Published in final edited form as:

Chem Res Toxicol. 2017 June 19; 30(6): 1317–1326. doi:10.1021/acs.chemrestox.7b00057.

Reactivity and Cross-Linking of 5'-Terminal Abasic Sites Within DNA

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Abstract

Nicking of the DNA strand immediately upstream of an internal abasic (AP) site produces 5'-terminal abasic (dRp) DNA. Both the intact and the nicked abasic species are reactive intermediates along the DNA base excision repair (BER) pathway and can be derailed by side reactions. Aberrant accumulation of the 5'-terminal abasic intermediate has been proposed to lead to cell death, so we explored its reactivity and compared it to the reactivity of the better-characterized internal abasic intermediate. We find that the 5'-terminal abasic group cross-links with the exocyclic amine of a nucleotide on the opposing strand to form an interstrand DNA-DNA cross-link (ICL). This cross-linking reaction has the same kinetic constants and follows the same pH dependence as the corresponding cross-linking reaction of intact abasic DNA, despite the changes in charge and flexibility engendered by the nick. However, the ICL that traps nicked abasic DNA has a shorter lifetime at physiological pH than the otherwise analogous ICL of intact abasic DNA, due to the reversibility of the cross-linking reaction coupled with faster breakdown of the 5'-terminal abasic species via β -elimination. Unlike internal abasic DNA, 5'-terminal abasic DNA can also react with exocyclic amines of unpaired nucleotides at the 3'-end of the nick, thereby bridging the nick by connecting DNA strands of the same orientation. The discovery and characterization of cross-links between 5'-terminal abasic sites and exocyclic amines of both opposing and adjacent nucleotides add to our knowledge of DNA damage with the potential to disrupt DNA transactions.

Introduction

Internal abasic (AP) DNA and 5'-terminal abasic (dRp) DNA are formed sequentially during cellular BER (Figure 1). Internal abasic DNA arises from the action of DNA glycosylases on damaged bases or spontaneously due to depurination of DNA. Nicking

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Supporting Information

Oligonucleotide annealing control reactions, cross-linking reactions between abasic groups and an upstream opposing A, effect of $MgCl_2$ on β -elimination reactions, cross-linking reactions between 5'-terminal abasic DNA and upstream nucleobases, summary of kinetic constants for β -elimination and cross-linking reactions.

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Notes

The authors declare no competing financial interest.

activity of a 5'-endonuclease upstream of the initial internal abasic site then results in 5'-terminal abasic DNA. The terminal abasic group is normally removed by the lyase domain of DNA polymerase β (Pol β). However, failure of Pol β to efficiently remove this lesion has been linked to hypersensitivity of cells to DNA alkylating agents, including the anticancer drug temozolomide, suggesting that accumulation of 5'-terminal abasic DNA is cytotoxic.¹⁻⁵ We investigated the reactivity of the 5'-terminal abasic DNA intermediate to better understand the possible consequences of its persistence.

Like small molecule aldehydes that form cross-links, the open chain aldehyde forms of both types of abasic species are reactive (Figure 1).⁶ The loss of a base from DNA provides a favorable pathway for β -elimination, which results in cleavage of the DNA backbone 3' to the abasic site. In addition, both internal and 5'-terminal abasic sites are susceptible to nucleophilic attack by amines. DNA-protein cross-links (DPCs) between abasic sites and active site amines are well-characterized intermediates in the mechanisms of bifunctional DNA glycosylases/lyases and dRp lyases that participate in DNA repair,⁷⁻¹⁰ but the relevance of other such DPCs that have been detected is less clear.¹¹⁻³⁵ Some may result from side reactions between the reactive abasic moieties and amine groups of proteins that bind to DNA or are abundant. Internal abasic sites also react with exocyclic amines of either upstream or downstream opposing nucleotides to form antiparallel ICLs,³⁶⁻⁴¹ but until now no DNA-DNA cross-links involving 5'-terminal abasic sites have been reported. The presence of such ICLs and DPCs in DNA may obstruct its repair, replication, recombination, and transcription.

Although the two abasic DNA species are chemically analogous, the presence of an upstream nick alters the flexibility and accessibility of the reactive aldehyde and changes the charge and connectivity of the phosphate group on the 5'-side of the abasic site. These differences may affect the β -elimination reactions and the tendency of each abasic site to cross-link with amines. The intrinsic reactivities of abasic sites are the subject of this work, but it is important to note that reactions of these DNA repair intermediates are known to be accelerated by lyases, AP endonucleases, nucleosomes, amine-containing peptides, and designed molecules.^{6, 42, 43}

5'-Terminal abasic DNA has been reported to be 10- to 100-fold more prone to β -elimination than internal abasic DNA, but the half-lives used to make this comparison were measured at different pH values, in different sequence contexts, and in separate studies.⁴⁴⁻⁴⁷ We systematically compared the reactivities of internal and 5'-terminal abasic DNA in the same sequence contexts as a function of pH, in the presence and absence of MgCl₂. We also investigated the extent to which 5'-terminal abasic sites, like their embedded counterparts, are vulnerable to potentially deleterious cross-linking with exocyclic amines of nucleotides. In the course of these studies we identified a new type of cross-link, between 5'-terminal abasic DNA and exocyclic amines of nucleotides upstream of the nick, in which the DNA strands that become joined are in parallel orientation.

Experimental Section

Synthesis and Purification of Oligonucleotides

All of the DNA oligonucleotides shown in Table 1 except for F_{int} and F_{term} were synthesized by Integrated DNA Technologies or the Keck Center at Yale University using standard protecting groups and deprotected according to the supplier's recommendations. They were desalted using Sephadex G-25 and purified using denaturing polyacrylamide gel electrophoresis as previously described.⁴⁸ Oligonucleotide concentrations were determined from their absorbances at 260 nm using calculated extinction coefficients, and the purified oligonucleotides were stored at -20°C .

Preparation of the Abasic Oligonucleotides F_{int} and F_{term} (Table 1)

F_{int} , with a centrally located (internal) abasic site, was prepared by combining 25 nmol of pre- F_{int} with 25 units of UDG (New England Biolabs, 5000 units/mL) at 37°C in 200 mM TrisCl (pH 8), 10 mM DTT, and 10 mM EDTA for 2.5 hrs. The UDG was then removed by phenol-chloroform extraction, and a NAP-5 gel filtration column (GE Healthcare) was used to exchange F_{int} into a storage buffer containing 10 mM NaMES (pH 6.5) and 40 mM NaCl. The extraction and buffer exchange steps for F_{int} were both performed at RT.

F_{term} , with a 5'-terminal abasic site, was prepared by incubating 12.5 nmol of pre- F_{term} with 250 units of UDG at 37°C in 200 mM NaMES (pH 6.5), 10 mM DTT, and 10 mM EDTA for 1 hr. The same extraction and buffer exchange steps described above for F_{int} were then performed for F_{term} at 4°C .

Denaturing PAGE of F_{int} and F_{term} samples that had been subjected to alkaline hydrolysis at 70°C showed that $>96\%$ of each substrate was cleaved to the expected 5'-P 17mer product, indicating that their UDG-catalyzed conversions from pre- F_{int} and pre- F_{term} were essentially complete (data not shown). Denaturing PAGE of unhydrolyzed samples that had been stabilized by reduction with 0.1 M sodium borohydride (NaBH_4) showed that F_{int} initially contained 3% 5'-P 17mer product and F_{term} initially contained 14% 5'-P 17mer product, due to β -elimination of the abasic oligonucleotides that occurred during preparation (data not shown). F_{int} and F_{term} concentrations were determined from their absorbances at 260 nm using calculated extinction coefficients, and aliquots of each abasic oligonucleotide were stored at -80°C .

Oligonucleotide Annealing Reactions

Duplex and nicked duplex DNA substrates in Figure 2 were prepared at RT in 10 mM NaMES (pH 6.5) and 40 mM NaCl directly before use in reactions. F_{int} or F_{term} was combined with a 1.5-fold excess of the indicated R oligonucleotide and, when applicable, a 3-fold excess of the indicated Up oligonucleotide. Doubling the amounts of R oligonucleotide (3-fold excess) or Up oligonucleotide (6-fold excess) in annealing control reactions had no detectable effect (Figure S1 and data not shown).

β -Elimination and Cross-Linking Reactions

Typical reactions contained 0.25 μ M DNA substrate and were carried out at 37 °C in 50 mM buffer, 100 mM NaCl, and 0 or 5 mM MgCl_2 . The following buffers were used at the indicated pH values: NaMES at pH 5.99 and 6.47, NaMOPS at pH 6.97 and 7.44, NaHEPES at pH 7.42 and 7.99, and NaTricine at pH 7.86 and 8.46. At least two independent reactions were performed for each substrate at each condition, and average values are reported.

Reactions were initiated by adding DNA to the remaining reaction components in a final volume of 20–60 μ L. Aliquots were withdrawn at various times and quenched with NaBH_4 to give a final concentration of 0.1 M. The 0.5 M NaBH_4 stock solution was stored in 10 mM sodium hydroxide at –20 °C to retain its potency. The quenched samples were incubated on ice for 30 min, to quantitatively reduce and thereby stabilize abasic sites, and then mixed with 2 volumes of formamide prior to gel analysis. Samples were run on 15 or 20% (w/v) polyacrylamide sequencing gels containing 6.6 M urea. Gels were scanned using a Typhoon Trio imager (GE Healthcare), and emission was measured with a 520BP40 filter after excitation of the fluorescein label at 488 nm. Fluorescence intensities of gel bands were quantified using ImageQuant TL (GE Healthcare), corrected for the amount of background signal, and converted to fraction abasic DNA (substrate), fraction 5'-P 17mer (β -elimination product), and fraction cross-link (cross-linking product).

The slopes of linear fits to the initial rate data for the β -elimination reactions of the internal abasic DNA substrates give k_{elim} directly. The faster β -elimination reactions of the 5'-terminal abasic DNA substrates proceeded to near completion, so these data were fit by a single exponential: fraction 5'-P 17mer = $0.14 + 0.82 \cdot (1 - \exp(-k_{\text{obs}}t))$, where k_{obs} is the observed rate constant and t is time. The equation includes the 5'-P 17mer starting fraction of 0.14 for this substrate (see above) and an end point of 0.96 (end point = starting fraction + maximum fraction formed = $0.14 + 0.82$), which was determined experimentally by following several reactions beyond 7 half-times. The reported k_{elim} values are the initial slopes of these curves: $k_{\text{elim}} = 0.82 \cdot k_{\text{obs}}$. The pH dependencies of β -elimination for the 5'-terminal abasic DNA substrates were fit to a single ionization event: $k_{\text{elim}} = k_{\text{elim}}^{\text{max}} / (1 + [\text{H}^+]/K_a)$.

Cross-linking and irreversible β -elimination of 5'-terminal abasic DNA occur on similar time scales, making it necessary to account for the loss of abasic DNA when analyzing the formation of the cross-linked species. We therefore used the ratio of cross-linked to unlinked substrate, rather than the fraction of cross-linked substrate, in our kinetic analyses (see Figure 3 for a side-by-side comparison of representative data plotted both ways). Only samples with 20% unlinked substrate remaining were included in these analyses, to ensure reproducible ratios.

Cross-linking end points were reached in **2P** reactions at $\text{pH} < 8$ and in **5P** reactions at $\text{pH} < 7$ (Table S1). We interpreted these end points to represent the equilibrium between overall formation and breakdown of the cross-link and fit these data sets to a single exponential: cross-link/abasic DNA = end point $(1 - \exp(-k_{\text{obs}}t))$, where k_{obs} is the observed rate constant for the approach to equilibrium and t is time. The reported k_{link} values are the initial slopes of these curves: $k_{\text{link}} = \text{end point} \cdot k_{\text{obs}}$. For conditions where equilibrium was not reached

(pH > 8 for **2P**; pH > 7 for **5P**), the slopes of linear fits to the initial rate data for the cross-linking reactions give k_{link} directly.

Cross-linking end points were also reached in reactions of **4P**, **6P**, **7P**, **8P**, and **9P** at pH 6.47 (Table S1). As for **2P** and **5P** reactions, we interpreted these end points to represent the equilibrium between overall formation and breakdown of the cross-link and fit these data sets to a single exponential: cross-link/abasic DNA = end point(1-exp(- $k_{\text{obs}}t$)), where k_{obs} is the observed rate constant for the approach to equilibrium and t is time. The reported k_{link} values are the initial slopes of these curves: $k_{\text{link}} = \text{end point} * k_{\text{obs}}$.

Results

Terminal abasic groups form ICLs that are analogous to those formed by internal abasic groups

Internal abasic sites in DNA cross-link with exocyclic amines of G, P, and A at some positions in opposing strands.³⁶⁻⁴¹ For example, the internal abasic group of the 35mer duplex substrate **2P** (Figure 2) cross-links with an upstream opposing P (Figure 3A, left). A small amount of a slower migrating product builds up at late time points when P is replaced with A in substrate **2A**, consistent with a different ICL between the internal abasic group and the exocyclic amine of one of the nearby natural opposing bases in this sequence context (Figure S2).

To find out if 5'-terminal abasic sites also cross-link with exocyclic amines, we prepared **5P** (Figure 2), the nicked equivalent of **2P**. A slowly migrating product forms in reactions of **5P** (Figure 3A, right). This species migrates faster than cross-linked **2P** (35+35 nt) on denaturing gels, as expected for cross-linked **5P** (35+18 nt) after dissociation of the 17mer upstream of the nick (Figure 3A, center). The ICL appears to involve P and not another nucleotide in the vicinity because it is absent when P is replaced with A in substrate **5A** (Figure S2). Substantially more of the 35+18 nt ICL accumulates in reactions of **4P**, which has no oligonucleotide upstream of the nick, and a small amount of a different ICL accumulates when the upstream opposing P is replaced with A in substrate **4A** (Figure S2). This suggests that the 5'-terminal abasic group can cross-link with exocyclic amines of P or of natural opposing bases, depending on its sequence context.

We simultaneously analyzed the β -elimination (Figure 3B) and cross-linking (Figure 3C) reactions of substrates that contained either internal or 5'-terminal abasic groups at 37 °C and pH 6.47. The accumulation of 5'-P 17mer product in β -elimination reactions of both single strand (**1**) and duplex substrates (**2A**, **2P**) with internal abasic groups was similarly slow. Likewise, there was little difference between the β -elimination reactions of single strand (**3**) and duplex substrates (**5A**, **5P**) with 5'-terminal abasic groups. However, k_{elim} values for the 5'-terminal abasic site substrates exceeded k_{elim} values for the internal abasic site substrates by ~100-fold under these conditions (Figure 3B, Table S1).

Although both the internal abasic group of **2P** and the 5'-terminal abasic group of **5P** cross-link with an upstream opposing P (Figure 3A), the two ICLs accumulate differently (Figure 3C). The amount of cross-linked **2P** plateaus at ~3% of the total labeled DNA, whereas

cross-linked **5P** increases to ~2% of the total before decreasing back to zero. The rise and fall of the **5P** ICL occurs on the same time scale as depletion of **5P** by β -elimination (Figure 3B, 3C), so we accounted for the loss of substrate over time by plotting the ratio of cross-linked to unreacted abasic substrate at each time point in Figure 3D. This analysis reveals that both the **5P** and **2P** cross-linking reactions reach end point ratios of ~0.03 with $t_{1/2}$ ~40 min. Little or no ICL was detected in reactions of duplex abasic substrates with an upstream opposing A (**2A**, **5A**) or in reactions of single strand abasic substrates (**1**, **3**).

Our side-by-side comparison of the reactivities of internal and 5'-terminal abasic DNAs of matched sequence at pH 6.47 shows greater susceptibility of the latter to β -elimination, while the respective cross-linking reactions with **P** proceed similarly (Figure 3). To better understand these observations we extended our analysis to cover a range of pH values.

Systematic comparison of β -elimination and cross-linking of internal and terminal abasic groups within the same sequence context as a function of pH

The effect of pH on k_{elim} , the rate constant for β -elimination, is shown in Figure 4A. As previously noted at pH 6.47, a 5'-terminal abasic group undergoes β -elimination at a faster rate than the corresponding internal abasic group at all pH values, and a given abasic group reacts similarly whether it is single stranded or within a duplex. However, while there is little variation in k_{elim} from one end of the pH range to the other for the internal abasic group, k_{elim} increases with increasing pH until it levels off above pH 7.5 for the 5'-terminal abasic group. The pH dependence of β -elimination of the 5'-terminal abasic group is consistent with a faster reaction of its dianionic 5'-phosphate form than of its monoanionic form, with a pK_a of ~7 for protonation of the 5'-phosphate (Figure 4B). In contrast, the 5'-phosphate of the internal abasic group is monoanionic due to its embeddedness in DNA, accounting for the slower, pH-insensitive β -elimination of the internal abasic group. Reactions that include 5 mM MgCl_2 show the same overall pH effects for β -elimination, albeit with a shift in the pK_a for protonation of the 5'-phosphate of the 5'-terminal abasic group from ~7 to ~6.5 (Figure S3).

The effect of pH on k_{link} , the rate constant for cross-linking of the abasic group with an upstream opposing **P**, is shown in Figure 4C. The internal abasic group of **2P** and the 5'-terminal abasic group of **5P** have matching k_{link} values over the entire pH range, and these values fall steeply with increasing pH. The k_{link} data in Figure 3C is well-represented by a line with a slope of -1, and this pH dependence is likely due to acid catalysis in the general mechanism of imine formation (Figure 4D). Slightly lower k_{link} values are measured when 5 mM MgCl_2 is present, but the same pH dependence is observed (Figure 4C). The propensity of the exocyclic amine of **P** to react with the aldehyde moiety of an abasic group is not affected by an upstream nick or by Mg^{2+} coordination, as evidenced by similar k_{link} values and pH dependencies for substrates with internal and 5'-terminal abasic groups in the presence and absence of MgCl_2 .

Cross-linking of 5'-terminal abasic DNA is affected by the identity of the upstream oligonucleotide

Internal abasic DNA is embedded within a defined sequence context. In contrast, a polymerase may add nucleotides to the 3'-end of a nicked duplex, which alters the local sequence context of 5'-terminal abasic DNA. To test the effects of context on reactivity of 5'-terminal abasic DNA, we varied the oligonucleotide that was upstream of the nick.

β -Elimination of 5'-terminal abasic DNA was very similar in all contexts that were investigated (Figure 5A). However, the cross-linking end point was 5-30-fold greater for **4P**, which has no upstream oligonucleotide, than for nicked duplex substrates (Figure 5B; Table S1), indicating that upstream oligonucleotides make the equilibrium for cross-linking less favorable. Comparison of the nicked duplex substrates reveals similar cross-linking parameters for the duplexes without an overhanging 3'-terminal nucleotide (**5P**) or with an overhanging 3'-terminal C (**7P**), whereas cross-linking endpoints and k_{link} values are both reduced for duplexes with an overhanging 3'-terminal A (**6P**), G (**8P**), or T (**9P**) (Figure 5C; Table S1). These reductions suggest that access of the exocyclic amine of P to the reactive aldehyde of the abasic site is impeded by the bulk of a 3'-overhanging purine or by base pairing between a 3'-terminal T and the central A of the R_{PA} template.

A novel cross-link between 5'-terminal abasic DNA and exocyclic amines of upstream nucleotides

The incised backbone of terminal abasic DNA may provide it with enough flexibility to cross-link with DNA that is upstream of the nick. While studying the effect of upstream oligonucleotides on the 35+18 nt cross-link between P and a terminal abasic site on opposing strands, we detected a new gel band in some nicked duplex reactions (Figure S4A). To rule out participation of the exocyclic amine of P in this new reaction, we replaced the R_{PA} template with R_{AA} in substrates (Figure 6).

We surveyed all 4 natural nucleotides upstream of the nick, opposite the central A of R_{AA} (**6A-9A**). These are the repair intermediates that would result from either accurate (**9A**) or inaccurate (**6A-8A**) DNA polymerization prior to lyase activity (Figure 7). A new product was observed only when R_{AA} was present and when the 3'-terminal nucleotide of the upstream oligonucleotide was A, C, or G (Figure 6A, lanes 2, 4, 5). Its mobility was consistent with a 36mer, suggesting that it is an 18+18 nt species that contains a cross-link between an exocyclic amine of a 3'-terminal nucleotide and the 5'-terminal abasic site (Figure 6B). Consistent with this hypothesis, the expected 25+18 nt cross-link formed in an analogous reaction that contained an upstream oligonucleotide with A at the 3'-terminus and a 5'-extension of 7 nt (Figure 6A, lane 3), and no cross-link formed with a 3'-terminal T, which lacks an exocyclic amine, at the nick (Figure 6A, lane 6).

We followed the appearance of the 18+18 nt cross-links with time (Figure 6C). β -Elimination depleted the 5'-terminal abasic DNA substrates before the cross-linking reactions reached equilibrium; slopes of linear fits to the initial rate data were therefore used to determine k_{link} values of $\sim 10^{-5} \text{ min}^{-1}$. Very similar results were obtained for appearance of the 18+18 nt cross-links that form opposite the R_{PA} template (Figure S4C).

The absence of an exocyclic amine on the 3'-terminal T at the nick likely explains why no 18+18 nt cross-link forms in the 9A reaction, but base pairing between the T and the central A of the R_{AA} template may also affect cross-linking. To distinguish between these possibilities we surveyed all 4 natural nucleotides upstream of the nick when opposite the central G of R_{AG} (Figure S4D). For the R_{AG} template, like the R_{AA} template, no 18+18 nt cross-link formed with a 3'-terminal T at the nick, as expected if the cross-link requires an exocyclic amine. Cross-linked species were observed with 3'-terminal A or G at the nick, but not with C, presumably because base pairing between the 3'-terminal C and the central G of the R_{AG} template prevents cross-linking between the exocyclic amine of C and the reactive aldehyde of the abasic site.

Discussion

ICLs between internal abasic groups and exocyclic amines of nucleotides on the opposing strand were first reported by Gates and coworkers.³⁶ Our results show that 5'-terminal abasic groups within nicked DNA duplexes likewise react with exocyclic amines of nucleotides on the opposing strand to form ICLs. These ICLs are chemically analogous to the ICLs of internal abasic groups within intact DNA duplexes of the same sequence.

The two types of abasic groups are similarly reactive, cross-linking with the exocyclic amine of an upstream opposing P with matching rate constants over a wide pH range (Figure 4C). Both cross-linking reactions have pH dependencies with a slope of -1, which are attributed to acid-catalyzed dehydration (Figure 4D). Due to these negative slopes, the two cross-links accumulate ~10-fold more slowly at a physiological pH of 7.4 than at the representative pH of 6.47 shown in Figure 3. Finally, both cross-linking reactions are reversible: depletion of 5'-terminal abasic DNA by β -elimination decreases the amount of its ICL (Figure 3), and binding of internal abasic DNA by enzymes as it is liberated from the exocyclic amine shifts its equilibrium away from ICL formation.⁴¹

Although the intrinsic chemistry of cross-linking appears to be unaffected by the altered charge and connectivity of the 5'-terminal abasic group, these differences have several important implications for the fates of abasic intermediates.

1. ICLs between internal abasic groups and amines are expected to be longer-lived than the corresponding ICLs of 5'-terminal abasic groups. Both cross-linking reactions are reversible, so depletion of either abasic substrate leads to depletion of its ICL. The 5'-terminal abasic substrate breaks down through β -elimination ~100-fold faster than the internal abasic substrate at pH 7, due to the higher reactivity of its dianionic 5'-phosphate form (Figure 4), so the lifetime of its ICL is correspondingly shorter. Proteins that bind to and sequester or react with the abasic intermediates will affect the lifetimes of their respective ICLs (see below).
2. Internal abasic DNA is embedded within a defined sequence context, but 5'-terminal abasic DNA may reside within a simple nicked duplex, within a nicked duplex with one or more overhanging 3'-terminal nucleotides, or within a gapped duplex (i.e., missing one or more 3'-terminal nucleotides). The presence or absence and the identity of nucleotides upstream of the nick have large effects

on both the equilibrium and the rate constants for cross-linking of 5'-terminal abasic DNA with an upstream opposing P, as shown in Figure 5B.

3. The 5'-terminal abasic group has a wider range of cross-linking partners than the internal abasic group. The internal abasic group is covalently confined within a DNA sequence, with fixed upstream neighbors. In contrast, a polymerase may add nucleotides to the 3'-end of the nick upstream of the 5'-terminal abasic group, changing its immediate neighbors. We have demonstrated that exocyclic amines of such 3'-terminal nucleotides can react with the abasic group to form DNA-DNA cross-links in which the participating strands are in parallel orientation (Figure 6, Figure S4). It remains to be seen whether or not such "parallel" cross-links, which accumulate slowly and in small amounts in the DNA sequence context we examined, are biologically significant. Displacing DNA synthesis occurring at the nick would leave the 5'-terminal abasic group at the end of a flap,⁴⁹ and this flexible tethering of the abasic group may enable it to cross-link with other upstream, downstream or opposing nucleotides.

Our detection and analysis of DNA cross-linking by 5'-terminal abasic groups sheds light on several features of the BER pathway. Tight binding of internal abasic DNA by DNA glycosylases has been proposed to shield it from side reactions until it is further processed by downstream enzymes.⁵⁰ Similarly, AP endonuclease binds 5'-terminal abasic DNA, its reaction product, with high affinity,^{51, 52} which may prevent this intermediate from forming inappropriate cross-links with amines of nucleotides (ICLs), amine groups of proteins (DPCs), and other biological amines. Efficient removal of the 5'-terminal abasic group by the lyase activity of Pol β also eliminates this intermediate's cross-linking ability. The lyase activity of Pol β normally precedes polymerase activity,⁵³ and this timing would limit formation of cross-links that bridge the nick, such as those we observed between overhanging 3'-terminal nucleotides and 5'-terminal abasic groups (Figure 6, Figure S4). However, levels of these and other DNA-DNA cross-links and DPCs are expected to increase when the 5'-terminal abasic DNA intermediate persists due to changes in flux through the BER pathway, for example, in cells expressing L22P Pol β , a tumor-associated variant that is lyase deficient.^{54, 55}

Although the reversibility of the cross-linking reactions allows released 5'-terminal and internal abasic sites in DNA to be protected or further processed by BER enzymes, their cross-links have a significant lifetime once formed. In the absence of an active mechanism for their breakdown, such cross-links could persist long enough to obstruct DNA processes including replication and transcription. Two oxidized abasic sites are also known to form reversible ICLs.^{43, 56, 57} The DNA glycosylase NEIL3 was recently shown to reverse a cross-link between an internal abasic site and the exocyclic amine of a downstream opposing A.⁵⁸ It is reasonable to predict that enzymes also repair cross-links that arise between internal or 5'-terminal abasic sites and other amines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the O'Brien lab for helpful discussions.

Funding

This work was supported by NIH grant RO1 GM108022 to P.J.O.

Abbreviations

AP	apurinic/apyrimidinic
dRp	5'-deoxyribose phosphate
BER	base excision repair
ICL	interstrand DNA-DNA cross-link
Polβ	DNA polymerase β
DPC	DNA-protein cross-link
UDG	uracil DNA glycosylase
P	2-aminopurine
NaMES	sodium 2-(<i>N</i> -morpholino)ethanesulfonate
NaMOPS	3-(<i>N</i> -morpholino) propanesulfonate
NaHEPES	sodium <i>N</i> -(2-hydroxyethyl)piperazine- <i>N'</i> -(2-ethanesulfonate)
NaTricine	sodium <i>N</i> -[tris(hydroxy-methyl)methyl]glycine

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Figure 1.

Base excision repair pathway for DNA. Internal abasic DNA and 5'-terminal abasic DNA intermediates and their reactive aldehyde forms are boxed. The numbered reactions correspond to the following enzyme activities: 1, DNA glycosylase; 2, AP endonuclease; 3, dRp lyase; 4, DNA polymerase; 5, DNA ligase. Internal abasic DNA and 5'-terminal abasic DNA are also known as apurinic/apyrimidinic (AP) DNA and 5'-terminal deoxyribose phosphate (dRp) DNA, respectively.

Figure 2.

Structural representations of internal and 5'-terminal abasic substrates used in this study. The DNA oligonucleotides annealed to construct each substrate are shown above or below the structures, and complete sequences are listed in Table 1. Abbreviations: ab, abasic site; P, 2-aminopurine; X = P or A; *, fluorescein label linked to the 3'-terminus of DNA via a phosphorothioate group.

Figure 3.

Internal and 5'-terminal abasic sites both cross-link with an opposing P. A. Slowly migrating cross-linked DNA forms in reactions of 2P (left) and 5P (right). Reactions contained 0.25 μ M DNA and were carried out at 37 °C and pH 6.47. Samples were reduced with NaBH₄ prior to separation on the same 15% denaturing polyacrylamide gel (intervening lanes have been omitted for clarity). The 5'-P 17mer that is visible at the bottom of all lanes arises from nonenzymatic β -elimination of the abasic sites prior to or during the reaction time course. The inset for the 5P reaction (right) is the gel at lower contrast, showing that the 5'-P 17mer is resolved from the 5'-terminal abasic strand. B. Fraction of 5'-P 17mer, the product of β -elimination, at time points in A, in duplicate reactions, and in reactions of related abasic substrates at pH 6.47 is shown. Some β -elimination occurs during preparation of the abasic DNA strands, so at time zero reactions of the internal abasic DNA substrates contain 3% 5'-P 17mer and reactions of the 5'-terminal abasic DNA substrates contain 14% 5'-P 17mer (see Experimental Section). Curves are linear fits to the initial rate data for the internal abasic DNA substrates (squares) and exponential fits to the data for the 5'-terminal abasic DNA substrates (circles). C. Fraction of cross-linked DNA at time points in A, in duplicate reactions, and in reactions of related abasic substrates at pH 6.47 is shown. This cross-linked DNA does not form when the opposing strand is absent (**1**, **3**) or contains A in place of P

(**2A**, **5A**). D. To account for the loss of the abasic substrates due to β -elimination during the time courses, the ratio of cross-linked to unlinked substrate is shown for the same reactions of **2P** and **5P** as in B and C (see Experimental Section). End point ratios of 0.036 and 0.033 and k_{obs} of 0.019 min^{-1} and 0.014 min^{-1} for the approach to the end point were obtained from exponential fits to the data for **2P** and **5P**, respectively. All error bars represent one standard deviation from the mean of at least two independent data points.

Figure 4.

Effect of pH on reactivity of abasic DNA species. A. The pH dependence of β -elimination for internal (squares) and 5'-terminal (circles) abasic DNA substrates. Non-linear least squares fits to a single ionization event give overlapping curves with pK_a values of 7.0, 7.0, and 7.1 for **3**, **5A**, and **5P**, respectively. B. Analogous chemical mechanisms for β -elimination of internal (left) and 5'-terminal (right) abasic DNA substrates. Protonation of the 5'-phosphate of the terminal abasic DNA substrate makes it less susceptible to β -elimination. C. The pH dependence of cross-linking for internal (squares) and 5'-terminal (circles) abasic DNA substrates, in the absence (black) or presence (red) of 5 mM MgCl_2 . A line with a slope of -1 is drawn through the data. D. General mechanism for imine formation. The step depicting acid-catalyzed protonation of oxygen, which promotes subsequent dehydration, is italicized. Imine formation in the cross-linking reaction involves attack of the exocyclic amine of P on the aldehyde moiety of an internal or 5'-terminal abasic group of DNA.

Figure 5.

Effect of DNA context on reactions of 5'-terminal abasic DNA variants at pH 6.47. A. The fraction of 5'-P 17mer, the product of β -elimination, in reactions is shown. Curves are exponential fits to the data for each substrate and give similar k_{elim} values (0.0011–0.0013 min^{-1} ; Table S1). B. The ratio of cross-link to unlinked abasic DNA is plotted for each substrate. End point ratios and k_{link} values were obtained from exponential fits to each data set (Table S1). C. Magnified version of Figure 5B, with the scale of the y-axis adjusted and

the outlier substrate 4P omitted for clarity. All reactions were performed in duplicate, and error bars represent one standard deviation from the mean value at each time point.

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Figure 6.

Exocyclic amines of upstream nucleotides cross-link with 5'-terminal abasic DNA. **A.** Cross-links form in reactions that have an upstream strand with a 3'-overhanging A, C, or G, when templated by R_{AA} (lanes 2–5). For size comparison, the end lanes (S) contain the 35 nt oligonucleotide pre-F_{int} and a 49 nt oligonucleotide of similar sequence.⁵⁹ Reactions contained 0.25 μ M DNA and were incubated at 37 °C and pH 6.47 for 12 hrs, at which time samples were reduced with NaBH₄ and separated on a 15% denaturing polyacrylamide gel. Only the upper portion of the gel where cross-linked species migrate is shown. **B.** Structural representation of the 18+18 nt cross-linked species visible in lanes 2, 4, and 5 of Figure 6A, where V is A, C, or G, respectively. The cross-linked species is shown annealed to its template strand. The analogous 25+18 nt cross-link in lane 3 has an upstream 5'-extension of 7 nt. **C.** The ratio of 18+18 nt cross-link to unlinked abasic DNA is plotted for each substrate. Curves are linear fits to each data set and give k_{link} values of $1.4 \times 10^{-5} \text{ min}^{-1}$ for **6A**, $6.5 \times 10^{-6} \text{ min}^{-1}$ for **7A**, and $6.3 \times 10^{-6} \text{ min}^{-1}$ for **8A**. Error bars represent one standard deviation from the mean of at least two independent data points.

Figure 7. Possible variation in reaction order in BER. Intermediates capable of cross-linking with the 5'-terminal abasic site will form if DNA polymerase activity precedes dRp lyase activity (red arrows).

Table 1

DNA Oligonucleotides

Name	Oligonucleotide Sequence ^{a,b}
Forward (F) ^c	
pre-F _{int}	5'-TTGCCCAGATAGCATCC <u>TU</u> CCTTCTCTCCATGCGTC*
pre-F _{term}	5'- <u>pU</u> CCTTCTCTCCATGCGTC*
F _{int} ^d	5'-TTGCCCAGATAGCATCC <u>Tab</u> CCTTCTCTCCATGCGTC*
F _{term} ^d	5'- <u>ab</u> CCTTCTCTCCATGCGTC*
Reverse (R) ^c	
R _{PA} ^e	3'-AACGGGCTATCGTAGG <u>PA</u> GGAAGAGAGGTACGCAG
R _{AA}	3'-AACGGGCTATCGTAGG <u>AA</u> GGAAGAGAGGTACGCAG
R _{AG}	3'-AACGGGCTATCGTAGG <u>AG</u> GGAAGAGAGGTACGCAG
Upstream ^c	
Up ₁₇	5'-TTGCCCAGATAGCATCC <u>T</u>
Up _{17A}	5'-TTGCCCAGATAGCATCC <u>TA</u>
Up _{17C}	5'-TTGCCCAGATAGCATCC <u>TC</u>
Up _{17G}	5'-TTGCCCAGATAGCATCC <u>TG</u>
Up _{17T}	5'-TTGCCCAGATAGCATCC <u>TT</u>
Up _{24A}	5'-GACATGATTGCCCAGATAGCATCC <u>TA</u>

^aAbbreviations: U, uracil; lowercase p, phosphate group; uppercase P, 2-aminopurine; ab, abasic site; *, fluorescein linked to the 3'-terminus of DNA via a phosphorothioate group.

^bOligonucleotides are offset and displayed in 5' to 3' or 3' to 5' orientation to align complementary regions.

^cForward, reverse, and upstream oligonucleotides were annealed to construct the DNA substrates represented in Figure 2. For example, F_{term}, R_{PA}, and Up₁₇ are the components of the nicked duplex **5P**.

^dMade by treating the corresponding pre-F oligonucleotide with UDG (see Experimental Section).

^ePrevious work showed robust cross-linking between an internal abasic site and P in the 5'-Tab/3'-PA sequence context,³⁷ with a faster approach to equilibrium than for other sequences known to cross-link.⁴¹ These factors made this context favorable for seeking the analogous cross-link between P and the less stable 5'-terminal abasic site.