairpin complex was measured to be ~1.5 mM (Fig. 5A) under these conditions (1.00 M KCl, 10 mM PBS, pH 7.4). This value is ~100-fold larger than K_D values reported in the literature [7]; though, the key difference between the current results and those in the literature is the difference in salt concentration. In the current studies, 1.00 M KCl was used as the electrolyte and increased salt concentrations have previously been shown to decrease polyamine DNA interactions [7]. Therefore, the larger K_D value measured in these studies can be explained.

Titration studies were also conducted for Spd and Put. The titration of the hairpin with Spd yielded similar data as observed when the hairpin was titrated with Spm (Fig. 4B). Further, the measured K_D was also ~1.5 mM for Spd (Fig. 5A). Again, this high value was anticipated due to the 1.00 M KCl electrolyte used in this study. Previous studies have also demonstrated that Spm and Spd bind duplex DNA with similar K_D values [10]. When hairpin sample was titrated with Put, the event frequency did not change as a function of Put concentration. At 4 mM Put some fleeting events that can be characterized as Type 3 were observed, but their abundance was <5% (Fig. 4C). These results with Put further support the inability to study these hairpin DNA-diamine interactions with the α -HL nanopore.

These titration studies led to an important observation with respect to studying polyamine DNA interactions with the α -HL nanopore. As the Spd or Spm were titrated into the solution of hairpin DNA being analyzed, the event frequency was found to decrease in response to the added polyamine. Specifically, going from 0 mM (~5 events/sec) up to 4 mM (~0.7 events/sec) Spd or Spm caused the event frequency to decrease by ~10-fold (Fig. 5C). Interestingly, this same effect was not observed for the addition of Put, in which the event frequency remained nearly constant across the titration profile. This observation further supports a conclusion that Put and the DNA hairpin do not interact or the interactions are highly dynamic under these conditions.

Voltage ramp study of the hairpin DNA Spm interaction

Inspired by the observation that Type 3 events resulting from capture of hairpin DNA-Spm complexes were not capable of unzipping, a voltage ramp study was conducted on these complexes. In these experiments, the DNA-Spm complexes were captured at 60 mV (*trans* vs. *cis*) and the voltage was increased by 20 mV increments up to 180 mV to determine if the complex would unzip (Fig. S4). On the basis of these experiments, the hairpin DNA-Spm complexes cannot be dissociated throughout the voltage range studied. One possible explanation for this observation is that the Spm neutralizes charge on the DNA leading to a decrease in the electrophoretic force on DNA that cannot be unzipped in the vestibule. The practical consequences of this observation apply to using polyamines, such as Spm or Spd, to slow the translocation of heterosequenced DNA through the α -HL nanopore. Heterosequenced ssDNA will undoubtedly contain hairpin secondary structures where polyamines can bind, and once trapped by the α -HL nanopore, will interfere with the unzipping process and alter the translocation profile.

Conclusions

In summary, biogenic polyamine interactions with a DNA hairpin were studied with the α -HL nanopore. The addition of Spm or Spd produced a new current-time population (Type 3, Fig. 3) for the hairpin that was not observed in their absence. The addition of Put did not lead to a new population (Fig. 4C). Because the new population was unique, titration studies were conducted to observe the dose-dependent response in the event frequencies as Spd and Spm were added to a hairpin sample studied with α -HL (Figs. 4 and 5). From these studies, it was found that Spd and Spm at 4 mM concentration gave exclusively event types that were characterized as the hairpin DNA-polyamine complex being trapped by the nanopore. These studies also provided K_D values for Spd and Spm under these conditions that were determined to be ~ 1.5 mM for both polyamines. Further, the new population observed when Spm or Spd was bound to the hairpin was not capable of unzipping and translocating through the nanopore at voltages up to 180 mV. This observation suggests that addition of polyamines to heterosequenced ssDNA during α -HL nanopore analysis can lead to highly stabilized structures that can be challenging to unzip and translocate through the pore. Moreover, as Spd or Spm were titrated from 0 to 4 mM concentration, the total event frequency decreased by ~ 10 -fold (Fig. 5C). These observations will impose practical limitations for studies of DNA with Spm or Spd present using the α -HL nanopore.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (R01GM093099). The nanopore instrument and software were generously donated by Electronic BioSciences, San Diego, CA (<http://electronicbio.com>). Discussions with Prof. H. S. White (University of Utah) are gratefully acknowledged.

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Fig. 1. Structures of spermine, spermidine, and putrescine and their pK_a values.

Fig 2.
Structure and dimensions for the α -HL nanopore (pdb 7AHL) [17] embedded in a lipid bilayer.

Fig 3.

Current vs. time heat plots observed when the hairpin DNA was studied without or with Spm present and a model was developed to describe the events. (A) Heat plots observed for translocation of the hairpin DNA without Spm (left) and with 1 mM Spm (right), in which the presence of Spm caused a new event type to be observed (Type 3). (B) Developed model for interaction of the hairpin DNA with the α -HL nanopore. Note: The heat plots shown in panel A have slightly different currents for the Type 2 events. This difference results from the variability of the currents recorded between two different proteins. Examples for the distribution of these currents can be found in Fig. S17.

Fig. 4. Heat plots for titration of Spm, Spd, or Put into the hairpin DNA analyzed with the α -HL nanopore. (A) Profile observed for titration of Spm. (B) Profile observed for titration of Spd. (C) Profile observed for titration of Put. (D) Heat plots observed for the single-stranded poly-dC₈₇ control.

Fig. 5.

Dose-dependent changes in the event type and frequency observed for hairpin DNA interactions with α -HL nanopore in the presence of Spd, Spm, or Put. (A) Heat plot for event types. (B) Fraction of event types measured with titration of Spm. (C) Fraction of event types measured with titration of Spd. (D) Event frequency vs. polyamine concentration.