Interaction of DNA Containing Fapy·dA or Its C-Nucleoside Analogues with Base Excision Repair Enzymes. Implications for Mutagenesis and Enzyme Inhibition†

Carissa J. Wiederholt,‡ Michael O. Delaney,‡ and Marc M. Greenberg*

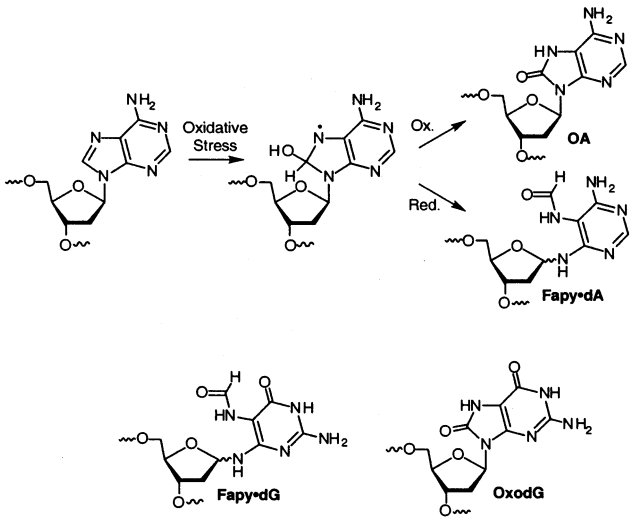
Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523

Received April 1, 2002; Revised Manuscript Received October 18, 2002

ABSTRACT: Fapy·dA is produced in DNA as a result of oxidative stress. Recently, this lesion and its C-nucleoside analogues were incorporated in chemically synthesized oligonucleotides at defined sites. The interaction of DNA containing Fapy·dA or nonhydrolyzable analogues with Fpg and MutY is described. Fpg efficiently excises Fapy·dA (K_m = 1.2 nM, k_{cat} = 0.12 min^{-1}) opposite T. The lesion is removed as efficiently from duplexes containing Fapy·dA:dA or Fapy·dA:dG base pairs. Multiple turnovers are observed for the repair of Fapy·dA mispairs in a short period of time, indicating that the enzyme does not remain bound to the product duplex. MutY does not incise dA from a duplex containing this nucleotide opposite Fapy·dA, nor does it exhibit an increased level of binding compared to DNA composed solely of native base pairs. MutY also does not incise Fapy·dA when the lesion is opposite dG. These data suggest that Fapy·dA could be deleterious to the genome. Fpg strongly binds duplexes containing the β-C-nucleoside analogue of Fapy·dA (β-C-Fapy·dA) opposite all native nucleotides (K_D < 27 nM), as well as the α-C-nucleoside (α-C-Fapy·dA) opposite dC (K_D = 7.1 ± 1.5 nM). A duplex containing a β-C-Fapy·dA:T base pair is an effective inhibitor (K_I = 3.5 ± 0.3 nM) of repair of Fapy·dA by Fpg, suggesting the C-nucleoside may have useful therapeutic properties.

Exposure of DNA to reactive oxygen species (e.g., hydroxyl radical) and other forms of oxidative stress (e.g., UV irradiation) result in the formation of base lesions (*1*−*3*). The DNA lesions can be cytotoxic and/or premutagenic. Consequently, their formation and accumulation in genomic DNA is implicated in aging and genetic diseases, such as cancer (*4*−*6*). An elaborate network of repair enzymes and cofactors that is conserved from prokaryotes to humans exists to defend cells against DNA lesions (*7*−*10*). The base excision repair (BER) pathway protects against DNA base damage. The initial step in this process involves hydrolysis of the lesion's glycosidic bond by an appropriate glycosylase (BER enzyme), which may also be able to effect a subsequent lyase reaction of the remaining abasic site, resulting in a strand break. Subsequent steps in the repair process utilize other enzymes that remove sugar fragments, fill in the excised nucleotide, and religate the fragmented biopolymer. The significance of this chemistry is underscored by the fact that excision of a base lesion from a mispair followed by filling in and ligation produces a genetic mutation. In vitro examination of the interaction between base lesions and appropriate BER enzymes provides important information concerning their biological effects. Similar analysis of designed analogues may provide the basis for using these molecules as therapeutic agents targeted to BER enzymes (*10*). We report on the interaction between the formamidopyrimidine lesion (Fapy·dA, Scheme 1) produced from deoxyadenosine (dA) with two of the three BER enzymes

Scheme 1: Exposure of DNA to Oxidative Stress Produces Formamidopyrimidine and 8-Oxopurine Lesions from a Common Intermediate



that make up the "GO" system. These enzymes are responsible for the repair of oxidized purines (*7*). One enzyme, formamidopyrimidine DNA *N*-glycosylase (Fpg, MutM), bears the name of the lesion of interest. Fpg is a bifunctional enzyme that hydrolyzes the glycosidic bond (glycosylase) of damaged purines and effects a β,δ-elimination (lyase) of the remaining abasic site. The other enzyme that has been examined is the mismatch repair enzyme MutY, a glycosylase that removes the incorrect nucleotide inserted opposite the modified purine (*7*). We also describe the use of C-nucleoside analogues of Fapy·dA as probes of the interaction of the lesion with Fpg.

† Supported by NIH Grant CA-74954.

* To whom correspondence should be addressed: Department of Chemistry, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218. E-mail: mgreenberg@jhu.edu.

‡ These authors contributed equally to this research.

10.1021/bi025903e CCC: $22.00
Published on Web 11/27/2002

Chart 1: Structures of Lesions and Duplex Substrates Used in This Study

5′-d(CGT T CAA CGT GCA C T X ACA GCA CGT CCC A T)
3′-d(G CA AGT TGC A CGT G A Y T GT CGT GCA GGG T A)

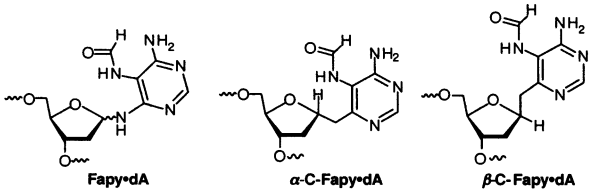
1a-d

a: X = Fapy•dA, Y = T
b: X = Fapy•dA, Y = dA
c: X = Fapy•dA, Y = dG
d: X = dA, Y = dG

5′-d(A GGCGTT CA A CGG CT C T GX GTC GTA CGT CCC A TGGT)
3′-d(T CCG CAAGT T GC CGAG ACY C AGC ATGC AGGG TA CCA)

2a-e

a: X = β-C-Fapy•dA, Y = T
b: X = β-C-Fapy•dA, Y = dA
c: X = β-C-Fapy•dA, Y = dG
d: X = β-C-Fapy•dA, Y = dC
e: X = α-C-Fapy•dA, Y = dC
f: X = dA, Y = T



The formamidopyrimidines (Fapy·dA and Fapy·dG) are derived from the purines in DNA as a result of oxidative stress. These molecules and the respective 8-oxopurine lesions (OxodA and OxodG) are believed to arise from a common intermediate (Scheme 1). The 8-oxopurines have been well studied using in vitro and in vivo methods. The latter types of experiments, carried out in several systems, consistently indicate that OxodG gives rise predominantly to G → T transversions (*11*−*13*). OxodA exhibits a lower mutation frequency (*11*, *13*, *14*). The formamidopyrimidines are produced in greater amounts under O_2 deficient conditions, but considerably less is known about their interactions with polymerases and repair enzymes than the effects of 8-oxopurines on these enzymes (*1*−*3*, *7*, *9*, *11*−*24*). This imbalance can be attributed to the absence of a method for preparing oligonucleotides containing the formamidopyrimidines at defined sites, which facilitates in vitro and in vivo studies on DNA lesions (*25*). We recently described the synthesis of oligonucleotides containing formamidopyrimidine lesions at defined sites. Fapy·dA and Fapy·dG were introduced using phosphoramidite chemistry as anomeric mixtures (*26*, *27*). The use of mixtures was not considered detrimental because the formamidopyrimidines rapidly epimerize in water (*28*, *29*). Prior to the synthesis of oligonucleotides containing Fapy·dA or Fapy·dG, studies on these lesions relied upon their random production in DNA or introduction of N-methylated analogues (*21*, *23*, *24*). Experiments using a template containing Fapy·dA at a defined site show that the frequency of misincorporation of deoxyadenosine (dA) or deoxyguanosine (dG) opposite the lesion by Klenow exo$^-$ increases by ∼50% compared to an otherwise identical template containing dA in place of the formamidopyrimidine (*30*). For comparison, incorporation of thymidine (T) or deoxycytidine (dC) is inhibited ∼4−6-fold. The kinetic preferences exhibited by Klenow exo$^-$ correlate with duplex stability as determined by van't Hoff analysis of UV melting studies (*30*). Moreover, the frequency of misinsertion of dA or dG opposite Fapy·dA is ∼8- or 3-fold greater, respectively, than the frequency of incorporation of these nucleotides opposite OxodA under similar conditions (*14*). Comparable experiments using templates containing α- or *β*-C-nucleoside analogues of Fapy·dA (α- or *β*-C-Fapy·dA, respectively) revealed that these molecules are significant blocks of Klenow exo$^-$ (*27*, *30*). The relative preferences for nucleotide insertion directed by *β*-C-Fapy·dA closely match those exhibited by Fapy·dA, indicating that the analogue and lesion present similar hydrogen bonding patterns to the polymerase. In contrast, a template containing α-C-Fapy·dA strongly prefers that Klenow incorporate dC opposite it. These data also suggest that despite the fact that oligonucleotides are synthesized containing readily epimerizable anomeric mixtures of Fapy·dA, it is the *β*-anomer that is responsible for the observed effects on Klenow exo$^-$. The ability of Fapy·dA, α-C-Fapy·dA, and *β*-C-Fapy·dA to instruct nucleotide incorporation by Klenow exo$^-$ raises the issue of whether the lesions are recognized by BER enzymes in duplex DNA.

EXPERIMENTAL PROCEDURES

Materials and General Methods. All H_2O was obtained from a Nanopure Barnstead still. Commercially available DNA synthesis reagents were obtained from Glen Research Inc. Radionuclides were obtained from Amersham. T4 polynucleotide kinase was obtained from New England Biolabs. Fpg was either a gift from J. Milligan of the University of California at San Diego (La Jolla, CA) or purchased from Trevigen. MutY was provided by S. David of the University of Utah (Salt Lake City, UT). Concentrations of Fpg and MutY were determined using the Bradford assay. Oligonucleotides were synthesized on an Applied Biosystems Inc. 394 DNA synthesizer. Oligonucleotides containing Fapy·dA or its C-nucleoside analogues were prepared as described previously (*26*, *27*). All others were synthesized using standard protocols. DNA manipulations were carried out using standard procedures (*31*). Radioactive samples were counted via Cerenkov counting, using a Packard Tri-Carb 1500 scintillation counter. Phosphor imaging analysis was carried out using a Storm 820 Molecular Dynamics Phosphorimager equipped with Imagequant software (Version 5.1). All duplexes containing Fapy·dA were hybridized at 60 °C (5 min), followed by slow cooling to room temperature. All other duplexes were hybridized at 90 °C (5 min).

Fapy·dA Excision and Inhibition Kinetics. A 2× solution of Fpg protein (final concentration of 1.755 nM, 1×10^{-4} unit) was added to a 2× solution containing **1a** in which the strand containing Fapy·dA was labeled with ^{32}P at its 5′-terminus (final concentration of 0.2−2.0 nM) and inhibitor (**2a**, final concentration of 0−7.5 nM) in 2× Rec buffer [20 mM HEPES/KOH (pH 7.4), 200 mM KCl, 20 mM EDTA, and 0.2 mg/mL BSA]. The inhibitor concentration was kept constant throughout a series of reactions while the substrate concentration was varied. When Fapy·dA excision was being assessed, the reactions were quenched after 5 min with formamide loading buffer (20 *μ*L). Inhibition reactions were quenched after 30 s. The samples were denatured (90 °C for 1 min and immediately cooled to 0 °C) prior to separation on a 20% denaturing PAGE gel and quantitative analysis by phosphorimaging. The amount of cleavage product due to Fpg was corrected for the amount of cleavage seen in the

Table 1: Effect of the Base Pair on Excision of Fapy·dA from **1** by Fpg

duplex	base pair	K_m (nM)	V_{max} (nM min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (nM^{-1} min^{-1})
1a	Fapy·dA:T	1.16 ± 0.20	0.21 ± 0.08	0.12 ± 0.05	0.10 ± 0.05
1b	Fapy·dA:dA	3.34 ± 0.25	0.33 ± 0.22	0.19 ± 0.13	0.06 ± 0.04
1c	Fapy·dA:dG	4.63 ± 0.41	0.97 ± 0.03	0.55 ± 0.02	0.12 ± 0.01

controls (those samples with no Fpg protein present). The apparent K_m/V_{max} values were obtained from a nonlinear least-squares analysis of a plot of the initial velocity versus substrate concentration. The K_I was determined by plotting the apparent K_m/V_{max} versus the inhibitor concentration (*32*). At least three experiments were run for each inhibitor concentration.

Fpg Turnover. An enzyme/substrate solution containing Fpg protein (1.755 nM), 1× Rec buffer, and **1a**−**c** (10 nM) was incubated at 37 °C. Aliquots (10 μL) were taken at 0, 1, 2, 5, 10, 15, and 30 min and the reactions quenched with formamide loading buffer (20 μL). Samples were denatured (90 °C for 1 min and immediately cooled to 0 °C) before separation by 20% denaturing PAGE.

MutY Cleavage. A solution of duplex DNA containing a mispair in which one of the strands was labeled at its 5′-terminus with ^{32}P (**1b**−**d**, 20−100 nM) was equilibrated at 37 °C in 20 mM Tris, 10 mM EDTA, and 0.1 mg/mL BSA (pH 7.5). MutY (100 nM) was added, and the solution was incubated at 37 °C with aliquots removed between 1 and 120 min (60 min for **1d**). The aliquots (10 μL) were added to 1 M NaOH (2 μL) and heated at 90 °C for 5 min. Formamide loading buffer (10 μL) was added, and the samples were heat denatured (90 °C for 3 min and immediately cooled to 0 °C). Cleavage products were separated by 20% denaturing PAGE and analyzed quantitatively via phosphorimaging. The amount of deglycosylation was determined by subtracting the amount of cleavage measured in a reaction mixture that was not treated with enzyme from those exposed to MutY.

Determination of the Extent of Binding of DNA by MutY and Fpg via Gel Shifts. Duplex DNA (**1b**, 50 pM) was equilibrated at 37 °C in 20 mM Tris, 10 mM EDTA, 0.1 mg/mL BSA, and 10% glycerol (pH 7.5) before addition of varying concentrations (run in triplicate) of MutY (0−500 nM). The protein/DNA solution was incubated at 37 °C for 20 min. Nondenaturing glycerol loading buffer (2 μL, 30% glycerol, 0.5× TBE, 1% xylene cylanol, and 1% bromophenol blue) was added to the solutions (20 μL) before they were loaded on a 6% nondenaturing gel run at 500 V for 2 h in a 4 °C refrigerator. Binding of DNA containing α- and β-C-Fapy·dA by Fpg was assessed in a similar manner, except that the Fpg concentration was varied from 0 to 200 nM and equilibrations were carried out at 25 °C.

UV Melting Experiments. The samples (see Table 3) for UV melting studies contained a 1:1 molar ratio of complementary oligonucleotides and were composed of a total oligonucleotide concentration ranging from 1 to 14.0 μM. The samples were prepared by the addition of appropriate amounts of complementary oligonucleotide stock solutions to 200 μL of PIPES buffer [20 mM PIPES (pH 7.0), 20 mM $MgCl_2$, and 200 mM NaCl] followed by dilution to 400 μL with H_2O. Samples were hybridized at 90 °C for 5 min and allowed to cool to room temperature overnight. The absorbance of the samples was then monitored at 260 nm while the temperature was increased at a rate of 1.0 °C/min over a range of 60 °C (25−85 °C). Melting temperatures were calculated by a computer fit of the first derivative of absorbance with respect to T^{-1} (*33*).

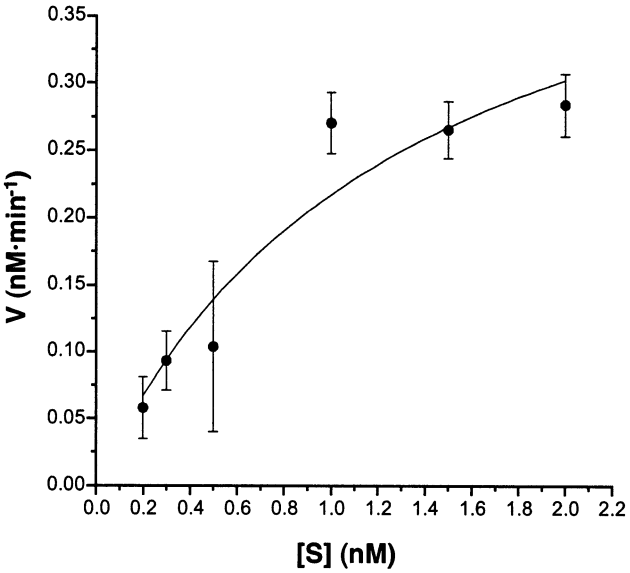


FIGURE 1: Excision of Fapy·dA when opposite T as a function of substrate concentration (S is **1a**).

RESULTS

Interaction of Fapy·dA with Fpg and MutY. In vitro experiments showed that Klenow exo$^-$ incorporated dA and dG in addition to thymidine opposite Fapy·dA at elevated levels compared to templates containing dA at the same site (*30*). The velocity of Fapy·dA excision by Fpg from duplexes containing the lesion opposite these three nucleotides, which are considered most likely to be incorporated opposite it, was measured as a function of DNA concentration (Figure 1 and Table 1). Fapy·dA repair was assessed by quantifying the formation of direct strand breaks using denaturing gel electrophoresis. The 3′-termini of the oligonucleotide fragments were composed entirely of phosphates and are consistent with the β,δ-lyase activity of Fpg (*7*). Kinetic parameters were extracted from a nonlinear least-squares analysis of a plot of the initial velocity versus substrate (DNA) concentration. These experiments showed that Fpg did not discriminate (k_{cat}/K_m, Table 1) between the duplexes containing the three Fapy·dA base pairs. In addition, multiple turnovers were rapidly observed under conditions where DNA was in excess (Figure 2), indicating that product inhibition was not an issue (*15*). For comparison, we verified the literature report and determined that OxodA is removed extremely slowly by *Escherichia coli* Fpg when it is opposite T (*16*).

The facile excision of Fapy·dA by Fpg regardless of the nucleotide opposite it was in stark contrast to its reaction with MutY. MutY (100 nM) did not incise dA (37 °C, 2 h) from a duplex (**1b**, 20 nM) containing the nucleotide opposite

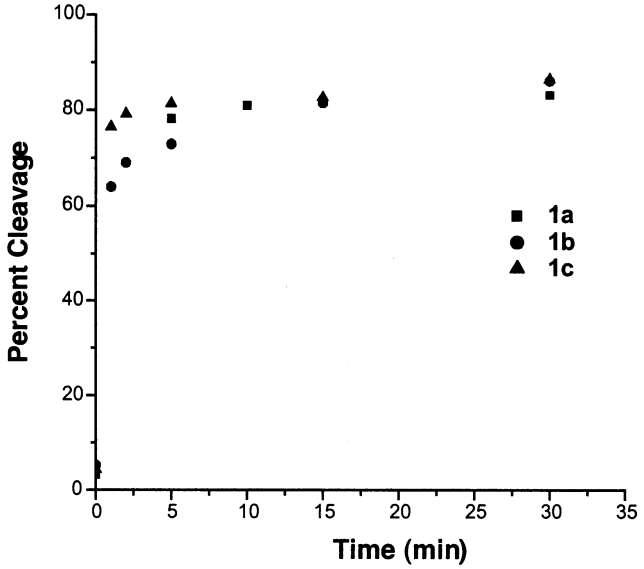


FIGURE 2: Excision of Fapy·dA as a function of time. [**1a**] = 10 nM, and [Fpg] = 1.755 nM.

Fapy·dA. Less than 3% incision was detected after 15 h. Furthermore, no incision of Fapy·dA was observed under identical conditions when the lesion was opposite dA (**1b**) or dG (**1c**). Examination of an otherwise comparable duplex containing a dG:dA base pair (**1d**) showed that 77% of dA was incised under identical conditions in 1 h, indicating that the observations involving oligonucleotides containing Fapy·dA could not be attributed to technical issues. The lack of incision by MutY of oligonucleotides containing Fapy·dA was reinforced by gel shift experiments, which showed that the enzyme bound the duplex (**1b**) containing the Fapy·dA:dA base pair (K_D = 44.0 ± 3.9 nM) significantly more weakly than DNA containing a dG:dA or OxodG:dA base pair (*22*). MutY binding of the Fapy·dA:dA base pair was comparable to the enzyme's affinity for duplexes composed entirely of native base pairs.

Binding of Fpg by and UV Melting Studies of DNA Containing C-Nucleoside Analogues of Fapy·dA. Although Klenow exo$^-$ incorporates dNTPs opposite β-C-Fapy·dA significantly more slowly than it does opposite Fapy·dA, the relative frequency of insertion is similar (*30*). These data were interpreted to mean that the analogue presents a hydrogen bonding pattern similar to that of Fapy·dA. Modeling experiments are consistent with this proposal (data not shown), and we anticipated that the interaction between DNA containing β-C-Fapy·dA and Fpg would follow suit. Gel shift experiments utilizing duplexes containing β-C-Fapy·dA and Fpg support this hypothesis. Strong binding of duplexes containing β-C-Fapy·dA opposite all four native nucleotides is observed (Table 2). In contrast, Fpg exhibits specific binding to a comparable duplex containing a α-C-Fapy·dA:dC base pair (**2e**, Figure 3), but not to duplexes in which the analogue is opposite any other nucleotide. Specific binding is not observed for an analogous duplex containing a dA:T base pair (**2f**). The low K_D determined for Fpg binding by duplexes containing β-C-Fapy·dA was corroborated by inhibition studies (Figure 4). Determination of the apparent K_m and V_{max} for Fapy·dA excision by Fpg in the presence of fixed concentrations of a comparable duplex containing a β-C-Fapy·dA:T base pair (**2a**) gave rise to a K_I of 3.5 ± 0.3 nM.

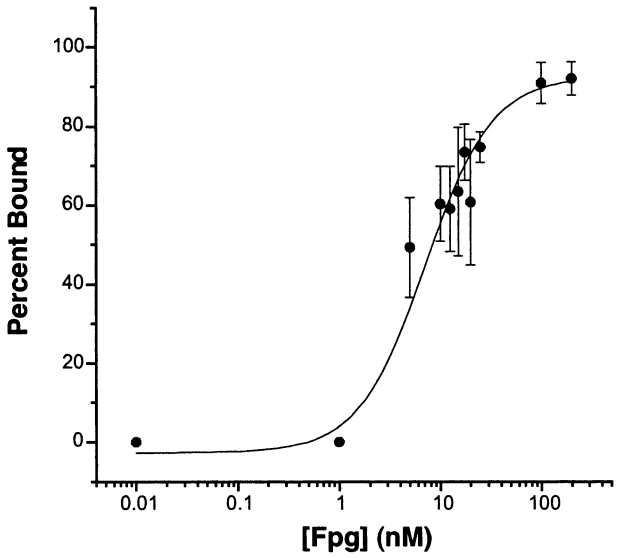


FIGURE 3: Gel shift analysis of DNA containing α-C-Fapy·dA:dC (**2e**) base pair binding by Fpg.

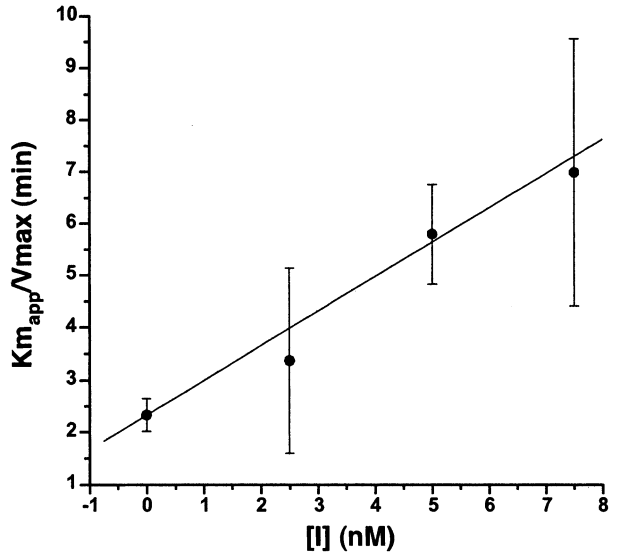


FIGURE 4: Inhibition of Fpg-mediated excision of Fapy·dA in **1a** by DNA containing a β-C-Fapy·dA:dC base pair (**2a**). Plot of the apparent K_m/V_{max} as a function of inhibitor concentration (I is **2a**).

Table 2: Assessment of the Fpg Binding of Duplexes Containing C-Nucleoside Analogues of Fapy·dA (α-C-Fapy·dA and β-C-Fapy·dA) in **2**

duplex	base pair	K_D (nM)
2a	β-C-Fapy·dA:T	10.5 ± 1.3
2b	β-C-Fapy·dA:dA	12.8 ± 2.9
2c	β-C-Fapy·dA:dG	26.5 ± 3.5
2d	β-C-Fapy·dA:dC	10.9 ± 1.3
2e	α-C-Fapy·dA:dC	7.1 ± 1.5
2f	dA:T	170 ± 37.5

UV melting thermodynamics were determined for dodecamers containing α- and β-C-Fapy·dA (Table 3). The local sequences were the same as those in DNA containing the C-nucleoside analogues that were used in the experiments described above and were compared to a template containing dA. Duplexes containing α-C-Fapy·dA:T or β-C-Fapy·dA:T base pairs were destabilized considerably relative to the native template. Additionally, unlike in Fapy·dA, the extent of duplex stabilization was not measurably increased when

Table 3: UV Melting Thermodynamics of Duplexes Containing α-C-Fapy·dA and β-C-Fapy·dA Compared to dA

5′-d(G C T C T G X G T C G T)
3′-d(C G A G A C Y C A G C A)

base pair	ΔH (kcal/mol)	ΔS (eu)	ΔG_{298} (kcal/mol)	T_M[a] (°C)
α-C-Fapy·dA:T	80.0	222.4	13.7	46.8
α-C-Fapy·dA:dA	66.9	182.2	12.6	46.6
α-C-Fapy·dA:dG	73.1	204.4	12.1	41.8
α-C-Fapy·dA:dC	73.9	203.9	13.1	46.4
β-C-Fapy·dA:T	77.8	215.6	13.5	46.8
β-C-Fapy·dA:dA	81.9	229.2	13.5	46.4
β-C-Fapy·dA:dG	76.0	213.5	12.4	42.6
β-C-Fapy·dA:dC	77.5	215.8	13.2	45.6
dA:T	92.2	252.0	17.0	56.6
dA:dA	75.1	207.6	13.2	46.7
dA:dG	73.9	202.1	13.6	48.3
dA:dC	87.2	246.7	13.6	44.7

[a] Total oligonucleotide concentration of 4.4 μM.

the C-nucleoside analogues were opposite any of the other three native nucleotides (*30*).

DISCUSSION

Fapy·dA is efficiently excised by formamidopyrimidine DNA glycosylase (Fpg, MutM). Fapy·dA and OxodA are produced from a common intermediate, but Fpg repairs the formamidopyrimidine much more efficiently when the lesions are opposite thymidine. This is consistent with previous studies on the repair of OxodA by Fpg protein (*16*, *19*, *20*). It is interesting to note that Fpg excises Fapy·dA as rapidly as OxodG (*16*, *17*). The relative preference of Fpg for these lesions is also qualitatively consistent with that reported by Dizdaroglu using DNA substrates containing randomly produced lesions by a variety of forms of oxidative stress (*20*). The K_m and V_{max} values measured in those experiments implied considerably less efficient repair overall. However, in those experiments, many other lesions were present in the DNA substrate and presumably competed for the BER enzyme.

A significant distinction between Fpg repair of Fapy·dA and OxodG concerns the activity of the enzyme on duplexes containing mispairs. Other than dC, polymerases are most likely to insert dA opposite OxodG. Fpg excises OxodG from OxodG:dA base pairs ~20−200 times more slowly than from a comparable duplex containing an OxodG:dC base pair (*16*, *17*). Removal of a lesion from a premutagenic base pair leaves the incorrect nucleotide as the sole source of genetic information and makes the polymerase's infidelity permanent. The inefficiency of OxodG removal from a mispair with dA by Fpg in effect protects the genome against G → T transversions. Our in vitro results suggest that the genome would not be protected in a similar manner against Fapy·dA. Experiments using Klenow exo⁻ showed that dA and dG are the nucleosides most likely to be misincorporated opposite Fapy·dA. However, the specificity with which Fpg excises Fapy·dA from duplexes in which the lesion is mispaired with dA or dG is equal within experimental error to that when it is opposite T (Table 1). This observation suggests that the formamidopyrimidine could be more deleterious to the genome than one would predict solely on the basis of polymerase fidelity experiments. The possible adverse biological effect of Fapy·dA is magnified by the inability of MutY to incise dA opposite the lesion. In comparison, MutY incises dA from DNA containing OxodG:dA base pairs and remains bound to the product, presumably to protect the duplex from the subsequent action by Fpg, which would result in a double-strand break (*15*, *22*). MutY also recognizes and removes dA from duplexes containing OxodA:dA base pairs (*22*). It appears that although Fpg treats the presence of Fapy·dA in DNA as dangerous, MutY does not recognize this lesion at all.

Why Fapy·dA is treated in a manner different from that of OxodG and OxodA is uncertain. We have considered two possible explanations. Perhaps Fpg has not evolved to prevent conversion of Fapy·dA:dA(dG) base pairs into permanent mutations, because they are not formed in vivo in significant amounts. This thought is consistent with some but not all related observations. The observation that Fapy·dA is formed in at least 10-fold smaller amounts than Fapy·dG in γ-irradiated human leukemia cells is consistent with this hypothesis, but inconsistent with irradiation of chromatin (*1*, *3*). When viewed in the context of OxodG, the absolute values of k_{cat}/K_m for Fpg excision of Fapy·dA may also suggest that this process is not biologically relevant. Fpg excises OxodG from DNA when it is paired with dG or T significantly more efficiently than when the lesion is opposite either of the two native nucleotides (dC and dA) that are actually incorporated translesionally by polymerases (*16*). Although it could be a coincidence, Fapy·dA removal by Fpg is comparable in efficiency to the excision of OxodG when it is opposite biologically irrelevant nucleotides (dG and T).

The biological relevance of in vitro polymerase experiments involving Fapy·dA can be interpreted either way. On one hand, Fapy·dA induces Klenow exo⁻ to misinsert nucleotides opposite it more often than does OxodA, but the misinsertion frequency is significantly lower than that for OxodG (*12*, *14*, *30*). If mispairs involving Fapy·dA are not formed frequently in vivo, then it is unnecessary for BER enzymes to evolve to properly repair them. In vivo experiments indicate that OxodA is at most weakly mutagenic, with OxodG being more deleterious (*34*−*36*). The question that remains unanswered is whether the in vivo mutation frequency of Fapy·dA is sufficiently high to warrant suitable protection by BER enzymes.

We also considered the possibility that the duplexes containing Fapy·dA:dA and Fapy·dA:dG base pairs are efficiently recognized by Fpg because either the lesion is more likely to adopt a conformation in which it is extrahelical or less energy is required for Fpg to "flip" the lesion (*37*). This scenario is consistent with UV melting studies of duplexes containing Fapy·dA opposite T, dA, or dG (*30*). Duplexes containing Fapy·dA opposite these nucleotides undergo similar changes in free energy upon melting, but considerably less energy is needed to melt them than an otherwise identical duplex containing a dA:T base pair. UV melting and Fpg binding studies on β-C-Fapy·dA show similar trends. Gel shift experiments reveal tight binding of duplexes containing the C-nucleoside analogue opposite any of the four native nucleotides (Table 2). UV melting studies do not reveal any stabilization by specific base pairs involving β-C-Fapy·dA, and all are considerably destabilized compared to the control duplex containing a dA:T base pair

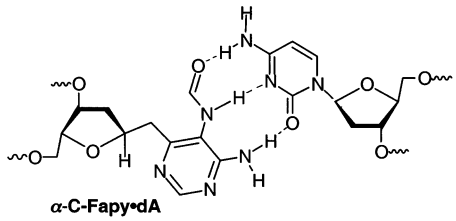


FIGURE 5: Hypothetical base pairing between α-C-Fapy·dA and dC.

(Table 3). Unfortunately, a similar correlation is not observed between Fpg recognition of OxodG and thermal stability studies of duplexes containing this lesion (*16*, *38*).

In addition, the recognition of the *β*-C-Fapy·dA by Fpg (Table 2) is significant independent of the above issues. Tight binding of duplexes containing the C-nucleotide opposite any of the four native nucleotides mirrors the observed excision of Fapy·dA opposite T, dA, and dG. The binding studies also indicate that *β*-C-Fapy·dA presents a hydrogen bonding pattern to the enzyme comparable to that of the lesion and that the glycosidic nitrogen is not involved in base pairing. The tight binding of Fpg by DNA containing dC opposite α-C-Fapy·dA is difficult to interpret. One can draw the α-C-Fapy·dA:dC base pair like a dG:dC base pair in which the analogue adopts the *syn* conformation, but this should induce significant distortion in the helix (Figure 5). Although UV melting experiments do not substantiate this concept, it is pertinent to note that the binding of DNA containing an α-C-Fapy·dA:dC base pair by Fpg is not anomalous. When α-C-Fapy·dA is present in a DNA template, it preferentially directs Klenow exo$^-$ to incorporate dC opposite itself (*30*). Hence, experiments with polymerase and repair enzymes indicate that there is something unique about the α-C-Fapy·dA:dC base pair.

The strong binding of Fpg by duplexes containing the *β*-C-nucleoside analogue of Fapy·dA (*β*-C-Fapy·dA) is comparable to that of nonhydrolyzable analogues of other DNA lesions (*18*, *39*). The independently measured inhibition constant for a duplex containing a *β*-C-Fapy·dA:T base pair indicates that the molecule could have useful therapeutic properties provided it can be incorporated enzymatically into DNA. As has been pointed out, inhibition of BER is a mostly unexplored process vis-à-vis therapeutic applications (*10*). It is possible if BER enzymes are unable to repair DNA containing molecules such as *β*-C-Fapy·dA, these analogues may be lethal.

CONCLUSIONS

The combined observations of the interactions of Fapy·dA-containing DNA with the Fpg and MutY repair enzymes suggest that miscoding by polymerases resulting in Fapy·dA:dA and Fapy·dA:dG base pairs will result in mutations. These observations need to be verified using in vivo methods in which the formamidopyrimidine lesion is introduced at a defined site in a plasmid, which is then transfected into a host (*11*, *13*). The observations described above evoke the question of whether if the genome is not protected from Fapy·dA by the GO system as well as it is from OxodG there is another repair pathway that recognizes the formamidopyrimidine. In addition, given the conflicting reports concerning the prevalence of Fapy·dA lesions and that the in vitro misincorporation frequency by a polymerase is lower for this lesion than for OxodG, is it possible that repair systems have not evolved to protect the genome against the formamidopyrimidine because it is unnecessary (*1*, *3*)? This question may be addressed by judicious use of in vivo experiments.

Experiments utilizing the nonhydrolyzable analogues of Fapy·dA are of mechanistic value and indicate that these molecules are potentially useful in other avenues. Exploitation of inhibition of BER by nucleotides incorporated in DNA is a therapeutic approach that is largely unexplored. The C-nucleosides studied here show promise in this respect.

ACKNOWLEDGMENT

We thank Dr. Jamie Milligan for a gift of Fpg and Professor Sheila David for providing MutY.

REFERENCES

1. Gajewski, E., Rao, G., Nackerdien, Z., and Dizdaroglu, M. (1990) *Biochemistry 29*, 7876−7882.
2. Doetsch, P. W., Zastawny, T. H., Martin, A. M., and Dizdaroglu, M. (1995) *Biochemistry 34*, 737−742.
3. Pouget, J.-P., Douki, T., Richard, M.-J., and Cadet, J. (2000) *Chem. Res. Toxicol. 13*, 541−549.
4. Lindahl, T., and Wood, R. D. (1999) *Science 286*, 1897−1905.
5. Hoeijmakers, J. H. J. (2001) *Nature 411*, 366−374.
6. Finkel, T., and Holbrook, N. J. (2000) *Nature 408*, 239−247.
7. David, S. S., and Williams, S. D. (1998) *Chem. Rev. 98*, 1221−1261.
8. Schärer, O. D., Deng, L., and Verdine, G. L. (1997) *Curr. Opin. Chem. Biol. 1*, 526−531.
9. Laval, J., Jurado, J., Saparbaev, M., and Sidorkina, O. (1998) *Mutat. Res. 402*, 93−102.
10. Lindahl, T. (2001) *Progress in Nucleic Acid Research and Molecular Biology, Vol. 68, Base Excision Repair* (Moldave, K., Ed.) pp xvii−xxix, Academic Press, San Diego.
11. Tan, X., Grollman, A. P., and Shibutani, S. (1999) *Carcinogenesis 20*, 2287−2292.
12. Shibutani, S., Takeshita, M., and Grollman, A. P. (1991) *Nature 349*, 431−434.
13. Delaney, J. C., and Essigmann, J. M. (2001) *Biochemistry 40*, 14968−14975.
14. Shibutani, S., Bodepudi, V., Johnson, F., and Grollman, A. P. (1993) *Biochemistry 32*, 4615−4621.
15. Porello, S. L., Leyes, A. E., and David, S. S. (1998) *Biochemistry 37*, 14756−14764.
16. Tchou, J., Bodepudi, V., Shibutani, S., Antoshechkin, I., Miller, J., Grollman, A. P., and Johnson, F. (1994) *J. Biol. Chem. 269*, 15318−15324.
17. Castaing, B., Geiger, A., Seliger, H., Nehls, P., Laval, J., Zelwer, C., and Boiteux, S. (1993) *Nucleic Acids Res. 21*, 2899−2905.
18. Zharkov, D. O., Rosenquist, T. A., Gerchman, S. E., and Grollman, A. P. (2000) *J. Biol. Chem. 275*, 28607−28617.
19. Tchou, J., Kasai, H., Shibutani, S., Chung, M.-H., Laval, J., Grollman, A. P., and Nishimura, S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4690−4694.
20. Karakaya, A., Jaruga, P., Bohr, V. A., Grollman, A. P., and Dizdaroglu, M. (1997) *Nucleic Acids Res. 25*, 474−479.
21. Asagoshi, K., Yamada, T., Terato, H., Ohyama, Y., Monden, Y., Arai, T., Nishimura, S., Aburatani, H., Lindahl, T., and Ide, H. (2000) *J. Biol. Chem. 275*, 4956−4964.
22. Bulychev, N. V., Varaprasad, C. V., Dormán, G., Miller, J. H., Eisenberg, M., Grollman, A. P., and Johnson, F. (1996) *Biochemistry 35*, 13147−13156.
23. Asagoshi, K., Yamada, T., Okada, Y., Terato, H., Ohyama, Y., Seki, S., and Ide, H. (2000) *J. Biol. Chem. 275*, 24781−24786.
24. Graziewicz, M.-A., Zastawny, T. H., Oli'nski, R., and Tudek, B. (1999) *Mutat. Res. 434*, 41−52.
25. Ide, H. (2001) *Progress in Nucleic Acid Research and Molecular Biology, Vol. 68, Base Excision Repair* (Moldave, K., Ed.) pp 207−222, Academic Press, San Diego.
26. Haraguchi, K., and Greenberg, M. M. (2001) *J. Am. Chem. Soc. 123*, 8636−8637.

27. Haraguchi, K., Delaney, M. O., Wiederholt, C. J., Sambandam, A., Hantosi, Z., and Greenberg, M. M. (2002) *J. Am. Chem. Soc. 124*, 3263−3269.
28. Greenberg, M. M., Hantosi, Z., Wiederholt, C. J., and Rithner, C. D. (2001) *Biochemistry 40*, 15856−15861.
29. Berger, M., and Cadet, J. (1985) *Z. Naturforsch. 40b*, 1519−1531.
30. Delaney, M. O., Wiederholt, C. J., and Greenberg, M. M. (2002) *Angew. Chem., Int. Ed. 41*, 771−773.
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
32. Silverman, R. P. (2000) *The Organic Chemistry of Enzyme-Catalyzed Reactions*, Academic Press, San Diego.
33. Breslauer, K. J. (1994) in *Protocols for Oligonucleotide Conjugates: Synthesis and Analytical Techniques* (Agrawal, S., Ed.) Humana Press, NJ.
34. Wood, J. L., Esteve, A., Morningstar, M. L., Kuziemko, G. M., and Essigmann, J. M. (1992) *Nucleic Acids Res. 20*, 6023−6032.
35. Kamiya, H., Miura, H., Kamiya-Murata, N., Ishikawa, H., Sakaguchi, T., Inoue, H., Sasaki, T., Masutani, C., Hanaoka, F., Nishimura, S., and Ohtsuka, E. (1995) *Nucleic Acids Res. 23*, 2893−2899.
36. Tan, X., Grollman, A. P., and Shibutani, S. (1999) *Carcinogenesis 20*, 2287−2292.
37. Sugahara, M., Mikawa, T., Kumasaka, T., Yamamoto, M., Kato, R., Fukuyama, K., Inoue, Y., and Kuramitsu, S. (2000) *EMBO J. 19*, 3857−3869.
38. Plum, G. E., Grollman, A. P., Johnson, F., and Breslauer, K. J. (1995) *Biochemistry 34*, 16148−16160.
39. Chepanoske, C. L., Porello, S. L., Fujiwara, T., Sugiyama, H., and David, S. S. (1999) *Nucleic Acids Res. 27*, 3197−3204.

BI025903E