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Single-molecule detection of a guanine(C8) - thymine(N3) cross-link using ion channel recording¹

Anna H. Wolna, Aaron M. Fleming, and Cynthia J. Burrows

Department of Chemistry, University of Utah, 315 S 1400 East, Salt Lake City, UT 84112-0850, USA, Phone: 801-585-7290, Fax: 801-585-0024

Cynthia J. Burrows: burrows@chem.utah.edu

Abstract

The capability to identify and sequence DNA damage within the context of the genome is an important goal for medical diagnostics. However, currently available methods are not suitable for this purpose. Ion channel nanopore analysis shows promise as a potential single-molecule method to sequence genomic DNA in such a way that also allows detection of base or backbone modifications. Recent studies in human cell lines demonstrated the occurrence of a new DNA cross-link between guanine(C8) and thymine(N3) (5'-G*CT*-3'). The current work presents immobilization and translocation studies of the 5'-G*CT*-3' cross-link in a single-stranded oligodeoxynucleotide using the α -hemolysin (α -HL) ion channel. A 3'-biotinylated DNA strand containing the 5'-G*CT*-3' cross-link was incubated with streptavidin that allowed immobilization of the DNA in the β -barrel of α -HL. In this experiment, the 5'-G*CT*-3' cross-link was placed near the sensitive constriction zone of α -HL, yielding a 2.5% deeper blockage to the ion current level when compared to the unmodified strand. Next, free translocation of a cross-link-containing strand was studied, and an inverse relationship of the time constant with respect to an increase in the applied voltage was found, indicating that the cross-link can easily fit into the β -barrel and traverse through the ion channel. However, a modulation in the ion current level was not observed. These studies suggest that higher resolution ion channels or mechanisms to slow the translocation process, or both, might ultimately provide a mechanism for single-molecule sequencing for G-T cross-links.

Keywords

α -Hemolysin; Nanopore Sequencing; DNA Damage; Guanine-Thymine Cross-Link

INTRODUCTION

Inflammation and oxidative stress are linked with the initiation and progression of many diseases such as cancer^[1] and neurological disorders.^[2] Inflammation produces a variety of oxidizing species including carbonate radical anion ($\text{CO}_3^{\cdot-}$). This one-electron oxidant is derived from ONOO^- and HCO_3^- precursors that react to form nitrosoperoxycarbonate

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Correspondence to: Cynthia J. Burrows, burrows@chem.utah.edu.

(ONOOCO_2^-) that then undergoes spontaneous homolysis to yield the $\text{CO}_3^{\cdot-}$ radical under biological reaction conditions.^[3] The DNA nucleobase guanine (G) has the lowest redox potential, thus it is the site most susceptible to oxidative damage.^[4] G oxidation by $\text{CO}_3^{\cdot-}$ yields a wide range of products, shown in Figure 1, and their yields are dependent on the reaction context.^[5–7] In vitro studies have shown that photochemically generated $\text{CO}_3^{\cdot-}$ selectively oxidizes G in single-stranded DNA (ssDNA) contexts to produce a cross-link in high yield. In ssDNA, the cross-link was found in the sequence context 5'-GCT-3', in which the C8 position of G was covalently linked with the N3 position of T (5'-G*CT*-3').^[8] Interestingly, the cross-link yield is highest when the G and T bases are separated by C, and G is at the 5' end of the sequence context (Figure 1).^[8]

Recently, the G*T* cross-link, the detectable form of the 5'-G*CT*-3' cross-link after complete nuclease digestion, was observed in human cells under conditions mimicking inflammation.^[9]

Methods currently used to quantify damaged bases in genomic DNA samples employ either the comet assay^[10] or enzymatic digestion followed by HPLC-MS/MS analysis; the latter method was used to quantify the G*T cross-linked dinucleoside in human cells that was derived from the 5'-G*CT*-3' cross-link.^[9] However, neither of these methods provides sequence information. Recent advances in single-molecule DNA sequencing with α -hemolysin (α -HL) nanopore offer the potential to obtain the sequence context and identify the type of damaged DNA.^[11–15] This method harnesses the size-limiting properties of the α -HL nanopore to give ion current signatures for the DNA bases when ssDNA is allowed to pass through the pore under an electrophoretic potential.^[16–18] However, sequencing based on free translocation of ssDNA is problematic due to the fast rate at which the DNA passes through the pore, making it difficult to evaluate changes in current level with single-nucleotide resolution.^[12] To overcome this problem and to determine the ion current signature for the various bases, immobilization experiments have been employed in which ssDNA is suspended in the ion channel.^[15, 19–22] In this experiment, a biotin tag is added to the ssDNA terminus; upon binding streptavidin, the complex is too big to pass through the pore; therefore allowing the DNA strand to be captured for long time periods (Figure 2).

Previously, it was shown that placement of biotin 14 nucleotides in the 3' direction compared to the site of interest (i.e. the lesion) on ssDNA, positions that site in the most sensitive zone of α -HL, situated just past the 1.4-nm constriction zone at the top of the β -barrel (Figure 2).^[23, 24] Additionally, this trapped complex is stationary in the pore allowing the current level to be recorded for a sufficient period to be accurately signal averaged. Our laboratory has used this method to differentiate damaged bases from the canonical ones based on their unique current levels.^[15, 25] Specifically, the G-oxidation products spiroiminodihydantoin (Sp), guanidinohydantoin (Gh) and 8-oxo-7,8-dihydroguanine (OG; Figure 1), have been characterized using the immobilization experiment,^[26] as well as an abasic site,^[25] thymine dimer and thymine glycol.^[15] The unique chemical reactivity of abasic sites was harnessed to add chemical tags under appropriate reaction conditions that modulate the current signature well beyond that of all other bases, both canonical and lesion bearing;^[17] chemical modification to modulate the current signature has also been adopted in labeling 5-hydroxymethylcytosine, an epigenetic marker.^[27] In the present study, the

current-level signature for the 5'-G*CT*-3' cross-link was determined in an immobilization experiment when the lesion was placed in the most sensitive region of α -HL, as well as dangling it deeper into the β -barrel to analyze positional effects in the ion current flow. Next, the cross-link was synthesized in the middle of a 53-mer unbiotinylated strand, and free translocation experiments were then conducted to determine if this lesion could be detected during a simulated translocation/sequencing experiment.

EXPERIMENTAL

Materials

All chemicals were purchased from commercial suppliers and used without further purification, except for the DNA strands. The DNA oligomers were synthesized by the DNA-peptide core facility at the University of Utah and were purified by ion-exchange HPLC prior to their use. The 5'-G*CT*-3' cross-link was synthesized according to published procedures and purified by ion-exchange HPLC.^[7, 8, 30] For the immobilization experiments, the 40-mer sequences 5'-CCCCCCCCCCC CCCCCCCCCC CCAACGCTA CCCCCCCCCC-Btn-3' and 5'-CCCCCCCCCCC CCCCCCCCCA ACGCTACCCC CCCCCCCCCC-Btn-3' were studied, and to avoid any unwanted oxidation reactions on the biotin tag, desthiobiotin was used. For the free translocation experiments, a 53-mer 5'-CCCCCCCCCCC CCCCCCCCCC CCAACGCTAC CCCAAAAAAA AAAAAAAAAA AAA-3' sequence was studied. Full details of the oxidation reactions and representative HPLC and ESI-MS data can be found in supplementary information.

Ion Channel Recordings

The ion channel recordings were conducted with a custom built amplifier and data acquisition system designed by Electronic Bio Sciences (EBS), San Diego, CA. The glass nanopore membrane (GNM) was fabricated using previously established procedures.^[31, 32] The immobilization events were collected in a pH 7.9 electrolyte solution (1 M KCl, 1 mM EDTA, 25 mM Tris), under a 120 mV bias (*trans* vs. *cis*) with a 10 kHz filter, and data acquisition rate of 50 kHz. For the immobilization experiments, the DNA-streptavidin (DNA-Strep) complex was obtained by mixing a 200 nM solution of biotinylated DNA with a 50 nM solution of streptavidin in the electrolyte solution for 10 min at 22 °C prior to adding this sample to the analysis chamber. In the immobilization experiments, a 200 nM DNA-streptavidin complex containing the lesion was added to the chamber. After recording >200 events, the same amount of a non-lesion bearing DNA-streptavidin complex was added to determine the current modulation of the cross-link relative to the native-DNA sequence. Lastly, the 3'-biotinylated 40-mer poly-C sequence was added as an internal standard for referencing the new ion currents to those obtained previously.^[25, 26] The data were analyzed using software donated by EBS. The events were extracted using QUB 2.0.0.20.

In the translocation studies, a 5 μ M DNA solution of ssDNA was added to the chamber and >1000 translocation events were collected at a 100, 120 and 140 mV (*trans* vs. *cis*) bias using a 100 kHz filter and a 500 kHz data acquisition rate. In these studies, both lesion-containing and non-lesion containing DNA strands were analyzed in a 1M KCl, 10 mM

NaP_i (pH 7.4), 1 mM EDTA electrolyte solution. The data was analyzed using the same software as previously mentioned.

RESULTS AND DISCUSSION

The most commonly occurring G*T* cross-link observed from CO₃^{•-} oxidation is one in which the G and T nucleotides are separated by a C (5'-G*CT*-3').^[8] More specifically, the Geacintov laboratory has studied this cross-link in the sequence context 5'-CCATCGCTACC-3'.^[33] Therefore, we elected to embed this sequence (substituting the 5' T with A to prevent formation of the 5'-T*CG*-3' cross-link) in a poly-C 40-mer background that was 3'-desthiobiotinylated, positioning the cross-link at the most sensitive region of α -HL, positions ω_{12-14} counting from the 3'-end. This strand will be noted throughout the text with the 5'-G*CT*-3' abbreviation, while the native undamaged sequence will be designated as 5'-GCT-3'. Next, ion channel recordings were commenced to determine the ion-current signature for the 5'-G*CT*-3' cross-link in wild-type α -HL that was embedded in a lipid bilayer suspended across a glass nanopore membrane.^[31, 32] When an electrical potential was applied across the channel, the Strep-Btn DNA complex was electrophoretically driven through the pore causing a deep current level blockage (I) to the open channel current (I_o). The deep-current level blockage was recorded for 1 s, followed by a reversal of polarity that drove the Strep-Btn DNA complex back into the bulk solution, restoring the open channel current. A typical current-time (i - t) trace is shown in Figure 3. The capture/release cycle was repeated >200 times to collect a population of events. The percentage residual current ($\%I/I_o$) for each event was calculated and plotted into a current-level histogram (Figure 4).

The current blockages for the 5'-G*CT*-3' cross-link, the native sequence (5'-GCT-3'), both located at ω_{12-14} , and the internal standard (C₄₀) were then evaluated. The C₄₀ current blockage level, which was used as a reference, was assigned a value of $\%I/I_o = 0$. Figure 4A shows a current level histogram for experiments in which the G of the 5'-GCT-3' sequence was placed at position ω_{14} for the lesion and native strands. In this sequence context the difference between the unmodified- and the 5'-G*CT*-3' cross-link containing strand was $\%I/I_o = 1.7\%$, with the cross-link being the more blocking to the current level. Additionally, this placed the 5'-G*CT*-3' strand at a value of $\%I/I_o = 0.5\%$ more blocking than the C₄₀ reference (Figure 4A). Figure 4B shows a histogram of the percent blockage current when the reactive G was placed deeper into the β -barrel at the position ω_{18} . This increased the observed difference between the cross-link and the unreacted standard. Placement of the cross-link deeper into the β -barrel gave a $\%I/I_o = 2.4\%$; again the cross-link displayed deeper blockage to the current in comparison to the unmodified strand. These results demonstrate that the 5'-G*CT*-3' cross-link is more blocking to the current level, and this effect is enhanced as the lesion is dangled deeper into the β -barrel.

The difference in the current blockage level measured between the unmodified and the cross-link strands is hypothesized to result from the increased rigidity and bulky shape of the 5'-G*CT*-3' cross-link. Consistent with this hypothesis is a previous molecular dynamics (MD) simulation conducted by the Broyde and Geacintov laboratories revealing that the 5'-G*CT*-3' cross-link greatly disturbs base stacking interactions;^[33] furthermore, this cross-link severely unwinds dsDNA based on MD analysis by introducing a severe kink in the

sugar-phosphate backbone, that ejects the intervening C base outwards toward solvent. It is anticipated that these distorting effects will exist in ssDNA as well, especially when the strand is confined in the sterically-demanding nanopore channel. Thus, the ion flow through the channel is perturbed more with the cross-link, and this is reflected in the greater degree of ion current blockage.

In both current-level histograms (Figure 4A and B), the 5'-G*CT*-3' strand showed a second peak that was close in $\%I/I_o$ current blockage to the C₄₀ reference. We hypothesize that this minor peak (~20% of the recorded events) might be due to the presence of the other G-oxidation product, 2Ih, that has been observed from CO₃^{•-} oxidations (Figure 1).^[7] Consistent with this hypothesis is a peak in the ESI-MS for this sample that has the same mass as 2-Ih and a similar relative concentration (~20%) as was detected in the immobilization experiment (Supporting Information). Unfortunately, due to the long length of this strand (40-mer), the cross-link and 2-Ih-containing DNAs were not resolvable, so further studies to confirm this hypothesis were not conducted.

The immobilization experiments presented above show that the 5'-G*CT*-3' cross-link can be differentiated from the unmodified 5'-GCT-3' strand based on current-level modulation (Figure 4A and B). Additionally, the 5'-G*CT*-3' cross-link was considerably more blocking to the ion current and increased as the strand was suspended deeper into the β -barrel. Because the same C₄₀ internal standard has been used in all of our previous studies, it can also be deduced that the cross-link is >2.0% more blocking than any of the natural bases within a poly-C background (Supporting Information).^[24, 26]

The next step was to examine the behavior of the 5'-G*CT*-3' cross-link in a single-stranded DNA construct as it translocated through the α -HL ion channel under an electrophoretic potential. During the translocation experiments, the DNA was allowed to freely pass through the protein channel from the *cis* to *trans* side of the pore, under a 100, 120 or 140 mV bias (*trans* vs. *cis*). The sequence 5'-C₂₀CCAACGCTACCCCA₂₀-3' was used for these experiments, and it was designed both to enhance the 5'-capture events at lower bias and to provide different current levels for 5' vs 3' translocation events. Capture of the 5'-end has previously been demonstrated to be a minor event type compared to 3'-capture at low bias for some sequence contexts.^[34–36] Additionally, in our previous studies it was determined that current modulation of adducts (18-crown-6 adducted to an abasic site) only modulated the current upon capture of the 5'-end.^[34] Thus, the sequence above was designed to optimize the chances of observing current modulation while the cross-linked lesions passed through the narrow constriction of the β -barrel in α -HL. It was previously demonstrated that 5'-entry is maximal with polyC while being minimal with polyA, thus the polyA tail was placed on the 3'-end and the polyC tail was placed on the 5'-end.^[34, 35]

Figure 5A shows *i-t* traces for single DNA molecule translocation events for unmodified and cross-linked 53-mer DNAs. The data collected from the translocation experiments were investigated with respect to directionality effects on entry and the time duration (t_D) for the translocation to occur. Consistent with the diffusional broadening of the translocation times, the t_D histogram of each population was described by a Gaussian curve with a mean peak value t_{max} . The majority of the events (75%) displayed a constant deep blockage current

level throughout the translocation (Figure 5A), with a small fraction of the events (25%) featuring a shoulder current level that preceded the deep blockage current level. The shoulder signature has previously been shown to result from the polyA tail interacting with the vestibule above the constriction zone.^[17] The individual *i-t* traces were then plotted into density plots that are shown in Figure 5B, in which two populations of events were observed that differed in the deep blockage current level. Based on literature reports, these two event populations represent the highest blockage to the current (-16 pA; $0.13 I/I_o$) to be the 3' polyA terminus entering first, and the lower blockage to the current (-22 pA; $0.18 I/I_o$) to be the 5' polyC terminus entering first.^[34, 35] Next, analysis was conducted to determine if there were any detectable differences between the reacted and unreacted strands.

First, the *i-t* traces for the cross-link and unmodified strands were inspected, and from Figure 5A, there did not appear to be any detectable differences in the current level in the *i-t* trace signature. Analysis of the *i-t* traces were conducted for both 3' and 5' entry (Supporting Information), and no observable differences were recorded. Next, analysis of the translocation time revealed that at 100 mV bias (*trans* vs. *cis*) both modified and cross-linked strands gave similar 3' vs. 5' frequencies of entry, and the $t_{\max}$ values (Figure 5C) were within the error limits of each other (5'-GCT-3' $t_{\max} = 0.08 \pm 0.01$ ms ; 5'-G*CT*-3' $t_{\max} = 0.09 \pm 0.01$ ms at 100 mV).

Finally, a voltage dependence study was performed to determine if the cross-link was small enough to fit and translocate across the α -HL ion channel. From these data (Figure 5C), it was observed that for both the standard and cross-link strands the translocation time decreased as the voltage increased. This is a diagnostic signature of a strand being able to translocate from the *cis* to *trans* side of α -HL.^[37] These results are interpreted to say that the 5'-G*CT*-3' cross-link is small enough to fit and translocate through the ion channel, and furthermore the presence of the cross-link does not detectably slow down the translocation speed; thus, this cross-link cannot be differentiated from an undamaged strand during free translocation based on either translocation current or time.

CONCLUSIONS

The studies described herein have shown that the α -HL ion channel has the ability to detect the 5'-G*CT*-3' cross-link in an immobilization experiment by changing the mean current level to one that is at least 2% more blocking than the native strand. However, during a free translocation experiment the cross-link could not be differentiated from an unmodified strand either in current level or translocation time. The latter result is surprising in that it suggests that the presence of a highly distorting base-to-base cross-link is insufficient to inhibit translocation through the α -HL β -barrel. However, use of protein or solid-state ion channels with narrower internal diameters may provide more dramatic current signatures for this lesion during translocation.

In contrast, the immobilization results are more promising because they provide a distinct current-level signature for the presence of this damage. Additionally, a bigger current difference can be observed when the cross-link was placed deeper inside the β -barrel. This deeper current level blockage will also show promise with methods that slow the rate of

translocation to a level that individual base assignments can be reliably made. Once single-molecule DNA sequencing is perfected, this study will provide baseline information for this cross-link that is likely to be observed during DNA sequencing experiments. Because it has been shown that cross-links are highly toxic to cellular processes that require an intact genome (i.e., replication and transcription),^[38] locating specific sites of lesion formation will aid in better understanding how oxidative and inflammatory stress damage the genome leading to disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Guanine (G) lesions observed from oxidation induced by $\text{CO}_3^{\cdot-}$. 8-Oxo-7,8-dihydroguanine (OG), 5-carboxamido-5-formamido-2-iminohydantoin (2Ih), spiroiminodihydantoin (Sp), guanidinohydantoin (Gh), 2,5-diamino-4H-imidazolone (Iz), and the intrastrand 5'-G*CT*-3' cross-link.

Figure 2.
Scale representation of α -HL (pdb 7AHL)^[28]/streptavidin (pdb 1MK5)^[29] Btn-DNA complex with a modeled ssDNA.

Figure 3.

A typical current-time trace for the immobilization experiment.

Figure 4.

Current level histograms for the 5'-G*CT*-3' cross-link, 5'-GCT-3' and the C₄₀ reference strand. (A) Histograms recorded when the G of the 5'-GCT-3' sequence was positioned at ω_{14} . (B) Histograms recorded when the G of the 5'-GCT-3' sequence was positioned at ω_{18} . In both histograms the 5'-G*CT*-3' cross-link is shown in blue, the unmodified 5'-GCT-3' sequence is shown in green, and the C₄₀ internal standard is shown in red.

Figure 5.

Translocation studies of the G*CT* cross-link in a 5' C₂₀CCAACGCTACCCCA₂₀ sequence. (A) Typical *i-t* traces of a single DNA molecule translocating through the pore. (B) Density plots of the unmodified and cross-linked strands at 120 mV (*trans* vs. *cis*). (C) Voltage dependence study showing the t_{max} vs. voltage plot.