Removal of Hydantoin Products of 8-Oxoguanine Oxidation by the *Escherichia coli* DNA Repair Enzyme, FPG†

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Received August 1, 2000; Revised Manuscript Received October 2, 2000

ABSTRACT: An intriguing feature of 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG) is that it is highly reactive toward further oxidation. Indeed, OG has been shown to be a "hot spot" for oxidative damage and susceptible to oxidation by a variety of cellular oxidants. Recent work has identified two new DNA lesions, guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp), resulting from one-electron oxidation of OG. The presence of Gh and Sp lesions in DNA templates has been shown to result in misinsertion of G and A by DNA polymerases, and therefore, both are potentially mutagenic DNA lesions. The base excision repair (BER) glycosylases Fpg and MutY serve to prevent mutations associated with OG in *Escherichia coli*, and therefore, we have investigated the ability of these two enzymes to process DNA duplex substrates containing the further oxidized OG lesions, Gh and Sp. The Fpg protein, which removes OG and a variety of other oxidized purine base lesions, was found to remove Gh and Sp efficiently opposite all four of the natural DNA bases. The intrinsic rate of damaged base excision by Fpg was measured under single-turnover conditions and was found to be highly dependent upon the identity of the base opposite the OG, Gh, or Sp lesion; as expected, OG is removed more readily from an OG:C- than an OG:A-containing substrate. However, when adenine is paired with Gh or Sp, the rate of removal of these damaged lesions by Fpg was significantly increased relative to the rate of removal of OG from an OG:A mismatch. The adenine glycosylase MutY, which removes misincorporated A residues from OG:A mismatches, is unable to remove A paired with Gh or Sp. Thus, the activity of Fpg on Gh and Sp lesions may dramatically influence their mutagenic potential. This work suggests that, in addition to OG, oxidative products resulting from further oxidation of OG should be considered when evaluating oxidative DNA damage and its associated effects on DNA mutagenesis.

Oxidative damage to DNA has been shown to be a relevant causative agent in carcinogenesis, aging, and neurological disorders (*1*, *2*). Oxidative DNA damage is mediated by reactive oxygen species (ROS)[1] such as superoxide, hydrogen peroxide, and hydroxyl radicals which are present in the cell as byproducts of endogenous reactions or as the result of external sources, such as ionizing radiation (*3*, *4*). The reactions mediated by ROS can lead to a wide variety of types of DNA damage, including DNA strand breaks, protein−DNA cross-links, abasic sites, and base lesions (*3*−*8*). All of these forms of DNA damage can be potentially detrimental to the cell; therefore, the activity of DNA repair systems is required to maintain the integrity of the genome (*9*). More than 50 different base lesions have been identified as products of oxidative DNA damage (*10*). However, the DNA base lesion receiving the most attention is arguably 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG), which is commonly used as a biomarker of oxidative DNA damage in the cell (*1*, *10*). When OG is present in a DNA template, insertion of A or C opposite OG occurs, depending on the specific polymerase that is involved (*11*, *12*). In bacterial and mammalian cells, OG has been shown to produce high levels of G:C → T:A transversion mutations (*12*−*14*).

The prevention of mutations caused by OG in *Escherichia coli* relies on the "GO" repair system (*15*, *16*) that utilizes two-base excision repair (BER) glycosylases (*17*), Fpg (MutM) and MutY. Fpg catalyzes the removal of the OG from an OG:C base pair, while the MutY enzyme provides a second line of defense by removing the aberrant adenine from an OG:A base pair. The subsequent action of AP endonuclease(s), a repair DNA polymerase, and DNA ligase results in restoration of the G:C base pair (*17*). A third enzyme, MutT, prevents incorporation of OG into DNA via the dNTP pool by catalyzing the hydrolysis of d(OGTP) to d(OGMP) (*18*). Yeast (yOgg1) and human (hOgg1) functional homologues to Fpg have been identified (*17*); these

† This work was supported by grants from the Huntsman Cancer Institute at the University of Utah and the American Cancer Society (RPG-00-310-01-GMC) to S.S.D. and an NSF grant (CHE9818484) to C.J.B. S.S.D. is an A. P. Sloan Research Fellow (1998−2000), and M.D.L. is an NIH predoctoral trainee (GM08537). ESI-MS was supported in part by the NSF (CHE-9708413) and the University of Utah Institutional Funds Committee.

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[1] Abbreviations: BER, base-excision repair; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Gh, guanidinohydantoin; Ia, iminoallantoin; IPTG, isopropyl β-D-thiogalactoside; nt, nucleotide; OG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; LB, Luria-Bertani broth; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; ROS, reactive oxygen species; Sp, spiroiminodihydantoin; TBE, tris-borate-EDTA; Tris, tris(hydroxymethyl)aminomethane.

10.1021/bi0017982 CCC: $19.00
Published on Web 11/02/2000

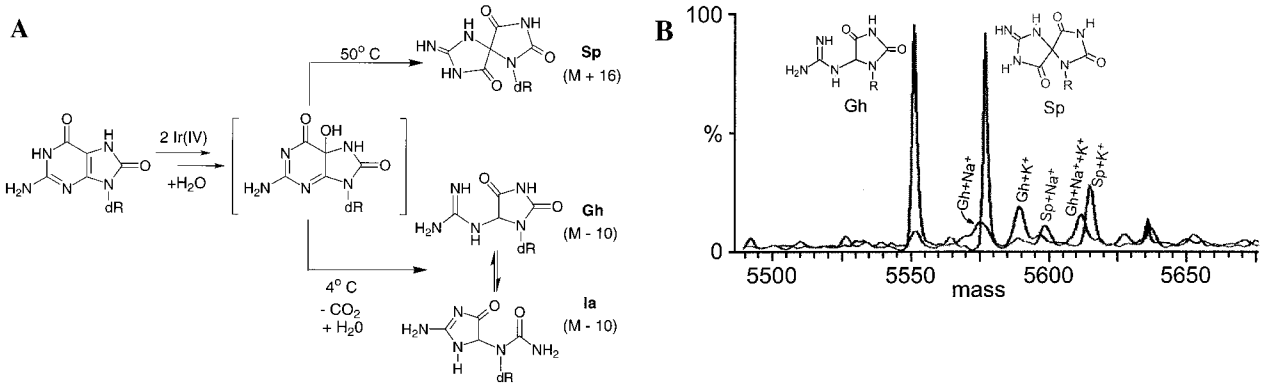

FIGURE 1: Oxidation of OG by Na_2IrCl_6 produces guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp) lesions. (A) Proposed pathway for formation of Gh and Sp by initial one-electron oxidation of OG that initially provides 5-hydroxy-OG. Rearrangement of 5-OH-OG leads to the Sp lesion, while hydration and decarboxylation provides the Gh lesion. Studies with nucleosides have shown that the Gh lesion equilibrates with an isomeric form, iminoallantoin (Ia). (B) ESI-MS analysis of 5′-TCATGGGTC(OG)TCGGTATA-3′ oxidized by Na_2IrCl_6 at 4 and 50 °C, yielding oligonucleotides containing Gh and Sp, respectively.

eukaryotic enzymes belong to a BER superfamily containing many BER glycosylases (*17*), notably, *E. coli* MutY and its human homologue (hMYH).

Fpg is a 30 kDa zinc finger-containing protein that utilizes the N-terminal proline in the displacement of the base in the glycosylase reaction (*19*−*22*). Fpg also catalyzes β- and δ-elimination on the resulting AP site (*23*) to produce a DNA strand scission event concomitant with catalysis of base removal. The versatility of the Fpg protein is also illustrated by its ability to catalyze the removal of 5′-terminal deoxyribose phosphates from DNA (dRPase activity) (*24*). In addition to OG, Fpg catalyzes the removal of a variety of damaged bases (*17*), including ring-opened forms of purines such as 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (Me-Fapy-G), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G), and 4,6-diamino-5-formamidopyrimidine (Fapy-A), bulky adducts such as aflatoxin B_1−Fapy adducts and aminofluorene adducts at C-8 of guanine, and oxidized pyrimidines such as 5-hydroxycytosine, 5-hydroxyuracil, and 5,6-dihydroxy-5,6-dihydrothymine. Recently, the crystal structure of an Fpg homologue from *Thermus themophilus* HB8 was reported, and this will provide a structural basis for understanding the recognition of these varied substrates by Fpg (*25*).

An important and increasingly recognized feature of OG is that it is highly reactive toward further oxidation, with several in vitro studies illustrating that OG is a "hot spot" for oxidative damage (*26*−*30*). Indeed, redox potentials for an OG nucleoside have been reported to be between 0.58 and 0.75 V versus the normal hydrogen electrode (NHE), significantly lower than that of the 2′-deoxyguanosine nucleoside (1.29 V vs NHE) (*26*, *31*, *32*). These redox potentials can be substantially modulated by the sequence environment of the DNA duplex. For example, the potential of G or OG may be lowered when flanked by another guanine in the duplex, and therefore, G-rich sites may be particularly susceptible to oxidative DNA damage (*33*−*36*). Recent studies have shown that OG is readily oxidized by the cellular oxidants $^1O_2$ (*37*, *38*) and peroxynitrite (*28*−*30*, *39*). There has also been considerable excitement over a variety of experiments that indicate that oxidative "hole" migration can occur over long distances within the DNA helix (*40*−*42*). This idea suggests that oxidative damage could occur at a remote site and eventually become "trapped" at the site of the lowest redox potential (*42*), such as OG. Such a mechanism would have the effect of concentrating further the oxidative damage at OG when it occurs by a one-electron process. Thus, it seems reasonable that lesions arising from further oxidation of OG may be commonplace in vivo and, therefore, may be important target sites for DNA repair enzymes.

One-electron oxidation is likely a common pathway for OG oxidation that may occur by type I photoprocesses, including ionizing radiation, reactions of transition metals, and mechanisms that involve attack of $RO^{\bullet}$ followed by loss of RO^- (*43*). Furthermore, the one-electron mechanism is the only one that would allow equilibration of oxidative damage elsewhere to the OG site. The Burrows laboratory has shown that, in vitro, specific one-electron oxidation of OG may be achieved using the convenient one-electron oxidant, Na_2IrCl_6 (*34*, *44*). This reagent has the appropriate redox potential to oxidize OG without resulting in oxidation of other undamaged DNA bases. Using electrospray mass spectrometry, two major pathways of OG one-electron oxidation have been proposed in which 5-hydroxy-OG is an intermediate that can isomerize to form spiroiminodihydantoin (Sp), or hydrate and decarboxylate to form guanidinohydantoin (Gh) as shown in Figure 1 (*45*). Using conditions that provided a mixture of these two lesions, the effects of the oxidation of OG on the activity of DNA polymerases have been evaluated (*46*). Primer extension assays using Klenow fragment exo− (Kf exo−) and an oligonucleotide containing a single oxidized OG lesion in the template showed incorporation of dAMP and dGMP opposite the lesion with transient inhibition of further extension after the modified base. Furthermore, these lesions block DNA synthesis when the eukaryotic polymerases calf thymus pol α and human pol β are used. These results suggest that further oxidation of OG in vivo could lead to blocks in DNA replication and point mutations leading to G:C → T:A and G:C → C:G transversion mutations. Therefore, cellular DNA damaged by oxidants likely contains OG and further oxidized forms (e.g., Gh), and the majority of resulting mutations would be G:C → T:A and G:C′ → C:G transversions. Will et al. (*47*) have reported that these mutations account for 90% of the base substitutions when CHO cells are exposed to the photosensitizer Ro19-8022 and light. In addition, Ono et al. (*48*) demonstrated that the presence of the oxidant

menadione in an *E. coli* strain lacking MutM resulted in a predominance of G:C → C:G mutations, followed by G:C → T:A mutations. Moreover, these two mutations were also observed in DNA from peroxy radical-treated cells (*49*), and in this case, no OG was detected in the analysis of the cellular DNA. These results, taken together with the low oxidation potential of OG, provide circumstantial evidence that Gh and Sp lesions may be important lesions leading to mutagenesis; therefore, determining the modes of possible repair of these lesions will be exceedingly important.

Since Fpg and MutY serve to counter the problems associated with OG in *E. coli*, we have investigated the effects of oxidation of OG-containing substrates on the activity of these enzymes. In addition, we have investigated the effect of the base opposite the lesion by measuring the rates of OG, Gh, and Sp removal by Fpg under single-turnover ([E] > [S]) conditions. This current work demonstrates that wild-type Fpg can remove OG and its oxidized products Gh and Sp when positioned opposite each of the four DNA bases in duplex DNA. In contrast, MutY was unable to remove A or G opposite Gh or Sp. The rate of damaged base excision by Fpg is highly dependent upon the identity of the base opposite the OG, Gh, or Sp lesion; as expected, OG removal from an OG:C substrate is considerably faster than from an OG:A substrate. However, when adenine is paired with Gh or Sp, a significant increase in the rate of removal of the damaged lesion by Fpg was observed compared to the rate of removal of OG from an OG:A mismatch. This observation could have important implications for an increase in G:C → T:A mutational frequency caused by the removal of Gh and Sp by Fpg.

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. Deionized, distilled water was further purified with a Milli-Q PF system and used for all manipulations. Fpg 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 4.2a) on a Windows NT 4.0 system was used to quantify storage phosphor autoradiograms. ESI-MS spectra were obtained with the use of a Micromass Quattro II tandem mass spectrometer equipped with a Z-spray API source. Electrophoresis experiments were performed using tris-borate-EDTA (TBE) buffer (90 mM Tris base, 90 mM boric acid, and 1 mM EDTA).

Fpg Purification. The purification of Fpg was adapted from several previously reported methods (*21*, *22*). *E. coli* JM109 cells harboring the *fpg* gene overexpression plasmid (pKKFapy2) were grown at 37 °C in LB medium containing 100 μg/mL ampicillin to an OD_{600} of 0.9. After addition of IPTG (1 mM) and 50 μM $ZnCl_2$, the cells were grown for 4 h at 30 °C. After centrifugation (8000 rpm for 10 min at 4 °C), the pelleted cells were resuspended in buffer A [50 mM Tris-HCl (pH 8), 2 mM EDTA, 5% glycerol, 5 mM DTT, and 250 mM NaCl] supplemented with 1 mM PMSF and stored at −80 °C. Upon thawing, the cells were disrupted by sonication (Branson Sonic Power Co., model 350, 70% pulse, 30 s on followed by 30 s off, repeated six times) and centrifuged (10 000 rpm for 5 min at 4 °C). Streptomycin sulfate was added to the sonication supernatants to 19% (w/v). The sample was centrifuged (14 000 rpm for 30 min at 4 °C), and ammonium sulfate (40%) was added to the supernatant. The pellet was collected after centrifugation (10 000 rpm for 10 min at 4 °C) and was resuspended in buffer B [25 mM Hepes/KOH (pH 7.6), 1 mM EDTA, and 10% glycerol] containing 100 mM NaCl. The protein sample was loaded on a Pharmacia HiPrep 26/10 desalting column equilibrated with buffer B. The protein-containing fractions were collected, pooled, and loaded onto a Bio-Rad EconoPak High S cation exchange column equilibrated with buffer B, washed, and then eluted with buffer B containing 1 M NaCl. After a 10-fold dilution with buffer B, the protein fractions were loaded onto a Pharmacia HiTrap Heparin column equilibrated with buffer B. The Fpg protein eluted at approximately halfway through a linear gradient with buffer B containing 1 M NaCl. The sample was diluted 3-fold with buffer C [2 mM EDTA, 100 mM Hepes/KOH (pH 7.6), 2 M NaCl, and 1.6 M ammonium sulfate] and loaded onto a HiPrep 16/10 Phenyl low substitution column (Pharmacia Biotech) equilibrated with buffer D [25 mM Hepes/KOH (pH 7.6), 1 mM EDTA, 1 M NaCl, and 0.8 M ammonium sulfate]. Fpg eluted at 81% buffer B during a linear gradient to buffer B. The eluted sample (25 mL) was concentrated in an Amicon model 8050 ultrafiltration cell to a final volume of 10 mL, then diluted to 15 mL with storage buffer (500 mM NaCl with buffer B containing 50% glycerol and 1 mM PMSF), and stored in 1 mL aliquots in liquid nitrogen until it was used. SDS−PAGE and silver stain analysis show the protein to be >95% pure. Concentrations of Fpg were determined using the absorbance at 280 nm and the reported extinction coefficient [$\epsilon_{280} = 3.9 \times 10^4$ L mol^{-1} cm^{-1} (*21*)]. N-Terminal Edman sequencing gave the unique sequence PELPEVET, confirming the identity of the purified protein as Fpg. The active site concentration of Fpg was estimated to be 25% using a DNA binding assay with a THF-containing oligonucleotide (*50*). Concentrations listed throughout are the total protein concentration based on the absorbance at 280 nM.

MutY Purification. MutY used for these assays was purified as described previously (*50*). Active site titration methods used for MutY (*51*) determined that the MutY preparation contained an active site concentration that was 50% of the total protein concentration, as determined by the method of Bradford (*52*).

Oligonucleotides. The oligonucleotide sequences listed below were used: d(5′-TCATGGGTCXTCGGTATA-3′) with complement d(3′-AGTACCCAGYAGCCATAT-5′), where X is OG and Y is C, A, T, or G. The DNA strands were synthesized on an Applied Biosystems Inc. 392B automated synthesizer using the manufacturer's protocols and purified via polyacrylamide gel electrophoresis (*46*) or ion exchange HPLC (Waters, Protein-Pak DEAE 8HR column). Oligonucleotides containing OG were deprotected in the presence of β-mercaptoethanol.

$IrCl_6^{2-}$ Treatment of OG-Containing DNA. All guanidinohydantoin- or spiroiminodihydantoin-containing DNA

strands were synthesized from the X-containing strand listed above, where X is OG. To generate the guanidinohydantoin lesion, Na_2IrCl_6 (final concentration of 100 μM) was added to the stock DNA solution [final concentrations, 12 μM ss-DNA, 10 mM sodium phosphate buffer (pH 7), and 100 mM NaCl], and the reaction mixture was placed in a water bath at 4 °C for 1 h and then the reaction quenched by addition of a stock solution of EDTA (final concentration of 50 mM). The resulting solution was dialyzed (2 kDa molecular mass cutoff) against water for 24 h. The spiroiminodihydantoin reaction was identical but was performed at 50 °C. Samples analyzed by electrospray mass spectrometry were prepared as previously described (*46*).

Substrate DNA Preparation. Five 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 strand was added in slight excess (10−25%). The annealing step was performed in buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM EDTA, and 150 mM NaCl. This solution (100 nM duplex) was heated to 95 °C and then slowly cooled over ≥3 h to anneal the duplex.

Single-Turnover Glycosylase Assays with Fpg. Single-turnover assays were performed using the 18 bp duplexes described above, where the X-containing strand was ^{32}P-labeled. In each case, the total reaction volume was 60 μL with a final duplex DNA concentration of 10 nM. 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. Aliquots (5 μL) were removed at 0, 0.25, 0.5, 1, 2, 4, 8, 15, 30, 60, and 90 min for all reactions except OG:A and at 0, 0.5, 1, 2, 4, 8, 15, 30, 60, and 120 min for the OG:A reaction. Each reaction was immediately quenched by addition to an equal volume of formamide denaturing loading dye (80% formamide, 0.025% xylene cyanole, and 0.025% bromophenol blue in TBE buffer) preheated to 90 °C. The combined mixture was placed at 90 °C for 4 min to heat denature before loading onto a 20% polyacrylamide gel containing 8 M urea to separate reactants and products. The substrate and product DNA were resolved after running the gel in TBE buffer for 2 h at 1600 V. Gels were exposed to a storage phosphor screen for ≥12 h. The resulting image was quantified using ImageQuaNT version 4.2a. GraFit version 4.06 was used to fit the data.

The raw data were analyzed using eq 1

$$[P]_t = A_o[1 - \exp(-k_{obs}t)] \quad (1)$$

where A_o represents the amplitude of the exponential phase and k_{obs} is the observed rate constant associated with that process. The final Fpg concentration was varied from 50 to 800 nM and resulted in no significant differences in k_{obs}, indicating that the appropriate experimental conditions had been achieved. A proposed minimal kinetic scheme for Fpg under these conditions is shown below:

$$\text{Fpg} + (\text{DNA})_s \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Fpg}\cdot(\text{DNA})_s \xrightarrow{k_2} \text{Fpg}\cdot(\text{DNA})_p$$

The initial binding of the DNA substrate is described by K_d, defined as k_{-1}/k_1, while k_2 defines the overall rate constant for steps involving chemistry of base removal and subsequent β- and δ-elimination strand cleavage reactions. Contributions due to the release of Fpg from the product DNA can be disregarded under these conditions. The observed rate constant under pseudo-first-order conditions ([Fpg] > [DNA]) is given by eq 2, assuming that enzyme−substrate binding is in a rapid equilibrium (i.e., $k_{-1} \gg k_2$).

$$k_{obs} = \frac{[E]_o}{K_d + [E]_o} k_2 \quad (2)$$

Additionally, since the enzyme concentration is assumed to be well above K_d, eq 2 simplifies to $k_{obs} = k_2$.

Glycosylase Assays with MutY. The adenine glycosylase assays with MutY were performed in a manner similar to that reported previously (*51*), using the duplex sequence above with the Y-containing strand (Y being G or A) radiolabeled.

RESULTS

Preparation of Gh- and Sp-Containing Substrates. The oxidized OG lesions were found to be highly sensitive to alkaline conditions, and therefore, it was not possible to synthesize oligonucleotides containing these species directly using the phosphoramidite method. In pursuing alternate approaches to the synthesis, we found that the relative amounts of Gh and Sp formed by one-electron oxidation of OG-containing oligonucleotides are highly dependent on reaction temperature. Thus, the sensitivity of the OG oxidation reaction to temperature provided a convenient means of synthesizing the two lesions in high yield in an oligodeoxynucleotide. Electrospray mass spectrometric analysis of the products formed from oxidation of OG within a single-stranded oligonucleotide at low temperatures (4 °C) indicated formation of a product with a mass consistent with guanidinohydantoin as the major product formed (Figure 1).[2] However, if this oxidation reaction is performed at higher temperatures (≥50 °C), a different product is observed that has an observed mass consistent with a spiroiminodihydantoin compound (Figure 1). Recent MS/MS and ^{18}O-labeling studies using an OG ribonucleoside as well as independent synthesis are consistent with the structural formulation of the spiroiminodihydantoin (*45*) and guanidinohydantoin compounds (W. Luo, J. G. Muller, E. Rachlin, and C. J. Burrows, unpublished results).

By performing the oxidation of a single-stranded OG-containing oligonucleotide at low versus high temperatures prior to annealing to the complementary strand, we found it possible to prepare duplex substrates for Fpg and MutY that contain >95% of either the guanidinohydantoin (Gh)[2] or the spiroiminodihydantoin (Sp) lesion.

Removal of Gh and Sp from DNA Duplex Substrates by Fpg. Fpg has previously been shown to catalyze both

[2] Note that Gh may further rearrange to an isomer, iminoallantoin (Ia). Both Gh and Ia have been characterized in nucleoside studies (W. Luo, J. G. Muller, E. Rachlin, C. J. Burrows, unpublished results), but the predominant isomer in duplex DNA has not yet been identified. For convenience, only the abbreviation Gh will be used to designate both Gh and its Ia isomer.

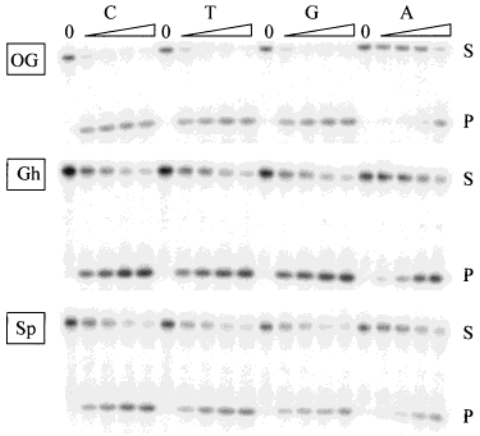

FIGURE 2: Storage phosphor autoradiogram of substrate processing of Gh (middle panel) and Sp (bottom panel) relative to OG (top panel) opposite C, T, G, and A. Reactions were performed at 37 °C, with a DNA concentration of 10 nM and an Fpg concentration of 300 nM. Lanes with 0 indicate controls without enzyme, and triangles indicate an increase in incubation time from left to right with time points of 0.25, 1, 5, and 30 min. The base opposite the OG, Gh, or Sp is designated at the top as C, T, G, and A. The label "S" designates the band corresponding to the 18 nt fragment resulting from the substrates, and "P" designates the band resulting from the 9 nt product oligonucleotide fragment.

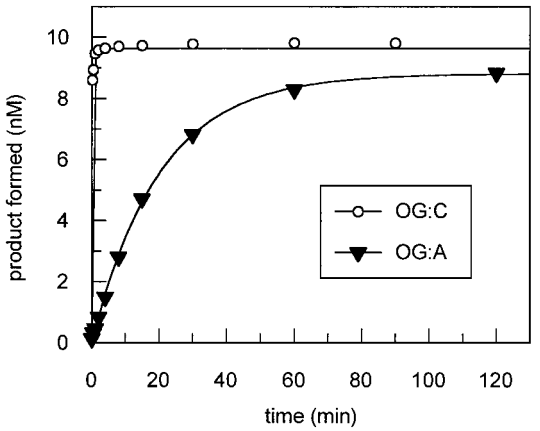

FIGURE 3: Comparison of the reaction of Fpg with an OG:C- and OG:A-containing duplex at 37 °C. In this particular experiment, 10 nM DNA duplex and 300 nM Fpg were used. The data have been fitted to eq 1 as described in the text, and the resulting rate constants averaged from at least four separate determinations are listed in Table 1.

removal of OG and β- and δ-elimination reactions on AP sites to achieve strand scission. The ability of Fpg to process Gh and Sp relative to OG was determined using 18 bp duplex substrates containing a centrally located X:Y base pair, where X is OG, Gh, or Sp and Y is C, A, T, or G. The X-containing strand was ^{32}P-end-labeled prior to annealing to the complementary strand. The 12 different duplexes that were formed were incubated separately with Fpg, and aliquots were removed and the reactions quenched at 0.25, 1, 5, and 30 min. The ^{32}P-end-labeled nine-nucleotide fragment resulting from the product was resolved from the labeled 18-nucleotide fragment of the substrate by denaturing polyacrylamide gel electrophoresis. The amount of product relative to substrate was visualized and quantitated using storage phosphor autoradiography (Figure 2). Importantly, Gh and Sp are substrates for Fpg opposite all of the four natural DNA bases. The efficiency of removal appears to be dependent on the base opposite Gh and Sp, which is discernible by visual inspection of the storage phosphor autoradiogram. The general trends for the dependence of the base opposite for removal of Gh and Sp mirror the results with OG. In addition to catalyzing removal of the base, Fpg catalyzed both β- and δ-elimination to yield a 3′-phosphate end, and this was confirmed by migration of these bands relative to Maxam−Gilbert sequencing reactions and base-treated controls (data not shown).

A number of laboratories have measured standard Michaelis−Menten parameters (k_{cat} and K_m) for Fpg with a variety of substrates (*53*, *54*). However, on the basis of the qualitative data depicted in Figure 2, where Fpg was in molar excess of the substrate, it is clear that some of the substrates, such as OG:A, are quite slow, and therefore, it will be difficult to measure k_{cat} and K_m parameters for all of the substrates due to the large amount of time that would be required to establish the steady state. Prompted by these initial observations, single-turnover experiments were used to evaluate the intrinsic rate of Fpg's glycosylase and lyase reactions. The reaction of Fpg with all of the duplex substrates under conditions where [Fpg] > [DNA] followed first-order kinetics, leading to nearly quantitative conversion to products. A representative plot of the reaction of Fpg with OG:C- and OG:A-containing substrates is shown in Figure 3. The data were analyzed as described in Materials and Methods. Accordingly, the reaction of Fpg with the OG:A duplex yielded a k_2 of 0.045 ± 0.007 min^{-1}. In the case of the OG:C duplex (as well as the OG:T and OG:G duplexes), the reaction was too fast for our manual assay; hence, an accurate value for k_{obs} (and therefore k_2) could not be determined. However, on the basis of the first time point assayed and the extent of conversion to product at that time point, a lower limit for k_2 of 7 min^{-1} was estimated for the OG:C-containing duplex. In the case of OG:C-, OG:G-, and OG:T-containing duplexes, when the reaction was slowed by lowering the temperature to 4 °C, the entire time course could be monitored, and the resulting k_2 values for all three substrates were found to be similar ($k_2 = 0.4 \pm 0.1$ min^{-1}). Thus, the intrinsic rate of removal of OG by Fpg is not affected by the replacement of C with G or T. However, the k_2 values indicate that OG:A is the poorest substrate for Fpg, and the measured rate is approximately 100 times lower than that for the OG:C, OG:T, or OG:G substrate at 37 °C. This trend in base-opposite dependence of OG removal by Fpg where OG:C, OG:T, and OG:G are preferred substrates compared to OG:A is completely consistent with trends observed previously based on qualitative measurements (*56*). In particular, the large difference in the rates of removal of OG from the OG:C substrate relative to the OG:A substrate is consistent with previous studies (*55*, *56*).

The k_2 values for the activity of Fpg toward Sp- and Gh-containing substrates relative to the OG counterparts are listed in Table 1. These results confirm the general trends observed in the qualitative experiments as well as provide a clearer picture of the differences in substrate processing by Fpg. Clearly, Fpg removes Gh and Sp lesions when paired opposite all four bases (Table 1). Somewhat surprisingly, similar rates for removal of Gh relative to that of Sp are observed. In the case of duplexes where the opposite base is C, T, or G, the observed rates of Gh or Sp removal are somewhat slower (approximately 5-fold) than with the

Table 1: Rate Constants Determined under Single-Turnover Conditions for Fpg with OG-, Gh-, and Sp-Containing Substrates at 37 °C

central mispair[a]	k_2 (min^{-1})[b]	central mispair[a]	k_2 (min^{-1})[b]
OG:C	>7	OG:G	>6
Gh:C	1.4 ± 0.4	Gh:G	1.9 ± 0.2
Sp:C	1.3 ± 0.2	Sp:G	1.4 ± 0.2
OG:T	>6	OG:A	0.045 ± 0.007
Gh:T	1.7 ± 0.6	Gh:A	0.34 ± 0.06
Sp:T	1.6 ± 0.3	Sp:A	0.34 ± 0.04

[a] Designates central base pair of the 18 bp duplex. [b] All k_2 values represent an average of at least four separate experiments.

corresponding OG-containing duplexes (Table 1). The overall preference for removal of the damaged guanine lesion opposite C, T, and G over A is similar for Gh/Sp and OG; however, the magnitude of differences due to the base opposite the oxidized OG lesions is considerably smaller. Specifically, the rate of Gh or Sp removal opposite A is only approximately 3-fold slower than the rate of removal opposite C; in contrast with OG, the same comparison indicates a more than 100-fold difference. A striking consequence of the altered substrate processing is that the Gh and Sp lesions opposite A are removed approximately 8-fold faster than OG opposite A. This suggests that incorporation of A opposite Gh or Sp or oxidation of OG in OG:A base pairs may enhance removal of "oxidized" OG. Such scenarios would always be promutagenic and therefore potentially deleterious to the cell.

Effects of Oxidation of OG on the Glycosylase Activity of MutY. Previously, using single-turnover experiments, we have measured the intrinsic rates for removal of A from both OG:A and G:A substrates (*51*). In addition, MutY has been shown to exhibit a weak activity toward removal of G from an OG:G base pair (*57*). Thus, on the basis of the fact that Kf exo− inserted G and A opposite Gh and Sp and the observed purine glycosylase activity of MutY, the ability of MutY to remove A and G opposite Gh and Sp relative to OG was examined. In these experiments, the same 18 bp duplex substrates were used; however, the "Y"-containing strand, where Y is G or A, was ^{32}P-end-labeled prior to annealing of the duplex. Excess MutY was incubated with each of the six duplexes, and aliquots were removed at 1, 15, 30, and 60 min. The reactions were quenched with NaOH which inactivates MutY and also serves to cleave the abasic site produced by the monofunctional glycosylase activity of MutY, and products were resolved from substrates by denaturing PAGE (Figure 4). Under these conditions, the OG:A duplex was almost completely converted to product within the first time point of 1 min. Nearly negligible glycosylase activitiy was observed toward A opposite Gh or Sp; after 60 min, the adenine removal that was observed was 6 and 2% opposite Gh and Sp, respectively. The activity of MutY with the OG:G duplex was considerably slower such that only 50% conversion was observed after 60 min; however, essentially no conversion to product (less than 2%) was observed with the Gh:G or Sp:G duplexes. Gel retardation experiments were performed on an OG:A duplex that had been oxidized with Na_2IrCl_6 under conditions that provided a mixture of Gh and Sp. The measured dissociation constant (K_d) with this duplex was 150 ± 90 nM, and this value is essentially identical to that observed for binding of MutY to a duplex containing all Watson−Crick base pairs (*50*).

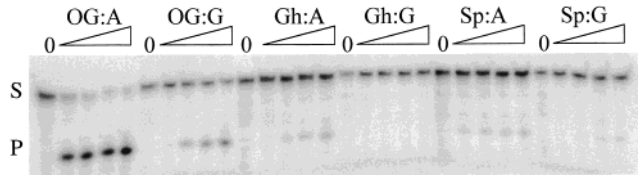

FIGURE 4: Storage phosphor autoradiogram of the removal of A and G from duplex substrates opposite OG, Gh, and Sp. Reactions were performed at 37 °C, with a DNA concentration of 10 nM and a MutY concentration of 30 nM (active site concentration). The central base pair is indicated at the top of the gel. Lanes with 0 indicate controls without enzyme, and triangles indicate an increase in incubation time from left to right with time points of 1, 15, 30, and 60 min. The label "S" designates the band corresponding to the 18 nt fragment resulting from the substrates, and "P" designates the band resulting from the 9 nt product oligonucleotide fragment.

DISCUSSION

Previous work (*45*; W. Luo, J. G. Muller, E. Rachlin, and C. J. Burrows, unpublished results) has identified spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh) as new DNA lesions that result from further oxidation of the common biomarker of oxidative damage, 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG). Importantly, conditions have been identified to prepare and characterize oligonucleotide duplexes containing a single site-specific Sp or Gh lesion, and the effects of these lesions on DNA polymerases and DNA repair enzymes can now be more readily evaluated. Herein, we have demonstrated that these two new DNA lesions are excellent substrates for the DNA repair enzyme, Fpg. Both lesions are efficiently removed opposite all four natural bases. Significantly, the ability of Fpg to remove the oxidized OG lesions Gh and Sp opposite A is enhanced over that for removal of OG opposite A. This work also represents the first quantitative measurement under single-turnover conditions of the relative glycosylase/lyase activity of Fpg with these substrates, and this provides further insight into the intrinsic properties of recognition and repair by Fpg.

The single-turnover rate constants (Table 1) indicate that the base opposite the OG is important, as OG:C, OG:T, and OG:G substrates are converted to product two orders of magnitude more efficiently than the OG:A substrate. These results are similar to those obtained by Castaing et al. (*55*), who showed that Fpg removed OG from an OG:X-containing duplex with the following relative preference: X = C > T > G ≫ A. In addition, specificity constants k_{cat}/K_m determined under conditions of multiple turnover (*56*) indicate a similar general trend for Fpg as reported herein. In all cases, Fpg has been found to be particularly sluggish toward the removal of OG from an OG:A base pair. These data are completely consistent with the biological role of Fpg in the "GO" repair pathway and the mutational properties of OG. Since OG:C and OG:A base pairs are the important base pairs encountered by Fpg in vivo, removal of OG from an OG:A base pair would be mutagenic, and therefore, an intrinsic preference of Fpg for removal of OG from OG:C base pairs is logical. Interestingly, the human functional homologue of Fpg, hOgg1, while following the same trend, is much more specific for OG when paired with pyrimidines (*58*−*62*); hOgg1 exhibits a limited ability to remove OG

opposite G, and no activity toward OG in an OG:A base pair. The crystal structure of the inactive K249Q hOgg1 enzyme bound to an OG:C-containing duplex shows that the enzyme has a binding pocket specific for the extrahelical OG, as well as amino acids that make specific hydrogen bond contacts to the orphaned cytosine (*63*). The ability of Fpg to readily remove OG opposite G and T as well as C suggests that the recognition of the opposite base by Fpg may occur in a more indirect fashion. Indeed, less efficient recognition of the OG:A base pair by Fpg has been suggested to be a result of the lack of specific recognition elements of OG in the correct DNA groove due to the syn conformation of OG in an OG:A base pair (*57*). Specifically, the OG(syn):A(anti) conformation of the base pair places the 8-oxo group of the OG in the DNA minor groove rather than in the major groove as in the OG(anti):C(anti) base pair.

A fascinating feature of Fpg is that it has been shown to remove a wide variety of substrates resulting from oxidative reactions (*18*). This also suggests that the damage recognition properties of Fpg may be significantly different from those of other BER enzymes. The results herein show that the OG oxidation products Gh and Sp are also removed opposite C, T, and G by Fpg with rate constants that are only slightly lower than those measured for OG. The ability of Fpg to retain the ability to recognize and remove Gh and Sp may be due to the fact that these substrates retain the appropriate functionalities of the "8-oxo" and "N(7)H" group of OG. Indeed, the crystal structure of K249Q hOgg1 bound to OG:C substrate DNA suggests the OG is recognized in part by a specific hydrogen bond between hOgg1 and the N(7)H of OG (*63*). However, the reduced efficiency of the removal of the Gh and Sp relative to OG may be due to the loss of other important functional groups for recognition and/or catalysis, such as the C6 carbonyl of OG. A particularly surprising result was that Gh and Sp are removed with almost identical efficiencies by Fpg even though their structures are strikingly different. Indeed, the Sp lesion would be expected to adopt a nonplanar structure, and result in significant DNA distortion. In contrast, the Gh lesion would be expected to be considerably less perturbing to the DNA helix due to the fact that Gh can enolize to a planar structure. Structural information about the Sp and Gh adducts within DNA duplexes should provide insight into the recognition properties of Fpg with these new lesions.

An interesting consequence of oxidation of OG to form Gh and Sp is that these oxidized forms of OG are excised 8 times more efficiently than OG when opposite adenine. The presence of an OG:A mispair in DNA results in minimal perturbation of the double-helical structure and provides for duplex stabilities rivaling that of a Watson−Crick base pair (*64*−*66*). Preliminary duplex melting experiments indicate that DNA duplexes containing a single Gh or Sp lesion opposite all of the four natural bases are less stable than the corresponding OG-containing duplexes (L. Sanderson, R. P. Hickerson, and C. J. Burrows, unpublished results). Thus, it is possible that the altered stability and possible lack of hydrogen bonding between Sp or Gh and A may result in more efficient recognition of these lesions than OG mispaired with adenine.

Though it has been known for some time that OG is readily further oxidized, such products have not gained as much notoriety as their parent lesion OG. In addition to removal of Gh and Sp shown herein, Fpg has also been shown to remove the G oxidation product oxazolone from a 15 bp duplex containing a single oxazolone:C base pair (*67*). Furthermore, qualitative studies employing long incubation times have illustrated that Fpg removes some but not all products of OG oxidation with peroxynitrite (*68*). Specifically, using duplexes containing single site-specific lesions derived from peroxynitrite, Fpg was shown to be capable of removing oxazolone and oxaluric acid products, but not cyanuric acid (*68*). Thus, the common use of Fpg as a reagent to estimate the amount of guanine oxidation and, in particular, the amount of OG may be problematic. Use of Fpg as an analytical tool requires a more detailed analysis of the types of substrates removed by Fpg and the relative efficiency of removal of these substrates.

The mutational spectra associated with oxidative damage include G:C → A:T transitions and G:C → T:A and G:C → C:G transversions, wherein the frequency for each type of mutation depends on particular agents mediating the oxidative DNA damage (*69*). The different types of DNA mutations likely result from the large number of diverse types of oxidative lesions formed, as well as the possibility of multiple base insertions opposite a specific lesion by DNA polymerases. The observation of G:C → T:A transversion mutations is usually thought to derive from the presence of OG; however, the insertion of A opposite a variety of lesions has been observed (*70*, *71*). For example, oxazolone causes misinsertion of A, and therefore may also lead to G:C → T:A mutations (*67*). In addition, the oxidation of OG to form Sp and Gh lesions may also lead to G:C → T:A transversion mutations based on observed misinsertion of A opposite Gh or Sp by Kf exo− observed in in vitro studies (*67*). Similarly, the Gh and Sp lesions would also generate G:C → C:G mutations, and such mutations cannot be ascribed to OG. Thus, it is possible that the Gh and Sp lesions are contributing to the observed mutations observed with a variety of oxidizing agents.

Another important factor that will influence the mutational spectra is the efficiency of recognition and removal of oxidized lesions from nonmutagenic and mutagenic base pairs by DNA repair enzymes. This situation may be even further exacerbated by sequence context effects (*53*, *72*). The efficient removal of Gh or Sp opposite C prior to DNA replication will prevent mutations caused by OG oxidation. In contrast, since Sp and Gh opposite both G and A are efficiently removed by Fpg, the action of Fpg on the base pairs produced with these lesions during DNA replication would secure the DNA mutation event. Thus, the action of Fpg, as well as other repair enzymes, on these oxidized OG lesions may be playing an important role in mutagenesis. In the case of OG, the MutY enzyme serves as a backup system for removing misincorporated A residues from OG:A base pairs. The presence of the oxidized OG lesions Gh and Sp may be more likely to lead to G:C → T:A mutations since the oxidation of OG enhances its removal by Fpg, and A opposite Gh or Sp is not removed by MutY. Notably, it is also possible that other repair enzymes may be involved in mediating the mutagenic potential of Gh and Sp lesions. Studies designed to address the mutagenic potential of Gh and Sp in different repair backgrounds are underway and should shed further light on these issues.

The miscoding properties of Gh and Sp and their ability to be removed by Fpg suggest that they may be important biological lesions. It will be important to investigate the processing of the Gh and Sp lesion by other *E. coli* DNA repair enzymes as well as eukaryotic DNA repair enzymes. Such studies are currently in progress. It is also exciting to speculate that oxidation of OG may serve as a protective mechanism for the cell since the Sp and Gh products serve to stall DNA polymerases, and therefore may serve as signals for recruiting the DNA repair machinery.

ACKNOWLEDGMENT

We are especially appreciative of the help of Dr. Marie-Pierre Golinelli in the purification of Fpg and Dr. Scott Williams with the initial assays of Fpg and MutY with oxidized OG duplexes. We also thank Dr. Mark Michaels for generously sharing plasmids and *E. coli* strains. The help of Dr. Elliot Rachlin with electrospray mass spectrometry analyses is also much appreciated.

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BI0017982