Probing the Configurations of Formamidopyrimidine Lesions Fapy·dA and Fapy·dG in DNA Using Endonuclease IV†

Jennifer N. Patro, Kazuhiro Haraguchi, Michael O. Delaney, and Marc M. Greenberg*

Department of Chemistry, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218

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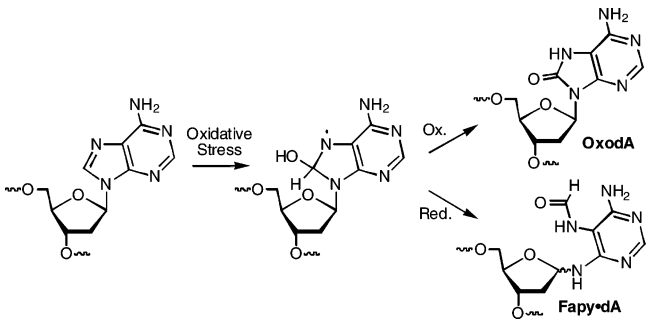
ABSTRACT: The formamidopyrimidines Fapy·dA and Fapy·dG are produced in DNA as a result of oxidative stress. These lesions readily epimerize in water, an unusual property for nucleosides. The equilibrium mixture slightly favors the β-anomer, but the configurational status in DNA is unknown. The ability of endonuclease IV (Endo IV) to efficiently incise α-deoxyadenosine was used as a tool to determine the configuration of Fapy·dA and Fapy·dG in DNA. Endo IV incision of the C-nucleoside analogues of Fapy·dA was used to establish selectivity for the α-anomer. Incision of α-C-Fapy·dA follows Michaelis−Menten kinetics ($K_m = 144.0 \pm 7.5$ nM, $k_{cat} = 0.58 \pm 0.21$ min^{-1}), but the β-isomer is a poor substrate. Fapy·dA incision is considerably slower than that of α-C-Fapy·dA, and does not proceed to completion. Endo IV incision of Fapy·dA proceeds further upon rehybridization, suggesting that the lesion reequilibrates and that the enzyme preferentially cleaves duplex DNA containing α-Fapy·dA. The extent of Fapy·dA incision suggests that the lesion exists predominantly (∼90%) as the β-anomer in DNA. Endo IV incises Fapy·dG to less than 5% under comparable reaction conditions, suggesting that the lesion exists almost exclusively as its β-anomer in DNA.

Cells are constantly subjected to exogenous (e.g., UV light, γ radiolysis) and endogenous (e.g., aerobic metabolism, lipid peroxidation) DNA-damaging agents, which create a variety of base lesions. The accumulation of mutagenic and/or cytotoxic lesions in DNA is involved in aging and various diseases, including cancer (*1*−*10*). The lesions formed can be divided into two families, abasic sites and those containing modified nucleobases. Abasic lesions are typically highly lethal, but purines are preferentially incorporated opposite them when they are bypassed in vitro or in *Escherichia coli* (*11*−*14*). In contrast, the lethality and mutagenicity of lesions containing modified bases vary widely (*15*−*17*). The formamidopyrimidines Fapy·dA and Fapy·dG[1] are a biologically significant and chemically interesting family of nucleobase-modified lesions. Fapy·dA and Fapy·dG are formed in competition with the 8-oxopurines OxodG and OxodA from common intermediates (Scheme 1) (*18*). The outcomes of the interactions of formamidopyrimidines with DNA polymerase and repair enzymes are similar to those reported for the 8-oxopurines (*19*−*25*). However, the formamidopyrimidines exhibit an unusual property for nucleosides in that they readily isomerize at their anomeric centers (eq 1). The free nucleosides exist predominantly in their pyranose forms, a structure that is unattainable in DNA (*26*, *27*). Formamidopyrimidine derivatives suitably 5′-protected to prevent rearrangement to their pyranose form exist as a mixture of α- and β-anomers (*28*, *29*). Equilibration occurs over several hours at 25 °C, and the β-anomer is slightly favored. Interactions within the helix could alter the slight preference for the respective β-anomer of these lesions. Determining the distribution of formamidopyrimidine anomers in duplex DNA is an important aspect of their structural characterization. Herein we describe experiments using Endo IV as a mechanistic probe which indicate that the β-isomers of Fapy·dA and Fapy·dG are strongly favored in DNA.

Scheme 1: Formamidopyrimidine and 8-Oxopurine Lesions Arise from a Common Intermediate



Interest in the formamidopyrimidines is cultivated in part by their structural and mechanistic relationship with the 8-oxopurines. The latter are perhaps the most well studied family of DNA lesions, and OxodG is often used as a biomarker (*30*, *31*). Formamidopyrimidines are formed in vitro in yields comparable to those of the mechanistically related 8-oxopurines, particularly under O_2-deficient conditions (*32*−*35*). Measurements of these lesions in vivo are more variable (*36*, *37*). Fapy·dG is present in greater amounts than OxodG in human leukemia cells exposed to ionizing

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* To whom correspondence should be addressed. E-mail: mgreenberg@jhu.edu. Phone: (410) 516-8095. Fax: (410) 516-7044.

[1] Abbreviations: Fapy·dG, 2′-deoxyguanosine formamidopyrimidine; Fapy·dA, 2′-deoxyadenosine formamidopyrimidine; MeFapy·dG, *N*7-methyl-2′-deoxyguanosine formamidopyrimidine; OxodG, 8-oxodeoxyguanosine; OxodA, 8-oxodeoxyadenosine; α-dA, α-deoxyadenosine; Fpg, formamidopyrimidine DNA glycosylase; BER, base excision repair; Endo IV, endonuclease IV.

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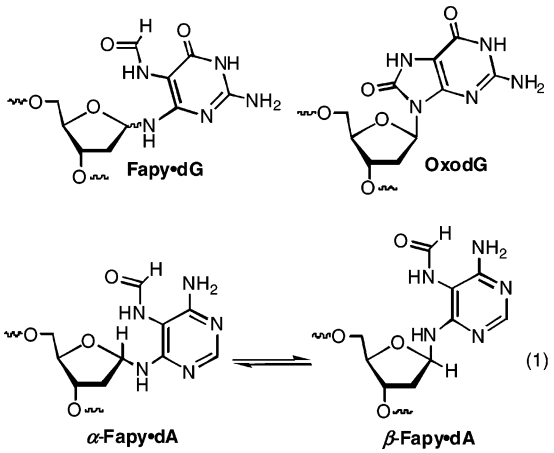


radiation, but endogenous levels depend on the cell type (*38*−*40*). In vitro experiments suggest that Fapy·dG influences biological processes in much the same way that OxodG does. For instance, randomly generated formamidopyrimidines are efficiently excised by many of the same BER enzymes that recognize OxodG (*41*−*43*). Comparisons between Fapy·dG and OxodG are further substantiated in studies concerned with the recognition of Fapy·dG by two BER enzymes, Fpg and Mut Y (*24*). Fapy·dG is excised ∼17-fold more rapidly by Fpg when it is opposite dC than dA. Furthermore, dA incision by Mut Y is efficient when it is opposite Fapy·dG. Experiments using a template containing Fapy·dG and a polymerase suggest that the selectivity exhibited by the BER enzymes evolved to protect against a mutagenic lesion (*19*, *21*).

Investigating the biochemistry of Fapy·dG and Fapy·dA was greatly accelerated by the development of a method for chemically synthesizing oligonucleotides containing the lesion (*44*, *45*). Fpg excises Fapy·dA even more rapidly than OxodG or Fapy·dG, but the enzyme is not discerning with respect to the opposing nucleotide (*23*). Fpg may not need to discriminate between various base pairs containing Fapy·dA, because it is unlikely that promutagenic base pairs containing the lesion will be found in vivo (*46*, *47*). This hypothesis is consistent with experiments which show that Fapy·dA has a very small effect on the fidelity of Klenow exo$^-$ (*20*).

Despite the varied and interesting interactions between polymerases or BER enzymes and these lesions, structural data on DNA containing formamidopyrimidines are unavailable. The lack of structural data makes it difficult to rationalize the lesions' interactions with enzymes. The interest in structural information is heightened by the possibility that the formamidopyrimidines may exist as a mixture of anomers in duplex DNA. Since NMR or X-ray data are unavailable, we sought to use a more inferential tool, Endo IV. We hypothesized that Endo IV, which incises α-deoxyadenosine and α-thymidine, could offer insight into the distribution of formamidopyrimidine anomers in DNA by selectively incising the α-anomer (*41*, *48*−*50*).

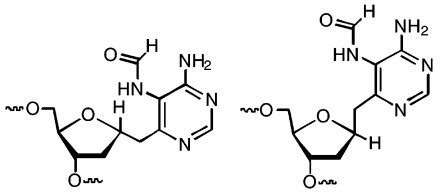
EXPERIMENTAL PROCEDURES

Materials and General Methods. Oligonucleotides were prepared on an Applied Biosystems Inc. 394 DNA synthesizer. Commercially available DNA synthesis reagents were obtained from Glen Research Inc. Oligonucleotides containing Fapy·dG, Fapy·dA, α-C-Fapy·dA, or β-C-Fapy·dA were prepared as previously described (*44*, *45*). All others were synthesized using standard protocols. DNA manipulations were carried out using standard procedures. T4 polynucleotide kinase was obtained from New England Biolabs. Radionuclides were obtained from Amersham Pharmacia. Endo IV was obtained from Trevigen.

5'-d(AGG CGT TCA ACG GCT CTG **X**GT CGT ACG TCC CAT GGT)
3'-d(TCC GCA AGT TGC CGA GAC TCA GCA TGC AGG GTA CCA)

1a,**b**

a: X = α-C-Fapy•dA
b: X =β-C-Fapy•dA



5'-d(CGT TCA ACG TGC ACT **Fapy•dA**AC AGC ACG TCC CAT)
3'-d(GCA AGT TGC ACG TGA T TG TCG TGC AGG GTA)

2

5'-d(AGG CGT TCA ACG TGC AGT **Fapy•dG**AC AGC ACG TCC CAT GGT)
3'-d(TCC GCA AGT TGC ACG TCA **X** TG TCG TGC AGG GTA CCA)

3a,**b**

a: X = C
b: X = G

*Time Course Incision of Fapy·dA (**2**), α-C-Fapy·dA (**1a**), β-C-Fapy·dA (**1b**), and Fapy·dG (**3a**, **3b**) by Endo IV.* Duplexes **1a**, **1b**, **2**, and **3** were prepared by hybridizing the 5′-^{32}P-labeled lesion containing oligonucleotide (200 nM) with the respective complement (600 nM). A 2× enzyme solution (40 μL) containing 200 nM (or 20 nM) Endo IV, 0.2 mg/mL BSA, 20 mM Hepes−KOH (pH 7.4), and 200 mM KCl was added to a 20 nM DNA solution (40 μL). Reactions were incubated at 37 °C. Aliquots (5 μL) were removed at the appropriate times, added to 10 μL of 95% formamide loading buffer, and placed on ice. Samples were denatured for 1 min at 90 °C and separated by 12% denaturing PAGE. To account for the deglycosylation of Fapy·dA, a control was done where substrate (10 nM) was treated with 0.1 M NaOH in a total volume (10 μL) and incubated at 37 °C for 20 min. The samples were neutralized with 1.0 M HCl (1 μL) and diluted with 95% formamide loading buffer (20 μL). The amount of cleavage in the control (adventitious cleavage) was subtracted from the amount of cleavage in the reactions to find the total amount of Fapy·dA cleaved from the duplex by Endo IV. Reactions were carried out in triplicate.

*Kinetics for Cleavage of α-C-Fapy·dA (**1a**) by Endo IV.* Radiolabeled **1a** was formed by hybridizing (55 °C) the appropriate oligonucleotide to its complement (1.5 equiv) at a final concentration of 5.1 μM. Kinetic data for α-C-Fapy·dA were obtained by preparing a 2× enzyme solution containing 1.5 nM Endo IV, 20 mM Hepes−KOH (pH 7.4), 200 mM KCl, and 0.2 mg/mL BSA. The enzyme solution (5 μL) was added to solutions containing varying concentrations (10−300 nM) of **1a** to make a final volume of 10 μL. Reactions were carried out at 37 °C for 15 min and quenched with 95% formamide loading buffer (20 μL). Samples were

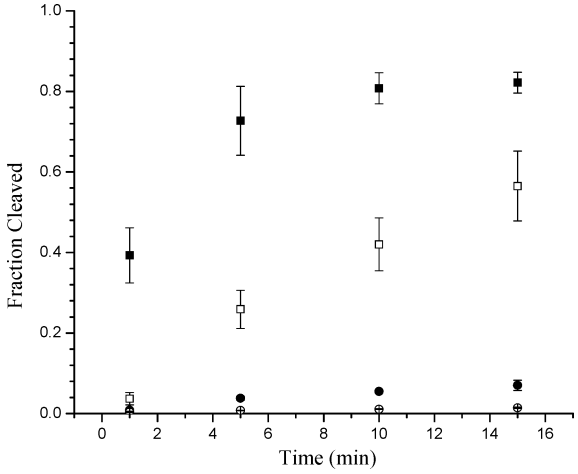


FIGURE 1: α-C-Fapy·dA (**1a**) and β-C-Fapy·dA (**1b**) incision by Endo IV as a function of time when the lesions are opposite dT: α-C-Fapy·dA (squares); β-C-Fapy·dA (circles); [Endo IV] = 100 nM (filled symbols); [Endo IV] = 10 nM (unfilled symbols).

denatured for 1 min at 90 °C and separated on a 12% denaturing PAGE. Kinetic parameters for α-C-Fapy·dA were extracted by nonlinear curve fitting (Origin 6.1) of velocity versus substrate concentration measurements. Kinetic constants reported are averages of three separate experiments each carried out in triplicate.

Incision of Fapy·dA (2) with Reannealing. Radiolabeled **2** was formed by hybridizing (55 °C) the appropriate oligonucleotide (20 nM) to its complement (10 equiv) by heating for 5 min at 55 °C and cooling slowly to room temperature in the Eppendorf Mastercycler 1 deg every 2.5 min. An enzyme solution (65 μL) containing 200 nM Endo IV, 0.2 mg/mL BSA, 20 mM Hepes−KOH (pH 7.4), and 200 mM KCl was added to a 65 μL of **2** (20 nM). The reaction was incubated for 20 min, at which time an aliquot (5 μL) was removed and quenched with formamide loading buffer (10 μL). The remaining reaction mixture was denatured for 1 min at 90 °C and placed on ice. The sample was reannealed by heating in an Eppendorf Mastercycler at 55 °C for 30 min, followed by cooling to room temperature at a rate of 1 deg every 2.5 min. Endo IV (0.5 μL) was added to the reannealed solution to a concentration of 100 nM. Incubation at 37 °C was carried out as above. Following removal of an aliquot, the process was repeated. A control was prepared as described above to account for adventitious cleavage. An aliquot was removed at the end of each cycle. The Endo IV incision data were corrected by subtracting the cleavage of the control from the cleavage from the reactions. Reactions were carried out in triplicate.

RESULTS

Incision of Fapy·dA C-Nucleoside Analogues by Endo IV. Incision of configurationally stable analogues of Fapy·dA was gauged as a function of time in the presence of equimolar Endo IV (Figure 1). Incision of α-C-Fapy·dA (**1a**) reached 56.5 ± 8.7% within 15 min and more than 80% when the enzyme was present in 10-fold excess. In contrast, less than 2% of DNA containing β-C-Fapy·dA (**1b**) was cleaved by equimolar Endo IV in 15 min, and only 7.0 ± 1.3% when the enzyme was present in 10-fold excess. Under the latter conditions, incision of **1b** reached a maximum of 11.0 ± 1.9% when allowed to proceed for 30 min. Although the

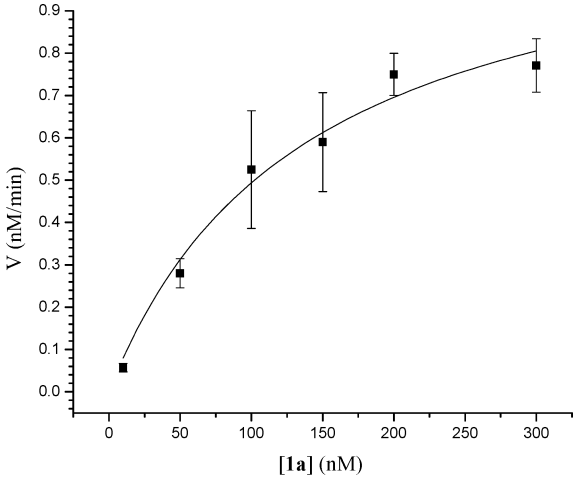


FIGURE 2: Michaelis−Menten kinetic analysis of α-C-Fapy·dA:dT incision by Endo IV.

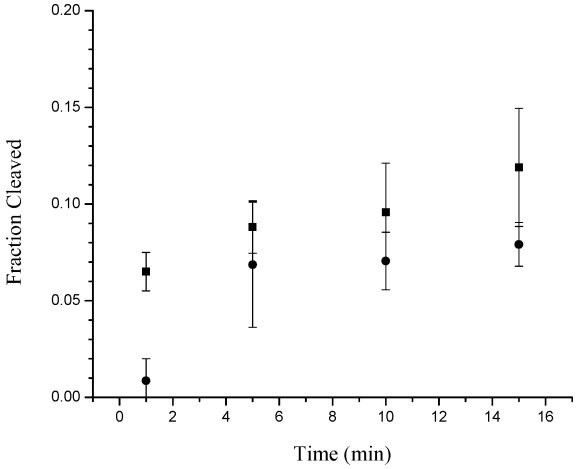


FIGURE 3: Fapy·dA:dT (**2**) incision by Endo IV as a function of time: [Endo IV] = 100 nM (squares); [Endo IV] = 10 nM (circles).

β-anomer was not a suitable substrate, incision of α-C-Fapy·dA was examined under Michaelis−Menten conditions (Figure 2). The lesion was incised from duplex **1a** with a K_m = 144.0 ± 0.9 nM and k_{cat} = 0.6 ± 0.2 min^{-1} (k_{cat}/K_m = (4.1 ± 0.2) × 10^{-3} nM^{-1} min^{-1}).

Fapy·dA Incision by Endo IV. When the incision of Fapy·dA (**2**) was measured, the amount of cleavage was corrected for the presence of any abasic sites present due to adventitious hydrolysis of the lesion's glycosidic bond. Such adventitious cleavage (typically <7%) was detected by treating an aliquot of the sample with NaOH (0.1 M, 37 °C, 20 min), which is known to cleave the abasic site but not Fapy·dA (*45*). Cleavage of duplex DNA containing Fapy·dA in the presence of equimolar Endo IV was only slightly greater (7.1 ± 1.5%) than that observed using the β-C-nucleoside substrate (Figure 3). Increasing the ratio of Endo IV to Fapy·dA-containing duplex to 10 results in a modest increase in strand scission in the same 15 min time period (11.9 ± 3.1%, Figure 3). Additional aliquots of Endo IV also did not increase the amount of strand scission in **2**. However, additional aliquots of Endo IV added after the duplex containing Fapy·dA was rehybridized did increase strand scission (Figure 4). Due to the need to add additional enzyme to the actual reaction mixture, the oligonucleotide containing the lesion was hybridized at the concentration (10 nM) at which the incision reaction was carried out. To drive

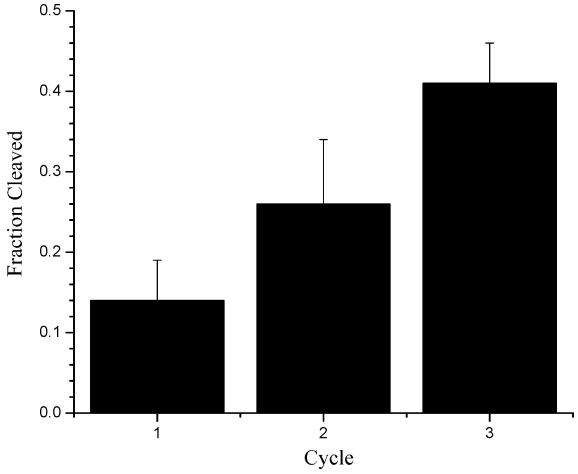


FIGURE 4: Stepwise incision of Fapy·dA:dT (**2**) by Endo IV after repeated reannealing/reequilibration of **2** and addition of fresh enzyme.

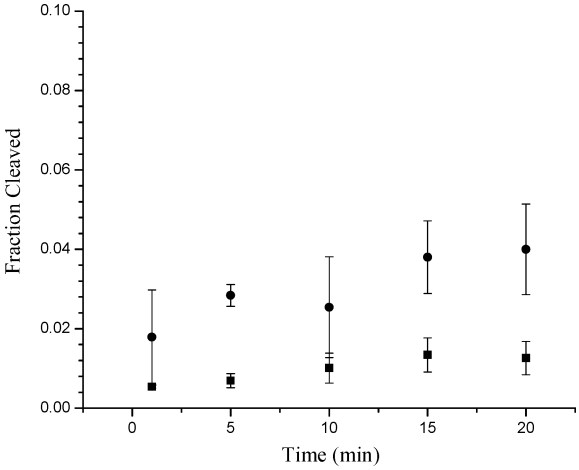


FIGURE 5: Fapy·dG:dC (**3a**; squares) and Fapy·dG:dG (**3b**; circles) incision by Endo IV as a function of time.

the formation of **2** to completion, a 10-fold excess of the complementary oligonucleotide was employed. The amount of strand scission produced in **2** during each cycle (~14%) was approximately twice the extent that duplex DNA containing β-C-Fapy·dA (**1b**) was cleaved under identical conditions.

Fapy·dG Incision by Endo IV. Fapy·dG proved to be an even poorer substrate than Fapy·dA for Endo IV. Extended reaction (20 min) of duplex DNA containing Fapy·dG:dC (**3a**; 10 nM) with Endo IV (100 nM) gave rise to 2.2 ± 0.4% cleavage (Figure 5). Incision of **3b** in which Fapy·dG was opposed by dG proceeded only slightly more proficiently (4.0 ± 1.1%) under identical reaction conditions (Figure 5).

DISCUSSION

In vitro analyses of the interactions of Fapy·dG and Fapy·dA present at defined sites in chemically synthesized oligonucleotides with DNA polymerase and repair enzymes have revealed interesting and potentially biologically significant effects of these lesions. Unfortunately, structural data that help rationalize these observations are lacking. UV−melting experiments revealed that an Fapy·dG:dC base pair destabilizes duplex DNA relative to a dG:dC base pair, but a dodecamer containing the lesion opposite dA is considerably more stable than one containing a dG:dA base pair (*19*). Furthermore, the ΔG for melting a duplex containing Fapy·dG:dA is equal, within experimental error, to that containing the lesion opposite dC. Fapy·dA exhibits similar effects on the thermal stability of duplex DNA (*20*). This lesion forms less stable duplexes than dA when opposite dT. However, the ΔG of melting is approximately equal when the lesion is opposite dT or dG. Providing a structural explanation for the base pairing effects and enzyme interactions is complicated, because monomeric formamidopyrimidines exist as mixtures of anomers in which the respective β-isomers are slightly favored (*28*, *29*).

It is not known whether the monomeric equilibrium reflects the distribution of configurational isomers in DNA. To provide insight into this structural issue, we utilized Endo IV as a probe. β-Deoxyribonucleotides are not substrates for the enzyme. Endo IV incises α-deoxyadenosine (K_m = 8.6 nM, k_{cat} = 1.2 min^{-1}) as efficiently as it does the tetrahydrofuran analogue (F) of an AP site (*48*). A more recent study using a shorter duplex describes incision occurring with a higher K_m (*49*). NMR analysis of this same α-dA:dT duplex confirms previous proposals that the lesion forms a reverse Watson−Crick base pair, but indicates that the base pairing is weak and that α-dA induces a kink in the duplex. The latter is believed to facilitate flipping out of the lesion for its incision by the enzyme. Although the structure of the α-dA-containing DNA−Endo IV complex is unavailable, it is believed that the α-dA is well accommodated within the enzyme's active site. Presumably, other α-nucleotides would also be better fits for the enzyme than the respective β-anomers.

Incision of DNA containing the configurationally stable C-nucleoside analogues of Fapy·dA (**1a**, **1b**) are consistent with this hypothesis. These analogues were examined as substrates for Endo IV to test the feasibility of using the enzyme as a probe for the configuration of the formamidopyrimidine lesions in DNA. Overlaying energy-minimized structures suggests that structural differences between the Fapy·dA isomers and their C-nucleoside analogues are minor (see the Supporting Information) (*51*). Furthermore, the lesions overlap well with α- or β-deoxyadenosine. The most significant difference between the C-nucleosides and the true lesions is rotation of the pyrimidine ring, which is attributable to substituting a sp^3-hybridized carbon for the glycosidic nitrogen. The C-nucleosides were used previously to examine the recognition of formamidopyrimidine lesions by Fpg (*23*, *24*). Fpg binding of the β-C-nucleoside analogues of Fapy·dA and Fapy·dG correlated well with excision of the true lesions. β-C-Fapy·dA bound strongly to Fpg regardless of the native nucleotide opposite it, whereas specific binding was observed only for β-C-Fapy·dG:dC base pairs. In contrast, only the α-C-Fapy·dA:dC base pair formed a tight complex with the enzyme. It is unclear why Fpg recognizes α-C-Fapy·dA opposite dC, but the aggregate of these experiments suggests that the β-isomers of the Fapy lesions are recognized by Fpg, and that the C-nucleosides are suitable models. However, the experiments do not provide information regarding the distribution of Fapy·dA(dG) configurational isomers in the DNA.

A semiquantitative experiment (Figure 1) demonstrated that α-C-Fapy·dA was a significantly better substrate for Endo IV than β-C-Fapy·dA. However, steady-state analysis

revealed that α-C-Fapy·dA is a poorer substrate for Endo IV than is α-dA. α-C-Fapy·dA incision exhibited an approximately 2-fold lower k_{cat} and an almost 17-fold higher K_m than that of α-dA (*48*). *β*-C-Fapy·dA was too poor of a substrate for Endo IV, and kinetic analysis could not be carried out on it. *β*-C-Fapy·dA (10 nM) incision in the presence of excess Endo IV (100 nM) did not show an initial burst in cleavage product, but rather a slow increase that reached 7.0 ± 1.3% in 15 min. In contrast, more than 80% of α-C-Fapy·dA-containing DNA was cleaved under comparable conditions.

The ability of Endo IV to distinguish between α- and *β*-C-Fapy·dA suggested that the enzyme would be a suitable probe for examining the configuration of Fapy·dA in duplex DNA. Fapy·dA incision showed a rapid burst, reaching ~7% in 1 min, but only ~12% within 15 min. The overall amount of incision is greater than that observed for DNA containing *β*-C-Fapy·dA and whose cleavage by Endo IV does not exhibit a burst. We believe that the presence of a burst is indicative of incision of α-Fapy·dA, but the modest amount of incision overall implies that Fapy·dA exists mostly as its *β*-anomer in duplex DNA. One could advance other explanations for the incision of Fapy·dA-containing DNA (**2**). For instance, one could suggest that Endo IV recognition of Fapy·dA is qualitatively different from that of the configurationally stable analogues, and that the similarity to incision of the *β*-C-nucleoside is fortuitous. Alternatively, one could suggest that Fapy·dA exists entirely as its *β*-anomer when it is intrahelical, and that incision by Endo IV is attributable to isomerization of the lesion when it is extrahelical. The lesion would be expected to flip out from the helix more readily than a native nucleotide due to weaker base pairing. However, the fact that Fapy·dA incision practically ceases and is not reinvigorated by additional Endo IV argues against these explanations. Moreover, experiments in which **2** is repeatedly treated with fresh Endo IV following reannealing are far more consistent with the interpretation that the α-Fapy·dA present in the duplex is selectively incised than either of these alternative explanations. Using the rate constants for interconversion of 5′-phosphate diesters α- and *β*-Fapy·dA, we presume that the lesion reequilibrates under the hybridization conditions (*28*). The fact that comparable amounts of duplex are cleaved during each cycle is consistent with this reequilibration. If all of the incision were due to cleavage of the α-anomer, then the Endo IV experiments would indicate an upper limit of ~14% of this isomer in the duplex. However, *β*-C-Fapy·dA is incised slowly by Endo IV. If *β*-Fapy·dA is incised at a rate comparable to that of *β*-C-Fapy·dA, a lower limit for α-Fapy·dA is estimated to be the difference (~7%) between the observed cleavage (~14%) and the expected amount of cleavage (~7%) of *β*-C-Fapy·dA under these conditions.

Modeling studies suggest that α-Fapy·dG and α-Fapy·dA adopt comparable structures, and we assume that both would be incised by Endo IV (see the Supporting Information). Fapy·dG incision is significantly less efficient that that of Fapy·dA, suggesting that the lesion exists almost exclusively (~98%) as the *β*-anomer when opposite dC. Slightly greater cleavage (~4%) is observed when the lesion is opposite dG, and this is consistent with the idea that the lesion is more weakly base paired opposite dG. Weaker base pairing could facilitate accommodation of the α-configuration. One could also propose (as above) that the more rapid Fapy·dG incision opposite dG is due to more facile flipping out of the nucleotide, followed by reaction with Endo IV. Unfortunately, the incision levels for Fapy·dG were too low to carry out the type of reannealing experiment described above for Fapy·dA. It is interesting to note that there are conflicting reports regarding the ability of Endo IV to incise MeFapy·dG. According to Saparbaev, MeFapy·dG is incised with efficiency comparable to that of α-C-Fapy·dA, which would suggest that the lesion exists at least partly as its α-anomer in DNA (*52*). However, Ide does not detect MeFapy·dG incision by the enzyme and believes that the lesion exists entirely as its *β*-anomer in DNA (*41*, *53*). These two investigators produced oligonucleotides containing MeFapy·dG using different methods, and it is possible that the conflicting conclusions are a consequence of the differing preparative conditions.

CONCLUSIONS

Fapy·dG and Fapy·dA are biologically interesting lesions that readily anomerize in aqueous solution, an unusual property for nucleotides. Biochemical studies have been carried out using Endo IV due to the absence of atomic level structural data on DNA containing the formamidopyrimidines. Although enzymes are routinely used to detect DNA lesions, or as probes for DNA binding, this is to our knowledge the first time that an enzyme has been used to probe the stereochemistry of a DNA lesion. The experiments described above suggest that Fapy·dG exists almost exclusively as its *β*-anomer, and that *β*-Fapy·dA is also the major stereoisomer (~90%) present in duplex DNA. Knowledge of the isomeric distribution of formamidopyrimidine lesions in DNA is important because there is a growing appreciation that diastereomeric lesions can interact with polymerase and repair enzymes differently (*54*−*56*). More detailed structural studies are needed to ascertain why the equilibria shift toward the *β*-anomers in DNA.

ACKNOWLEDGMENT

We thank the reviewers for helpful comments.

SUPPORTING INFORMATION AVAILABLE

Overlays of molecular modeling results showing similarities in energy-minimized structures of native nucleotides, formamidopyrimidines, and C-nucleoside analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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