Recognition and Removal of Oxidized Guanines in Duplex DNA by the Base Excision Repair Enzymes hOGG1, yOGG1, and yOGG2[†]

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ABSTRACT: 8-Oxo-7,8-dihydroguanine (OG) is susceptible to further oxidation in vitro to form two secondary oxidation products, guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp). Previous work from this laboratory has shown that OG, Gh, and Sp are recognized and excised from duplex DNA substrates by the *Escherichia coli* DNA repair enzyme Fpg. In this report, we extend these studies to the functionally related eukaryotic OG glycosylases (OGG) from yeast and humans: yOGG1, yOGG2, and hOGG1. The hOGG1 enzyme was active only toward the removal of 8-oxoguanine, exhibiting a 1000-fold faster rate of removal of 8-oxoguanine from OG·C-containing duplexes relative to their OG·A counterparts. Duplexes containing Gh or Sp opposite any of the four natural bases were not substrates for the hOGG1 enzyme. In contrast, both yOGG1 and yOGG2 enzymes removed Gh and Sp in a relatively efficient manner from an 18 bp duplex. No significant difference was observed in the rate of reaction of Gh- and Sp-containing duplexes with yOGG1. However, yOGG2 removed Sp at a faster rate than Gh. Both yOGG enzymes exhibit a negligible dependence on the base opposite the lesion, suggesting that the activity of these enzymes may be promutagenic. Surprisingly, in the 18 bp sequence context, both yOGG enzymes did not exhibit OG removal activity. However, both removed OG in a 30 bp duplex with a different sequence surrounding the OG. The wide range of repair efficiencies observed by these enzymes with different substrates in vitro suggests that this could greatly affect the mutagenicity of these lesions in vivo. Indeed, the greater efficiency of the yOGG proteins for removal of the further oxidized products, Gh and Sp, over their 8-oxoguanine parent, suggests that these lesions may be the preferred substrates in vivo.

All organisms experience oxidative DNA damage from a variety of sources, such as ionizing radiation, environmental pollutants, and normal respiration (*1*). Increased levels of damage or inappropriate repair has been linked to conditions such as colorectal (*2*, *3*), lung (*4*, *5*), kidney (*6*), head and neck (*7*), and breast cancers in humans (*8*), as well as aging in mice (*9*−*12*). One of the most prevalent oxidative damage products, 7,8-dihydro-8-oxoguanine (OG),[1] results from guanine oxidation (*13*). In *Escherichia coli*, a system employing three different enzymes has evolved for the repair of OG (*14*). Fpg catalyzes the removal of OG from an OG·C base pair. MutT hydrolyzes dOGTP to dOGMP to remove it from the nucleotide pool used by DNA polymerases, thus preventing its incorporation opposite cytosine or adenine in the nascent strand. Failure to remove OG from duplex DNA before DNA replication can result in the misincorporation of adenine opposite OG to form OG(syn)·A(anti) base pairs leading to G·C to T·A transversion mutations in subsequent replication events (*15*). As a second line of defense, MutY removes the mispaired adenine from an OG·A base pair. This provides DNA polymerase another chance to correctly recreate the OG(anti)·C(anti) base pair substrate for Fpg. The importance of preventing mutations associated with oxidized guanines in human health has been recently highlighted by the discovery of a direct link between mutations in the human MutY homologue (MYH) and colorectal cancer (*3*).

Eukaryotic functional homologues to Fpg, such as yOGG1 and yOGG2 in *Saccharomyces cerevisiae* or hOGG1 in humans, lack sequence or structural similarity to Fpg. On the basis of sequence and structural homology, the OG glycosylases (OGGs) appear to belong to an enzyme superfamily containing *E. coli* endonuclease III and AlkA, characterized by a helix−hairpin−helix motif, a Gly-Pro-rich loop, and a conserved aspartate necessary for efficient catalysis (*16*). The OGG proteins catalyze base removal through their glycosylase action, followed by β-elimination on the resulting AP site to effect phosphodiester backbone strand scission 3′ to the original damaged base. Like other enzymes in the superfamily, but unlike Fpg, the OGG enzymes do not catalyze δ-elimination. *S. cerevisiae* is unusual in that it does not contain homologues to MutY or MutT. Bruner et al. have suggested that the presence of two

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[1] Abbreviations: BER, base excision repair; bp, base pair; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fpg, formamidopyrimidine glycosylase or MutM; Gh, guanidinohydantoin; hOGG1, human OG glycosylase 1; IPTG, isopropyl β-D-thiogalactoside; nt, nucleotide; OG, 8-oxo-7,8-dihydroguanine; LB, Luria−Bertani broth; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; Sp, spiroiminodihydantoin; TBE, Tris−borate−EDTA; Tris, tris(hydroxymethyl)aminomethane; yOGG, yeast OG glycosylase.

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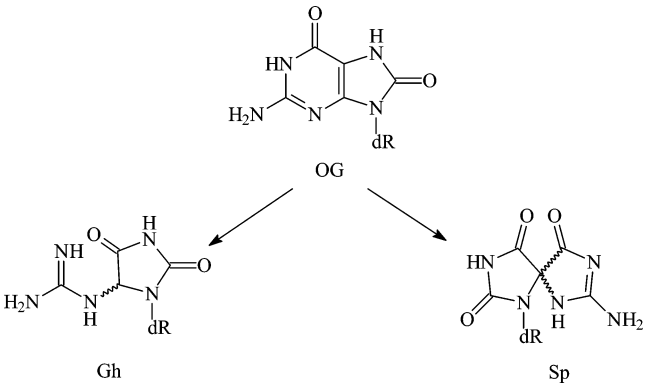

FIGURE 1: Structures of the OG lesion and its secondary oxidation products Gh and Sp. Note that Sp is a nonplanar lesion and, therefore, might be expected to distort the DNA duplex more significantly than OG or Gh.

functional OGGs in this organism compensates for the absence of the other two OG repair enzymes (*17*). The yOGG1 enzyme is proposed to be the primary OGG responsible for the removal of OG in the parent strand when paired with cytosine, while yOGG2 (Ntg1) is proposed to catalyze removal of OG resulting from incorrect incorporation of dOGTP by DNA polymerase opposite adenine during daughter strand formation (*15*, *18*, *19*).

Insights into the complexities of DNA damage recognition by the OG glycosylases have been elucidated by a number of researchers. As the name implies, each OGG has been shown qualitatively to remove OG; however, the efficiency of OG removal is greatly affected by the opposing base in the base pair. For example, various reports in the literature have found that hOGG1 (*20*) and yOGG1 (*21*−*23*) are much more specific for OG when paired with pyrimidines, barely acting upon an OG·G substrate, and not processing an OG·A substrate at all. By comparison, in some studies, yOGG2 has been shown to remove OG paired with both purines and pyrimidines, with a slight preference for the purine-containing base pair (*17*, *24*, *25*). In some reports, the ability of yOGG2 to remove OG has not been observed, prompting a different name for the enzyme (Ntg1) (*26*, *27*). Thus, quantitative analysis of the reactions of the OGG enzymes with OG substrates is clearly needed.

It has been demonstrated that OG has an even lower oxidative potential than guanine and therefore may be specifically oxidized when present in DNA (*28*). Tretyakova et al. identified several oxidation products of OG, including cyanuric acid, upon reaction of OG-containing duplexes with peroxynitrite (*29*). Luo et al. have shown in nucleoside and duplex DNA studies that OG can be specifically converted to spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh) (Figure 1) (*30*, *31*). Additionally, recent work by several laboratories has demonstrated that Sp can be formed directly from guanine by a variety of oxidative conditions, such as methylene blue (*32*) and riboflavin photooxidation (*33*) as well as oxyl radicals derived from the photolysis of hydroxyacetophenone (*34*). Indeed, investigators have now demonstrated that the proposed 4-OH-OG lesion originally identified by Cadet et al. (*35*) is actually Sp, based on HPLC, MS, and NMR experiments (*32*, *34*).

Since the Gh and Sp lesions (Figure 1) can originate from OG, and previous work from this laboratory (*36*) has shown that Fpg can excise all three lesions in a variety of base-pairing contexts, we have now examined whether yOGG1, yOGG2, and hOGG1 can recognize and excise Gh and Sp from duplex DNA and compared these activities to those for OG removal. In addition, we have quantitatively reinvestigated the effect of the opposite base on OG removal that had previously been investigated using qualitative assays. Glycosylase activity of Fpg, hOGG1, yOGG2, and yOGG1 with these various lesion-containing substrates was measured under single- and multiple-turnover conditions. This work demonstrates that hOGG1 can remove OG, but not Gh or Sp, from all four base-pairing contexts. Removal of Gh and Sp by the two yOGG enzymes was observed when either was positioned opposite each of the four DNA bases in duplex DNA, without regard to the base opposite the lesion. There is an observable difference in the rate of removal of Gh and Sp by yOGG2, with the latter being removed more quickly with all base pairs tested. Interestingly, both yOGG enzymes were more efficient at removal of Gh and Sp than OG; indeed, the OG removal activity of these enzymes was found to be sensitive to the sequence of the DNA duplex containing OG. This work is beginning to provide insight into the types of different substrates that may be processed by OG glycosylases. Such analyses are important for relating the effects of repair by specific enzymes to the mutagenic potential of these lesions in vivo.

MATERIALS AND METHODS

General Methods. All chemicals were purchased from Fisher Scientific, Sigma, or USB. [γ-^{32}P]ATP was purchased from Amersham Life Sciences. New England Biolabs was the source for T4 polynucleotide kinase. 7,8-Dihydro-8-oxo-2′-deoxyguanosine phosphoramidite was purchased from Glen Research, while standard 2-cyanoethyl phosphoramidites were purchased from Applied Biosystems Inc. A Milli-Q PF system was used to further purify deionized, distilled water which was then used to make all buffers and used in all reactions. All buffers were passed through a 0.45 μm filter before use. OGG purification was performed on a Bio-Rad Biologic medium-pressure liquid chromatography system. A Molecular Dynamics Storm 840 phosphorimager was used for storage phosphor autoradiography. ImageQuaNT software (version 5.2a) on a Windows 2000 system was used to quantify the images from storage phosphor autoradiograms. All electrophoresis experiments used Tris−borate−EDTA (TBE) buffer (90 mM Tris base, 90 mM boric acid, and 1 mM EDTA). Fpg and endonuclease IV were purified as previously described (*36*, *37*).

OGG Purification. E. coli BL21(DE3)pLysS cells were transformed with the hOGG1 (*20*), yOGG1 (inserted using *Xba*I and *Hin*dIII sites), or yOGG2 (*17*) pET30a plasmid (all three plasmids being generous gifts of the Verdine laboratory) encoding the wild-type OGG with an N-terminal His_6 tag. The transformed cells were grown at 37 °C in 8 L of LB media containing 34 μg/mL kanamycin and 34 μg/mL chloramphenicol to $OD_{600} = 0.5-0.7$ and then induced with 1 mM IPTG, and the temperature was lowered to 30 °C. After 3 h of growth at 30 °C, the cells were pelleted by centrifugation (8000 rpm, 10 min, 4 °C), resuspended in 150 mL of buffer A (20 mM Tris-HCl, pH 7.5, 10% glycerol) supplemented with 1 mM PMSF, and then stored at −80 °C.

Upon thawing, the cells were supplemented with 5 mM imidazole and 300 mM NaCl, then disrupted by sonication (Branson Sonic Power Co., model 350, 70% pulse, 30 s on followed by 30 s off, repeated six times), and centrifuged (10000 rpm, 5 min, 4 °C). The sonication supernatant was split into groups of 40 mL, 1 mL of Ni-NTA resin (Qiagen) was added to each group, and the samples were placed on a rocking platform at 4 °C for 1 h. The samples were then combined and poured into two empty columns to collect the resin. Each sample of column resin was then washed with 7 mL of buffer A containing 5 mM imidazole to remove any remaining proteins nonspecificially bound to the resin. Next, the resin was washed with 5 mL of buffer A containing 500 mM imidazole to elute the specifically bound proteins. The resulting solutions were combined and then passed through a 0.45 μm filter. A Pharmacia HiPrep 26/10 desalting column was attached to the Biologic medium-pressure liquid chromatography system and equilibrated with 0 M NaCl buffer B. The protein sample was then loaded onto the column. The protein-containing fractions were collected, pooled, and loaded onto a Pharmacia HiTrap heparin column equilibrated with 0 M NaCl buffer A. The hOGG1 protein eluted at 490 mM NaCl during a linear gradient to 1 M NaCl buffer A; the yOGG1 protein eluted at 500 mM NaCl, while the yOGG2 protein eluted at 400 mM NaCl. The yOGG1 preparation included a second peak eluting at 390 mM NaCl which was resolved by the heparin column; the amount of this protein varied from preparation to preparation. This second protein is presumably misfolded or partially degraded yOGG1, as it retained some activity against Gh·Y or Sp·Y substrates but none against OG·Y substrates. The eluted sample (12 mL) was diluted to 25 mL with glycerol, concentrated in an Amicon Model 8050 ultrafiltration cell to 10 mL, and then stored in 0.5 or 1 mL aliquots in liquid nitrogen until used. It should be noted that the yOGG1 protein was less stable to storage than yOGG2 or hOGG1, so it was stored in 15 μL single-use aliquots at −80 °C. SDS−PAGE and silver stain analysis show all three proteins to be >95% pure (data not shown). The N-terminal His_6 tag was not removed prior to kinetic analysis, as analysis of hOGG1 lacking the His_6 tag (gift of G. Verdine) demonstrated no difference in single-turnover rate constants (data not shown). Initial protein concentrations were determined by Bradford assays using BSA as a standard. Active site titration assays were performed using an OG·C duplex for hOGG1 and yOGG1 or Gh·C duplex for yOGG2 (*38*). Each purification yielded protein which was 50−70% active; all OGG protein concentrations given below or in figure captions are active enzyme concentrations.

Oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems Inc. 392B automated synthesizer using the manufacturer's protocols and purified via polyacrylamide gel electrophoresis or ion- exchange HPLC (Waters, Protein-Pak DEAE 8HR column). Gh- and Sp-containing oligonucleotides were synthesized from the original OG-containing oligonucleotide as previously described (*36*). Sequences of the oligonucleotides used and the relevant DNA duplexes studied are shown in Figure 2.

Substrate DNA Preparation. Two and a half picomoles of the X-containing strand was 5′-^{32}P-end-labeled with T4 polynucleotide kinase according to the manufacturer's protocol. Excess [γ-^{32}P]ATP was removed using a Pharmacia MicroSpin G-25 spin column, as per the manufacturer's protocols. The unlabeled X-containing strand was supplemented with 5% 5′-^{32}P-end-labeled X-containing strand, and then the complementary Y-containing strand was added in excess (10−50%). The annealing was performed in buffer containing 20 mM Tris-HCl, pH 7.6, 10 mM EDTA, and 150 mM NaCl. This solution (200 nM labeled strand) was heated to 95 °C and then slowly cooled over at least 3 h to anneal the duplex.

Duplex 18a
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5'-TCATGGGTCXTCGGTATA-3'
3'-AGTACCCAGYAGCCATAT-5'
X = OG, Gh, Sp; Y = C, T, G, A
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Duplex 30a
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5'-TGTTCATCATGGGTCXTCGGTATATCCCAT-3'
3'-ACAAGTAGTACCCAGYAGCCATATAGGGTA-5'
X = OG; Y = C, T, G, A
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Duplex 30b
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5'-TGTTCAACGTGCAGTXAACGCACGTCCCAT-3'
3'-ACAAGTTGCACGTCAYTTGCGTGCAGGGTA-5'
X = OG; Y = C, T, G, A
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FIGURE 2: Sequences of the DNA duplexes studied. The 30a sequence has the 18a sequence as the central portion of the duplex. Comparing 18a with 30a will enable the effect of duplex length to be studied independently of sequence, while comparison of 30b with 30a will allow the effect of duplex sequence to be studied independent of length. Note that Gh and Sp were only studied in the 18a sequence; therefore, X = OG in the 30b and 30a sequences.

Single-Turnover Kinetics. Single-turnover experiments were performed using the 18 or 30 base pair duplexes described above, where the X-containing strand was ^{32}P-end-labeled. In each case, the total reaction volume was 60 μL with a final duplex DNA concentration of 20 nM. The only exception to this was yOGG1 with the 30b duplex containing a central OG·C base pair, where the substrate concentration yielding the highest rate was 4 nM. The final OGG concentrations ranged from 30 to 800 nM active enzyme, and no difference in rate was observed in that range. The reactions were performed at 37 °C under final buffer conditions of 26 mM Tris-HCl, pH 7.6, 13 mM EDTA, 21 mM NaCl, and 0.1 mg/mL BSA. Four microliter aliquots were removed at 0, 0.25, 0.5, 1, 2, 4, 8, 15, 30, and 60 min for the reaction of hOGG1 with OG·C and OG·T substrates; at 0, 0.25, 0.5, 1, 2, 4, 8, 20, 45, and 90 min for the reaction of hOGG1 with OG·G and yOGG1 and yOGG2 with all substrates; and at 0, 0.5, 1, 2, 4, 8, 15, 30, 60, and 120 min for the reaction of OG·A with hOGG1. Each aliquot was immediately quenched by addition to an equal volume of formamide denaturing loading dye (80% formamide, 0.025% xylene cyanole, 0.025% bromophenol blue in TBE buffer) preheated to 90 °C and then placed on dry ice. A set of reactions were performed in the same fashion but quenched with either 0.1 M NaOH and heat or including the presence of *E. coli* endonuclease IV. Specifically, this was performed with the reaction of OG·G and OG·A duplex 18a with hOGG1 and Fpg using a base quench. The reaction of Gh·T and Gh·A with yOGG2 was run in the presence of endonuclease IV. In both cases, no difference in the rate of the reaction was detected.

When the enzyme reaction was too fast to accurately measure using manual methods, rapid-quench experiments were performed using an RQF-3 rapid quench-flow machine from KinTek Corp. The initial DNA mixture conditions (2×) were 20 nM substrate DNA, 52 mM Tris-HCl, pH 7.6, 26 mM EDTA, 42 mM NaCl, and 0.2 mg/mL BSA. The initial

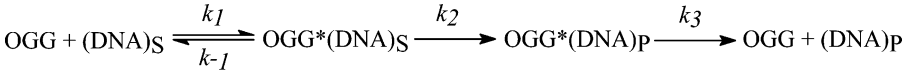

FIGURE 3: Minimal kinetic scheme used for the analysis of the glycosylase/lyase activity of OGG proteins.

enzyme mixture conditions (2×) were 200 nM active protein, 25 nM unlabeled 30 bp nonspecific duplex DNA (*38*; nonspecific DNA used to stabilize the proteins before mixing with substrate DNA), 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 20% glycerol. During the course of the experiment, equal volumes of the DNA mixture and enzyme mixture at 37 °C were combined for varying time points (0.5, 1, 2, 4, 6, 8, 10, 20, 50, and 100 s); then this mixture was quenched with an equal volume of 0.5 M NaOH and then immediately placed at 90 °C for 4 min. The combined mixture was placed at 90 °C for 4 min to heat denature before being loaded onto a 20% polyacrylamide gel containing 8 M urea to separate reactants and products. The substrate and product DNA were resolved after the gel was run in TBE buffer for 2 h at 1600 V. Gels were exposed to a storage phosphor screen for at least 12 h. The resulting autoradiogram was quantified using ImageQuaNT version 5.2a. GraFit version 4.06 was used to fit the data as previously described (*36*, *38*).

Multiple-Turnover Kinetics. Multiple-turnover assays were performed under the same reaction conditions as the single-turnover assays, except the active enzyme concentration was significantly (typically 10-fold) less than the concentration of the DNA substrate. The time points used were 0, 0.25, 0.75, 1, 1.25, 1.5, 1.75, 2, 3, 4, 8, 20, 40, and 60 min. Biphasic kinetics were also observed with yOGG1; however, due to the lower stability of the yOGG1 protein compared to yOGG2 or hOGG1, detailed multiple-turnover analysis of its various substrates was not attempted. The data analysis was performed as described in Porello et al. (*38*). In cases where the difference in k_2 and k_3 was 10-fold or less, k_3 was calculated as in Chmiel et al. (*39*).

RESULTS

General Considerations of Glycosylase Assays. The glycosylase activity of the various OG glycosylases was analyzed using the three duplex substrates shown in Figure 2. In each case, the lesion-containing strand was end-labeled with ^{32}P, and the activity was assessed by the extent of strand scission at the lesion site. The OG glycosylases catalyze an associated β-lyase activity, and in most cases, this activity of the enzymes was used to provide strand scission at the abasic site. To confirm that the β-lyase activity was not limiting the observed rates of reactions, the glycosylase activity alone was analyzed with representative enzyme and substrate pairs using either NaOH/heat for the base-stable OG lesion or *E. coli* endonuclease IV for the base-sensitive Gh and Sp lesion to provide strand scission at the abasic sites. In all cases tested, no difference was observed in the measured rate constants (k_2). When an enzyme exhibited no measurable cleavage with a given substrate, the assays were also repeated by quenching the reactions with NaOH or in the presence of *E. coli* endonuclease IV to ensure that any AP sites formed would result in observable strand scission.

The eukaryotic OG glycosylases hOGG1, yOGG1, and yOGG2 exhibit biphasic kinetic behavior under conditions of substrate excess with the majority of their substrates. This biphasic behavior is characterized by the observation of an initial burst of product formation (due to the first turnover) followed by a slow linear phase for product formation (due to multiple subsequent turnovers). We previously observed similar behavior with the *E. coli* MutY enzyme and therefore have utilized a similar type of analysis of the kinetic data (*38*). The data from experiments performed under single- and multiple-turnover conditions were fitted with the appropriate rate equations using the kinetic scheme shown in Figure 3 (*38*) to extract the rate constants for the steps including chemistry (k_2) and those relating to product release (k_3). In practice, k_2 was determined from single-turnover experiments since this is generally more accurate. The k_3 rate constant was measured from multiple-turnover experiments.

Table 1: Rate Constants (k_2) Determined at 37 °C under STO Conditions for Fpg, hOGG1, yOGG1, and yOGG2 with 18a Duplexes

	k_2 (min^{-1})			
substrate	Fpg	hOGG1	yOGG1	yOGG2
OG·C	15 ± 2	30 ± 6	1.2 ± 0.4[b]	NC
OG·T	22 ± 2	28 ± 8	0.3 ± 0.1[b]	NC
OG·G	27 ± 2	0.3 ± 0.1	0.3 ± 0.1[b]	0.09 ± 0.03[b]
OG·A	0.045 ± 0.007[a]	0.03 ± 0.01	NC	NC
Gh·C	1.4 ± 0.4[a]	NC	1.1 ± 0.3	0.3 ± 0.1
Gh·T	1.7 ± 0.6[a]	NC	1.1 ± 0.4	0.2 ± 0.1
Gh·G	1.9 ± 0.2[a]	NC	1.0 ± 0.3	0.2 ± 0.1
Gh·A	0.34 ± 0.06[a]	NC	0.5 ± 0.2	0.4 ± 0.2
Sp·C	1.3 ± 0.2[a]	NC	1.1 ± 0.3	1.0 ± 0.6
Sp·T	1.6 ± 0.3[a]	NC	1.6 ± 0.9	0.9 ± 0.3
Sp·G	1.4 ± 0.2[a]	NC	3 ± 1	1.2 ± 0.6
Sp·A	0.34 ± 0.04[a]	NC	0.5 ± 0.3	1.6 ± 0.4

[a] Values previously reported in Leipold et al. (*36*). [b] Rates determined with the 30b DNA duplex. NC = no significant cleavage observed after 90 min; upper limit rate of <0.0008 min^{-1} was estimated.

Removal of Oxidized Guanine Lesions by hOGG1 and Fpg: Determination of k_2. Rate constants (k_2) from single-turnover experiments were determined for the base removal reaction of hOGG1 with duplex 18a containing Gh, Sp, or OG opposite C, T, G, and A. As shown in Table 1, hOGG1 does not excise Gh or Sp in any of the four base-pairing contexts. No additional cleavage was observed when the assays were repeated in the presence of endonuclease IV. Table 1 also demonstrates a marked difference in cleavage efficiency between the four OG-containing substrates. As expected, OG opposite a pyrimidine is excised much more efficiently than when opposite a purine (*5*). As previously demonstrated, the rate of reaction of hOGG1 with OG·C and OG·T substrates is too fast to measure accurately by manual methods at 37 °C (*40*); for this reason, the glycosylase reaction with these substrates was monitored using a rapid-quench instrument. Under these conditions, the measured rate constants k_2 for removal of OG from OG·C and OG·T base pairs by hOGG1 were found to be the same within error (k_2 = 30 ± 6 and 28 ± 8 min^{-1}, respectively). The quantitative data (Table 1) also reveal that the OG·A substrate is processed 1000 times less efficiently than the corresponding OG·C- or OG·T-containing substrates. Moreover, hOGG1 discriminates between A and G by removing OG opposite G 10-fold more efficiently than opposite A.

To directly compare the OG removal activity of hOGG1 to Fpg, rapid-quench experiments with Fpg were also performed since the reaction is too fast to measure manually (*36*). Somewhat surprisingly, inspection of the measured rate constants (Table 1) indicates that Fpg-catalyzed removal of OG from an OG·C base pair ($k_2 = 15 \pm 2$ min^{-1}) is considerably slower than from an OG·T or OG·G base pair ($k_2 = 22 \pm 2$ min^{-1} and 27 ± 2 min^{-1}, respectively). However, as expected, Fpg processing of OG·C substrates was 400-fold faster than the corresponding OG·A substrate. OG removal from the OG·C substrate by hOGG1 is 2-fold faster than Fpg, while the glycosylase reactions proceed with similar rates with the OG·T base pair substrate for both enzymes.

Removal of Oxidized Guanine Lesions by yOGG1: Determination of k_2. In marked contrast to the results obtained with hOGG1, Gh- and Sp-containing duplexes were substrates for the yOGG1 enzyme, and the rate constants (k_2) measured under single-turnover conditions are listed in Table 1. Surprisingly, the data indicate that there is little discrimination for the base opposite the lesion. There appears to be a slight discrimination against removal of Gh or Sp opposite A; however, the measured values are extremely close if the experimental error is taken into account. There also appears to be no significant difference in the rate of removal of Gh relative to Sp when paired with C, T, or A. However, Sp is removed opposite G three times faster than Gh opposite G.

Surprisingly, even though Gh and Sp are removed opposite all four bases in the 18a duplex sequence by yOGG1, no detectable removal of OG was observed in any case in this duplex sequence. However, examination of OG removal in a different sequence context with a longer duplex (30b) revealed the ability of yOGG1 to catalyze removal of OG. It is also noteworthy that yOGG1 exhibited a higher rate of cleavage when the OG·C-containing duplex 30b was at 4 nM concentration, rather than the 10 or 20 nM used with the other OG duplexes. Using conditions that provided optimal rates for OG removal by yOGG1 with the 30b duplex, the observed trends are consistent with the literature reports (*21*−*23*). Indeed, OG was excised more rapidly from OG·pyrimidine base pairs than from the duplexes containing the OG·purine base pair. On the basis of our results, yOGG1 processed the OG·C-containing duplex 30b approximately 4-fold faster than the corresponding OG·T- and OG·G-containing duplexes. The OG·A-containing duplex did not appear to be a substrate for the yOGG1, since no significant cleavage in the gel assay was observed even after 90 min. Notably, the rate of removal of OG from the 30b duplex substrates was approximately equal to or less than that for removal of Gh or Sp in the 18a duplex sequence. Thus, even though OG removal was observed, it was not robust.

Removal of Oxidized Guanine Lesions by yOGG2: Determination of k_2. Similar to yOGG1, yOGG2 removes Gh and Sp lesions from all base pairings tested. There is no significant difference in the rate of removal within each Gh·Y and Sp·Y set. Interestingly, there is a difference in the rate of removal of Gh compared to Sp, with the latter being removed three to six times more efficiently depending on the opposite base. Thus, Gh, either alone or when base paired, must have a different set of structural determinants than Sp which yOGG2 uses to recognize the base for excision.

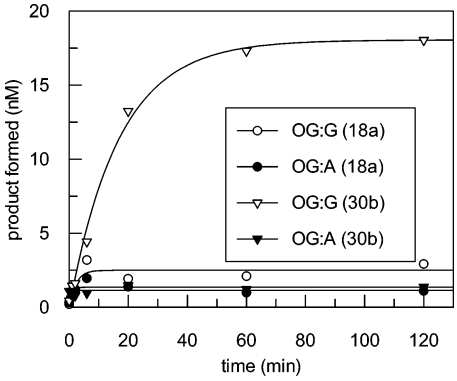

FIGURE 4: Representative plot of DNA product formation as a function of time for the yOGG2 enzyme at 37 °C. Note that duplexes containing an OG·A base pair are not cleaved in either the 18a or 30b sequence, while the duplex containing an OG·G base pair was completely cleaved by yOGG2 in the 30b sequence but not the 18a sequence. [DNA] = 20 nM, and [yOGG2] = 440 nM active protein; reactions were performed at 37 °C.

Remarkably, as observed with yOGG1, no OG removal for yOGG2 was observed in the 18a duplex (Figure 4). However, OG removal from an OG·G bp was observed in the 30b sequence, albeit with a fairly unimpressive efficiency (Figure 4 and Table 1). The 18a and 30b duplexes differ both in length and in the sequence context flanking the lesion base pair. Interestingly, in the reaction of yOGG2 with the OG·G 30a duplex, which contains the 18a duplex, a maximum of 30% cleavage was observed even after prolonged incubation (data not shown). On the basis of the fact that neither the 18a nor 30a duplex containing OG was converted to more than 20−30% product by yOGG2 while OG removal proceeded at a faster rate and to completion in the OG·G 30b duplex, the sequence context flanking the OG lesion strongly influences the ability of yOGG2 to catalyze 8-oxoguanine removal.

Removal of Oxidized Guanines under Conditions of Multiple Turnover with hOGG1 and yOGG2:[2] *Measurements of k_3.* The human OG glycosylase hOGG1 exhibited biphasic behavior only with OG·C and OG·T substrates. It should be noted that the rate derived from fitting of the exponential burst phase in multiple-turnover experiments with hOGG1 with these two substrates is approximately an order of magnitude less than the observed rate measured by single-turnover experiments. Doubling the concentration of hOGG1 and substrate DNA in multiple-turnover experiments resulted in no change in the rate for the exponential burst phase. This result suggests that encounter of hOGG1 and substrate is not limiting the glycosylase activity under these conditions. One possible explanation for the slower burst phase is substrate inhibition when substrate is in excess. Even with the lower rate for the exponential burst phase with OG·C and OG·T substrates, the product release rate was sufficiently slow that it remained rate-limiting, and a biphasic curve was observed. Thus, values for the rate constant k_3 (Table 2) could be accurately measured for the OG·C and OG·T substrates. These values of k_3 suggest that there is no significant difference in the rate of product release by hOGG1 between OG·C and OG·T substrates.

[2] We observed that yOGG1 was less stable to long-term storage than hOGG1 or yOGG2. Due to this observation, experiments under multiple turnover were not attempted with this enzyme.

Table 2: Rate Constants (k_3) Determined at 37 °C under MTO Conditions for hOGG1 and yOGG2 with 18a Duplexes

	k_3 (min^{-1})	
substrate	hOGG1	yOGG2
OG·C	0.04 ± 0.02	NC[b]
OG·T	0.04 ± 0.02	NC
OG·G	nd[a]	nd
OG·A	nd	NC
Gh·C	NC	0.03 ± 0.01
Gh·T	NC	0.006 ± 0.002
Gh·G	NC	0.06 ± 0.02
Gh·A	NC	0.018 ± 0.005
Sp·C	NC	0.03 ± 0.01
Sp·T	NC	0.02 ± 0.01
Sp·G	NC	0.008 ± 0.004
Sp·A	NC	0.014 ± 0.008

[a] nd = no significant cleavage was observed after 90 min, even though these duplexes exhibited measurable cleavage under STO conditions. [b] NC = no significant cleavage observed after 90 min; upper limit rate of <0.0008 min^{-1} was estimated.

The reaction of hOGG1 with the OG·G substrate is quite inefficient under these conditions of substrate excess; at 60 min, only 1% of the substrate has been cleaved. Due to the lack of substrate processing under these conditions, a value for the rate constant k_3 could not be determined for this substrate. Apparently, the presence of OG·G substrate in excess over hOGG1 reduced the rate of reaction, in a manner similar to that observed with OG·C and OG·T substrates, to an extent that minimal reaction is observed. Finally, the rate constant measured under single-turnover conditions (k_2) with the OG·A substrate was sufficiently small that multiple-turnover experiments were not attempted with hOGG1 for this substrate.

The yOGG2 enzyme exhibited biphasic behavior with all Gh- and Sp-containing substrates in the 18a duplex sequence, and values for the rate constant k_3 are listed in Table 2. Due to the inefficient removal of OG, multiple-turnover experiments with OG-containing substrates and yOGG2 could not be performed. As illustrated by the rate constants in Table 2, there is little or no difference in k_3 between Gh·C, Gh·G, or Gh·A substrates. However, for Gh·T, this rate is at least 3-fold slower.

DISCUSSION

This work represents the first quantitative measurements under single- and multiple-turnover conditions of the glycosylase activity of yOGG1, yOGG2, and hOGG1 with hydantoin and OG-containing substrates, as well as a refinement of the rates of excision of OG by *E. coli* Fpg (*36*). Rate constants measured utilizing rapid-quench methods demonstrate that Fpg removes 8-oxoguanine from an OG·C base pair 400 times faster than from an OG·A base pair. The results also show that Fpg excises OG from an OG·G base pair slightly faster than from an OG·T bp and 1.5−2-fold faster than from an OG·C bp. Tchou et al. observed similar trends in assays performed under turnover conditions, where the reactions with OG·G- and OG·T-containing substrates exhibit larger k_{cat}/K_m values than with the corresponding OG·C-containing substrate (*41*). Data obtained from Fpg−DNA cocrystal structures by Fromme and Verdine demonstrate that the arginine involved in specific contacts to the intrahelical base opposite the OG can make satisfactory hydrogen bonds to thymine and guanine in addition to cytosine (*42*). Both of these results, in conjunction with our data presented here, suggest that the ability of Fpg to excise OG from OG·T and OG·G substrates may be an unintended consequence of its specificity for the OG·C base pair and the lower thermal stability of OG·T- and OG·G-containing duplexes enabling the enzyme to more easily distort the helix to allow base flipping (*43*). Since thymine or guanine are seldom incorporated opposite OG (*15*), the ability of Fpg to remove OG in these contexts may not be biologically significant.

From Table 1, it becomes apparent that the identity of the oxidized guanine is of primary importance for hOGG1. The hOGG1 enzyme removes OG from duplex DNA but does not excise Gh or Sp. The removal of OG by hOGG1 also demonstrates that the enzyme distinguishes substrates on the basis of the identity of the base opposite OG in the base pair, as it cleaves OG·pyrimidine much more readily than OG·purine. This is most pronounced in the 1000-fold difference between OG·C and OG·A duplexes. These results are in agreement with qualitative results from the literature (*5*, *44*−*46*). The ability of hOGG1 to recognize and excise OG in a fashion dependent on the opposite base may be the result of specific contacts between the enzyme and the complementary base. Bruner et al. note in the K249Q hOGG1−duplex DNA cocrystal structure that the cytosine of the original OG·C base pair is recognized by Arg154 and Arg204 in bidentate hydrogen bonds specific for adjacent acceptor atoms, a pattern unique to cytosine among the four naturally occurring DNA bases (*16*).

In contrast to hOGG1, yOGG1 recognizes and excises Gh and Sp from all four base-pairing contexts. Remarkably, Gh and Sp are removed more efficiently than OG in the 18a duplex sequence. This suggests that removal of OG may be more sensitive to sequence context than the removal of Gh or Sp. This may be related to the fact that Gh and Sp are duplex-destabilizing lesions in the DNA helix as indicated by UV melting experiments (*47*). The 18a duplex contains a higher G·C content surrounding the lesion base pair when compared with the 30b sequence. In the 30b sequence, yOGG1 removed OG from OG·C 4-fold more quickly than from OG·T, which was comparable in rate to OG·G. The OG·A-containing duplex 30b was not a substrate at any enzyme or duplex concentration or ratio tested. A second point of note regarding yOGG1 is that, in most base-pairing contexts, it removes Gh and Sp with equal or greater efficiency than yOGG2 does. The sole exception to this trend is Sp·A, where yOGG2 is more efficient. This is perhaps not surprising, since the inability of yOGG1 to cleave OG·A suggests a lack of catalytic efficiency when the oxidized guanine species is paired with adenine. Indeed, Gh·A and Sp·A are cleaved by yOGG1 with the lowest rates among the Gh·Y and Sp·Y substrate sets.

As was observed with yOGG1, the ability of yOGG2 to excise OG exhibits a strong dependence on the identity of the duplex DNA. The only duplex that was found to be a substrate for yOGG2 was the OG·G-containing duplex 30b. The observed activity of yOGG2 toward OG·G in the 30b sequence and not the 30a or 18a sequence suggests that, for yOGG2, the sequence context has a marked influence on the OG removal activity. Indeed, the apparent controversy in the literature regarding the substrate specificity of yOGG2

may be due to sequence context effects. Bruner et al. reported removal of OG from OG·G base pairs in a 25 nt duplex containing five A·T base pairs in the regions flanking the lesion (*17*), whereas no OG removal was observed by You et al. from a 37 bp duplex containing only two A·T base pairs in proximity to the OG lesion (*27*). Another interesting feature of the reaction of yOGG2 in the 30b sequence is the activity toward this duplex containing an OG·G bp but not an OG·A bp. This is in contrast to some previous reports in the literature (*17*, *24*). The effect of sequence may also explain this substrate specificity difference, as well as the reports from other laboratories (*26*, *27*) which do not demonstrate OG removal by yOGG2.

The yOGG2 enzyme efficiently excises both Gh and Sp from duplex DNA. Unlike Fpg with Gh- or Sp-containing duplexes, yOGG2 does not exhibit any significant substrate discrimination due to the base opposite the lesion to be excised. However, yOGG2 demonstrated a difference in rate of excision based on whether the duplex contains Gh or Sp. In comparison of yOGG2 activity with OG·Y-18a duplexes (where Y = G, C, T, or A), Sp is excised 3−4-fold faster than Gh. Unlike OG, the structural properties of Gh and Sp within duplex DNA are not known. The discrimination between these two lesions by yOGG2 may be due to the fact that multiple tautomeric forms of Gh are possible, and the one predominanting under these conditions is unknown (*31*). Moreover, Sp will likely distort the DNA helix due to the sp^3 carbon linking its two five-membered rings, whereas Gh can adopt a planar tautomeric form. Therefore, the ability of yOGG2 to recognize and excise Sp with greater efficiency than with Gh may be due to greater local helix destabilization leading to kinking of the DNA or opening of the Sp-containing base pairs. Indeed, most crystal structures of BER glycosylases bound to DNA reveal large distortions in the DNA double helix in order to extrude the damaged base for recognition and catalysis. For example, the K249Q hOGG1−DNA cocrystal structure (*16*) shows a sharp kink of 63° and a flipped-out OG base localized in a protein pocket. Distortion induced by Sp may preorder the DNA toward more efficient yOGG2 binding. Indeed, previous work in the literature using atomic force microscopy shows that hOGG1 is localized with or causes bent DNA (*48*). If indeed yOGG2 is searching for areas of helix disturbance, this could also explain the relative inefficiency of yOGG2 on OG-containing duplexes, since the presence of OG leads to little, if any, helix distortion (*49*, *50*) or destabilization as measured by melting experiments (*43*, *47*).

We observed that hOGG1, yOGG1, and yOGG2 exhibit a slow step after chemistry consistent with rate-limiting product release. On the basis of the k_3 observed for hOGG1 with OG·C and OG·T substrates, the half-life of the hOGG1−product DNA complex was calculated to be 17 min; this is significantly shorter than the nearly 3 h half-life calculated for the MutY−OG·(AP site)−product DNA complex (*39*). It has been proposed that since the product of MutY acting on an OG·A base pair would be an OG·AP site which could be further recognized and processed by an OG glycosylase, MutY remains tightly bound to its product DNA to avoid the resulting double-strand break. However, the cleaved AP site product of hOGG1 is more toxic than the OG lesion, and therefore association of hOGG1 with the product duplex is advantageous. As has been observed with other glycosylases (*37*), the catalytic turnover of hOGG1 can be stimulated by APE1, the major AP endonuclease in humans (*51*−*53*).

In the case of turnover rates (k_3) measured with yOGG2, there was little or no difference in the rate of product release between Gh·C and Gh·A substrates. However, the rate of product release with Gh·T was 3−10-fold slower than with any of the other Gh·Y substrates. There appears to be a structural determinant in a Gh·T base pair that causes a markedly reduced rate of product release. This gives additional credence to the idea that the OG glycosylases rely on specific contacts to both the OG and opposing base for recognition, chemistry, and product release.

From the results presented in this work, a fundamental question arises: assuming that the Gh and Sp lesions do occur in human cellular DNA, which enzyme is responsible for repairing them, since no activity was observed for hOGG1? From the results with yOGG1 and yOGG2, it is apparent that recognition and repair of such lesions can occur in eukaryotes. One possibility is that another base excision repair enzyme such as human endonuclease III (hNTH1) or one of the new human Fpg (hFpg2; *54*) or endonuclease VIII (Neil1 and Neil2; *55*, *56*) analogues may perform this function in humans. Another possibility is that the lesions are removed by a more complex pathway such as transcription-coupled nucleotide excision repair. The removal of oxidatively damaged DNA bases by transcription-coupled NER has been demonstrated in the literature with OG, cyclobutane pyrimidine dimers, and thymine glycol lesions (*57*−*59*). It is also conceivable that there may be an effect of duplex length or sequence on the removal of Gh or Sp by hOGG1, similar to that seen with OG removal by the yOGG proteins. Alternatively, the action of polymerases involved in translesion synthesis may be more capable of inserting the correct nucleotide opposite these lesions and/or correctly bypassing polymerase-blocking lesions, as has been demonstrated for pol η with thymine−thymine cyclobutane dimers (*60*−*62*).

The overall mutagenic potential of any lesion in vivo is dependent upon the interplay of lesion formation, lesion repair, and translesion synthesis. The oxidized OG lesions, Gh and Sp, are more efficient blocks of primer extension by Klenow fragment exo^- than their parent lesion OG, and this may be an important feature for stalling the replication machinery under conditions of oxidative stress to allow for repair to occur (*47*, *63*). In addition, these in vitro assays indicated that guanine and adenine are inserted opposite both Gh and Sp (*47*, *63*). Significantly, recent work by Henderson et al. using an in vivo replication assay has shown that Gh is bypassed nearly as efficiently as OG, though it is more highly mutagenic, causing almost exclusively G → C transversions (*64*). In contrast, Sp was a stronger block to replication and caused a mixture of G → T and G → C mutations. These studies reveal that, in *E. coli*, Gh and Sp lesions are highly mutagenic and more mutagenic than their parent lesion OG. The activity of repair enzymes might actually facilitate mutagenesis in this context. Indeed, we previously observed removal of Gh and Sp opposite G and A by Fpg which would help to secure the G → C and G → T mutations, respectively (*36*). Thus, similar in vivo studies in repair-deficient *E. coli* might be illuminating on the role of the repair glycosylase in mutagenesis. Due to the lack of

dependence of the opposite base on removal of Gh and Sp by the yOGG enzymes, it might expected that repair may be similarly beneficial or detrimental in eukaryotes depending on the context of the damaged base.

In conclusion, this present work has demonstrated that hOGG1, yOGG1, and yOGG2 have significant differences in their ability to excise OG, Gh, or Sp. Each of the OGG enzymes has different characteristics in terms of the preference for the base opposite the lesion and the duplex sequence. It is also particularly important to note that all three enzymes exhibit biphasic kinetics, having high affinity to their products. Thus, the effects of downstream enzymes on the activity of the enzymes will be of interest. The lack of activity of hOGG1 on Gh·Y- and Sp·Y-containing substrates is particularly striking and begs for continued investigation of the repair properties of these and related lesions.

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