Substrate Specificity of Human Methylpurine DNA *N*-Glycosylase†

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ABSTRACT: The activity of human methylpurine DNA *N*-glycosylase (hMPG) for major substrates was directly compared using two types of substrates, i.e., natural DNA and synthetic oligonucleotides. By the use of ARP assay detecting abasic sites in DNA, we first investigated the activity on the natural DNA substrates containing methylpurines, ethenopurines, or hypoxanthine (Hx) prepared by the conventional methods. After the treatment with hMPG, the amount of AP sites in methylated DNA was much higher than that in DNA containing ethenopurines or Hx. The oligodeoxynucleotide having a single 7-methylguanine (7-mG) was newly synthesized in addition to 1,N^6-ethenoadenine (ϵA)-, Hx-, and 8-oxoguanine-containing oligonucleotides. 7-mG was effectively excised by hMPG, though it might be less toxic than the other methylated bases with respect to mutagenesis and cell killing. The kinetic study demonstrated that k_{cat}/K_m ratios of the enzyme for ϵA, Hx, and 7-mG were 2.5×10^{-3}, 1.4×10^{-3}, and 4×10^{-4} min^{-1} nM^{-1}, respectively. The oligonucleotides containing ϵA effectively competed against 7-mG, while Hx substrates showed unexpectedly low competition. Concerning the effect of the base opposite damage, hMPG much preferred Hx·T to other Hx pairs, and ϵA·C and ϵA·A pairs were better substrates than ϵA·T.

It has been reported that the cellular DNA is spontaneously alkylated in vivo by endogenous methyl donor compounds such as *S*-adenosylmethionine (*1*, *2*). The repair of *N*-alkylated bases is initiated by the action of damage-specific DNA glycosylases. Two distinct alkylpurine glycosylases have been identified in *Escherichia coli (3*−*5*). The *tag* gene product, 3-methyladenine DNA glycosylase I, exclusively recognizes and removes 3-alkylpurine (*3*, *4*, *6*, *7*), while the *alkA* gene product, 3-methyladenine DNA glycosylase II, can excise 7-alkylguanine in addition to 3-alkylpurines (*6*−*9*). In eukaryotic cells, however, only the latter type of *N*-methylpurine DNA glycosylase (MPG,[1] 3-methyladenine DNA glycosylase, alkylpurine DNA glycosylase) has been reported (*10*−*16*). MPG removes 3-methyladenine (3-mA), 7-methylguanine (7-mG), and 3-methylguanine (3-mG) from DNA as *AlkA* protein does. Hypoxanthine (Hx) and 1,N^6-ethenoadenine (ϵA) are spontaneously formed in cellular DNA by deamination of adenine residues and nitric oxide, respectively (*17*, *18*). Both *AlkA* protein and eukaryotic MPGs can also remove these damages from DNA (*19*−*23*). In addition, both human and mouse MPGs are shown to have an activity toward an oxidized guanine base, 7,8-dihydro-8-oxoguanine (8-oxoG) (*24*). Recent experiments using MPG-knockout mice, however, have demonstrated that the activity on 8-oxoG may not represent a major function of this enzyme (*25*, *26*).

In contrast with the *tag* gene product, MPG recognizes substrates with great structural diversity as mentioned above. Surprisingly, Berdal et al. reported that *AlkA* protein, *Saccharomyces cerevisiae* 3-methyladenine DNA glycosylase (MAG), and human MPG excised intact bases from DNA (*27*). Thus, the exact biological role of MPG is still to be elucidated. Most of the previous studies have shown that MPG prefers cytotoxic 3-mA to other substrates such as ϵA and Hx (*13*, *15*, *19*, *22*, *23*). However, the relative activities on individual substrates were investigated independently, and have never been directly compared. Furthermore, the enzyme activity was quantified using different detection methods or substrates prepared by a variety of treatment. In the case of methylpurines, radiolabeled substrates were usually prepared by treating DNA with [^{3}H]dimethyl sulfate (DMS), which produces 7-mG (80−90%) and 3-mA (ca. 10%) together with minor products including 3-mG, O^6-methylguanine, 1-me-

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[1] Abbreviations: ARP, aldehyde reactive probe; AP sites, apurinic/apyrimidinic sites; CAA, chloroacetaldehyde; DMS, dimethyl sulfate; ϵA, 1,N^6-ethenoadenine; hMPG, human methylpurine DNA *N*-glycosylase; Hx, hypoxanthine; 3-mA, 3-methyladenine; 3-mG, 3-methylguanine; 7-mG, 7-methylguanine; 8-oxoG, 7,8-dihydro-8-oxoguanine; MMS, methylmethanesulfonate.

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thyladenine, and 7-methyladenine (*28*, *29*). Substrate DNA containing ϵA was prepared by the treatment with chloroacetaldehyde (CAA), a metabolite of vinyl chloride which generates ϵA and 3,N^4-ethenocytosine along with minor products such as N^2,3-ethenoguanine and 1,N^2-ethenoguanine (*21*, *30*). On the other hand, Hx-DNA was synthesized by the primer-extension method using [^{3}H]dITP instead of dGTP. Excision of these bases was monitored by fluorescence (ϵA) or radioactivity (Hx and methylpurine) after HPLC separation (*10*−*16*, *20*−*22*). Because both DMS- and CAA-treated DNAs contain structurally different lesions recognized by MPG, the competing effect between the different types of lesions cannot be disregarded. The oligonucleotide containing a single damaged base at a unique position may be the most suitable substrate to compare the enzymatic activity in vitro. While several reports concerning the action of human MPG on oligonucleotide substrates containing ϵA and Hx are available, the activity on the synthetic methylpurine oligonucleotides has not been investigated, probably because methylated purines are fairly unstable.

In the present study, the activities of full-length human MPG were quantitatively examined and compared using oligonucleotides containing defined lesions. We also investigated the effects of the base opposite damage, which have not been examined in detail for full-length human MPG.

MATERIALS AND METHODS

Materials. 7-Methyl-2′-deoxyguanosine 5′-triphosphate was purchased from Sigma. Ultrapure 2′-deoxynucleoside 5′-triphosphates and 2′-deoxyinosine 5′-triphosphate (dITP) were obtained from Pharmacia and Boehringer Mannheim, respectively. T4 ligase, T4 polynucleotide kinase, and DNA polymerase I (Klenow fragment) were purchased from New England Biolabs.

Bacterial Strains and Plasmids. Escherichia coli MV1932 (*tag*$^-$ *alkA*$^-$) (*31*) was used for the expression of human *N*-methylpurine DNA glycosylase (hMPG). MV1932 and the plasmid containing full-length hMPG cDNA (phMPGFL2) were gifts from Dr. S. Mitra, University of Texas at Galveston. The plasmid pQE32 (Qiagen) was used for the construction of hMPG expression vector.

Construction of Human MPG Expression Vector. The plasmid phMPGFL2 was digested with *Eco*RI and *Bam*HI to obtain insert DNA containing full-length hMPG cDNA. The plasmid vector pQE32 was treated with *Sma*I/*Bam*HI, and the *Sma*I site was converted to the *Eco*RI site with a *Sma/Eco* linker (5′-AATTCCCGGG-3′ annealed with 5′-CCCGGG-3′), and then ligated with insert DNA using T4 ligase. The resulting expression plasmid pQE32hMPGFL2 encoded His_6-tagged protein with an additional 13 amino acids (MRGSHHHHHHGIH) at the amino terminus. The plasmid pQE32hMPGFL2 was transfected into MV1932 by electroporation using Cell Porator (BRL). The *E. coli* resistant to ampicillin was isolated and designated MV16-1.

Purification of hMPG. MV16-1 cells were grown at 37 °C in 1 L of 2YT (16 g/L Bacto-tryptone, 10 g/L Bacto-yeast extract, 5 g/L NaCl) containing 100 μg/mL ampicillin. At $A_{600} = 1.0$, isopropyl β-D-thiogalactopyranoside (1 mM) was added. After incubation for 4 h, cells were harvested and stored at −80 °C until use. About 3 g of cells was thawed and resuspended in 10 mL of extraction buffer [300 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mg/mL lysozyme, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiothreitol]. The suspension was subjected to five cycles of freeze and thaw treatments. The lysate was sonicated for 30 s and centrifuged at 30000*g* for 30 min at 4 °C. The supernatant was extensively dialyzed against MPG storage buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 10% glycerol]. The crude extract (8 mL) was applied on an Econo-pac heparin column (Bio-Rad) preequilibrated with MPG storage buffer. After the column was washed with 120 mL of MPG storage buffer omitting glycerol, elution was carried out with a linear gradient of 0−0.5 M NaCl (200 mL) in the same buffer. Fractions containing MPG activity (20 mL, 0.34−0.38 M NaCl) were pooled and dialyzed against Ni−NTA buffer [50 mM sodium phosphate (pH 8), 0.3 M NaCl, 0.5 mM PMSF]. The dialysate was then loaded onto a 5 mL His-Trap column (Pharmacia) preequilibrated with the same buffer. The column was washed with 24 mL of Ni−NTA buffer containing 1 mM imidazole and eluted with 17 mL of the same buffer containing 20 mM imidazole. The column elutant was dialyzed against MPG storage buffer (1 L) 4 times and 350 mL of buffer A [20 mM HEPES−KOH (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 0.1 M NaCl]. The dialysate was loaded onto a 1 mL POROS HS column (Perseptive) preequilibrated with buffer A at a flow rate of 0.5 mL/min. After the column was washed with 5 mL of buffer A, elution was performed with a linear gradient of 0.1−0.7 M NaCl (30 mL) in the same buffer.

Preparation of DNA Substrates. Partially depurinated DNA was prepared by acid−heat treatment as follows (*32*, *33*). Calf thymus DNA (Worthington Biochemicals) was treated with RNase A (100 μg/mL, Sigma) at 37 °C for 1 h and extracted with phenol−chloroform. The purified DNA (500 μg/mL) was dialyzed against AP buffer [10 mM sodium citrate (pH 5.0), 100 mM NaCl], heated at 70 °C for 10−50 min, and then precipitated with ethanol. For the preparation of the methylated DNA, the purified calf thymus DNA was treated with 59 mM methylmethanesulfonate (MMS, Sigma) in NTE buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl] at 37 °C for 1 h. To prepare DNA containing 1,N^6-ethenoadenine (ϵA), purified calf thymus DNA was treated with 0.5 M chloroacetaldehyde (CAA) in 0.1 M sodium cacodylate buffer (pH 7.0) at 37 °C for 12 h (*21*). Modified DNAs were precipitated with ethanol and dissolved in TE buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA] at a concentration of 1 mg/mL. M13 dsDNA containing hypoxanthine (Hx) was prepared according to Saparbaev and Laval with slight modification (*22*). In brief, single-stranded M13mp8 DNA (70 μg) was annealed with 17mer reverse sequence primer −41 (39 ng) in 140 μL of the Sequenase version 2 reaction mixture (Amersham). Primer extension reaction was carried out at 37 °C for 15 min using Sequenase version 2 according to the instructions. dITP were used instead of dGTP at a final concentration of 2.4 mM. The Hx-DNA was purified and recovered by ethanol precipitation.

Detection of AP Sites in hMPG-Treated DNA Using the Aldehyde Reactive Probe. To determine the glycosylase activity, the aldehyde reactive probe (ARP) assay for detecting abasic sites in DNA was performed as reported previously (*33*−*36*) with slight modifications. Various

concentrations of the DNA substrates containing methylpurines, etheno adducts, and Hx were incubated with 0.8 μg of purified hMPG in the presence of 5 mM ARP. Then DNA was extracted by phenol−chloroform, ethanol-precipitated, and dissolved in TE at a concentration of 1 μg/mL. The ARP-modified DNA was immobilized on the protamine-coated plates prepared as described previously (*35*−*37*). After the plate was washed with phosphate-buffered saline containing 0.5% Tween-20 (TPBS), 100 μL of 1:20 diluted ABC solution (PK-4100; Vector Laboratories) was added into each well. The plate was incubated at room temperature for 30 min and then washed with TPBS. Two hundred microliters of the substrate solution [1 mg/mL 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 51 mM Na_2HPO_4, 24 mM citric acid] was added into each well. After 120 min, the absorbance at 405 nm was measured. The ARP signals after subtracting background readings for control DNA were converted to the number of AP sites on the basis of the ARP signal obtained for standard acid/heat-treated calf thymus DNA containing the known amount of abasic sites.

5′-End-Labeling of Oligonucleotides. The sequence of the 17mer oligonucleotides used was 5′-AGCATTCG**X**GACTGGGT-3′. Four oligonucleotides (17mer) containing normal guanine, 1,N^6-ethenoadenine (ϵA), hypoxanthine (Hx), or 7,8-dihydro-8-oxoguanine (8-oxoG) at position **X** were obtained as follows. ϵA oligonucleotide was prepared by the reported method and purified by reversed phase HPLC (*38*). HPLC-purified oligonucleotides containing Hx and 8-oxoG were purchased from Pharmacia and Nippon Gene, respectively. The oligonucleotide containing normal guanine and the complementary 17mer oligonucleotides (the base opposite **X** was A, C, G, or T) were obtained from Bio Synthesis Inc. The oligonucleotides containing ϵA, Hx, or 8-oxoG (5 pmol) were 5′-end-labeled with 555 kBq of [γ-^{32}P]-ATP (111 TBq/mmol, NEN) using T4 polynucleotide kinase and annealed with a 2-fold excess of the complementary strands. The double-stranded (ds) oligonucleotides were purified by phenol−chloroform extraction followed by ethanol precipitation.

Synthesis of Oligonucleotide Containing 7-Methylguanine. Oligonucleotide containing 7-methylguanine (7-mG) was prepared by the primer extension method using 25mer template oligonucleotide (5′-GAGAGAAGG**C**AGGATGCTATCGATG-3′). 7-Methyl-dGMP was incorporated opposite the underlined cytosine. The oligonucleotide (12.5 pmol) was annealed with 5 pmol of ^{32}P-labeled primer (5′-CATCGATAGCATCCT-3′) in 20 μL of the annealing buffer [20 mM Tris-HCl (pH 7.5), 50 mM NaCl]. To the mixture were added 10 nmol of 7-methyl-dGTP, 1 nmol of dTTP, 1 nmol of dCTP, 1.5 units of *E. coli* DNA polymerase I (Klenow fragment), and 5 μL of 10× polymerase buffer [660 mM Tris-HCl (pH 7.6), 66 mM $MgCl_2$, 15 mM mercaptoethanol], and the volume was adjusted to 50 μL with water. The mixture was incubated at 25 °C for 40 min, and then 0.5 M EDTA was added at a final concentration of 10 mM. The 25mer oligonucleotides containing G or Hx instead of 7-mG at the same position were also synthesized by using dGTP (1 nmol) or dITP (10 nmol) in place of 7-methyl-dGTP. To determine whether 7-mG was incorporated, the purified 7-mG oligonucleotide was heated at 90 °C for 30 min in water. This treatment converted heat-unstable 7-mG to an abasic site, resulting in strand scission. The 7-mG oligonucleotide but not G and Hx oligonucleotides gave the nicked product with the expected length. Further details of the synthesis and characterization of the oligonucleotide containing 7-mG will appear elsewhere (Asagoshi et al., manuscript in preparation).

DNA Glycosylase Assay. Purified hMPG (5.9 or 11.8 ng) was reacted with various concentrations of ^{32}P-labeled oligonucleotide duplexes in 20 μL of MPG assay buffer [35 mM HEPES−KOH (pH 7.5), 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.1 M NaCl] in the presence of purified *E. coli* endonuclease IV (a gift from Dr. Y. W. Kow, Emory University). After an appropriate incubation period, the reaction was stopped by addition of an equal volume of loading buffer (0.1% xylene cyanol in formamide) followed by heating at 55 °C for 5 min. The samples were loaded onto a 20% polyacrylamide gel containing 7 M urea and electrophoresed in Tris/borate/EDTA buffer at 20−25 V/cm for 2 h. The gel was dried under vacuum, and the radioactivity of each band was measured by Beta Scope (Betagene).

RESULTS

Comparison of the hMPG Activities for DNA Substrates. Most of the single-base damages in DNA are effectively excised by specific DNA glycosylases, and apurinic/apyrimidinic (AP) sites are formed (*39*−*42*). Therefore, the activity of DNA glycosylase can be measured by quantifying the resulting AP sites. To compare the relative activity of hMPG, we employed the sensitive assay for AP sites, the aldehyde reactive probe (ARP) assay (*33*, *34*). For the substrates of hMPG, methylmethanesulfonate (MMS)- and chloroacetaldehyde (CAA)-treated calf thymus DNA and primer-extended M13 DNA containing hypoxanthine (Hx-DNA) were prepared as described under Materials and Methods. The number of damages in each substrate DNA was determined by measuring AP sites after exhaustive digestion of DNA with an excess amount of hMPG. The amounts of AP sites in MMS- and CAA-treated DNA and Hx-DNA were 1.4, 3.1, and 106 per 10^3 nucleotides, respectively. The reaction rate of hMPG for the substrate DNA was determined as a function of the damage concentration which was adjusted by diluting the substrate DNA with undamaged substrate. As shown in Figure 1, the reaction rate of hMPG was MMS-DNA ≫ CAA-DNA > Hx-DNA. Therefore, hMPG excised methylpurines approximately 4- and 6-fold more rapidly than etheno adducts and hypoxanthine, respectively.

Substrate Specificity of hMPG Determined with Oligodeoxynucleotides. Both the MMS- and CAA-treated DNA substrates used above contained several types of base damages at various levels. The employment of oligonucleotides containing a single damage at a defined position is probably the most suitable way to examine the substrate specificity of DNA *N*-glycosylases. Using synthetic substrates, several kinetic studies on mammalian methylpurine DNA *N*-glycosylases have been reported (*23*, *43*). To compare the enzymatic activities, we prepared a 25mer oligonucleotide containing a single 7-methylguanine (7-mG) and 17mer oligonucleotides containing either a single ϵA, Hx, or 8-oxoG. As shown in Figure 2, substantial amounts of the oligonucleotides containing 7-mG, 1,N^6-ethenoadenine (ϵA), and hypoxanthine (Hx) were cleaved by hMPG plus

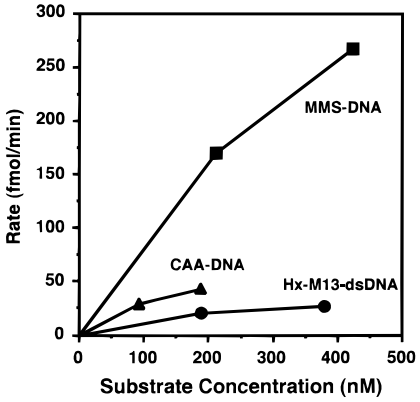

FIGURE 1: Comparison of substrate specificity of hMPG based on the detection of AP sites using ARP assay. Various concentrations of MMS- or CAA-treated calf thymus DNA or primer-extended M13 dsDNA containing hypoxanthine (Hx) were incubated with 0.8 μg of hMPG at 37 °C for 1 h in 200 μL of reaction mixture. After the incubation, DNA were deproteinized and recovered by ethanol precipitation. Then, AP sites were quantified by the ARP assay as described under Materials and Methods.

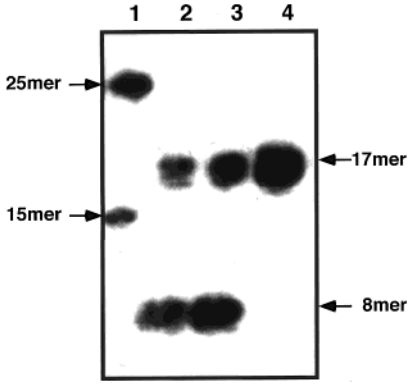

FIGURE 2: Activities of hMPG for oligonucleotide substrates. Five nanomolar samples of oligonucleotides containing 7-mG·C (lane 1), ϵA·T (lane 2), Hx·T (lane 3), or 8-oxoG·C pair (lane 4) were incubated with hMPG (11.8 ng) and Endo IV at 37 °C for 15 min. Then the products were separated by electrophoresis on a 20% polyacrylamide−7 M urea gel. The bands were visualized using Beta Scope (Betagene).

endonuclease IV treatment, while no product was observed for 8-oxoG oligonucleotide. Further examination of the activity of hMPG on 8-oxoG under a variety of conditions did not reveal any detectable activity to this damage, while *E. coli* 8-oxoG glycosylase (Fpg) showed the activity on this substrate (data not shown).

Kinetic Constants of hMPG for Excision of 7-mG, ϵA, *and Hx from Oligonucleotides*. To determine the optimum enzyme concentration for the kinetic study, 10 nM oligonucleotides containing 7-mG, ϵA, or Hx were incubated with various amounts of hMPG at 37 °C for 15 min. The concentration of hMPG chosen was 295 ng/mL for Hx and 590 ng/mL for 7-mG and ϵA, respectively. Under these conditions, less than 30% of oligonucleotides were cleaved. To determine the appropriate substrate concentration range, approximate K_m (Michaelis constant) values were estimated at substrate concentrations up to 160 nM. Then, we chose six substrate concentrations within (0.33−2.0)K_m and incubated the substrates with the appropriate amounts of hMPG at 37 °C for 5, 10, and 15 min. The fraction of nicked oligonucleotides increased in proportion to the incubation time in all reactions (data not shown). In Figure 3A,B, the

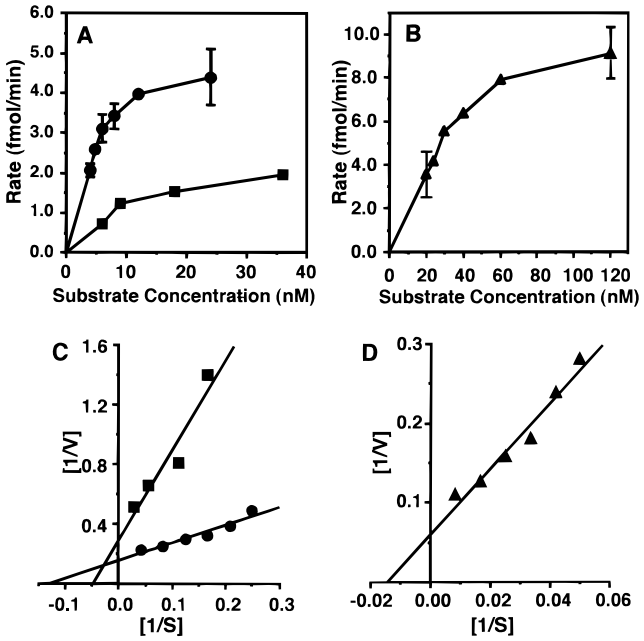

FIGURE 3: Repair kinetics of hMPG for oligonucleotides. Various concentrations of oligonucleotides containing 7-mG·C, ϵA·T, or Hx·T were incubated with hMPG at 37 °C for 5, 10, and 15 min. The reaction rates were calculated and plotted in (A) for ϵA·T (closed circles) and 7-mG·C (closed squares) and in (B) for Hx·T (closed triangles). Lineweaver−Burk plots of (A) and (B) are depicted in (C) and (D), respectively.

Table 1: Kinetic Constants of hMPG for Oligonucleotides Containing a 7-mG·C, ϵA·T, or Hx·T Pair[a]

substrate	K_m (nM)	V_{max} (fmol·min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (nM^{-1}·min^{-1})
7-mG·C	21	3.2	0.009	0.0004 (1.00)
ϵA·T	8	6.4	0.019	0.0025 (6.25)
Hx·T	69	16.5	0.097	0.0014 (3.50)

[a] Numbers in parentheses represent the relative ratio.

reaction rates were plotted as a function of substrate concentrations. According to these plots, hMPG removed ϵA more rapidly than 7-mG and Hx. Figure 3C,D shows Linweaver−Burk plots, and Table 1 summarizes the kinetic constants for these three substrates. The K_m values of hMPG for 7-mG, ϵA, and Hx suggest that apparent affinity is in the order of ϵA > 7-mG > Hx. On the other hand, the catalytic rate constants (k_{cat}) indicate that Hx is more rapidly removed from oligonucleotide by hMPG than the others once the enzyme−substrate complex is formed. The ratios k_{cat}/K_m indicate that hMPG prefers ϵA as compared with Hx and 7-mG.

Effect of Opposite Bases on the Activity of hMPG. To determine whether the base opposite the lesion influenced the enzyme activity, oligonucleotides (10 nM) containing ϵA or Hx paired with thymine (T), cytosine (C), adenine (A), or guanine (G) were incubated with hMPG (590 ng/mL) at 37 °C for 15 min. As shown by the nicked products (8mer) in Figure 4, the activity of hMPG on both substrates was notably affected by the opposed base. For more quantitative comparison, ϵA and Hx oligonucleotides were incubated with hMPG for increasing periods where the $S-V$ plots were in a linear range shown in Figure 3A or 3B. The results are summarized in Table 2. Interestingly, hMPG excised ϵA opposite C or A more quickly than opposite T, which is

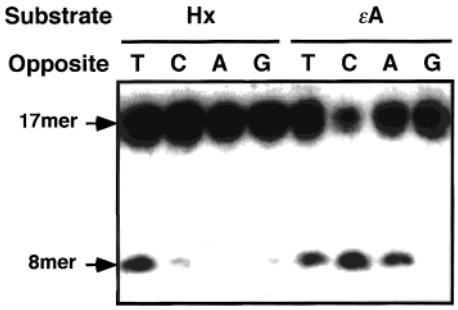

FIGURE 4: Influence of the base opposite Hx or ϵA on the activities of hMPG. 17mer oligonucleotides containing a Hx or ϵA paired with T, C, A, or C were prepared. 10 nM substrates were incubated with 11.8 ng of hMPG and Endo IV at 37 °C for 15 min. The reaction was stopped by addition of gel loading buffer, and the samples were analyzed by 20% polyacrylamide−7 M urea gel electrophoresis.

Table 2: Activities of hMPG on ϵA or Hx Paired with Different Bases[a]

	rate (fmol·min^{-1})	
opposite	ethenoadenine	hypoxanthine
thymine	2.17 (1.00)	2.83 (1.00)
cytosine	3.46 (1.59)	0.87 (0.31)
adenine	2.64 (1.22)	0.31 (0.11)
guanine	0.78 (0.36)	0.89 (0.31)

[a] The relative activities are shown in parentheses.

formed in natural DNA after modification with CAA. On the other hand, hMPG removed Hx (deamination product of adenine) opposite T much more efficiently than that paired with C, A, or G.

Competitive Effects of ϵA, *Hx, and 8-oxoG on the Activity of hMPG for 7-mG*. To compare the substrate preference of hMPG directly, 5 nM of the ^{32}P-labeled 7-mG oligonucleotide substrate was incubated with hMPG in the presence of various concentrations of cold duplex oligonucleotides containing ϵA, Hx, 8-oxoG, or guanine (control) at 37 °C for 30 min (Figure 5). The activity of hMPG on 7-mG was reduced to 40% by the addition of an equal amount of the ϵA oligonucleotide and completely abolished by a 5-fold excess of ϵA oligonucleotide (IC_{50} = 4.2 nM). Hx oligonucleotides significantly competed with 7-mG substrate, but a 17-fold excess amount was required for 50% inhibition (IC_{50} = 87 nM). The 8-oxoG oligonucleotide (IC_{50} = 161 nM) had virtually no additional inhibitory effects over the control G oligonucleotide (IC_{50} = 140 nM) on the activity of hMPG.

Figure 6 shows the results of the experiments in which a pair of ^{32}P-labeled oligonucleotide substrates were incubated with hMPG. Various combinations of 25mer (7-mG·C or Hx·C pair) and 17mer (Hx·T, Hx·C, or ϵA·T pair) substrates were incubated with 11.8 ng of hMPG in 20 μL of the reaction mixture at 37 °C for 15 min. The resultant products from 25mer and 17mer were readily separated by their sizes after gel electrophoresis. When oligonucleotides containing 7-mG·C and ϵA·T pairs were simultaneously incubated (combination A), the amounts of incised products for 7-mG and ϵA were 55 and 88% of those observed in the reaction of the single substrate alone, respectively. The activity on 7-mG·C substrate was inhibited more strongly by the presence of ϵA·T substrate than that of Hx·T oligonucleotide

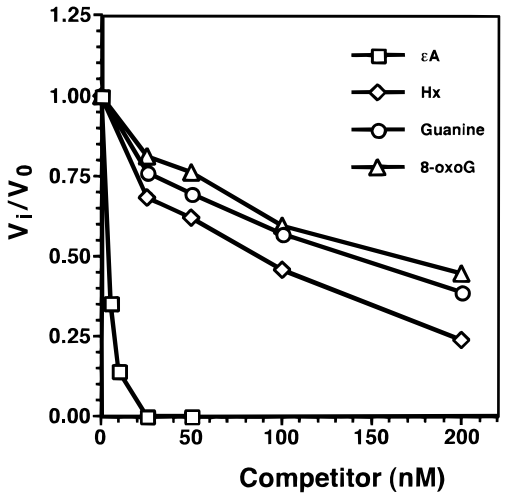

FIGURE 5: Competition of ϵA, Hx, or 8-oxoG for the excision of 7-mG by hMPG. Competitor oligonucleotides containing a single ϵA·T, Hx·T, 8-oxoG·C, or normal G·C pair at the concentrations indicated were added to the reaction mixture. The reaction mixture contained 11.8 ng of hMPG and 5 nM ^{32}P-7-mG-oligonucleotide. The initial velocity of enzyme reaction on the 7-mG-substrate in the presence (v_i) of competitors was compared with that in the absence (v_0) of competitor.

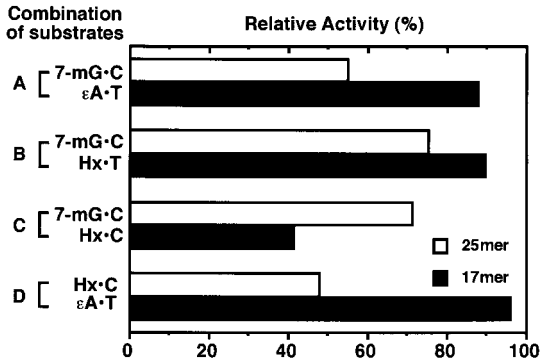

FIGURE 6: Comparative activity of hMPG in the presence of a pair of substrates. Various combinations of 25mer oligonucleotides (white columns) containing 7-mG·C or Hx·C pair and 17mer oligonucleotides (black columns) containing ϵA·T, Hx·T, or Hx·C pair were incubated with hMPG (11.8 ng). Remaining activities for each substrate relative to those in the absence of the competitor are shown.

(combinations A and B). The Hx·C oligonucleotide was less effectively recognized by hMPG than ϵA or 7-mG substrates (combinations C and D).

Miao et al. reported the effect of the length of synthetic Hx oligonucleotide substrate on the activity of hMPG (*43*). Therefore, we compared the activity of hMPG using 17mer and 25mer Hx oligonucleotides. The hMPG showed essentially the same activity on both substrates (data not shown). According to the data shown in Figure 6, hMPG seemed to slightly prefer the Hx·T pair to the 7-mG·C pair, which was in turn preferred over the Hx·C pair. Taken together, the substrate pair preference of hMPG is ϵA·T > Hx·T> = 7-mG·C > Hx·C.

DISCUSSION

DNA base excision repair (BER) is one of the important mechanisms to prevent mutation and cell death (*39*). The specific DNA glycosylases excise damaged bases in the first

step of the process. Certain enzymes such as uracil DNA glycosylase recognize a unique type of base alteration. However, many glycosylases are now known to have relatively wide substrate specificity (*39, 42*). Among them, hMPG (also designated as AAG or ANPG) has been reported to recognize a broad range of substrates including alkylpurines, 1,N^6-ethenoadenine (ϵA), hypoxanthine (Hx), and 8-oxoguanine (8-oxoG) (*19*−*24, 42*). In these studies, however, a variety of procedures were employed to introduce damaged bases into substrate DNA as well as to measure the enzyme activity. In addition, the kinetic properties of the enzyme might be also affected to certain extents by the different enzyme preparations employed in these experiments. In the present report, the substrate preference of hMPG was investigated by comparing the enzyme activity on the major substrates using the same assay systems.

Using ARP assay for detection of AP sites, it was shown that hMPG more rapidly removed methylpurines than etheno adducts or Hx from DNA (Figure 1). Since AP sites or incised AP sites are produced by all types of DNA glycosylases as intermediates of BER (*38*−*41*), ARP assay is considered to be a useful and convenient method for detecting BER intermediates (intact and incised AP sites) (*33*−*36*). In this study, conventional procedures were employed to prepare calf thymus and M13 DNA substrate. Under these conditions, MMS and CAA generate methylpurines [mainly 3-methyladenine (3-mA) and 7-methylguanine (7-mG)] and etheno adducts (mostly ϵA and 3,N^4-ethenocytosine) in DNA, respectively. Although MMS-treated DNA was the best substrate for hMPG when the reaction rates were compared (Figure 1), hMPG excised 7-methylguanine less efficiently than ϵA and Hx from oligodeoxyribonuceotide substrates (Table 1). Therefore, these results suggest that hMPG excises 3-mA much more effectively than other known substrates. We previously reported that AP sites were accumulated in DNA from HeLa cells about 3 h after MMS treatment (*36*). Recently, Elder et al. have reported that MMS induces A·T to T·A and G·C to T·A transversions in splenic T lymphocytes derived from MPG-knockout mouse (*46*). The G·C to T·A transversions are probably generated by mispairing 3-methylguanine with adenine because the most abundant 7-mG is probably not premutagenic lesion. The observed A·T to T·A transversions may resulted from 3-mA and AP sites. Because 3-mA which blocks DNA replication is a cytotoxic lesion (*44, 45*), it may be urgent for cells to remove the damage even though the discordant base excision repair process results in the accumulation of the harmful intermediate, an apurinic site.

The K_m value for 7-mG was similar to that reported by O'Connor (*47*), while the k_{cat} for 7-mG is about 35-fold smaller. On the other hand, the enzyme preparation by the same group was recently shown to have almost the same k_{cat} value for Hx oligonucleotide as that determined in the present study (*43*). Thus, it is not likely that the disagreement of k_{cat} resulted from the difference in the specific activity of hMPG employed. Since the previously reported k_{cat} for 7-mG was estimated using DMS-treated DNA which contained a variety of methylation products, it is possible that the enzyme activity was affected by other damages. The K_m values determined in the present study suggest that the affinity of hMPG for the substrates is in the order of ϵA > 7-mG > Hx. The turnover rate (k_{cat}) of hMPG for Hx paired with thymine is almost same as the reported value (*43*) and notably higher than 7mG·C and ϵA·T (Table 1). Since the turnover of hMPG is slow as in the case of most mammalian glycosylases, a relatively high enzyme concentration (vs substrate concentrations) was required to determine the enzymatic parameters for oligonucleotide substrates in the present experiments. However, it should be pointed that apparent V_{max} values were obtained in the substrate concentration range up to 160 nM for all oligonucleotide substrates. As suggested by Miao et al. (*43*), the length of oligonucleotides might have an influence on K_m in our experiments. When the hMPG activities on 17- and 25mer substrates containing a single Hx·C pair were compared, however, a very slight difference was observed between them (data not shown). The ratio of k_{cat}/K_m revealed that hMPG preferred ϵA over Hx or 7-mG. However, the difference in k_{cat}/K_m was at most 6-fold, suggesting that less toxic 7-mG is also actively removed by hMPG probably because of the potential toxicity after spontaneous conversion to more harmful damages, such as apurinic sites or formamidopyrimidines.

The observed effect of the base opposite ϵA (Table 2) essentially agrees with that reported by Saparbaev et al. (*23*), though the activity for ϵA·C was higher than that for ϵA·G by 4-fold in this study. The high activity to ϵA·C suggests an additional role of hMPG in the defense against the genotoxic effect arising from the misincorporation of ethenodATP formed in the nucleotide pool (*48*). The effect of the base opposite Hx has not been reported for hMPG. The activity of Alk A, an *E. coli* functional homologue of hMPG, was affected only partially by the base opposite Hx (*22*), while calf thymus Hx-glycosylase was much more active on the Hx·T pair than the Hx·C pair. In the present study, we have demonstrated that hMPG prefers Hx·T over the other pairs (Table 2). The K_m of hMPG for the Hx·C pair was about 120 nM (data not shown), which was 2 times higher than Hx·T (Table 1); thus, the base paired with Hx affects reaction kinetics of hMPG. Recently, Lau et al. reported the structure of truncated hMPG/AAG cocrystallized with oligonucleotide containing a pyrrolidine abasic site (*49*). The structure of truncated human hMPG/AAG revealed that there is no apparent structure domain directly interacting with the base opposite the pyrrolidine abasic site. However, it is probable that the base paired with the damaged base influences the enzyme activity by changing the strandedness, as suggested by Hang et al. (*50*). Since hMPG exclusively prefers double-stranded substrates, the strandedness and base stacking interactions around the damaged base may be an important factor of the reaction process. hMPG preferred the Hx·T pair to the more thermodynamically stable Hx·C pair; therefore, the paired effect cannot be solely explained by the thermodynamic property. The differential hMPG activities depending on the paired base for ϵA and Hx (Table 2) implicate the complex role of hMPG in repair, as is the case for Ogg1 and Ogg2 (*51*).

It should be noted that the control oligonucleotide showed slight but significant competition for the 7-mG substrate (Figure 5). The competing capacity of 8-oxoG against 7-mG was almost the same as the control oligonucleotide. It has been reported that mouse MPG binds to the undamaged double-stranded oligonucleotide (*52*). Therefore, it is probable that the enzyme may initially bind substrate DNA by a relatively nonspecific manner, and then slides to approach

the damage as suggested by the crystal structure presented by Lau et al. (*49*).

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