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Research Article

Selective Incision of the α-N^5-Methyl-Formamidopyrimidine Anomer by *Escherichia coli* Endonuclease IV

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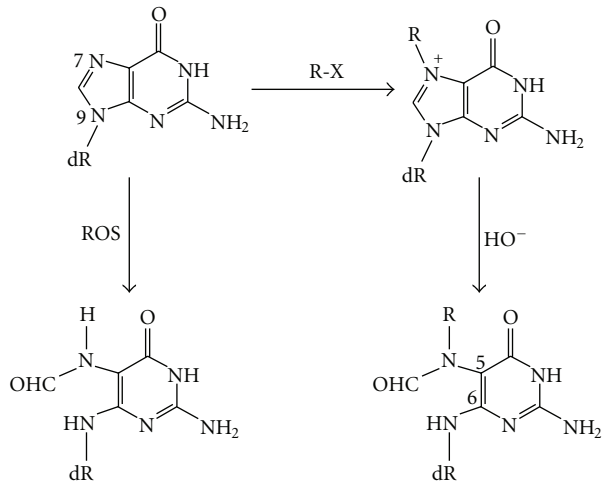
Formamidopyrimidines (Fapy) lesions result from ring opening of the imidazole portion of purines. Fapy lesions can isomerize from the natural β-anomeric stereochemistry to the α-configuration. We have unambiguously demonstrated that the α-methyl-Fapy-dG (MeFapy-dG) lesion is a substrate for *Escherichia coli* Endonuclease IV (Endo IV). Treatment of a MeFapy-dG-containing 24 mer duplex with Endo IV resulted in 36–40% incision. The catalytic efficiency of the incision was comparable to that of α-dG in the same duplex sequence. The α- and β-MeFapy-dG anomers equilibrate to ~21 : 79 ratio over ~3 days. Related studies with a duplex containing the α-Fapy-dG lesion derived from aflatoxin B_1 epoxide (α-AFB-Fapy-dG) showed only low levels of incision. It is hypothesized that the steric bulk of the aflatoxin moiety interferes with the binding of the substrate to Endo IV and the incision chemistry.

1. Introduction

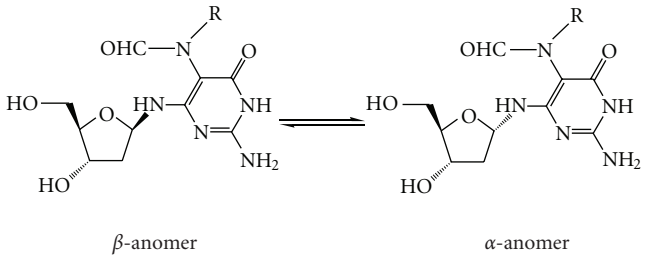
Formamidopyrimidines (Fapy) are DNA lesions that result from ring opening of the imidazole portion of purines. Fapy lesions derived from dG can form by either initial oxidation or N7-alkylation of the guanine base (Scheme 1) [1–6]. These are structurally related lesions that differ by the substitution at the N^5-formamide. The formyl nitrogen of the Fapy-dG derived from oxidative damage is unsubstituted, and it has been proposed that recognition of this lesion by human OGG involves hydrogen-bonding interactions between the protein and this functional group [7]. The formyl nitrogen is substituted with the alkylating agent from the alkylative pathway, and this substitution is likely to change the conformation of the adduct and its interaction with repair proteins and DNA polymerases. The corresponding Fapy-dG adducts were shown to be the persistent DNA lesions resulting from exposure to methylating agents (MeFapy-dG) and aflatoxin B_1 (AFB-Fapy-dG) [8–10].

An unusual property of Fapy lesions is that the C1′ stereochemistry can interconvert between the β- and α-anomers. This presumably occurs through the N^6-C1′ imine intermediate [11, 12]; the pyranose form can also result at the nucleoside level [13, 14]. The α-anomer of dA, dT, and dC results from γ-radiolysis of DNA [15, 16]; however, the α-stereochemistry of these lesions is static once it is formed.

Escherichia coli Endonuclease IV (Endo IV) is a double-strand specific AP endonuclease that hydrolyzes the phosphodiester bond on the 5′-side of abasic sites and accounts for ~10% of the AP endonuclease activity in *E. coli* [17–19]. The incision of the damaged strand results in a 5′-terminal ribosyl-5′-phosphate on the downstream fragment and a free 3′-hydroxyl group of the upstream fragment. Endo IV also possesses 3′-diesterase activity and can remove 3′-blocking groups such as 3′-phosphates, 3′-phosphoglycolate, and the 3′-(4-hydroxy-2-pentenal)-5-phosphate group that results from β-elimination of abasic sites. Endo IV was also found to possess 3′ → 5′ exonuclease activity and can generate a single base gap at the 5′-side of a damaged base [20, 21]. Endo IV contains three Zn^{2+} atoms in its active site and recognizes the damage site by a double nucleotide-flipping mechanism [22–24]. Oligonucleotides containing the α-dA, α-dT, α-dC, α-Fapy-dA, and α-Fapy-dG lesions as well as the configurationally stable N^6-carbon Fapy analogues



Scheme 1: Fapy-dG lesions derived from oxidative or alkylative damage to dG.



Scheme 2: The β- and α-anomers of Fapy-dG.

α-C-Fapy-dA have been shown to be substrates for Endo IV [25–29]. Oligonucleotides containing MeFapy-dG have also been examined as potential substrates for Endo IV. Asagoshi et al. reported that a MeFapy-dG-containing oligonucleotide was not a substrate for Endo IV and concluded that this lesion existed as the β-MeFapy-dG anomer [30, 31]. However,Ishchenko et al. [32] reported incision of the MeFapy-dG lesion with a catalytic efficiency comparable to that reported for α-C-Fapy-dA [29]. Interestingly, evidence that Endo IV plays a role in the repair of deoxyribosylurea lesions has also been reported [33]; this lesion can also undergo anomerization, and it is likely that the α-anomer is a substrate for Endo IV [34–36].

We recently described the synthesis of a phosphoramidite reagent of the MeFapy-dG lesion and its utility in the site-specific synthesis of modified oligonucleotides [11]. Two transitions were observed in the thermal melting curve of a 12-mer duplex containing a MeFapy-dG lesion. The lower melting transition was assigned as the α-MeFapy-dG anomer and the higher melting transition as the β-anomer. These assignments were based on a similar thermal melting profile of the AFB-Fapy-dG adduct for which the conformations of the α- and β-anomers (Scheme 2) have been determined by

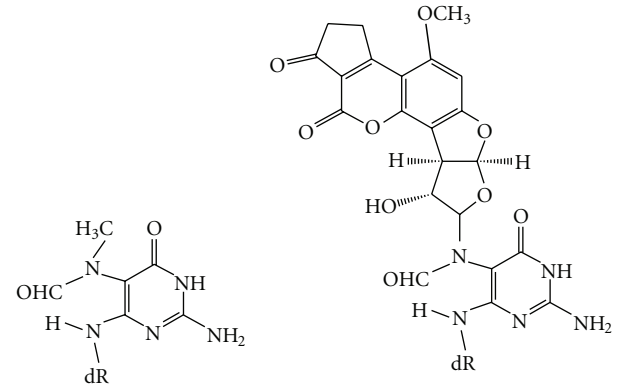


Figure 1: Structures of the methyl and aflatoxin B_1 Fapy-dG lesions.

NMR [37, 38]. The α : β anomeric ratio of the MeFapy-dG adduct was estimated to be ~40 : 60. We report here the selective incision of the α-MeFapy-dG anomer from duplex DNA by Endo IV.

2. Materials and Methods

α-dG was purchased from Berry and Associates, Inc. and was converted to its phosphoramidite reagent using standard protocols [39]. T4 polynucleotide kinase and *E. coli* Endo IV were purchased from New England Biolabs. γ-^{32}P-ATP was purchased from PerkinElmer Life Sciences. Unmodified oligonucleotides were purchased from Midland Certified Reagents. MALDI-TOF mass spectra were recorded using a 3-hydroxypicolinic acid (HPA) and ammonium hydrogen citrate matrix.

Oligonucleotide Synthesis. The MeFapy-dG and AFB-Fapy-dG modified oligonucleotides were synthesized as previously described [11, 40]. The α-dG-containing oligonucleotide was synthesized via the phosphoramidite method. The purity of all oligonucleotides was judged to be >99% by capillary gel electrophoresis. Oligonucleotides were characterized by MALDI-TOF mass spectrometry and enzymatic digestion.

5′-*ACCACGCTAGC-(α/β-MeFapy-dG)-AGTCCTAACAAC*-3′. It was purified by reversed-phase HPLC using gradient 1 and further purified by PAGE. MALDI-TOF MS (HPA) *m/z* calcd for (M-H), 7304.4; found 7305.4.

5′-*ACCACGCTAGC-(α-dG)-AGTCCTAACAAC*-3′. It was purified by reversed-phase HPLC using gradient 2. MALDI-TOF MS (HPA) *m/z* calcd for (M-H), 7272.2; found 7272.3.

5′-*ACCACTACTAT-(α-AFB-Fapy-dG)-ATTCATAACAAC*-3′. It was purified by reverse-phase HPLC using gradient 3.

MALDI-TOF MS (HPA) *m/z* calcd for (M-H), 7593.2; found 7594.1.

HPLC Purification. A YMC ODS-AQ column (250 × 4.6 mm, flow rate 1.5 mL/min; 250 × 10 mm, flow rate 5 mL/min) or Phenomenex Gemini-C18 column (250 × 4.6 mm, flow rate 1.5 mL/min; 250 × 10 mm, flow rate 5 mL/min) was used for oligonucleotide purifications and to monitor reactions. Oligonucleotides were detected by their UV absorbance at 254 nm. The mobile phase consisted of CH_3CN and 100 mM ammonium formate buffer (v/v).

Gradient 1. Initial conditions were 1% CH_3CN; a linear gradient to 8% CH_3CN over 5 minute; a linear gradient to 13% CH_3CN over 15 minute; a linear gradient to 80% CH_3CN over 2 minute; isocratic at 80% CH_3CN for 2 minute; then a linear gradient to the initial conditions over 3 minute.

Gradient 2. Initial conditions were 1% CH_3CN; a linear gradient to 5% CH_3CN over 5 minute; a linear gradient to 10% CH_3CN over 15 minute; a linear gradient to 80% CH_3CN over 2 minute; isocratic at 80% CH_3CN for 2 minute; then a linear gradient to the initial conditions over 3 minute.

Gradient 3. Initial conditions were 6% CH_3CN; a linear gradient to 25% CH_3CN over 25 minute; a linear gradient to 80% CH_3CN over 2 minute; isocratic at 80% CH_3CN for 2 minute; then a linear gradient back to the initial conditions over 3 minute.

LC-ESI-MS Analysis. MS analysis was performed in the Vanderbilt University facility on a Waters Acquity UPLC system connected to a Finnigan LTQ mass spectrometer (ThermoElectron) equipped with an Ion Max API source and a standard electrospray probe using an Acquity UPLC BEH C18 column (1 μm, 1.0 mm × 100 mm). LC conditions were as follows: buffer *A* contained 10 mM $NH_4CH_3CO_2$ plus 2% CH_3CN (v/v), and buffer *B* contained 10 mM $NH_4CH_3CO_2$ plus 95% CH_3CN (v/v). The following gradient program was used with a flow rate of 150 μL/min: initially 0% *B*, 3 minute linear gradient to 3% *B*, 1.5 minute linear gradient to 20% *B*, 0.5 minute linear gradient to 100% *B*, isocratic at 100% *B* for 0.5 minute, 1 minute linear gradient to 0% *B*, and isocratic at 0% *B* for 3 minute. The temperature of the column was maintained at 50°C, and the samples (10 μL) were infused with an autosampler. The electrospray conditions were as follows: source voltage: 4 kV; source current: 100 μA; N_2 was used as the auxiliary gas at a flow rate setting of 20; sweep gas flow-rate setting, 5: sheath gas flow setting: 34; capillary voltage: −49 V; capillary temperature, 350°C; and tube lens voltage: −90 V. The automatic gain control (AGC) setting in full MS was 10000. The maximum injection time in full MS was 10 ms. The MS data were acquired in negative mode. The number of μscan used for data acquisition in full MS was 2. Product ion spectra were acquired over the range of *m/z* 345–2000.

Oligonucleotide Labeling and Annealing. The labeling of the MeFapy-dG oligonucleotide was performed as previously described using T4 polynucleotide kinase and γ-^{32}P-ATP [41]. Electrophoresis gels were exposed to a PhosphorImager screen (Imaging Screen *K*, Bio-Rad) overnight. The bands were visualized with a PhosphorImaging system (Bio-Rad, Molecular Imager FX) using the manufacturer's Quantity One software, version 4.3.0. Unless otherwise noted, the oligonucleotide duplexes were formed by annealing the 5′-^{32}P-labeled MeFapy-dG-containing oligonucleotide (200 nM) with its complementary strand (600 nM) in Tris buffer (100 μL, 50 mM, pH 8.0) at 95°C for 5 minute; the solution was then slowly cooled to ambient temperature over 1 h.

Time Course Incision of the MeFapy-dG and α-dG-Containing Duplexes by Endo IV. The oligonucleotide duplex (4 μL, 0.8 pmols) was added to the Endo IV reaction buffer (74 μL of 1X : 100 mM NaCl, 50 mM Tris-HCL, 10 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.9) and warmed to 37°C. Endo IV (0.08 nM) was added; aliquots (5 μL) were removed at the appropriate times, added to 10 μL of 95% formamide loading buffer containing xylene cyanol and bromophenol blue dyes, and heated for 1 minute at 90°C. Aliquots (6 μL) from the samples were separated by electrophoresis on a denaturing gel.

To determine the extent of background deglycosylation of the lesion, aliquots of the MeFapy-dG-containing oligonucleotide were treated with 0.1 M NaOH and incubated at 37°C for 20 minute. The samples were neutralized by addition of 0.1 M HCl, diluted with loading buffer, and analyzed by gel electrophoresis. The amount of the cleavage in this control experiment was subtracted from the amount of incision in the Endo IV reactions. Reactions were carried out in duplicate. The same procedure was used for the incision of the 5′-^{32}P-labeled α-dG-containing duplex.

Incision of the α-AFB-Fapy-dG-Containing Duplex by Endo IV. The 5′-^{32}P-labeled α-AFB-Fapy-dG oligonucleotide (120 nM) was annealed to its complementary strand (160 nM) in Tris buffer (100 μL, 50 mM, pH 8.0) at room temperature. Aliquots (10 μL, 120 nM) were taken every 5 minute and added to a mixture of Endo IV enzyme buffer (10X, 6 μL), water (40 μL), and Endo IV (0.4 nM) at 37°C; the incision reactions were incubated at 37°C and pH 7.9. Aliquots (5 μL) were taken every 2 minute and added to 10 μL of 95% formamide loading buffer containing xylene cyanol and bromophenol blue dyes and heated for 1 minute at 90°C. Aliquots (6 μL) were separated by electrophoresis on a denaturing gel. The extent of background deglycosylation of the α-AFB-Fapy-dG lesion was determined as described above. The amount of the cleavage in the control experiment was subtracted from the amount of incision in the Endo IV reactions. Reactions were carried out in duplicate.

Kinetics for the Incision of the MeFapy-dG- and α-dG-Containing Duplexes by Endo IV. The duplex was annealed

as described above with the following modifications. The 5′-^{32}P-labeled MeFapy-dG and its complementary strand were 100 and 150 nM, respectively. Endo IV (0.08 nM) was added to varying concentrations of the DNA duplex (0.5–35 nM in 1X Endo IV reaction buffer, pH 7.9) to a final volume 50 μL. Reactions were run at 37°C for up to 10 minute. Aliquots (5 μL) were taken at 1 minute intervals, added to loading buffer (10 μL), and heated at 90°C for 1 minute. Separation was achieved by gel electrophoresis. The kinetics parameters were calculated using KaleidaGraph 4.0. Reactions were carried out in duplicate. The same procedure was used for incision of the α-dG-containing duplex.

Time Course Incision of the MeFapy-dG-Containing Duplex by Endo IV at pH 7.0. The oligonucleotide duplex (12 μL, 2.4 pmols) was added to the Endo IV reaction buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7, 222 μL), followed by addition of Endo IV (0.08 nM). The reaction was incubated at 37°C for 5 days. A fresh portion of Endo IV (0.08 nM) was added every 24 h. Aliquots (5 μL) were removed 30 minute after the Endo IV addition, added to 10 μL of 95% formamide loading buffer containing xylene cyanol and bromophenol blue dyes, and heated for 1 minute at 90°C. The incision reaction was analyzed by gel electrophoresis. The experiment was run in duplicate.

Incision of the MeFapy-dG-Containing Duplex after Denaturation and Reannealing. The DNA duplex was formed as described above with the following modifications. The 5′-^{32}P-labeled MeFapy-dG and its complementary strand were 200 and 400 nM, respectively. The oligonucleotide duplex (8 μL, 1.6 pmols) was added to 1X Endo IV reaction buffer (148 μL), followed by addition of Endo IV (0.08 nM). The reaction was run at 37°C for 40 minute and aliquots (5 μL) were removed periodically, added to 10 μL of 95% formamide loading buffer containing xylene cyanol and bromophenol blue dyes and heated for 1 minute at 90°C. The remaining reaction mixture was heated at 95°C for 5 minute, cooled to 75°C for 30 minute, then cooled to ambient temperature over 1 h. The reannealed DNA duplex incubated at 37°C and Endo IV (0.08 nM) was added. Aliquots were taken as previously described, and the level of incision was analyzed by gel electrophoresis. The denaturation and reannealing cycles were repeated one more time.

LC-ESI-MS Analysis of the Incision of the MeFapy-dG-Containing Duplex by Endo IV. MeFapy-dG-containing 24 mer oligonucleotide (0.774 nmol) was annealed to its complimentary strand (1.5 eq., 1.161 nmol) in Tris buffer (100 μL, 50 mM, pH 8.0) as described above. To this solution Endo IV reaction buffer (13 μL, 10X) was added followed by addition of Endo IV (0.8 nM). The reaction mixture was incubated at 37°C for 2 h. The reaction mixture was then passed through a spin column (Bio-spin 6 Tris columns) and lyophilized. The white residue was dissolved in water and analyzed by LC-ESI-MS.

Incision of the MeFapy-dG-Containing Duplex by Endo IV at pH 7.0. The DNA duplex was annealed as described above in Tris buffer (50 mM, pH 7.0). The oligonucleotide duplex (12 μL, 2.4 pmols) was added to the Endo IV reaction buffer (222 μL, 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.0). The reaction was incubated at 37°C. Aliquots (50 μL) were removed after 1, 3, 5, 7, and 10 days and treated with Endo IV (0.08 nM). Aliquots (8 μL) were added to 10 μL of 95% formamide loading buffer containing xylene cyanol and bromophenol blue dyes and heated for 1 minute at 90°C. The experiment was run in duplicate.

Thermal Melting (T_m) Analysis of the MeFapy-dG-Containing 12 mer Duplex. A 1 : 1 mixture of MeFapy-dG containing 12 mer oligonucleotide and its complementary strand (0.25 A_{260} units) were placed in melting buffer (0.5 mL, 10 mM Na_2HPO_4/NaH_2PO_4, 1.0 M NaCl, and 50 μM Na_2EDTA, pH 7.2). The UV absorbance of the duplex was monitored 260 nm as a function of temperature. Absorbance measurements were taken at 1 minute intervals with a 1°C/min temperature gradient. The temperature was cycled between 15 and 85°C. The first derivative of the melting curve was used to establish T_m values. After running the first T_m, the sample was incubated at 37°C for 5 days, then T_m experiment was repeated.

3. Results and Discussion

3.1. Incision of MeFapy-dG-Containing Duplex by Endo IV. The MeFapy-dG lesion was site specifically incorporated at position 12 of the 24 mer oligonucleotide shown in Figure 2 (X = MeFapy-dG) and annealed to its complementary strand. The activity of Endo IV toward the MeFapy-dG lesion when paired with dC was examined. PAGE analysis of the reaction mixture typically showed between 36% and 40% of the 5′-^{32}P-ACCACGCTAGC-3′ incision product after 30 minute (Figure 2(a)). Increasing the initial concentration of Endo IV or addition of a second portion of enzyme after the initial 30 minute incubation period did not increase the amount of incised product. The incision reaction was also followed by LC-ESI-MS with nonradiolabeled oligonucleotides (Figure 3). In addition to the MeFapy-dG-containing oligonucleotide (m/z = 1825.4 [M-4H] and 1460.3 [M-5H]) and its complementary strand (m/z = 1484.9 [M-5H] and 1237.2 [M-6H]), the 5′-ACCACGCTAGC-3′ (m/z = 1094.1 [M-3H] and 820.3 [M-4H]) and 5′-p(MeFapy-dG)-AGTCCTAACAAC-3′ (m/z = 1345.2 [M-3H] and 1009.0 [M-4H]) incision products were observed.

After the initial incision of the MeFapy-dG-containing duplex, the remaining 24 mer duplex, which was presumably highly enriched in the β-MeFapy-dG anomer, was denatured by heating to 90°C, then slowly cooled to reanneal the duplex. The heating and annealing cycle is expected to equilibrate the α- and β-anomers of the MeFapy-dG lesion [29]. Additional Endo IV was added and the 5′-^{32}P-ACCACGCTAGC-3′ incision product was found to be ~59% (Figure 2(b)); the 23% increase of the incision

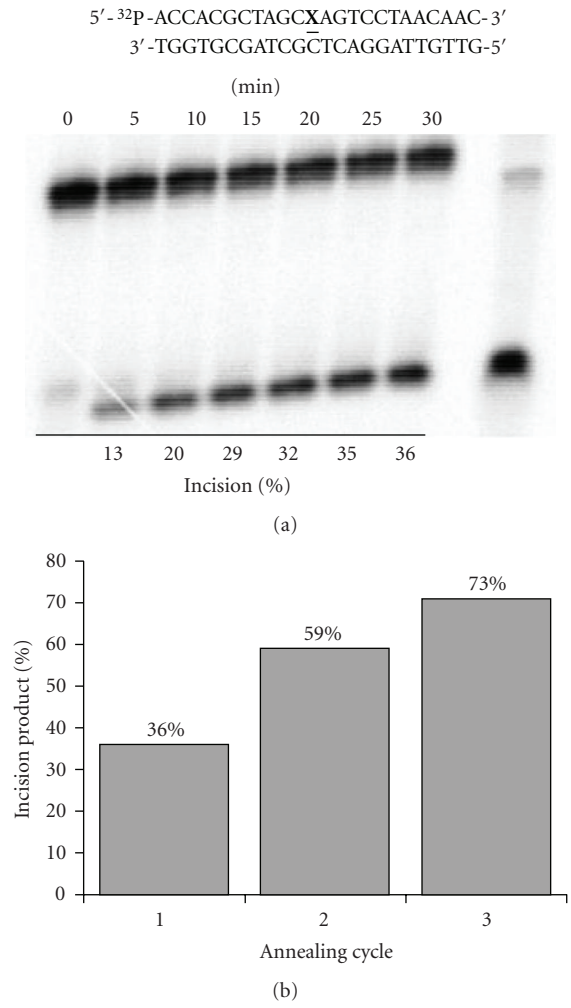


Figure 2: Gel electrophoretic analysis of the incision of the MeFapy-dG containing duplex with *E. coli* Endo IV. (a) Incision after the annealing of the 5′-^{32}P-labelled MeFapy-dG containing oligonucleotide (24 mer) with its complement. The right lane is a standard of the 5′-^{32}P-ACCACGCTAGC-3′ incision product. (b) Percentage of the incision product after subsequent denaturation-reannealing and additional Endo IV.

products represents ∼36% cleavage of the MeFapy-dG duplex remaining (64%) after the initial Endo IV incision. A subsequent denaturation-reannealing cycle followed by addition of a third aliquot of Endo IV resulted in an additional 14% increase of the incision product (73% total), representing ∼34% cleavage of the MeFapy-dG-containing duplex remaining after the prior two rounds of Endo IV treatment (41%). These results are similar to those reported by Patro et al. for the incision of the Fapy-dA containing duplex and is consistent with selective incision of the α-MeFapy-dG anomer [29].

The MeFapy-dG-containing oligonucleotide was also hybridized to complementary strands in which the lesion was mismatched with dG, dA, and dT. Treatment of these duplexes with Endo IV and PAGE analysis resulted in 30%, 32%, and 30% incision, respectively. The reactions products

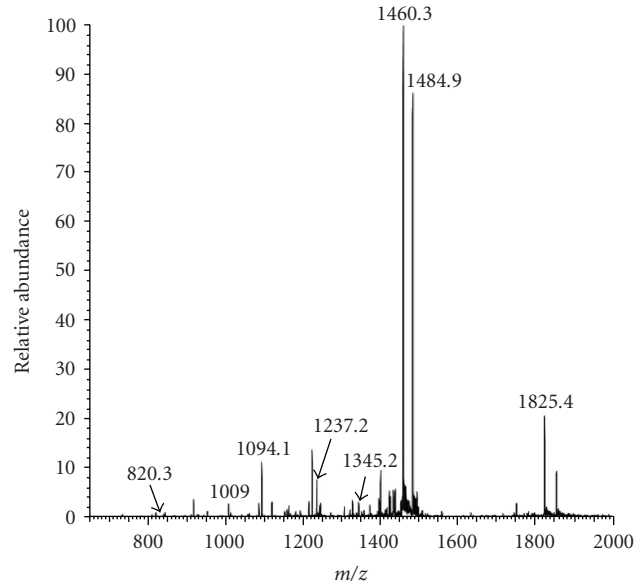


Figure 3: Full-can mass spectrum of the Endo IV incision of the MeFapy-dG containing duplex; *m/z* 1825.4 [M-4H] and 1460.3 [M-5H] are the 5′-ACCACGCTAGC-(MeFapy-dG)-AGTCCTAACAAC-3′, *m/z* 1484.9 [M-5H] and 1237.2 [M-6H] are the 5′-GTTGTTAGGACTCGCTAGCGTGGT-3′, *m/z* 1094.1 [M-3H] and 820.3 [M-4H] are the 5′-ACCACGCTAGC-3′, and *m/z* 1345.2 [M-3H] and 1009.0 [M-4H]) is the 5′-p(MeFapy-dG)-AGTCCTAACAAC-3′ oligonucleotide.

from the mismatched duplexes were also characterized by LC-ESI-MS analyses.

3.2. Steady-State Analysis for the Endo IV Incision of the MeFapy-dG-Containing Duplex. Figure 4 shows the steady-state kinetic analysis for the incision of MeFapy-dG strand when paired with dC. The modified strand was incised with a $k_{cat} = 5.5 \pm 0.5\,min^{-1}$ and $K_m = 17.8 \pm 3.8\,nM$ ($k_{cat}/K_m = 0.33 \pm 0.12\,nM^{-1}\,min^{-1}$; filled circles). When the DNA concentration used in the rate calculation was adjusted to reflect only the amount of the α-MeFapy-dG adduct (∼40%, open circles), the catalytic efficiency improved to $0.78 \pm 0.33\,nM^{-1}\,min^{-1}$ by virtue of a lower K_m ($7.1 \pm 1.5\,nM$). This treatment of the rate data assumes that the β-MeFapy-dG-containing duplex does not inhibit Endo IV and is based on the observation that the incision of duplex DNA containing α-dA was not inhibited by the addition of an unmodified duplex, even at concentrations 30-fold higher than the substrate DNA [25]. For comparison, the phosphoramidite reagent of α-dG was prepared and incorporated into the same 24 mer sequence; α-dG was chosen for this comparison because of the commercial availability of the nucleoside. The catalytic efficiency for the incision of the α-dG-containing duplex (k_{cat}/K_m) was $0.69 \pm 0.5\,nM^{-1}\,min^{-1}$ ($k_{cat} = 4.9 \pm 0.5\,min^{-1}$ and $K_m = 7.1 \pm 1.0\,nM$) (Figure 4 (b)).

3.3. Equilibration of the MeFapy-dG Anomers in DNA. The α-MeFapy-dG lesion was excised from the 24 mer duplex, and the equilibration of the remaining β-MeFapy-dG to

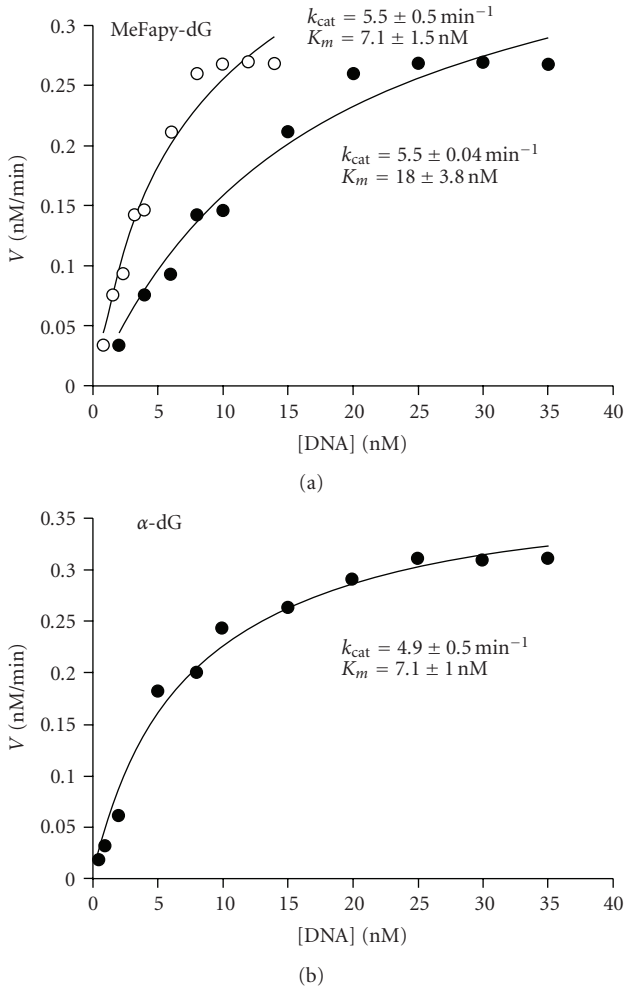


Figure 4: Steady-state kinetic analysis for the incision of the MeFapy-dG-(a) and α-dG-(b) containing duplexes when the modified base was opposite dC. The Endo IV concentration was 0.08 nM.

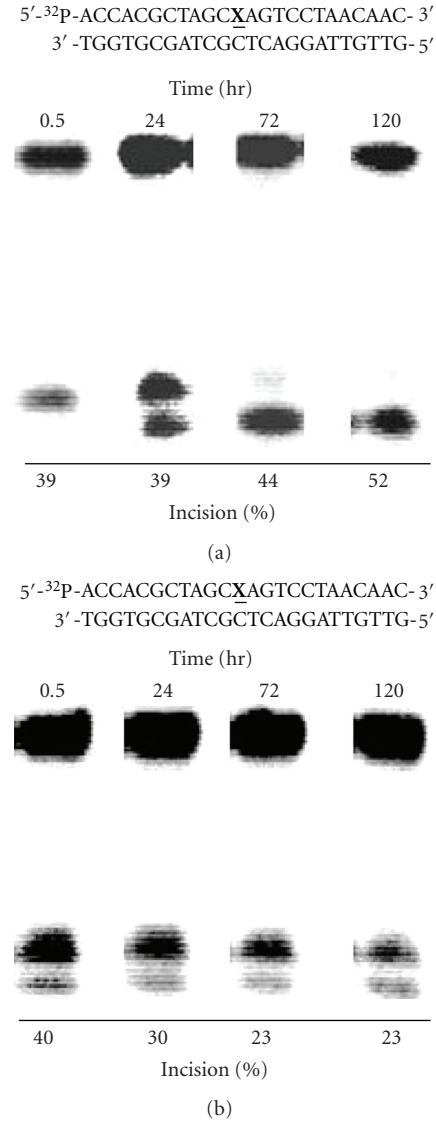


Figure 5: Gel electrophoretic analysis of the equilibration of the β- and α-MeFapy-dG anomers in duplex DNA. (a) Anomerization of the β-MeFapy-dG after initial incision with Endo IV. Aliquots were treated with additional Endo IV every 24 h; the increase in the incision product is attributed to equilibration of the β-MeFapy-dG to the α-anomer. (b) Treatment of the MeFapy-dG-containing duplex with Endo IV after incubation at 37°C, pH 7.0 for up to 5 days.

a mixture of α- and β-anomers was monitored at 37°C (Figure 5). Aliquots were taken every 24 h after the initial incision reaction and additional Endo IV was added; the increase in the 5′-^{32}P-ACCACGCTAGC-3′ was attributed to the anomerization of the β-MeFapy-dG remaining after initial incision. The prolonged exposure of the incision product to Endo IV resulted in its conversion to a product one nucleotide shorter. We attribute this observation to the 3′ → 5′ exonuclease activity of Endo IV, and the product is presumably 5′-^{32}P-ACCACGCTAG-3′ [20]. Virtually no anomerization was observed at pH 8.0, consistent with studies that showed that the anomerization of the AFB-Fapy-dG lesion was acid catalyzed [40]. We found that the anomeric stereochemistry of the β-MeFapy-dG equilibrated over ~5 days to an α : β ratio of ~21 : 79 at pH 7.0 and 37°C. To insure that the additional cleavage of the duplex was not due to fortuitous deglycosylation of the MeFapy-G base, the MeFapy-dG-containing duplex was incubated at 37°C for 5 days, then *E. coli* Endonuclease V was added, which is known to cleave abasic sites. No cleavage was observed.

The duplex that resulted from annealing the MeFapy-dG modified oligonucleotide with its complement initially contained ~36%–40% of the α-MeFapy-dG. The equilibration of the α-MeFapy-dG anomer to the β-anomer was monitored by incubating the annealed duplex at 37°C and treating aliquots with Endo IV (Figure 5 (b)). We observed that the α : β anomeric ratio was ~30 : 70 after 24 h and 23 : 77 after 3 days; the reaction was monitored up to 10 days, and the percentage of incision product remained at ~23%. These results are in good agreement with those starting from the β-anomer.

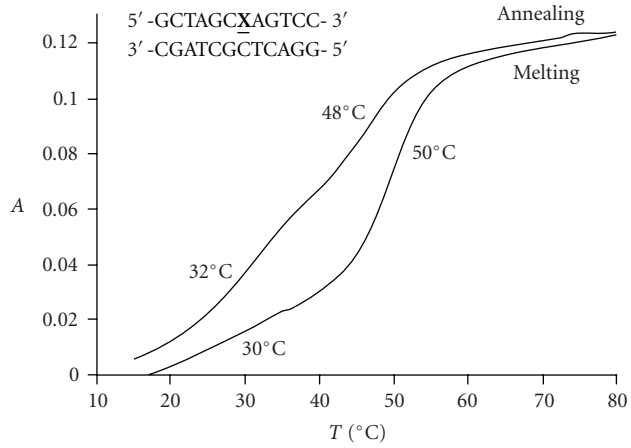


Figure 6: Thermal melting analysis of the 12 mer duplex containing the MeFapy-dG lesion opposite dC after 5 days at 37°C. The T_m for the unmodied duplex was 59°C.

We initially reported that the thermal melting (T_m) curve of a 12 mer duplex containing the MeFapy-dG lesion did not change over time [11]; these studies were conducted at 5°C. The thermal melting temperature of the 12 mer duplex 5′-GCTAGC-(MeFapy-dG)-AGTCC-3′ · 5′-GGACTCGCTAGC-3′ was reexamined by monitoring the hyperchromicity of the DNA UV absorption at 260 nm as a function of temperature (Figure 6). This duplex has the same local sequence as the 24 mer duplex used for the Endo IV incision studies. The duplex was incubated at 37°C for 5 days then analyzed. The T_m values were determined by taking the first derivative of the melting and annealing curves. As previously reported, two transitions were present in the melting cycle; however, the relative proportions of the transitions differed after 5 days at 37°C. The higher melting transition ($T_m = 50$°C), which was assigned as the β-MeFapy-dG anomer, made up ~80% of the duplex after the incubation period. The T_m curve for the annealing cycle was displaced from the heating cycle, reflecting that the reannealing of the MeFapy-dG oligonucleotide with its complement resulted in a different $\alpha : \beta$ anomeric ratio than after equilibration for 5 days. Similar melting and annealing profiles were recently reported for a duplex containing the AFB-Fapy-dG lesion [38]. These studies are consistent with the Endo IV incision data.

The initial anomeric ratio (~36 : 64) after annealing the MeFapy-dG-containing oligonucleotide with its complement may reflect the ratio in single-strand DNA. The α- and β-MeFapy-dG anomers equilibrate over several days and reach a final $\alpha : \beta$ ratio of ~21 : 79. The equilibration studies may provide insight to the conflicting reports by Asagoshi et al. regarding the Endo IV incision of the MeFapy-dG lesion in DNA. The MeFapy-dG-containing duplexes were prepared differently. Ishchenko et al. prepared the MeFapy-dG lesion by treating a single-stranded oligonucleotide containing a single dG with a methylating agent [32]; the resulting 7-methyl-dG was then subjected to base treatment to generate the MeFapy-dG lesion. Annealing of the MeFapy-dG-containing oligonucleotide is expected to contain both the α- and β-anomers as demonstrated in our studies. Asagoshi et al. prepared a DNA duplex by incorporating 7-methyl-dGTP opposite a template strand containing a single dC with a DNA polymerase [30, 31]; alkali treatment of the duplex produced the MeFapy-dG lesion. It is likely that ring opening of β-7-methyl-dG in duplex DNA produced largely or exclusively the β-MeFapy-dG anomer. We demonstrated that the anomerization of the β-MeFapy-dG anomer is slow at pH 7.0 and is expected to be slower at pH 7.5, the conditions reported by Asagoshi et al. for the attempted Endo IV incision [30]. It is possible that the β-MeFapy-dG anomer had not equilibrated when treated with Endo IV, and consequently, no incision was observed.

The MeFapy-dG lesion was shown to be the persistent DNA lesion in rats treated with methylnitrosourea, 1,2-dimethylhydrazine, and N,N-dimethylnitrosamine [8, 9]. The MeFapy-dG lesion was reported to be present 21 days after exposure; these studies detected the MeFapy-G base, thus information regarding the anomeric stereochemistry was lost. As noted above, the MeFapy-dG lesion is likely to be initially formed as the β-anomer; our observation that β-MeFapy-dG will slowly equilibrate to ~21% of the α-anomer over 3–5 days suggests that the α-anomer is present in cells due to the long-lived nature of this lesion and therefore may be biologically relevant.

3.4. Incision of AFB-Fapy-dG by Endo IV. The 24 mer oligonucleotide containing the AFB-Fapy-dG lesion, 5′-ACCACTACTAT-(AFB-Fapy-dG)-ATTCATAACAAC-3′, was synthesized as previously described, and the α- and β-AFB-Fapy-dG-containing oligonucleotides were separated by HPLC [38]. The oligonucleotide containing the α-AFB-Fapy-dG lesion was 5′-^{32}P-end labeled and hybridized to its complementary strand in which the lesion was opposite dC. Treatment of this duplex (20 nM) with Endo IV (0.08 nM) under the same conditions used for incision of the MeFapy-dG-containing duplex resulted in ~5% incision of α-AFB-Fapy-dG modified strand after 30 minute (Figure 7); the level of incision did not increase after 90 minute. The level of incision reached ~ 9% when the Endo IV concentration was increased by 10-fold.

The activity of Endo IV toward the AFB-Fapy-dG lesion was low in comparison to the MeFapy-dG lesion. The α-AFB-Fapy-dG was reported to have completely isomerized to the β-anomer over several days at pH 7.0 and 5°C [37]. It is possible that the low level of incision observed for the AFB-Fapy-dG-containing 24 mer duplex was due to rapid isomerization of the α-anomer to the β; however, HPLC analysis indicated that the level on the β-AFB-Fapy-dG anomer was less than 5% over the 90 minute reaction time and conditions used to ^{32}P-labeled the substrate. In Figure 1 a recent NMR structure of the α-AFB-Fapy-dG leson in a 5′-CTAT-(AFB-Fapy-dG)- ATTCA-3′ · 5′-TGAATCATAG-3′ 10 mer duplex revealed that the AFB moiety was intercalated to the 5′-side of the modified α-Fapy-dG base [38] and well stacked (Figure 8). The modified α-AFB-Fapy-dG base was

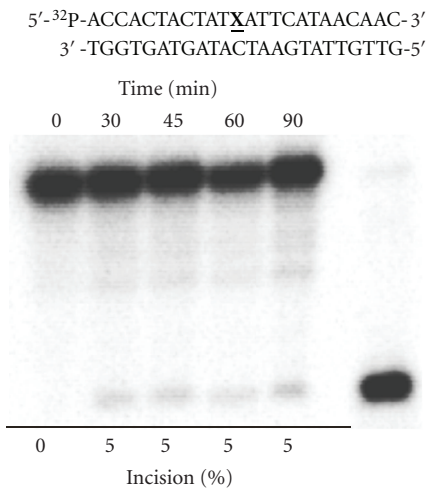


Figure 7: Gel electrophoretic analysis of the incision of the α-AFB-Fapy-dG-containing 5′-32P-labeled 24 mer duplex by Endo IV. The right lane is a standard of the 5′-32P-ACCACTACTAT-3′ incision product.

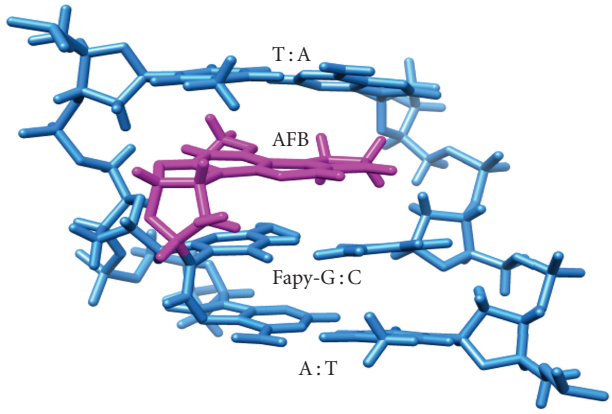


Figure 8: Conformation of the α-AFB-Fapy-dG-containing 10 mer duplex. The AFB moiety (magenta) was intercalated to the 5′-side of the Fapy-G base, which was paired with its complement (pdb code 2KH3).

paired with its complementary dC, and both bases were intrahelical. The 10 mer sequence possesses the same local sequence as the AFB-Fapy-dG containing 24 mer used in this study. The insertion of the Tyr-72 side chain of Endo IV into the DNA helix plays an important role in the recognition and catalytic mechanism by promoting the flipping of the abasic nucleotide along with its partner nucleotide into the active site of the enzyme, stabilizing a 90° bend in the DNA and positioning the phosphate for hydrolysis. The position of the bulky AFB group may severely interfere with the binding of the substrate DNA to Endo IV and the incision reaction [22, 23]. Alternatively, a solvent accessible pocket in the active site of Endo IV is hypothesized to accommodate α-nucleotides, and it is plausible that bulky lesions such as the α-AFB-Fapy-dG cannot be readily accommodated in this pocket [22]. Chromatographic evidence for a minor conformation of the α-AFB-Fapy-dG in single strand was reported [38]; the nature of this conformation is not clear but the possibilities include geometric isomers of the amide or atropisomers [14, 40, 42]. It is possible that Endo IV is selectively incising this minor α-AFB-Fapy-dG conformer.

4. Conclusion

We unambiguously observed that the α-MeFapy-dG lesion is a substrate for Endo IV. This activity was exploited to probe the anomeric configuration of the MeFapy-dG lesion in duplex DNA. Endo IV was used to follow the equilibration of the α- and β-MeFapy-dG lesions, and we estimate the α : β-ratio to be ~21 : 79. Although the equilibration was slow, it was faster than the repair of the MeFapy-dG lesion from the DNA of rats. We conclude that the α-MeFapy-dG will be present in mammalian cells, and its repair and replication are therefore of interest. Only low levels of incision were observed for the α-AFB-Fapy-dG-containing duplex. It is likely that the bulk of the AFB moiety, which was intercalated on the 5′-side of the modified base, interferes with substrate recognition and the incision reaction by Endo IV.

The MeFapy-dG lesion has been shown to be a substrate for the Fpg/Nei family of glycosylases [3, 30, 43–45]. It is possible that Endo IV and the Fpg/Nei glycosylases have distinct roles during the repair of the MeFapy-dG with Endo IV incising the α-anomer and Fpg/Nei glycosylases excising the β-anomer. The specificity of Endo IV for the α-MeFapy-dG anomer may provide a valuable tool for uncovering the mechanistic details of the repair of MeFapy-dG and related lesions.

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