Efficient Removal of Formamidopyrimidines by 8-Oxoguanine Glycosylases[†]

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ABSTRACT: Under conditions of oxidative stress, the formamidopyrimidine lesions (FapyG and FapyA) are formed in competition with the corresponding 8-oxopurines (OG and OA) from a common intermediate. In order to reveal features of the repair of these lesions, and the potential contribution of repair in mitigating or exacerbating the mutagenic properties of Fapy lesions, their excision by three glycosylases, Fpg, hOGG1 and Ntg1, was examined in various base pair contexts under single-turnover conditions. FapyG was removed at least as efficiently as OG by all three glycosylases. In addition, the rates of removal of FapyG by Fpg and hOGG1 were influenced by their base pair partner, with preference for removal when base paired with the correct Watson−Crick partner C. With the FapyA lesion, Fpg and Ntg1 catalyze its removal more readily than OG opposite all four natural bases. In contrast, the removal of FapyA by hOGG1 was not as robust as FapyG or OG, and was only significant when the lesion was paired with C. The discrimination by the various glycosylases with respect to the opposing base was highly dependent on the identity of the lesion. OG induced the greatest selectivity against its removal when part of a promutagenic base pair. The superb activity of the various OG glycosylases toward removal of FapyG and FapyA in vitro suggests that these enzymes may act upon these oxidized lesions in vivo. The differences in the activity of the various glycosylases for removal of FapyG and FapyA compared to OG in nonmutagenic versus promutagenic base pair contexts may serve to alter the mutagenic profiles of these lesions in vivo.

A plethora of modifications of the heterocyclic DNA bases are produced when the biopolymer is exposed to oxidative conditions (*1−4*). Oxidative DNA base damage and inefficient remediation via repair are intimately linked to the initiation and progression of carcinogenesis (*4, 5*). The oxidation product of guanine, 7,8-dihydro-8-oxo-guanine (OG)[1,2] has been the focus of investigations on the effects of oxidative damage (*4, 6−9*). However, ring-opened formamidopyrimidine lesions, 2,6-diamino-4-hydroxy-5-for-

mamidopyrimidine (FapyG) and 4,6-diamino-5-formamidopyrimidine (FapyA) are formed in vitro in yields comparable to the respective 8-oxopurines from common radical intermediates (Figure 1) (*10−13*). Oxygen-rich conditions favor the 8-oxopurines, while reductive conditions favor production of Fapy lesions (*12, 13*). For example, FapyG was observed to be the major product in mammalian chromatin or a human leukemia cell line exposed to γ-radiolysis under anoxic conditions (*13, 14*). In addition, FapyG and OG are formed endogenously in DNA, with the relative amounts highly dependent on the cell type (*15*). Similarly, oxidation of adenine in mammalian chromatin by γ-radiolysis under O_2 deficient conditions produces FapyA in greater amounts than 7,8-dihyro-8-oxoadenine (OA). However, the levels of OA present in cells are an order of magnitude less than OG (*14*).

The wealth of attention paid to OG stems in part from the ease with which oligonucleotides with OG at defined positions may be obtained. This is due to the well-established synthetic procedures available to make the phosphoramidite monomer for automated DNA synthesis (*16*). Information regarding the mutagenic potential of FapyG and FapyA lesions is beginning to emerge due to the recent development of synthetic methods for preparing oligonucleotides containing these lesions at a defined location (*17−19*). In vitro replication studies using *Escherichia coli* DNA polymerase I Klenow fragment (KF) show that FapyG is bypassed less efficiently than OG (*20, 21*). Similar to OG, the presence of FapyG results in misinsertion of dAMP as well as correct insertion of dCMP by KF (*20, 22*). The KF misinsertion

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[1] Abbreviations: BsFpg, *Bacillus stearothermophilus* Fpg; BER, base excision repair; bp, base pair; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; exo, exonuclease; FapyA, 4,6-diamino-5-formamidopyrimidine; FapyG, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; Fpg, formamidopyrimidine glycosylase or MutM; hOGG1, human OG glycosylase; IPTG, isopropyl *β*-D-thiogalactoside; KF, Klenow fragment of DNA polymerase I; NER, nucleotide excision repair; nt, nucleotide; OA, 8-oxo-7,8-dihydroadenine; OG, 8-oxo-7,8-dihydroguanine; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; TBE, tris-borate-EDTA; Tris, tris(hydroxymethyl)aminomethane; yOGG2 (Ntg1), yeast OG glycosylase.

[2] The nucleotides referred to throughout this manuscript are 2′-deoxynucleotides, although not explicitly designated in terms of the abbreviations used. For simplicity's sake, the same abbreviation is used for the base and the nucleotide. The context makes it clear which is being referred to.
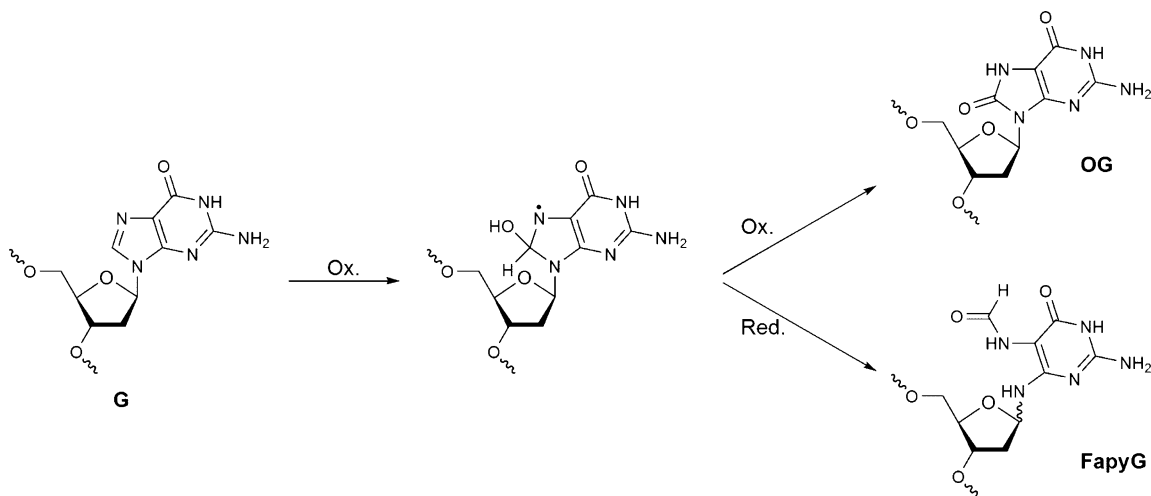
FIGURE 1: Formation of FapyG and OG from a common intermediate. FapyA and OA are also formed from a common intermediate in an analogous mechanism.

frequency opposite FapyG is also reduced compared to OG (*21*). Experiments using a template containing FapyA at a defined site show low levels of misincorporation of dAMP and dGMP opposite the lesion by KF exo⁻ (*23*). The studies with isolated enzymes are consistent with the mutational response to OG and FapyG observed in cellular experiments. Numerous investigations have shown that the presence of OG in bacterial, yeast and mammalian cells mediates G → T transversion mutations (*2, 3*). The mutagenic potential of the formamidopyrimidine lesions relative to oxopurine lesions was studied in simian kidney cells using a shuttle phagemid vector containing the lesions at defined positions (*24*). These experiments revealed that FapyG induces G →T mutations at a higher frequency than OG, while FapyA is only weakly mutagenic and induces A → C mutations. In contrast, in *E. coli*, the frequency of G → T transversions induced by FapyG were minimal, and clearly below the amount observed for OG (*21*).

Important features that influence the mutagenic potential of a given lesion are the efficiency of its repair and the sensitivity of the repair enzymes to the correct base-pairing context. In *E. coli*, the mutagenic effects of OG are significantly reduced by the synergistic activities of three enzymes that constitute the "GO" repair pathway (*25*). Two of these are base excision repair (BER) glycosylases that act upon DNA lesions within duplex DNA. Any OG present in OG:C base pairs is targeted by the formamidopyrimidine-DNA glycosylase (Fpg/MutM). Fpg carries out the first step of BER by catalyzing removal of the damaged OG base. The BER machinery then acts appropriately to reconstitute the original undamaged DNA sequence (*26–28*). If OG is not removed prior to formation of an OG:A base pair, the MutY adenine glycosylase removes the misincorporated A, providing another opportunity to recreate an appropriate OG:C substrate for Fpg. Functionally similar enzymes are present in most organisms (*28*).

Fpg was originally discovered on the basis of its ability to mediate the release of ring-opened 7-methylguanine (MeFapyG) from DNA treated with alkylating agents followed by alkali (*29*). Thus, many reports of BER glycosylases, such as Fpg, have used MeFapy as a convenient model for FapyG (*30–33*). Other studies utilized DNA that had been treated with ionizing radiation or chemical agents to randomly produce FapyG and FapyA (and many other oxidized bases) in high-molecular-weight DNA (*34–37*). In addition to the bacterial enzyme Fpg, such studies have revealed a number of glycosylases capable of releasing FapyG or MeFapyG, including the human OG glycosylase (hOGG1) (*38*), the yeast OG glycosylases, yOGG1 and Ntg1 (yOGG2) (*39–41*) and the mammalian "Nei-like" enzymes, NEIL1 and NEIL2 (*37, 42*). Previous work showed that Fpg excises FapyG and FapyA localized at a specific site in a synthetic oligonucleotide duplex (*43*). While Fpg excises FapyG opposite C approximately 20-fold more efficiently than when opposite A, it indiscriminately removes FapyA opposite the four native nucleotides. A more recent investigation of Fapy lesion repair by mammalian cell extracts using synthetic oligonucleotides revealed that OGG1 and NTH1 are the major DNA glycosylases responsible for removing FapyG and FapyA, respectively (*44*).

In an effort to further understand features influencing recognition and removal of FapyG and FapyA lesions relative to OG, we examined the base excision activity of three BER enzymes (Fpg, hOGG1 and Ntg1) on synthetic duplexes containing these lesions in various base-pairing contexts. By carrying out experiments under single-turnover conditions, the intrinsic properties of the base recognition and removal process were revealed because complications associated with product release are removed. Indeed, with most BER glycosylases, product release is rate limiting. Therefore, k_{cat} measured under steady-state conditions is primarily a reflection of the rate of product release rather than the steps involved in base excision. The quantitative measurements demonstrate that Fapy lesions are excellent substrates for these repair enzymes. Indeed, FapyG was found to be as good or better as a substrate than OG for all three enzymes. Moreover, these studies revealed subtle differences between the various glycosylases with respect to the selectivity for the base opposite the FapyG and FapyA lesions. Based on the results reported herein, the action of the glycosylase enzymes would be anticipated to alter the mutagenic properties of these lesions.

MATERIALS AND METHODS

General Materials and Instrumentation. Radiolabeling was done using [γ-³²P]ATP purchased from Amersham Life

Duplex 1   5'-AGGCGTTCAACGTGCAGT<u>X</u>ACAGCACGTCCCATGGT-3'
             3'-TCCGCAAGTTGCACGTCA<u>Y</u>TGTCGTGCAGGGTACCA-5'

where X = FapyG or OG and Y = C and A

Duplex 2   5'-CGTTCAACGTGCACT<u>X</u>ACAGCACGTCCCAT-3'
             3'-GCAAGTTGCACGTGA<u>Y</u>TGTCGTGCAGGGTA-5'

where X = FapyA or OG and Y = C, A, T and G

FIGURE 2: Sequence of the duplexes used in this study. Duplex 1 is a 36-base-pair duplex with a centrally located FapyG or OG lesion. Duplex 2 is a 30-base-pair duplex with a centrally located FapyA or OG lesion.

Sciences with T4 polynucleotide kinase which was obtained from New England BioLabs. A Milli-Q PF system was used to purify distilled deionized water that was used to make all the buffers. All buffers were passed through a 0.45 μm filter before use. Storage phosphor autoradiography was performed on a Typhoon 9400 phosphorimager system. Data analysis was performed using ImageQuaNT software (version 5.2a), and the rate constants were determined using GraFit 5.0 software. All other chemicals used for these experiments were purchased from Fisher Scientific, VWR, or Sigma. Fpg, hOGG1 and Ntg1 were purified as previously described (*45, 46*).

Oligonucleotides. The FapyG and FapyA containing oligonucleotides were synthesized as previously described (*17−19*). The oligonucleotides containing OG and the complementary strands were synthesized at the University of Utah oligonucleotide synthesis core facility. These samples were then purified via HPLC on a Beckman Gold Nouveau system with a Dionex 100 ion exchange column. Sequences of the oligonucleotides used and the relevant duplexes studied are shown in Figure 2.

Substrate DNA preparation. For all experiments, 2.5 pmol of the X-containing strand was radiolabeled on the 5' end using [γ-^{32}P]-ATP by T4 kinase at 37 °C. Excess [γ-^{32}P]-ATP was removed using a Pharmacia Microspin G-50 spin column according to the manufacturer's protocol. For the glycosylase activity assays, additional nonradioactive X-containing DNA was added to the labeled strand to yield a solution containing 5% labeled DNA. This was then annealed to the complement (added at 20% excess) by heating at 55 °C for 5 min for FapyG and FapyA (to prevent degradation) and 90 °C for OG in annealing buffer (20 mM Tris-HCl (pH 7.6), 10 mM EDTA, and 150 mM NaCl), followed by cooling overnight. To determine the effect of different annealing temperatures on the extent of duplex formation, the OG:A duplex annealed under both conditions was analyzed via native gel (no urea) electrophoresis followed by quantification with storage phosphor autoradiography. This analysis indicated only a slightly lower amount of duplex formed at the lower duplex annealing temperature (98% vs 100%).

Single-Turnover Kinetics. Single-turnover experiments, where [Enz] > [DNA], were performed using the base pair duplexes in Figure 2 to evaluate the glycosylase activity of the enzymes. In each case, the total reaction volume was 60 μL with a final duplex DNA concentration of 20 nM. The duplex was incubated with either 200 nM active Fpg or 100 nM active hOGG1/Ntg1 in an assay buffer (20 mM Tris-HCl, pH 7.6, 10 mM EDTA, 0.1 mg/mL BSA, and 30 mM NaCl, (70 mM NaCl for hOGG1)) at 37 °C. Aliquots were removed at various times (15 s to 60 min) and quenched by

the addition of 5 μL of formamide denaturing dye (80% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue in TBE buffer) (for the glycosylase/lyase activity) and placed on dry ice. To measure only the base excision (glycosylase) activity, aliquots were quenched by the addition of 5 μL of 0.5 N NaOH, heated to 55 °C (90 °C for OG) for 2 min and then frozen with dry ice, until ready for gel loading. A control experiment with the OG:A duplex annealed at 55 °C gave identical results.

For reactions under single-turnover conditions in which the glycosylase reaction was too fast to measure manually, a Rapid Quench Flow instrument (RQF-3) from Kintek was used in a manner similar to that previously reported (*46, 47*). The relevant enzyme was mixed with the DNA duplex (20 nM final). Aliquots were removed at various time points between 0.1 s and 5 min and quenched by the addition of 0.5 M NaOH. The samples were then heated for 2 min at 55 °C for Fapy lesions and 90 °C for OG, and then the formamide denaturing dye was added. The samples were then run on a 15% denaturing polyacrylamide gel in 1X TBE at 1600 V for 2 h. The separation of the product and the substrate was visualized using autoradiography by exposure to a storage phosphor screen overnight. In all cases, the data are reported as the average of at least three separate experiments, and the error is reported as the standard deviation of the sample set.

RESULTS

General Considerations of Glycosylase Assays. The glycosylase activity of the three enzymes was analyzed using the DNA duplexes shown in Figure 2, utilizing methods similar to those reported previously (*45−47*). In each case, the lesion-containing strand was end-labeled with [γ-^{32}P]-ATP, and the activity was assessed by the extent of strand scission at the lesion site. All three glycosylases have lyase activities that lead to strand scission. The base excision activity can be monitored using the enzyme to provide strand scission or by quenching with NaOH to cleave abasic sites. The glycosylase and lyase activities are tightly coupled in Fpg such that both types of experiments provide identical amounts of strand scission. However, with hOGG1 and Ntg1, a greater extent of strand scission is observed with the NaOH quench, indicating a decoupling of the glycosylase and β-lyase activities, with the former being more efficient. A representative storage phosphor autoradiogram of a PAGE analysis with hOGG1 is shown in Figure 3A and 3B.

Previous work showed that following the rate of strand scission at OG mediated by Fpg, hOGG1 and Ntg1 under single-turnover conditions ([E] > [DNA]) provides data that follows first-order kinetics and may be fitted to a single
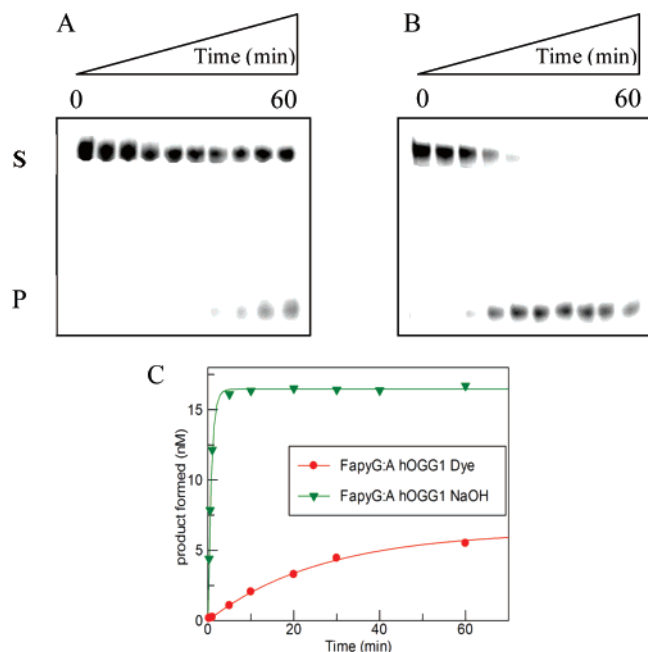
FIGURE 3: Representative assays for glycosylase/lyase and glyco-sylase actvity of hOGG1. (A) Storage phosphor autoradiogram of the removal of FapyG opposite A by hOGG1 with a dye quench to determine the glycosylase/lyase activity. Reactions were performed at 37 °C, with a DNA concentration of 20 nM and a hOGG1 concentration of 100 nM (active site concentration). The first lane is a control without the enzyme, and the triangle indicates an increase in incubation time of up to 60 min. "S" and "P" refer to the bands arising from the substrate and product duplex, respec-tively. (B) Storage phosphor autoradiogram of the removal of FapyG opposite A by hOGG1 with a NaOH quench to determine the glycosylase activity. Reactions were performed at 37 °C, with a DNA concentration of 20 nM and a hOGG1 concentration of 100 nM (active site concentration). The first lane is a control without the enzyme, and the triangle indicates an increase in incubation time of up to 60 min. (C) Representative plot of the removal of FapyG from FapyG:A base-pair-containing duplex 1 by hOGG1 with a dye (red circles) or a NaOH quench (green inverted triangles). The graph was derived from the quantitation of the storage phosphor autoradiograms shown in panels A and B. The data were fit to a single exponential to determine k_{gl} or k_g. Rate constants from several experiments are averaged to provide the values in Table 1.

Scheme 1: Minimal Kinetic Scheme Used in Analysis of Glycosylase Activity of Fpg, hOGG1 and Ntg1[a]
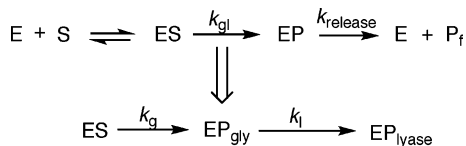
[a] In the case of Fpg, the lyase step is fast so it is not possible to measure the lyase rate. In contrast, glycosylase and lyase steps are uncoupled in hOGG1 and Ntg1. The k_g denotes the rate constant for the glycosylase step determined by quenching the reaction with a NaOH/heat treatment. The strand cleavage step is determined by quenching the reaction with a formamide dye treatment and is denoted by k_{gl}. In the case of Fpg, $k_{gl} = k_g$, while with hOGG1 and Ntg1 $k_{gl} = k_l$.

exponential (46). Under these conditions the observed rate constant associated with this process (k_{obs}) is equal to k_{gl} (Scheme 1). The rate constant k_{gl} includes the base excision (glycosylase) and the strand scission (β-lyase) reactions. The rate constants k_g and k_{gl} are determined from reaction using NaOH or formamide dye quenching, respectively. Notably, Fapy lesions are more sensitive to NaOH treatment at 90 °C than OG. As a result, the quenching steps for all the Fapy

Table 1: Rate Constants Determined at 37 °C under Single-Turnover Conditions for Fpg, hOGG1 and Ntg1 with FapyG and OG in Duplex 1

	Fpg	hOGG1		Ntg1	
substrate	k_g (min^{-1})	k_{gl} (min^{-1})	k_g (min^{-1})	k_{gl} (min^{-1})	k_g (min^{-1})
FapyG:C	86 ± 10	0.23 ± 0.01	57 ± 6	0.24 ± 0.04	0.9 ± 0.4
FapyG:A	17 ± 1	<0.02	1.2 ± 0.1	0.29 ± 0.05	2.4 ± 0.3
OG:C	21 ± 1	0.12 ± 0.06	57 ± 7	MC[a]	MC
OG:A	0.6 ± 0.1	<0.01	<0.02	MC	MC

[a] MC: minimal cleavage (<5% after background correction at all time points).

lesions were carried out at 55 °C to reduce degradation. Under these conditions, in the absence of a glycosylase enzyme, minimal cleavage at the FapyG lesion site was observed. However, 20−30% of the FapyA substrate strand was cleaved under these conditions. Consequently, the extent of cleavage at the FapyG or FapyA site under the reaction conditions in the absence of the enzyme was subtracted from the amount of cleavage produced in the presence of enzyme to correct for the nonenzyme catalyzed degradation of the substrate.

Removal of FapyG by Fpg, hOGG1 and Ntg1. Steady-state experiments show that polymerases incorporate dAMP opposite FapyG more readily than opposite G (20). More-over, mutagenesis studies in COS-7 cells and *E. coli* indicate that FapyG causes G → T transversions, thus also implicating the formation of FapyG:A base pairs (21, 24). These investigations indicated that base pairs consisting of FapyG opposite C and A are potential substrates for BER enzymes. The efficiency of base-excision of these lesions by the three glycosylases (Fpg, hOGG1 and Ntg1) was determined from single-turnover experiments with duplex 1 containing either FapyG or OG opposite C or A (Table 1). When the lesions are paired with C in this duplex, Fpg catalyzes the removal of FapyG with an ∼4-fold faster rate than OG. Quenching with NaOH or relying upon Fpg to provide strand scission gave similar observed rates, consistent with the tight coupling of the enzyme's glycosylase and lyase activities. With the FapyG:A-containing substrate, the rate constant for base removal ($k_g = 17 ± 1$ min^{-1}) represented a 30-fold increase over the rate constant observed for OG removal from the corresponding OG:A substrate ($k_g = 0.6 ± 0.1$ min^{-1}). A comparison of the relevant rate constants indicates a 5-fold preference of Fpg for removal of FapyG from the FapyG:C- over the FapyG:A-containing duplex. This is compared to a 35-fold greater rate of removal of OG by Fpg from the OG:C over the OG:A substrate.

A significant difference between Fpg and hOGG1 or Ntg1 is that the glycosylase (base excision) step is faster than the lyase (strand cleavage) step in the eukaryotic enzymes. This is evident in the differences in the rate constants k_g and k_{gl} (Table 1). With hOGG1, the rate constant for FapyG removal from FapyG:C bps ($k_g = 57 ± 6$ min^{-1}) is more than 200-fold greater than the rate constant observed for the combined reaction ($k_{gl} = 0.23 ± 0.01$ min^{-1}). The difference in the two rate constants indicates that k_{gl} is the rate constant for the β-lyase step. With the hOGG1 enzyme, the rate constant for the FapyG glycosylase activity from FapyG:C is within error of that for OG glycosylase activity from the corre-sponding OG:C-containing duplex ($k_g = 57 ± 7$ min^{-1}). In contrast, hOGG1 removes FapyG from FapyG:A bps ($k_g =$

Table 2: Rate Constants Determined at 37 °C under Single-Turnover Conditions for Fpg, hOGG1 and Ntg1 with FapyA and OG in Duplex 2

substrate	Fpg k_g (min^{-1})	hOGG1 k_{gl} (min^{-1})	hOGG1 k_g (min^{-1})	Ntg1 k_{gl} (min^{-1})	Ntg1 k_g (min^{-1})
FapyA:G	64 ± 6	MC[b]	< 0.02	0.36 ± 0.03	69 ± 6
FapyA:A	99 ± 7	MC	0.34 ± 0.04[a]	0.4 ± 0.1	99 ± 2
FapyA:T	103 ± 7	MC	0.21 ± 0.03[a]	0.41 ± 0.02	141 ± 8
FapyA:C	84 ± 2	0.05 ± 0.01	1.9 ± 0.3	0.4 ± 0.1	77 ± 5
OG:G	14 ± 1	< 0.02	0.030 ± 0.002	0.04 ± 0.01	0.10 ± 0.03
OG:A	0.57 ± 0.01	MC	< 0.02	MC	MC
OG:T	14 ± 1	0.08 ± 0.02	57 ± 2	MC	MC
OG:C	14 ± 1	0.06 ± 0.01	50 ± 7	MC	MC

[a] Reaction only 50% complete after 60 min. [b] MC: Minimal cleavage (<5% after background correction at all time points).

1.2 ± 0.1 min^{-1}) with a rate constant that is 60-fold greater than for OG from OG:A base pairs (k_g < 0.02 min^{-1}). By comparing the rate constants, FapyG removal by hOGG1 from the FapyG:C substrate is 47-fold more efficient than from the corresponding FapyG:A substrate. The magnitude of the effect of the opposite base for FapyG removal is reduced compared to the activity of hOGG1 on the corresponding OG-containing duplexes, where there is 3000-fold preference of hOGG1 for OG:C over OG:A base pairs.

As reported previously (46), Ntg1 does not catalyze removal of OG opposite either C or A. However, Ntg1 removes FapyG in both base-pairing contexts. As observed with hOGG1, the rate of the glycosylase step with Ntg1 is much faster than the β-lyase step. The rate constant for the glycosylase reaction with FapyG:A (k_g = 2.4 ± 0.3 min^{-1}) is almost 8-fold larger than the rate constant for the β-lyase step (k_{gl} = 0.29 ± 0.05 min^{-1}). Interestingly, the enzyme-catalyzed strand scission (k_{gl}) shows no preference for the base opposite the lesion (Table 1). However, in monitoring the glycosylase step, Ntg1 catalyzes removal of the FapyG lesion from the promutagenic FapyG:A base pair 3-fold faster than from the corresponding FapyG:C base pair duplex (k_g = 0.9 ± 0.4 min^{-1}).

Removal of FapyA by Fpg, hOGG1 and Ntg1. In vitro experiments show that KF exo$^-$ incorporates dAMP and dGMP opposite FapyA more readily than it does opposite A, suggesting that base pairs containing FapyA opposite A, G and T are relevant biological substrates for BER glycosylases (23). The efficiency of base-excision of the FapyA lesion in the potential biological contexts by the OG glycosylases (Fpg, hOGG1 and Ntg1) was determined from single-turnover experiments in the sequence context of duplex 2 (Table 2). In addition, the FapyA excision activity was also evaluated in an FapyA:C base pair context to further elaborate the sensitivity to the opposite base and the potential role of base pair stability. To provide a benchmark for the comparison of the data with FapyG, the rate constants for the corresponding duplex 2 substrates containing OG were also determined.[3] Like FapyG, FapyA is a much better substrate for Fpg than OG. However, in contrast to the processing of FapyG, Fpg shows very little discrimination with respect to the identity of the opposing base when excising FapyA. For example, the rate constant for FapyA removal from the FapyA:A substrate (k_g = 99 ± 7 min^{-1}) is similar to that measured with the FapyA:T substrate (k_g = 103 ± 7 min^{-1}). This feature of the removal of FapyA by Fpg makes the difference in processing of FapyA relative to OG quite different depending on the base pair context being compared. For example, the rate constant for the FapyA glycosylase activity of Fpg with the FapyA:G-containing substrate is more than 4-fold faster than its OG glycosylase activity with OG:G-containing substrate (Table 2). However, relative to the activity of Fpg with OG:A, removal of FapyA when paired with A is 150-fold more efficient.

Some activity of removal of FapyA by hOGG1 was observed in all four base pair contexts tested, although the reaction with the FapyA:G duplex was minimal (k_g < 0.02 min^{-1}) (Table 2). Notably, the reaction was only observed to go to completion with the FapyA:C duplex. The exact reason for the lack of complete reactions with the FapyA:A and FapyA:T substrates is not clear, but may be a consequence of the slow rate of the reaction and instability of the duplex under the reaction conditions. The rate constant for the glycosylase reaction with the FapyA:C substrate (k_g = 1.9 ± 0.3 min^{-1}) is considerably slower than the corresponding rate for removal of OG in the corresponding duplex (k_g = 50 ± 7 min^{-1}). As observed in duplex 1, the lyase activity for OG removal by hOGG1 is considerably slower than the glycosylase step. For example, with the OG:T-containing duplex 2, the rate constant for the glycosylase step (k_g = 57 ± 2 min^{-1}) is ~600-fold faster than the lyase step (k_{gl} = 0.08 ± 0.02 min^{-1}).

A distinctive feature of the glycosylase activity of Ntg1 is that the activity toward FapyA is greater than toward FapyG. With the FapyA:A-containing duplex, the rate constant for the glycosylase reaction (k_g = 99 ± 2 min^{-1}) is nearly 45-fold faster than with the FapyG:A substrate (k_g = 2.4 ± 0.3 min^{-1}). Like hOGG1, Ntg1 also catalyzes deglycosylation much more readily than the strand scission reaction. With the FapyA:A substrate, the glycosylase step (k_g = 99 ± 2 min^{-1}) is nearly 300-fold faster than the lyase step (k_{gl} = 0.4 ± 0.1 min^{-1}). Similarly, with another biologically relevant base pair substrate, FapyA:G, the glycosylase step (k_g = 69 ± 6 min^{-1}) is nearly 200-fold faster than the lyase step (k_{gl} = 0.36 ± 0.03 min^{-1}). In addition, like Fpg, excision of FapyA by Ntg1 is insensitive to the identity of the opposite base.

DISCUSSION

Studies on DNA containing randomly generated lesions established that FapyG and FapyA are substrates for various OG glycosylases (34, 35). However, due to limitations in

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[3] Though FapyA is structurally similar to OA, this was not used as a benchmark since OA was found to be a poor substrate for these glycosylases (Voth, A, David, S. S., unpublished results).

studying randomly generated lesions, the absolute efficiency of repair of these lesions was unknown. Moreover, the magnitude of the effects of the base opposite the lesions could not be established. Using defined oligonucleotide duplex substrates containing formamidopyrimidine lesions and quantitative kinetics, such features have now been revealed. An important aspect of the analysis of the activity of the glycosylases in this study was the evaluation of the glycosylase reaction separately from the strand scission (lyase) reaction. With Fpg these two reactions are tightly coupled; however, with hOGG1 and Ntg1 the glycosylase step is much faster than the lyase step. In fact, the extent of the activity of these enzymes may be underestimated or differences in substrate preferences may not be revealed if the enzyme is used to provide strand scission. This can be illustrated with hOGG1 in which the relative preference for C over A opposite the lesion is larger when comparing the rate constants for the glycosylase reaction (k_g) than the lyase reaction (k_{gl}). Similarly, the preference of Ntg1 for removal of FapyG in the promutagenic FapyG:A bp over the FapyG:C bp is only revealed when analyzing the glycosylase reaction.

Importantly, this approach revealed the robust activity of Fpg and hOGG1 for removal of FapyG lesions. Indeed, the measured rate constants show that Fpg excises FapyG lesions much more efficiently than OG. Similarly, FapyG was removed as efficiently as OG by the human BER glycosylase hOGG1. The fact that both lesions are substrates for Fpg and hOGG1 may be rationalized by the structural similarity between FapyG and OG. Indeed, though the structures of hOGG1 and Fpg are completely different from each other, the strategy for OG recognition by the two enzymes is similar (*9*). Both enzymes recognize OG by extracting the lesion base from the helix for placement within a lesion specific pocket where contacts are made to NH7 of OG which distinguishes it from G (*48, 49*). X-ray structural studies of *Lactococcus lactis* Fpg (LlFpg) bound to lesion-containing DNA have shown that a carbocyclic analogue of FapyG is specifically recognized within the OG base-binding site by residues that are strictly conserved among Fpg enzymes from different species (*48, 50*). However, the manner of recognition of FapyG within the LlFpg active site was found to be significantly different than that observed for OG recognition by *Bacillus stearothermophilus* Fpg (BsFpg). The flexibility to adopt alternate recognition complexes may be a feature that enables Fpg to recognize a wide variety of substrates (*26*). The ability of Fpg to remove FapyA and FapyG with similar efficiencies also is consistent with the idea that the Fpg base recognition site is open and plastic. In contrast, X-ray structural studies of hOGG1 bound to DNA substrates have revealed a rigid, preformed site into which OG fits in snugly (*9, 49*). There are many contacts with the Watson−Crick face of OG, as well as residues that interact on opposite sides of the planar OG heterocycle to sandwich the base within the active site. Calculations have also indicated the importance of favorable dipole−dipole interactions in hOGG1 recognition of OG (*51*). The minimal activity of hOGG1 toward removal of FapyA lesions is consistent with a restricted substrate scope that is limited to lesions that are very similar to OG. The "A"-like features of FapyA may not allow for proper engagement in the OG-specific pocket of hOGG1 to allow for efficient excision (*9, 51*). The fact that FapyA is only removed when paired with C is also consistent with the preference of hOGG1 for lesions derived from G that would be found paired with C.

The identity of the base opposite has a marked influence of the observed rate of lesion excision by Fpg and hOGG1 (Table 1). Single-turnover experiments revealed that both enzymes more readily remove FapyG or OG when paired with C over A. Similar results were previously observed in the steady-state kinetics studies with Fpg with FapyG containing substrates (*43*). This selectivity for FapyG and OG in the correct Watson−Crick context provides a means of protecting the genome from mutagenesis. Selective removal of FapyG or OG opposite C helps in restoring the original base pair, while slower removal of the lesions opposite A prevents G →T transversion mutations. In the structural studies, hOGG1 and Fpg provide specific contacts to the intrahelical C left behind after expulsion of OG (*49, 52*). However, the extent of C-specific interactions is more extensive with hOGG1. This is consistent with a more stringent selection against the presence of A opposite OG by hOGG1. This is illustrated by the relative rate constants (Table 1) that demonstrate a preference of hOGG1 for C over A for OG removal that is quite large (∼3000-fold). Notably, the relative preference of hOGG1 for C over A opposite FapyG is significantly reduced (47-fold). This is due primarily to the greater ability of hOGG1 to remove FapyG from FapyG:A base pairs than OG from OG:A base pairs. The relative preference of Fpg for C over A when the G lesion is FapyG is also reduced compared to OG; however the magnitude of the difference is not quite as large as was observed with hOGG1.

Though often thought of as an OG glycosylase, Ntg1 shows minimal excision activity with OG (*46*). However, Ntg1 catalyzes removal of both formamidopyrimidine lesions. In fact the observed rates show that the activity toward the FapyA lesion is quite efficient. The activity of Ntg1 toward FapyG is approximately 100-fold slower than FapyA; however the rate constants measured are similar to those of other glycosylases with standard substrates (*46, 47, 53*). Ntg1 is similar in terms of sequence and substrate specificity to the *E. coli* BER glycosylase endonuclease III and its human homologue, NTH1 (*26*). Endonuclease III has been previously shown to be capable of removal of FapyA and FapyG, with more activity toward FapyA (*54*). The fact that Ntg1 cleaves FapyG and FapyA is consistent with these lesions resembling pyrimidines. Though no structural data on Ntg1 is available, it is possible that the lack of activity toward OG is due to the inability of a large planar purine heterocycle to fit into the lesion-binding site. Another unusual feature of the glycosylase activity of Fpg and Ntg1 toward FapyA is insensitivity to the identity of the opposite base. For example, FapyA is removed as efficiently when paired with A, G or C as it is when base paired with T. Removal of a lesion from a promutagenic base pair leaves the incorrect nucleotide thus leading to permanent mutations. The fact that FapyA is weakly mutagenic may suggest that repair of this lesion may not be as crucial, and therefore highly discriminatory recognition of this lesion may not have been developed. Indeed, the activity of Fpg and Ntg1 toward removal of FapyA may be a consequence of the structural similarity of this lesion to other substrates (e.g., OG for Fpg and thymine glycol for Ntg1).

A factor that influences the recognition of a lesion and the opposite base is the stability of the lesion-containing base pair. If the lesion base pair is more easily disrupted, this may compensate for the absence of favorable interactions with the proper opposite base. Reduced selectivity for the base opposite a lesion has previously been observed in studies on Fpg excision of the helix-destabilizing hydantoin lesions, guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp) (45−47). Duplexes containing the FapyG lesion do not appear to be less stable than those containing OG (20), however, the local stability may be reduced due to the structural flexibility of the ring-opened FapyG relative to the planar OG. This property may also result in reduced stability at the damaged base pair upon interrogation by the glycosylase enzyme. Recent structural studies of BsFpg trapped via disulfide cross-linking to normal nonlesion DNA suggest that this glycosylase actively tests the robustness of base pairs as part of the damage search process (51). Notably, FapyA lesions are more duplex destabilizing than FapyG (23), and this may be a factor resulting in the lack of opposite base sensitivity with both Fpg and Ntg1.

An important aspect of the prevention of mutations associated with OG is the removal of misincorporated adenines from OG:A base pairs by the adenine glycosylase MutY. This enzyme intercepts this promutagenic base pair and allows for recreation of a proper substrate for Fpg. MutY has been shown to remove adenine from FapyG:A base pairs (43). The rate of adenine removal mediated by MutY from FapyG:A bps is not as fast as from OG:A bps, but faster than when G is paired with A. Surprisingly, however, recent in vivo studies in *E. coli* have shown that FapyG is not particularly mutagenic, and moreover this mutagenesis is not enhanced in *mutM− mutY− E. coli*. This suggests that the activity of MutY toward FapyG:A base pairs may not be as important for preventing mutagenesis as the activity toward OG:A. The origin of the low mutagenesis of FapyG was proposed to be due to the processing by the DNA polymerase. This proposal was based on detailed analysis of the kinetics of KF exo$^+$ processing of FapyG-containing DNA templates (21). The observed inefficient extension of Fapy-G:A base pairs coupled with the inherently lower insertion frequency would provide an opportunity for the exonuclease activity of the DNA polymerase to correct its mistakes. It is also possible that removal of FapyG may occur via alternative BER glycosylases or other repair pathways (e.g., NER). Redundant mechanisms for preventing mutations from a given lesion may make observing the effects of a specific repair enzyme difficult. Moreover, such redundancies are likely important to mitigate the properties of mutagenic and toxic lesions, like FapyG.

However, the striking observation that FapyG is more mutagenic in simian kidney (COS-7) cells than in *E. coli* suggests that the processing of FapyG by polymerases and repair enzymes may be distinctly different in eukaryotic cells. Moreover, the presence of FapyG was also more mutagenic than OG in simian kidney cells. This is particularly interesting in light of the more relaxed specificity of hOGG1 for the proper opposite base when the lesion is FapyG. In fact, this may contribute to the higher mutation frequency of FapyG over OG lesions in COS-7 cells. Analysis of the details of the activity of other human glycosylases toward removal of FapyG and the effects of the base opposite may

be quite illuminating. In particular, the mammalian NEIL1 glycosylase has been shown to be able to remove Fapy lesions (37). This glycosylase appears to remove mainly damaged pyrimidines (55) and therefore one might find that this enzyme also has activity toward FapyG in promutagenic contexts. Thus, detailed analysis of the kinetics of FapyG with other BER glycosylases in defined base pairing and sequence contexts may provide insight into their role as potential modifiers of mutagenesis caused by FapyG lesions. Moreover, such studies would aid in the design of cellular experiments to evaluate how such effects translate to a cellular environment.

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