Distinct Repair Activities of Human 7,8-Dihydro-8-oxoguanine DNA Glycosylase and Formamidopyrimidine DNA Glycosylase for Formamidopyrimidine and 7,8-Dihydro-8-oxoguanine

(Received for publication, September 10, 1999, and in revised form, November 30, 1999)

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7,8-Dihydro-8-oxoguanine (8-oxoG) and 2,6-diamino-4-hydroxyformamidopyrimidine (Fapy) are major DNA lesions formed by reactive oxygen species and are involved in mutagenic and/or lethal events in cells. Both lesions are repaired by human 7,8-dihydro-8-oxoguanine DNA glycosylase (hOGG1) and formamidopyrimidine DNA glycosylase (Fpg) in human and Escherichia coli cells, respectively. In the present study, the repair activities of hOGG1 and Fpg were compared using defined oligonucleotides containing 8-oxoG and a methylated analog of Fapy (me-Fapy) at the same site. The $k_{\text{cat}}/K_m$ values of hOGG1 for 8-oxoG and me-Fapy were comparable, and this was also the case for Fpg. However, the $k_{\text{cat}}/K_m$ values of hOGG1 for both lesions were approximately 80-fold lower than those of Fpg. Analysis of the Schiff base intermediate by NABHI trapping implied that lower substrate affinity and slower hydrolysis of the intermediate for hOGG1 than Fpg accounted for the difference. hOGG1 and Fpg showed distinct preferences of the base opposite 8-oxoG, with the activity differences being 19.8- (hOGG1) and 12-fold (Fpg) between the most and least preferred bases. Surprisingly, such preferences were almost abolished and less than 2-fold for both enzymes when me-Fapy was a substrate, suggesting that, unlike 8-oxoG, me-Fapy is not subjected to paired base-dependent repair. The repair efficiency of me-Fapy randomly incorporated in M13 DNA varied at the sequence level, but orders of preferred and unpreferred repair sites were quite different for hOGG1 and Fpg. The distinctive activities of hOGG1 and Fpg including enzymatic parameters ($k_{\text{cat}}/K_m$), paired base, and sequence context effects may originate from the differences in the inherent architecture of the DNA binding domain and catalytic mechanism of the enzymes.

Reactive oxygen species and photosensitized oxidation of DNA generate two major guanine lesions, i.e. 7,8-dihydro-8-oxoguanine (8-oxoG) and 2,6-diamino-4-hydroxyformamidopyrimidine (Fapy). 8-oxoG is mutagenic and forms an 8-oxoG (syn-form): A mispair during DNA replication, hence leading to G:C $\rightarrow$ T:A transversions in bacterial and mammalian cells (reviewed in Ref. 2). The deoxyribonucleoside 5′-triphosphate of 8-oxoG resulting from oxidation of dGTP in the nucleotide pool is also incorporated opposite template A and induces A:T $\rightarrow$ C:G transversions via the 8-oxoG:A mispair (3–5). To avoid such genotoxic effects of 8-oxoG, cells have a unique repair mechanism, namely the GO system (6, 7). The GO system of Escherichia coli is comprised of three enzymes as follows: Fpg/ MutM, a glycosylase/lyase removing 8-oxoG from 8-oxoG:C pairs; MutY, a monofunctional glycosylase excising A from 8-oxoG:A mispairs; and MutT, a triphosphatase degrading the triphosphate of 8-oxoG to the innocuous monophosphate. Recently, human homologs of Fpg, MutY, and MutT have been cloned and designated as hOGG1/hMMH (reviewed in Ref. 8), MYH (9), and MTH1 (10), respectively. Thus, the genotoxic effects and repair enzymes of 8-oxoG have been characterized fairly well, whereas those of Fapy have been less clarified (11, 12). The major reason is a lack of the method that enables specific incorporation of the Fapy lesion into DNA or oligonucleotides.

For introduction of Fapy as a unique lesion, the use of ionizing radiation, oxidizing chemical reagents, or photooxidation is impractical since these agents are known to produce not only Fapy but also other damage (1). Until now, most studies on the genotoxicity/cytotoxicity (13, 14) and repair (15–17) of Fapy have been conducted using 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (me-Fapy), an N-methylated analog of Fapy, since me-Fapy can be introduced into DNA as a major damage (15). To introduce me-Fapy, DNA was first treated with methylyating agents followed by base open the imidazole ring. Although me-Fapy is a major product formed in this treatment, complete conversion of the target guanine to me-Fapy is practically impossible without introducing other unintended damage. The primary reason is that methylation of DNA generates not only 7-methylguanine (m7G), a precursor of me-Fapy but also O- and N-alkylated bases and methyl phosphates simultaneously (18). In addition, certain methylated purines such as 3-methyladenine (m3A) and 3-methylguanine (m3G) readily undergo depurination, thereby generating abasic sites. Therefore, certain precaution is necessary to interpret DNA glycosylase; Endo IV, endonuclease IV; m7G, 7-methylguanine; m7dGTP, 7-methyl-2′-deoxyguanosine 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; HhH, hairpin-helix-hairpin; Pol I Kf, polymerase I Klenow fragment; BSA, bovine serum albumin; AP, apurinic/apyrimidinic.

* This work was supported by grants-in-aid from the Ministry of Education, Science, and Culture of Japan (to H. I.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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The abbreviations used are: 8-oxoG, 7,8-dihydro-8-oxoguanine; Fapy, 2,6-diamino-4-hydroxyformamidopyrimidine; me-Fapy, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine; Fpg, formamidopyrimidine DNA glycosylase; hOGG1, human (yeast) 7,8-dihydro-8-oxoguanine DNA glycosylase; Endo IV, endonuclease IV; m7G, 7-methylguanine; m7dGTP, 7-methyl-2′-deoxyguanosine 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; HhH, hairpin-helix-hairpin; Pol I Kf, polymerase I Klenow fragment; BSA, bovine serum albumin; AP, apurinic/apyrimidinic.

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the results obtained with DNA substrates prepared by the conventional method.

Repair enzymes that recognize 8-oxoG also act on Fapy and me-Fapy and excise them from DNA. *Saccharomyces cerevisiae* Ntg1/OGG2 protein was originally reported to recognize me-Fapy but not 8-oxoG (19, 20). However, the recent studies on Ntg1/OGG2 have suggested that both lesions are substrates, and the activity for 8-oxoG varies depending on the paired base (21, 22). Therefore, 8-oxoG DNA glycosylases from bacteria (Fpg), yeast (OGG1 and Ntg1/OGG2), and human (hOGG1/hMMH) so far identified recognize me-Fapy as well. Among these enzymes, OGG1 and Fpg are functionally similar enzymes, and cellular repair activity for 8-oxoG and me-Fapy primarily relies on these enzymes. Despite such a functional similarity, their primary amino acid sequences are quite different. Moreover, OGG1, but not Fpg, is a member of endonuclease III superfamily that contains a hairpin-helix-hairpin (HhH)-GPD motif (reviewed in Ref. 8). For hOGG1, Lys-249 and Asp-268 in the HhH-GPD motif are likely to be involved in the glycosylase/AP (apurinic/apyrimidinic)-lyase activity (23), whereas for Fpg, a proline residue in the N-terminal region is responsible for it (24). Therefore, it is still equivocal how these structurally unrelated proteins can recognize and catalyze excision of both 8-oxoG and me-Fapy. Such a mechanistic question can be best addressed by comparing the intrinsic activities of OGG1 and Fpg for 8-oxoG and me-Fapy using common substrates.

In the present work, we have prepared oligonucleotide and DNA substrates containing me-Fapy as unique lesions. The oligonucleotide substrate was tested for hOGG1 and Fpg, and the enzymatic parameters, influences of the paired base, and reaction mechanisms were compared with those for 8-oxoG embedded in the same site. Furthermore, the effects of surrounding sequence contexts on the recognition of me-Fapy by the two enzymes were compared using M13 DNA containing randomly distributed me-Fapy lesions.

**EXPERIMENTAL PROCEDURES**

**Enzymes**—*E. coli* DNA polymerase I Klenow fragment (Pol I Kf) and T4 polynucleotide kinase were purchased from Life Technologies, Inc., and New England Biolabs, respectively. Formamidopyrimidine DNA glycosylase (Fpg) and endonuclease IV (Endo IV) were overexpressed in *E. coli* cells harboring plasmids containing the fpg or nfo gene (gifts from S. S. Wallace and Z. Hatahet, University of Vermont) and purified as described (25). Human 8-oxo-guanine glycosylase (hOGG1/MutM homolog (hMMH)) was purified as described (26–28). Preliminary studies with the protein prepared by the method of Roldan-Arjona et al. (26) or Abrutani et al. (27). The results presented in this paper were obtained with hOGG1/hMMH (type 1a isoform) prepared by the method of Monden et al. (28). The purified hOGG1/hMMH contained 5 additional N-terminal amino acid residues (GPLGS) derived from glutathione S-transferase. The repair enzymes used in this study (Fpg, Endo IV, and hOGG1) were apparently homogeneous in SDS-PAGE analysis. Liquid chromatography-mass spectrometry (LC-MS) analysis showed that the purity of hOGG1/hMMH was over 99%.

**Oligonucleotides and DNA**—Oligonucleotides used in this study are listed in Table I. Oligonucleotides 15PRM, 25PRM, 25COM-N (N = A, G, C, T), 30COM-C, 25G, and 25OX containing 8-oxoG were synthesized by the phosphoramidite method and purified by reversed phase high pressure liquid chromatography. The duplex substrate 25MG/25COM-C containing a single 7-methylguanine (m7G) lesion at the specific position was prepared by the DNA polymerase reaction using 7-methyl-2'-deoxyguanosine 5'-triphosphate (m7dGTP) as a substrate. m7dGTP was previously shown to serve as a substrate for Sequanase (T7 DNA polymerase deficient in 3'-5' exonuclease activity) (29). 15PRM was an end-labeled with [γ-32P]ATP (110 Tbp/monomer units, Pharmacia Biotech) and T4 polynucleotide kinase and purified as described (30). 15PRM annealed to the template/primer DNA (25 pmol as template/ primer) in buffer A (200 mM) was extended by Pol I Kf (25 units) in the presence of dATP, dCTP (both 20 mM), and m7dGTP (200 μM, Sigma) at 25 °C for 40 min. The composition of buffer A was 66 mM Tris-HCl (pH 7.5), 1.5 mM mercaptoethanol, and 6.6 mM MgCl2. The reaction was terminated by the addition of EDTA (final concentration 50 mM). The control duplex substrate 25G/25COM-G containing G at the same position of m7G was also prepared by a similar DNA polymerase reaction, except that dGTP (20 μM) was used instead of m7dGTP. The reaction mixture was extracted with phenol, and DNA was recovered by ethanol precipitation. DNA was resuspended in water, purified by a Sephacryl G-20 column, and finally recovered by ethanol precipitation. The duplex substrate 25FP/25COM-C containing a me-Fapy lesion was prepared by the alkali treatment of 25MG/25COM-C. 25MG/25COM-C in a microdialysis cup was dia lyzed against 20 mM phosphate buffer (pH 11.4) containing 2 mM EDTA at room temperature for 10 h, and then against 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA at room temperature for 12 h, and finally against the same buffer at 4 °C for 12 h. Duplex substrates containing me-Fapy paired with four different bases (A, G, C, and T) were prepared as follows. 25MG/30COM-C was prepared by the DNA polymerase reaction using 32P-labeled 15PRM (primer) and 30COM-C (template) as described for 25MG/25COM-C and converted to 25FP/30COM-C by the alkali treatment. Complete conversion of m7G to me-Fapy in 25MG was essential, otherwise re-matching m7G underwent depuration during the subsequent purification step. 25FP/30COM-C was heat-denatured at 50 °C in gel loading buffer and separated by 16% PAGE. The use of 30COM-C in place of 25COM-C as a template facilitated separation of 25FP and the template. The band corresponding to 25FP was detected by autoradiography and excised from the gel. 25FP in the crashed gel was extracted by 500 mM ammonium acetate and 10 mM magnesium acetate, purified by a Sep-Pak cartridge, and finally annealed to appropriate complementary strands (25COM-A, -G, -C, -T). These substrates were resistant to Endo IV, indicating that contamination of oligonucleotides containing abasic sites was negligible (data not shown). The substrates containing 8-oxoG paired with four different bases were prepared by annealing 25OX to 25COM-A, -G, -C, -T.

Duplex M13 DNA containing randomly distributed m7G was prepared using the method similar to oligonucleotides with slight modifications. Single-stranded M13mp18 DNA (5 μg, ~2 pmol) was primed with 20PRM (5'-end-labeled, 4 pmol) and replicated by Pol I Kf (5 units) in buffer A (400 μl, MgCl2 was replaced by 0.5 mM MnCl2) by the presence of dATP, dCTP, dGTP (all 50 μM), dTP (10 μM), and m7dGTP (50 μM). m7dGTP was incorporated into DNA more uniformly in the presence of Mn2+ than Mg2+. The reaction was continued at 37 °C for 30 min. Purification of DNA and the alkali treatment to convert m7G to me-Fapy was performed as described for 25FP.

**Reaction with Repair Enzymes**—To follow the conversion of m7G to me-Fapy, untreated and alkali-treated 25MG/25COM-C (5 nm) were incubated with Fpg (100 ng) in buffer B (10 μl) at 37 °C for 30 min. The composition of buffer B was 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, and 0.1 mg/ml BSA. To reveal abasic sites potentially formed during substrate preparation, untreated and alkali-treated 25MG/ 25COM were also treated with Endo IV. Untreated and alkali-treated 25MG/25COM-C (5 nm) in buffer C (10 μl) was incubated with Endo IV (8 ng) at 37 °C for 30 min. The composition of buffer C was 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and a 50 mM NaCl.

**Enzymatic parameters of Fpg and hOGG1 for 8-oxoG and me-Fapy** were determined as follows. 25OX/25COM-C and 25FP/25COM-C were incubated with Fpg (0.5 ng) in buffer B (10 μl) at 37 °C for 3 min. For

### Table I

| Abbreviation | Sequence* |
|--------------|-----------|
| 15PRM | 5'-CATGATGATCCTCTCAGAGGGAAGAGAG |
| 25COM-A | 3'-GTAGCCTAGTCTAGGAGGAGAGAGAGAG |
| 25COM-G | 3'-GTAGCCTAGTCTAGGAGGAGAGAGAGAG |
| 25COM-C | 3'-GTAGCCTAGTCTAGGAGGAGAGAGAGAG |
| 25COM-T | 3'-GTAGCCTAGTCTAGGAGGAGAGAGAGAG |
| 30COM-C | 3'-AACTCGTACATGATCGAGGAGAGAGAG |
| 25G | 5'-CATGATGATCCTCTCAGAGGGAAGAGAG |
| 25MG | 5'-CATGATGATCCTCTCAGAGGGAAGAGAG |
| 25FP | 5'-CATGATGATCCTCTCAGAGGGAAGAGAG |
| 25OX | 5'-CATGATGATCCTCTCAGAGGGAAGAGAG |
| 20PRM | 5'-CTGACCACTTGGAAAGGAG |

* M, 7-methylguanine (m7G); F, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (me-Fapy); O, 7,8-dihydro-8-oxoguanine (8-oxoG).

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2 K. Asagoshi, H. Terato, Y. Ohyama, and H. Ide, unpublished results.
hOGG1, the substrates were incubated with hOGG1 (6 ng) in buffer D (10 μl) at 37 °C for 15 min. The composition of buffer D was 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 50 mM KCl, and 0.1 mg/ml BSA. The concentration ranges of the substrate used in the experiments were indicated in the figures. Parameters (V_{max} and K_{m}) were evaluated by a hyperbolic curve-fitting program. The activity of Fpg and hOGG1 to me-Fapy and 8-oxoG paired with four different bases was determined by an essentially similar manner. The substrates (25FP/25COM-C, 25OX/25COM-C) were treated with Fpg (2 ng) for 5 min or hOGG1 (6 ng) for 15 min at 37 °C.

M13 DNA containing me-Fapy (0.25 μg, ~0.1 pmol) was incubated with Fpg (5–20 ng) in buffer B (10 μl) or hOGG1 (50–500 ng) in buffer D (10 μl) at 37 °C for 30 min (Fpg) or 60 min (hOGG1). Reaction products were analyzed by PAGE as described below.

Heat and Piperidine Treatments—For chemical characterization of mG introduced into substrates, 25MG/25COM-C was heated in water at 90 °C for 1 h to induce depurination of mG and strand scission. Alternatively, substrates containing mG or me-Fapy were treated with 10% piperidine at 90 °C for 1 h. Piperidine was removed by repeated evaporation with water, and the sample was resuspended in gel loading buffer.

Product Analysis—After enzymatic or chemical reactions, the samples were mixed with gel loading buffer (0.05% xylene cyanol, 0.05% bromphenol blue, 20 mM EDTA, and 98% formamide), heated at 90 °C for 5 min, and separated by 8% (M13 DNA) or 16% (oligonucleotides) denaturing polyacrylamide gel electrophoresis. Harsh heat denaturation at high temperatures (i.e., boiling) was avoided to prevent break down of DNA containing the heat-labile lesions. The gel was autoradiographed at ~80 °C overnight. Alternatively, the radioactivity of the separated bands was analyzed by Fuji BAS 2000.

Cross-link Reaction with NaBH4—Cross-link reactions between repair enzymes (Fpg or hOGG1) and substrates (25G/25COM-C, 25MG/25COM-C, 25OX/25COM-C, 25FP/25COM-C) were performed as follows. An aqueous solution of NaBH4 (600 nm, 1 μl) was added to 8 μl of buffer B (Fpg) or D (hOGG1) containing the substrate (final concentration 2–40 nm). In NaBH4 reactions, the salts (NaCl and KCl) and BSA were omitted in buffer B and D. Immediately after, the solution was mixed with Fpg (1.5 ng, 1 μl) or hOGG1 (19.5 ng, 1 μl), and incubation was continued at 37 °C for 30 min. The reaction solution was mixed with an equal volume of SDS-loading buffer (100 mM Tris-HCl (pH 6.8), 8% SDS, 24% (v/v) glycerol, 4% 2-mercaptoethanol, 0.02% SERVA Blue G) and heat-denatured. The sample was electrophoresed on a 10% SDS-polyacrylamide gel. Autoradiography and quantitation of the radioactive activity were performed as described above. To analyze Schiff base intermediates with- out NaBH4 reduction, 25OX/25COM-C (5 nm) was incubated with Fpg and hOGG1 (both 4, 15, 45 ng) in buffer B (Fpg) or D (hOGG1) at 37 °C for 5 min. The sample was subjected to SDS-PAGE as described above.

RESULTS

Preparation and Characterization of Substrates—The presence of mG in 25MG prepared by the DNA polymerase reaction was confirmed by the heat or piperidine treatment. In the heat treatment, 25MG/25COM-C and control 25G/25COM-C (prepared by the same polymerase reaction) was heated at 90 °C for 1 h, and products were analyzed by PAGE (Fig. 1). The mG with a heat-labile N-glycosidic bond underwent depurination, and the resulting abasic site was totally cleaved (lane 6). By comparison with the gel mobility of 15PRM (lane 1), the products were identified as β- (minor bands) and δ-elimination (major band) products. In the hot piperidine treatment, 25MG was totally converted to the δ-elimination product migrating slightly faster than 15PRM (lane 7). The complete conversion of 25MG to the β- and δ-elimination products indicated that mG was quantitatively incorporated in the designed position of 25MG. 25G similarly prepared by Pol I Kf was also cleaved under the δ-elimination condition (lanes 3 and 4).

25FP/25COM-C containing me-Fapy was prepared by alkali treatment (pH 11.4) of 25MG/25COM-C. To follow the alkali-catalyzed ring opening reaction of mG, oligonucleotides before and after the alkali treatment were analyzed with Fpg that specifically recognizes me-Fapy. Before the treatment, 25MG (paired with 25COM-C) was not cleaved by Fpg (Fig. 2A, lane 3) but was almost quantitatively converted to the δ-elimination product after the alkali treatment for 10 h (lane 6). These results indicate quantitative conversion of mG to me-Fapy by the alkali treatment. Partial depurination of mG (yielding abasic sites) or fission of the imidazole ring (yielding me-Fapy) in untreated 25MG was ruled out by the resistance to Endo IV (lane 1). 25FP/25COM-C was treated by alkali for 10 h was also resistant to Endo IV (lane 7), showing the absence of abasic sites in this substrate. Fig. 2B shows the time course of the conversion of mG in 25MG to me-Fapy during the alkali treatment. The percent of me-Fapy in the oligonucleotide was determined by quantifying the nicked and unnicked products by Fpg. The proportion of me-Fapy increased with the incubation time with alkali and reached a plateau (~96%) around 10 h. Thus, me-Fapy lesion was specifically introduced into DNA by the present method.

FIG. 1. Characterization of mG in 25MG, 25G and 25MG containing G and mG, respectively, at the same position were synthesized by DNA polymerase reactions using 15PRM (5′-end labeled)25COM-C (Table I) as primer/template. Purified 25G and 25MG (as duplexes with 25COM-C) were treated by heat (90 °C, 1 h) or 10% piperidine (90 °C, 1 h) and analyzed by denaturing PAGE. Lane 1, 15PRM (marker); lane 2, untreated 25G; lane 3, heat-treated 25G; lane 4, piperidine-treated 25G; lane 5, untreated 25MG; lane 6, heat-treated 25MG; lane 7, piperidine-treated 25MG. β- and δ-elimination products are indicated by arrows.
ination products bearing different 3’-terminal deoxyribose modifications.

To obtain kinetic parameters, the initial velocities of the reaction (V) were determined at varying substrate concentrations (S). The S-V plots for Fpg and hOGG1 are shown in Fig. 4. The enzymatic parameters are summarized in Table II.

NADH, Trapping of the Reaction Intermediates—hOGG1 and Fpg are known to form transient imine intermediates (Schiff base) in the reaction with substrates containing 8-oxoG (reviewed in Ref. 8). Reduction of the imine bond by NaBH₄ leads to formation of irreversibly cross-linked complexes between the enzyme and substrate. To verify the proposed mechanism further, NaBH₄-trapping experiments were performed using the substrate containing me-Fapy. 25FP/25COM-C and 25OX/25COM-C were treated with Fpg or hOGG1 and analyzed by denaturing PAGE. The products formed by Fpg (lanes 8 and 9) or hOGG1 (lanes 10 and 11) were further treated by Endo IV (lanes 12–14) to analyze the nature of 3’-termini. The substrate and combination of the enzyme are indicated on the top of the gel. The β- and δ-elimination products and that carrying 3’-OH are indicated by arrows.

FIG. 2. Conversion of m7G in 25MG to me-Fapy by alkali treatment. A. 25MG (5′-end labeled)/25COM-C was treated by alkali for 10 h as described under “Experimental Procedures.” To reveal specifically me-Fapy and abasic sites, untreated and alkali-treated 25MG/25COM-C were digested by Fpg (lanes 3 and 6) or Endo IV (lanes 4 and 7) and analyzed by denaturing PAGE. The treatment without (0 h) and with alkali (10 h) and enzymes used for digestion are indicated on the top of the gel. Lane 1, 15PRM (marker); lanes 2 and 5, untreated and alkali-treated 25MG/25COM-C without enzyme digestion, respectively. The products carrying OH and phosphate groups at the 3’ termini are indicated by arrows with 3’-OH and δ, respectively. B, time course of the conversion of m7G in 25MG to me-Fapy. 25MG/25COM-C was treated by alkali for the indicated time. The fraction (%) of me-Fapy converted from m7G in 25MG was determined by exhaustive Fpg digestion and plotted against the incubation time.

FIG. 3. Products formed by Fpg and hOGG1. 25G/25COM-C for control (lanes 2–4), 25OX/25COM-C containing 8-oxoG (lanes 5–7), and 25FP/25COM-C containing me-Fapy (lanes 8–10) were incubated with Fpg or hOGG1 and analyzed by denaturing PAGE. The enzymes used are indicated by plus signs on the top of the gel. Lane 1, 15PRM (marker); lanes 5 and 8, 25OX/25COM-C and 25FP/25COM-C without enzyme treatment, respectively. The products formed by Fpg (lanes 6 and 9) or hOGG1 (lanes 7 and 10) were further treated by Endo IV (lanes 11–14) to analyze the nature of 3’-termini. The substrate and combination of the enzyme are indicated on the top of the gel. The β- and δ-elimination products and that carrying 3’-OH are indicated by arrows.

FIG. 4. S-V plots for evaluation of enzymatic parameters. The indicated concentrations of 25OX/25COM-C and 25FP/25COM-C were incubated with Fpg (0.5 ng) or hOGG1 (6 ng) at 37 °C for 3 min (Fpg) or 15 min (hOGG1). The products were separated by PAGE and quantified by Fuji BAS 2000 as described under “Experimental Procedures.” The initial velocity of the reaction (V) was plotted against the substrate concentration ([S]). The combination of the enzyme and damage is Fpg/8-oxoG (A), Fpg/me-Fapy (B), hOGG1/8-oxoG (C), and hOGG1/me-Fapy (D). The data points were based on 3 independent experiments. Standard deviations are shown by error bars.
were incubated with Fpg or hOGG1 in the presence of NaBH4.

lanes 9

lanes 25COM-C

lanes 3

25OX/25COM-C

25G/25COM-C

25OX/25COM-C

25FP/25COM-C

25OX containing 8-oxoG (Fig. 5, lane 4).

Cross-linked complexes between 25FP and Fpg (Fig. 5, lane 10) were migrated faster than that for hOGG1 (39 kDa) due to the molecular mass difference of the cross-linked enzymes. Similar upper shifted bands were also observed for 25OX containing 8-oxoG (Fig. 5, lanes 6 and 7) but not for intact 25G (Fig. 5, lanes 3 and 4), indicating that the Schiff base formation was specific to the lesioned substrates (25FP and 25OX). The amount of cross-linked products was determined at varying substrate concentrations to obtain parameters for the Schiff base formation. The correlation between the concentrations of the substrate and enzyme used are shown at the top. Cross-linked complexes are indicated by arrows.

Cross-linked complexes between 25FP and Fpg (Fig. 5, lane 9) or hOGG1 (Fig. 5, lane 10). The complex for Fpg (molecular mass = 30 kDa) migrated faster than that for hOGG1 (39 kDa) due to the molecular mass difference of the cross-linked enzymes. Similar upper shifted bands were also observed for 25OX containing 8-oxoG (Fig. 5, lanes 6 and 7) but not for intact 25G (Fig. 5, lanes 3 and 4), indicating that the Schiff base formation was specific to the lesioned substrates (25FP and 25OX).

The amount of cross-linked products was determined at varying substrate concentrations to obtain parameters for the Schiff base formation. The correlation between the concentrations of substrate (S) and trapped Schiff base (SB) is shown in Fig. 6. For both 8-oxoG and me-Fapy, the amount of cross-linked products increased with the substrate concentration and reached plateaus. By applying simple Michaelis-Menten analysis to the data, approximate dissociation constants (Kd) were evaluated (Table III). For more detailed analysis of the data, see “Discussion.”

**Table II**

| Enzyme/substrate | Kd | Vmax | km | kcat/Km |
|------------------|----|------|----|---------|
| Fpg              |    |      |    |         |
| 8-OxoG           | 13 | 2.7  | 0.85| 0.14    |
| Me-Fapy          | 38 | 7.7  | 5.1 | 0.13    |
| hOGG1*           |    |      |    |         |
| 8-OxoG           | 23 | 0.51 | 0.034| 0.0015  |
| Me-Fapy          | 15 | 0.38 | 0.025| 0.0017  |

a Average of three independent experiments with standard deviations.

b Numbers in parentheses are relative values.

c Concentrations of Fpg and hOGG1 were 1.5 and 15 nM, respectively.

d Concentrations of Fpg and hOGG1 were 5 and 50 nM, respectively.

was a good substrate and excised 12-fold more efficiently than the 8-oxoG:A pair (Fig. 7A). Surprisingly, such a substrate preference was almost abolished for me-Fapy. The activity of Fpg for me-Fapy was virtually independent of the paired base and comparable to those for 8-oxoG paired with G, C, T. The activity difference was at most 1.7-fold between the most (C) and least (A) preferred bases. hOGG1 recognized 8-oxoG paired with C most efficiently and then T (Fig. 7B). Those paired with purines (A and G) were poor substrates. The relative activities for 8-oxoG paired with C, T, G, A were 19.8:4.3:1.5:1, respectively. These results are consistent with those reported for human (26, 27, 36) and yeast (37) OGG1. Although hOGG1 showed some preference for me-Fapy:C, the paired base effect was much less obvious than that for 8-oxoG. The activity difference was only ~2-fold between the most (C) and least (A) preferred bases.

**Table III**

| Enzyme/substrate | Kd | [SB]max | kSB | kSB/Kd |
|------------------|----|---------|-----|--------|
| Fpg              |    | 0.2     | 0.04| 0.04 (10) |
| 8-OxoG           | 1  | 0.2     | 0.04| 0.02 (7)  |
| Me-Fapy          | 2  | 0.2     | 0.04| 0.004 (1) |
| hOGG1*           |    | 0.2     | 0.04| 0.003 (1) |

a Calculated from the data in Fig. 6 by applying Michaelis-Menten kinetics.

b Maximum concentration of the Schiff base intermediate trapped by NaBH4.

c kSB = [SB]/[E], where [E] is the concentration of Fpg or hOGG1.

d Numbers in parentheses are relative values.

e Concentrations of Fpg and hOGG1 were 1.5 and 15 nM, respectively.

Sequence Context Effects on the Repair Efficiency—Duplex M13 DNA containing randomly distributed me-Fapy was treated with Fpg and hOGG1, and the repair efficiency of individual sites was analyzed by PAGE (Fig. 8). The elimination products formed by Fpg (lanes 3–5) migrated slightly faster than the corresponding elimination products by...
coli Fpg (34, 35, 38, 39) and eukaryotic OGG1 (17, 26, 27, 36) for addition, some conflicting data were obtained in these studies and 17%) were converted to me-Fapy in the substrates. In me-Fapy (34, 38). However, minor portions of the target G (11 min (B) at 37 °C, and the amount of incised products was determined by FAGE analysis. The percentages of incised products were plotted against the base pairs. The data were averages of three independent experiments. Standard deviations are shown by error bars.

hOGG1 (lanes 6–9) in the electrophoresis. me-Fapy lesions in M13 DNA were not equally removed so that the band intensity varied significantly depending on the site. The variation of the band intensity was also observed with the enzymes. For quantitative analysis, the gel was run for a longer time than shown in Fig. 8 to achieve better band separation (particularly for G doublets). The intensity of the individual band formed by 10 ng of Fpg (Fig. 8, lane 4) or 200 ng of hOGG1 (Fig. 8, lane 8) was divided by that of the corresponding piperidine-generated band (Fig. 8, lane 2). Since me-Fapy sites are quantitatively cleaved by the piperidine treatment, the intensity of the piperidine-generated band represents the actual lesion frequency of the individual site. This calculation corrects the site-dependent variation of the lesion frequency in the substrate DNA. It is also noted that the amount of nicked products increased with the increasing amount of Fpg (Fig. 8, lanes 3–5) and hOGG1 (Fig. 8, lanes 6–9), indicating that the reaction conditions were within a dynamic (i.e. not saturating) range. The corrected repair efficiency for up to the 20th position of G is summarized in Table IV together with the surrounding sequences. To compare the repair efficiency of the individual site by Fpg and hOGG1, a normalized repair efficiency ( nicked %/sum of nicked % for positions 1–20) was calculated from the data in Table IV and plotted against the sequence (Fig. 9). The normalized repair efficiency represents the distribution of the repair event if the two enzymes remove the same amount of me-Fapy as a whole from the region of interest (i.e. positions 1–20). According to this analysis, me-Fapy at positions 5, 8, 10, 11, 12, 15, and 19 was excised by Fpg and hOGG1 with comparable efficiencies, and that at 1, 2, 6, 14, 17, 18, and 20 was preferred by Fpg, and that at 3, 4, 7, 9, 13, and 16 was preferred by hOGG1.

**DISCUSSION**

**Reaction Kinetics of hOGG1 and Fpg**—The activities of E. coli Fpg (34, 35, 38, 39) and eukaryotic OGG1 (17, 26, 27, 36) for 8-oxoG and me-Fapy have been studied previously. To our knowledge, two comparative studies have been performed using oligonucleotide substrates containing a single 8-oxoG or me-Fapy (34, 38). However, minor portions of the target G (11 and 17%) were converted to me-Fapy in the substrates. In addition, some conflicting data were obtained in these studies with respect to the reaction efficiency ($k_{\text{cat}}/K_m$) of Fpg to 8-oxoG and me-Fapy. One study indicates the ratio of the reaction efficiency for 8-oxoG versus me-Fapy was 1.4:1 and the other 15.8:1. The discrepancy mainly originated from the difference in $k_{\text{cat}}$ for these lesions. The present results (Table II) have shown that Fpg has a roughly 3-fold higher affinity for 8-oxoG ($K_m = 13$ nM) than me-Fapy ($38$ nM). However, with respect to $k_{\text{cat}}$, me-Fapy ($k_{\text{cat}} = 5.1$ min$^{-1}$) is preferred to 8-oxoG ($k_{\text{cat}} = 1.8$ min$^{-1}$). Consequently, the opposite effects of the two parameters resulted in comparable $k_{\text{cat}}/K_m$ values, indicating similar reaction efficiencies to 8-oxoG and me-Fapy. In contrast to Fpg, the activities of eukaryotic OGG1 to me-Fapy and 8-oxoG have not been compared on the basis of the kinetic parameters using defined substrates. The parameters determined in the present study (Table II) indicate that hOGG1 has comparable affinities and reaction efficiencies for me-Fapy than 8-oxoG (both paired with C). The substrate preference ($k_{\text{cat}}/K_m$) of hOGG1 (8-oxoG versus me-Fapy = 0.9:1, Table II) is notably different from that obtained for yOGG1 (8-oxoG versus me-Fapy = 12.2:1) (17). The comparison for yOGG1 was made by measuring 8-oxoG and me-Fapy glycosylase activities (not...
kinetic parameters but the amount released products). In addition, the substrates were methylene blue/light-treated calf thymus DNA (8-oxoG) and conventionally prepared me-Fapy-containing poly(dG-dC), which may have contained other adducts interfering the analysis.

With respect to the reaction kinetics of Fpg and hOGG1, two sets of parameters were obtained from steady state reactions (Table II) and NaBH₄ cross-link reactions (Table III), although the latter data were approximate values due to the limited number of experimental points (Fig. 6). The parameters in Table II are for the overall repair reaction, which includes (i) association/dissociation of the enzyme (E) and substrate (S); (ii) formation of the Schiff base (ESₜₜ) between the enzyme and substrate resulting in release of the damaged base and strand cleavage; (iii) hydrolysis of the imine bond between the enzyme and DNA; and (iv) dissociation of the enzyme and product (P) (Fig. 10). The data in Table III represent dissected parameters for the events before hydrolysis of the imine bond, i.e. steps i and ii. The parameters in Table III were calculated by applying simple Michaelis-Menten analysis to steps i and ii. In the calculation, it was assumed that approximately the same proportion of Fpg and hOGG1 was inactivated during the trapping reaction and that the Schiff base intermediate was quantitatively converted to the stable cross-linked product by NaBH₄. The latter was confirmed by the absence of nicked products in the NaBH₄-trapping reactions (see Fig. 5). According to the dissociation constant (Kₐₑₛ) in Table III, the intrinsic affinity of Fpg for the substrates was severalfold greater than that of hOGG1, which was in contrast to the steady state parameters showing less obvious preferences between Fpg and hOGG1 (Kₑₛ in Table II). Fpg has much higher kₐₑₛ and kₑₛ/Kₑₛ than hOGG1 for the entire reaction (Table II). However, if the reaction is dissected, the relative rate constants for the Schiff base formation (kₑₛ) were essentially similar for Fpg and hOGG1 (note that kₑₛ should be read as relative values since the effective concentration of the enzymes in the trapping reaction was not known). Accordingly, the reactions before hydrolysis of the Schiff base (kₑₛ/Kₑₛ for Fpg versus hOGG1 = 10:1 (8-oxoG) or 7:1 (me-Fapy)) account for only a part of the difference in the overall reaction efficiency between Fpg and hOGG1 (kₑₛ/Kₑₛ for Fpg versus hOGG1 = 80:1). This suggests that hydrolysis of the imine bond and/or subsequent dissociation of hOGG1 proceeds more slowly (~10-fold) than that of Fpg. The slow hydrolysis of

### Table IV

| Position (nicked %) | Sequence (5'–3')     | Position (nicked %) | Sequence (5'–3')     |
|---------------------|-----------------------|---------------------|-----------------------|
| 1 (63.8)            | GGACA bật GAA        | 9 (91.1)            | CAGGCF CATAG          |
| 20 (52.6)           | AACCGF TATT          | 13 (88.6)           | GGCCTG TGCAC          |
| 17 (40.2)           | ATCCFF ACAG          | 8 (65.2)            | ACCAGF CGCAT          |
| 8 (38.4)            | ACCAGF CGCAT         | 11 (63.5)           | CATAGF CGTGAC         |
| 18 (33.6)           | GCCAFA ACGG          | 16 (62.9)           | CAGAGFT AATC          |
| 13 (32.5)           | GGGCFT CGTC          | 15 (55.2)           | ATCAAF GATTA          |
| 14 (30.0)           | TGGCTF ACCT          | 4 (48.1)            | GAAAGF GTAC           |
| 15 (27.7)           | ATCAAF GATTA         | 12 (42.4)           | AGGGCF GTGG           |
| 11 (27.6)           | CATAGF GGGG          | 3 (36.8)            | TGACCFT GTACG         |
| 12 (22.3)           | AGGCTF CCTGA         | 1 (35.8)            | GACAGFT GAGA          |
| 9 (21.8)            | CAGGCF CATAG         | 7 (35.7)            | GACCGF GCGG           |
| 19 (19.4)           | GAAAGF GATA          | 20 (33.7)           | ACCAGF TATT           |
| 2 (16.9)            | CAGATF ACGG          | 19 (27.4)           | GACCGF GATAT          |
| 4 (14.9)            | GACGTF GAGC          | 5 (26.0)            | AGGGCF TACG           |
| 7 (13.4)            | GCCAFA CGGCA         | 17 (25.3)           | ATCCFF ACAACG         |
| 3 (13.0)            | TGAGCF GTGA          | 10 (24.0)           | GCATAGF GTGG           |
| 5 (12.9)            | ACGGFT TACAG         | 14 (21.8)           | TGACCFT ACCT          |
| 6 (12.8)            | GTAACF ACGC          | 18 (13.0)           | GACAAFAACCG           |
| 10 (10.4)           | GCATAF GCTGG         | 6 (8.9)             | GTCAAF ACGG           |
| 16 (9.7)            | CAAGAFT AATC         | 2 (5.3)             | CAGATF ACGG           |

**Fig. 9. Normalized sequence context effects on the excision of me-Fapy.** The normal repair efficiency (nicked %/sum of nicked % for positions 1–20) were calculated for each site from the data in Table IV and plotted against the sequence. The sequence and damage positions (1–20) are shown below the graph. Open bar, Fpg; closed bar, hOGG1.

**Fig. 10. Reaction scheme for NaBH₄ trapping of the Schiff base intermediates.** E, enzyme; S, substrate; ES, enzyme-substrate complex before Schiff base formation; ES₁ₜₜ, Schiff base intermediate formed between enzyme and substrate; P, product.
Differential Damage Recognition by hOGG1 and Fpg

The imine bond of hOGG1 relative to Fpg was further supported by direct SDS-PAGE analysis of the reaction mixture without the NaBH₄ treatment (Fig. 11). In the SDS-PAGE analysis, a faint band migrating slower than the free substrate (25OX) was present with 45 ng of hOGG1 (lanes 4 and 6). The mobility of the band was comparable to the cross-linked product formed by NaBH₄ (lanes 1 and 3). Such a band indicates the Schiff base intermediate was not present when the same amount of Fpg was incubated in the absence of NaBH₄ (lanes 1 and 3). Therefore, these results are consistent with the prediction based on the kinetic parameter analysis. Consequently, the low reaction efficiency of hOGG1 relative to Fpg is likely due to the combined outcome of the low affinity to the substrate (Kₐ) and slow hydrolysis of the Schiff base intermediate.

Distinct Paired-base Effects on the Repair of 8-oxoG and me-Fapy—Although Fpg and hOGG1 recognize 8-oxoG and me-Fapy, they have distinct preferences for paired bases when acting on 8-oxoG. Fpg preferentially acts on 8-oxoG paired with G, C, T, but not A (34, 35), whereas human (27, 36) and yeast (37) OGG1 prefers 8-oxoG paired with pyrimidines (particularly C) over purines with an order of C > T > G, A. These preferences were also confirmed in the present study (Fig. 7).

Interestingly, the stringent paired base preferences of Fpg and hOGG1 were markedly relieved for me-Fapy (Fig. 7). me-Fapy in DNA constitutes a strong block to DNA replication but not a premutagenic lesion (13, 14). These data suggest that template-Fpg does not direct incorporation of any nucleotides opposite the lesion, hence arresting DNA replication. Therefore, me-Fapy (and probably its analog Fapy as well) is likely to exist exclusively as a me-Fapy (Fapy):C pair derived from a G:C pair in cells. Thus, unlike 8-oxoG potentially existing either as an 8-oxoG:C or an 8-oxoG:A pair in cells (6, 7), Fpg and hOGG1 may not need to discern the base opposite me-Fapy (and Fapy) strictly since only me-Fapy (Fapy):C is actually subjected to repair by these enzymes. Granting that this is the case, mechanistic questions still remain as to how the base opposite 8-oxoG and me-Fapy differentially affects the damage recognition by Fpg and so does by hOGG1. The situation is further complicated by their distinct activities for abasic sites. With respect to the paired base effect on abasic sites, human (36) and yeast (37) OGG1 have a preference similar to that for 8-oxoG (C > T > G, A), whereas Fpg shows little preference (37). Combining the present and previous results, it follows that hOGG1 shows a stringent paired base preference for 8-oxoG and abasic sites (C > T > G, A) but not for me-Fapy, whereas Fpg shows the preference only for 8-oxoG (G, C, T > A) but not for me-Fapy and abasic sites. Despite their functional similarity, primary amino acid sequences of Fpg and hOGG1 are totally different. Fpg is a metalloenzyme with a zinc finger motif located at the C terminus and is suggested to use a proline residue in the N-terminal region for catalysis (24, 40). In contrast, hOGG1 is a member of endonuclease III superfamily containing the HhH-GPD motif and is assumed to use Lys-249 and Asp-268 in the HhH-GPD motif for catalysis (23).

Thus, the architecture of the active site accommodating lesioned DNA and amino acid residues involved in catalysis is expected to be quite different between Fpg and hOGG1. However, it remains to be seen how these differences lead to the distinct damage recognition by hOGG1 and Fpg.

Distinct Sequence Context Effects on Damage Recognition—There is heterogeneity in DNA repair, and the heterogeneity in base excision repair has been mostly related to the sequence context (Refs. 41 and 42 and references cited therein). In the present study, the repair efficiency of individual me-Fapy varied 9.7–63.8% for Fpg and 5.3–91.9% for hOGG1 (Table IV), showing sequence-dependent variations of 6.6-fold for Fpg and 17.2-fold for hOGG1. According to the reported data for Fpg, the sequence-dependent variations of the in vitro repair rate of me-Fapy (43) and 8-oxoG (44) are 2- and 33-fold, respectively. The repair rate of me-Fapy by Fpg was previously compared using conventionally prepared 12-mer substrates with only four sequence contexts. A larger variation of the repair rate of me-Fapy observed in this study (6.6-fold for Fpg) is probably attributable to the more diverse sequence samples. Fpg has been also suggested to excise efficiently me-Fapy in G-rich regions (43). According to the present result (Table IV), me-Fapy flanked by 5'-G (i.e. 5'-GF-3', F = me-Fapy) was excised more efficiently than that flanked by 3'-G (i.e. 5'-FG-3') when the relative repair efficiencies of me-Fapy in the individual G doublet were compared (positions 3/4, 7/8, 10/11, 12/13, 19/20). The influences of 5'- and 3'-flanking G were common for Fpg and hOGG1. Thus, G flanking at 5' and 3' sides exerted opposite influences on the repair of me-Fapy. Efficiently and inefficiently repaired consensus sequences for 8-oxoG have been also reported (44). In the four consensus sequences poorly recognized by Fpg, three of them contain 8-oxoG flanked by 3'-A. We compared the present data and reported sequence data. However, the me-Fapy lesions flanked by 3'-A were not necessarily poor substrates in this study (Table IV), implying the subtle interplay of the lesion structure (8-oxoG versus me-Fapy) and surrounding sequences in the Fpg-DNA interaction. This was also the case for efficiently excised sequences when the present and reported (44) data were compared. The sequence context effect on the repair rate of me-Fapy by hOGG1 was quite different from that by Fpg (Table IV). For instance, me-Fapy at position 16 was poorly removed by Fpg but efficiently by hOGG1. Conversely, me-Fapy at positions 17 and 18 was efficiently removed by Fpg but poorly by hOGG1. Thus, no correlation was observed between the orders of preferred to unpreferred sequences for Fpg and hOGG1 (correlation coefficient (r) = 0.074). Although Fpg did not show any obvious consensus sequences for efficient or inefficient repair except for the G doublets mentioned above, hOGG1 showed a preference for 5'-GC/GFC-3' (positions 8, 9, 11, and 13 in Table IV). No exception was observed for this rule. As mentioned in the previous section, Fpg and hOGG1 are functionally similar with respect to the substrate specificity but are structurally unrelated.

![Fig. 11. Analysis of the reaction intermediate formed between hOGG1 and 25OX/25COM-C in the absence of NaBH₄.](image-url)
proteins. These differences probably resulted in distinct protein-DNA contacts in the close vicinity of the lesion as well as at the lesion and served as determinants for efficient and inefficient repair.

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Distinct Repair Activities of Human 7,8-Dihydro-8-oxoguanine DNA Glycosylase and Formamidopyrimidine DNA Glycosylase for Formamidopyrimidine and 7,8-Dihydro-8-oxoguanine

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J. Biol. Chem. 2000, 275:4956-4964.
doi: 10.1074/jbc.275.7.4956

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