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MUTYH prevents OGG1 or APEX1 from inappropriately processing its substrate or reaction product with its C-terminal domain

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ABSTRACT

MutY homolog (MUTYH) excises adenine opposite 8-oxoguanine (8-oxoG) in DNA, thus preventing occurrence of G:C to T:A transversion. In cell-free extract prepared from the thymocytes of wild type but not MUTYH-null mice, adenine opposite 8-oxoG in DNA was excised by MUTYH, however, the generated apurinic (AP) site opposite 8-oxoG mostly remained unincised. Recombinant mouse MUTYH (mMUTYH) efficiently excised adenine opposite 8-oxoG and prevented mouse AP endonuclease (mAPEX1) from incising the generated AP site. In contrast, an AP site opposite 8-oxoG created by uracil DNA glycosylase or tetrahydrofuran opposite 8-oxoG was efficiently incised by mAPEX1 in the presence of an excess amount of mMUTYH. Mutant mMUTYH with R361A or G365D substitution, excised adenine opposite 8-oxoG as efficiently as did wild-type mMUTYH, but failed to prevent mAPEX1 from incising the generated AP site. Wild-type mMUTYH bound duplex oligonucleotides containing A:8-oxoG pair with a lower apparent K_d than that of the mutants, and prevented OGG1 from excising 8-oxoG opposite adenine or the generated AP site. The G365D mutant failed to prevent OGG1 from excising 8-oxoG opposite the generated AP site, thus indicating that the protection of its own product by mMUTYH is an intrinsic function which depends on the C-terminal domain of mMUTYH.

INTRODUCTION

Cellular DNA is at high risk of being oxidized by reactive oxygen species, which are inevitably generated by normal metabolic functions such as mitochondrial respiration or by environmental exposure to ionizing radiation and chemicals. The oxidation of DNA appears to result in either spontaneous mutagenesis or cell death and, as a result, it is implicated in various age-related diseases such as cancer and neurodegeneration (1). Among the various types of oxidized damage in DNA, 8-oxoguanine (8-oxoG), an oxidized form of guanine, can pair with adenine as well as cytosine during DNA replication or transcription, and it is thus considered to be one of the spontaneous causes of mutagenesis or cell death (2,3).

Mutator mutants of *Escherichia coli* revealed that the *mutM* (*fpg*) gene encoding 8-oxoG DNA glycosylase which excises 8-oxoG opposite cytosine in DNA, and the *mutY* gene encoding the adenine DNA glycosylase which removes the adenine incorporated opposite 8-oxoG in template DNA, play important roles in the prevention of such spontaneous mutagenesis (4–6). It has been established that most eukaryotic cells also possess either a MutM homolog or its functional homolog, OGG1 for the repair of 8-oxoG, and a MutY homolog (MUTYH or MYH) for the repair of adenine opposite 8-oxoG (7–9).

Prokaryotic MutY and its mammalian homologs MUTYH proteins specifically recognize the adenine base that is misincorporated opposite 8-oxoG present in the template strand, and excise the adenine base by their intrinsic DNA glycosylase activity. The generated AP site opposite 8-oxoG by MUTYH is likely to be processed by the multi-enzyme base excision repair (BER) pathway to yield C:8-oxoG pair, which is then further processed by the BER pathway initiated by MutM or OGG1 to restore the C:G pair (10).

In *E.coli* and fission yeast, the absence of these repair enzymes resulted in significant increases in the spontaneous mutation rate, especially of G:C to T:A transversion mutation (11,12). Recently, familial alterations in the human *MUTYH* gene have been reported to be possible causative mutations for certain types of familial colorectal tumors without a germ-line mutation in the *APC* gene (13,14). We recently generated MUTYH-null mouse embryonic stem (ES) cells. In the

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MUTYH-null cells carrying no adenine DNA glycosylase activity, the spontaneous mutation rate increased 2-fold in comparison to wild-type cells, thus demonstrating that the absence of the MUTYH function in mammalian cells indeed results in a mutator phenotype (15).

In order to suppress G:C to T:A transversion mutation which is initiated by generation of 8-oxoG in template strand, MUTYH has to specifically excise adenine incorporated into the nascent strand only opposite the 8-oxoG, but not adenine in a template strand to which 8-oxoG is misinserted from the nucleotide pools. Therefore, it is proposed that MUTYH recognizes the nascent strand in association with various cellular proteins, such as PCNA or mismatch repair complex (16,17). After an excision of adenine opposite 8-oxoG, cytosine may be inserted opposite 8-oxoG during the repair synthesis, and thereafter OGG1 has to initiate an excision repair of 8-oxoG, thus resulting in a restoration of the G:C pair (18). In order to understand the molecular mechanism regulating how MUTYH accomplishes the appropriate repair processes, an *in vitro* reconstitution of such repair processes is essential.

In the present study, we examined the repair reaction by mMUTYH in cell-free extracts prepared from mouse thymus glands, and found that mMUTYH can excise adenine opposite 8-oxoG or guanine, however, an efficient incision was observed at the apurinic/apyrimidinic (AP) site generated only opposite guanine. Using recombinant mMUTYH and mAPEX1 proteins, we showed that mMUTYH excises adenine opposite 8-oxoG as well as guanine, and prevents mAPEX1 from incising the generated AP site only opposite 8-oxoG. Furthermore, we found that mutant mMUTYH (R361A or G365D) have a much higher dissociation rate than wild type from duplex DNA containing A:8-oxoG pair. As a result, the mutant protein is considered to lose the ability to prevent OGG1 from excising 8-oxoG opposite adenine or the generated AP site, as well as losing the ability to prevent mAPEX1 from incising the generated AP site opposite 8-oxoG.

Table 1. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]
mY5-D207N	GGT AAC CGG TGT GGT Gaa tGG GAA CGT TT
mY5-Q360A	GTC CTC CTG GTG gct AGG CCT GAC TC
mY3-Q360A	GAG TCA GGC CTa gcC ACC AGG AGG AC
mY5-R361A	CTC CTG GTG CAA gct CCT GAC TCA GGT C
mY3-R361A	GAC CTG AGT CAG Gag cTT GCA CCA GGA G
mY5-F373A	ACT GTG GGA Ggc tCC ATC TGT CAC CTT G
mY3-F373A	CAA GGT GAC AGA TGG agc CTC CCA CAG T
−10T7 promoter	CCC GCG AAA TTA ATA CGA CT
+59T7 terminator	ATA GTT CCT CCT TTC AGC AA
*A	FAM-AGC GGC CAT CGA TAC CGT C*A*A CCT CGA GGA ATT CC
*F	FAM-AGC GGC CAT CGA TAC CGT C*F*A CCT CGA GGA ATT CC
*U	FAM-AGC GGC CAT CGA TAC CGT C*U*A CCT CGA GGA ATT CC
*GO	FAM-GGA ATT CCT CGA GGT *GO* GA CGG TAT CGA TGG CCG CT
A	AGC GGC CAT CGA TAC CGT C*A*A CCT CGA GGA ATT CC
F	AGC GGC CAT CGA TAC CGT C*F*A CCT CGA GGA ATT CC
C	AGC GGC CAT CGA TAC CGT C*C*A CCT CGA GGA ATT CC
G	GGA ATT CCT CGA GGT *G*GA CGG TAT CGA TGG CCG CT
T	GGA ATT CCT CGA GGT *T*GA CGG TAT CGA TGG CCG CT
GO	GGA ATT CCT CGA GGT *GO* GA CGG TAT CGA TGG CCG CT
19-P	FAM-AGC GGC CAT CGA TAC CGT C-phosphate
19-OH	FAM-AGC GGC CAT CGA TAC CGT C-OH
15-P	FAM-GGA ATT CCT CGA GGT-phosphate
15-OH	FAM-GGA ATT CCT CGA GGT-OH

FAM, 5′ end was labeled with FAM; GO, 8-oxoG; F, tetrahydrofuran; U, uracil; phosphate, 3′ end was attached with a phosphate group; OH, 3′ end was with a hydroxy group.
[a]A lower-case letter indicates a base substituted for mutagenesis.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides shown in Table 1, were obtained from Greiner Japan, and the Hokkaido System Science.

Mouse

By means of gene targeting, we previously established *Mutyh* gene knockout mice (15), and heterozygous mice ($Mutyh^{+/-}$) backcrossed to C57BL6/J were maintained.

Cell-free extracts from thymocytes

Thymus glands were isolated from four-week old wild-type C57BL/6J and $Mutyh^{-/-}$ mice obtained by mating the $Mutyh^{+/-}$ mice. Isolated thymus glands (wet weight 1.6 g from 20 mice) were crushed in phosphate-buffered saline (PBS), and liberated thymocytes were collected by centrifugation at 2100 *g* for 10 min at 4°C. Thymocytes were suspended in 1.6 ml of the lysis buffer (20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.25 M NaCl, 1 mM dithiothreitol) on ice, and were disrupted by sonication. The cell-free extracts were clarified by centrifugation at 40 000 *g* for 15 min at 4°C, and were stored at −80°C (19).

Plasmids

For expression of mMUTYH in *E.coli*, wild-type mouse *Mutyh* cDNA was subcloned into pET32a(+) (Novagen), and resulted plasmid was designated as pET32a-mMUTYH which encodes a fusion protein with thioredoxin (Trx-mMUTYH) (15). cDNAs encoding mutant mMUTYH proteins, mMUTYH(D207N), mMUTYH(R361A), mMUTYH(G365D) and mMUTYH(F373A), which were generated by recombinant PCR (20), using oligonucleotides listed in Table 1, were also subcloned into pET32a(+) to obtain pET32a-mMUTYH (D207N), pET32a-mMUTYH(R361A), pET32a-mMUTYH (G365D) and pET32a-mMUTYH(F373A), respectively. mAPEX1 cDNA (21) was subcloned into an *E.coli* expression vector pQE30 (Qiagen) containing a His^6 tag at the N-terminus, and resulted plasmid was designated as pQE30-mAPEX1. For the expression of hOGG1-1a (hOGG1-1a), hOGG1-1a cDNA was subcloned into pET3d (Novagen), and the resulted plasmid was designated as pET3d-hOGG1-1a (22).

Preparation of recombinant mAPEX1 proteins

The expression of His-mAPEX1 was induced by the addition of 1 mM isopropyl β-D-thiogalactoside (Wako Pure

Chemicals) to the cultures of *E.coli* JM109 cells transformed with pQE30-mAPEX1 following incubation at 37°C for 4 h. Cells were suspended in buffer A [154 mM NaCl/5 mM β-mercaptoethanol/50 mM Tris–HCl (pH 7.5)/0.1 mM PMSF/leupeptin (0.5 μg/ml)/pepstatin (0.5 μg/ml)/chymostatin (0.5 μg/ml)], disrupted by sonication and cell lysates were clarified by centrifugation. His-mAPEX1 protein was purified by aid of TALON superflow metal affinity resin (Clontech), according to the manufacturer's instruction. Fractions contained APEX1 were loaded onto PD-10 desalting column (Amersham Biosciences) equilibrated with buffer B [50 mM Tris–HCl (pH 7.5)/50 mM NaCl/1 mM dithiothreitol/ 1 mM EDTA/5 mM $MgCl_2$/ 5% glycerol], and His-mAPEX1 protein was eluted in buffer B, and stored at −80°C.

Preparation of recombinant Trx-mMUTYH proteins

To obtain a soluble recombinant mMUTYH preparation, thioredoxin-fusion protein (Trx-mMUTYH) was prepared as described previously (23). Briefly, cells expressing Trx-mMUTYH were suspended in buffer C [200 mM KCl/5 mM β-mercaptoethanol/20 mM Hepes-KOH (pH 7.8)/0.1 mM PMSF/leupeptin (0.5 μg/ml)/pepstatin (0.5 μg/ml)/chymostatin (0.5 μg/ml)], were disrupted by sonication and cell lysates were clarified by centrifugation. By aid of the TALON superflow metal affinity resin, Trx-mMUTYH was purified, then fractions containing Trx-MUTYH protein were further separated through Superose 12/30 column (Amersham Biosciences) equilibrated with the buffer C containing 500 m M KCl and 10% glycerol, and fractions containing a single band of Trx-MUTYH were stored at −80°C.

Preparation of recombinant hOGG1-1a protein

The expression of hOGG1-1a was induced by adding 1 mM isopropyl β-D-thiogalactoside to the culture of *E.coli* BL21(DE3) cells transformed with pET3d-hOGG1-1a (24), following incubation at 37°C for 2 h. The cells suspended in lysis buffer [50 mM Tris–HCl (pH7.5)/500 mM KCl/20 mM DTT/0.1 mM EDTA], were disrupted by sonication and the cell lysates were clarified by centrifugation. The supernatant was applied onto DEAE–sepharose column (Amersham Biosciences) equilibrated with the lysis buffer, and proteins recovered in the flowthrough fraction was precipitated by ammonium sulfate (40–75% saturation). Protein was dissolved in buffer D [50 mM potassium phosphate (pH7.5)/10% glycerol/10 mM DTT/0.1 mM EDTA], and applied onto Hitrap-heparin column (Amersham Biosciences) equilibrated with buffer D, and was eluted by a linear gradient of KCl (0–1 M). Fractions containing hOGG1-1a was applied onto Mono-S column (Amersham Biosciences) equilibrated with buffer E [50 mM potassium phosphate (pH6.5)/10 mM DTT/0.1 mM EDTA] and bound protein was eluted by a linear gradient of KCl (0–1 M). Fractions containing hOGG1-1a was further applied onto Hiprep-200 (Amersham Biosciences) equilibrated with buffer F [100 mM potassium phosphate (pH7.8)/100 mM KCl/10 mM DTT/0. 1 mM EDTA], and fractions containing a single 40 kDa band were stored at −80°C.

Determination of the protein concentration

The protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad) and BSA as the standard.

Nicking assay

A fluorescent dye, 6-carboxy fluorescein-aminohexyl amidite (FAM) was attached to the 5′ end of the strand with a target base or damaged base. Duplex oligonucleotide substrates for a nicking assay were prepared as previously described (24), and the labeled strand was shown with an asterisk as *A, *F, *GO, and so on, as summarized in Table 1. Oligonucleotides with *AP:G or *AP:GO were prepared from oligonucleotides with *U:G or *U:GO reacted with *E.coli* uracil DNA glycosylase (Invitrogen), as previously described (22,25). The standard nicking assay was performed, as previously described (22,25). Briefly, 20 nM duplex oligonucleotide substrates were incubated in 12.5 μl of the reaction buffer (10 mM Tris–HCl pH 7.6, 5 μM $ZnCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 1.5% glycerol, 100 μg/ml BSA) with 60 μg of cell-free extract, or 40 nM Trx-mMUTYH and/or mAPEX1 or 100 nM OGG1 at 37°C for a given time, unless otherwise noted. In the presence of mAPEX1, 0.2 mM $MgCl_2$ was carried over from the buffer B in which mAPEX1 was stored. Next, the reactions were heated in the presence of 0.67N NaOH at 95°C for 5 min, and products were extracted with phenol/chloroform, and precipitated with ethanol. Precipitated products were dissolved in 30 μl of 40% formamide containing 3 μg/ml BlueDextran (Sigma) and 10 mM EDTA, then 3 μl of the mixture was fractionated on 8% long Ranger denatured gel (24 cm length) containing 7 M urea at 30 W for 2 h. Specifically nicked 19-base (MUTYH reaction), or 15-base (OGG1 reaction) length oligonucleotide, labeled with FAM was detected using the model 373 automated DNA sequencer and quantified with GeneScan 3.1 software (Perkin Elmer), according to the manufacturer's instructions.

Gel shift assay

FAM-labeled duplex oligonucleotides (20 nM) was incubated in the reaction buffer with 40 nM Trx-MUTYH and/or 200 nM APEX1 at 37°C for 60 min, unless otherwise noted. Next, the reactions products were fractionated on 4% native polyacrylamide gel (12 cm length) in 0.5 × TBE at 4 W for 3 h. Oligonucleotide labeled with FAM was detected using the model 373 automated DNA sequencer and then it was quantified with GeneScan 3.1 software. The results from one of several independent experiments are presented.

RESULTS

Inefficient incision of the AP site generated after the excision of adenine opposite 8-oxoG by mMUTYH in a cell-free extract

Cell-free extracts were prepared from the thymocytes of wild-type and MUTYH-null mice (Figure 1A, lanes 1, 2), and were incubated with duplex oligonucleotides containing adenine opposite 8-oxoG (*A:GO) or adenine opposite guanine (*A:G), in which the asterisk indicates a base in the FAM-labeled strand. Cleavage of *A-containing strand after NaOH treatment, which cleaves the generated AP sites by β/δ-elimination, was examined (Figure 1B). The cleaved fragments (labeled as 19-P and 19-OH) of both duplex oligonucleotides were detected in the reaction products with wild-type extract but not MUTYH-null extract (Figure 1B,

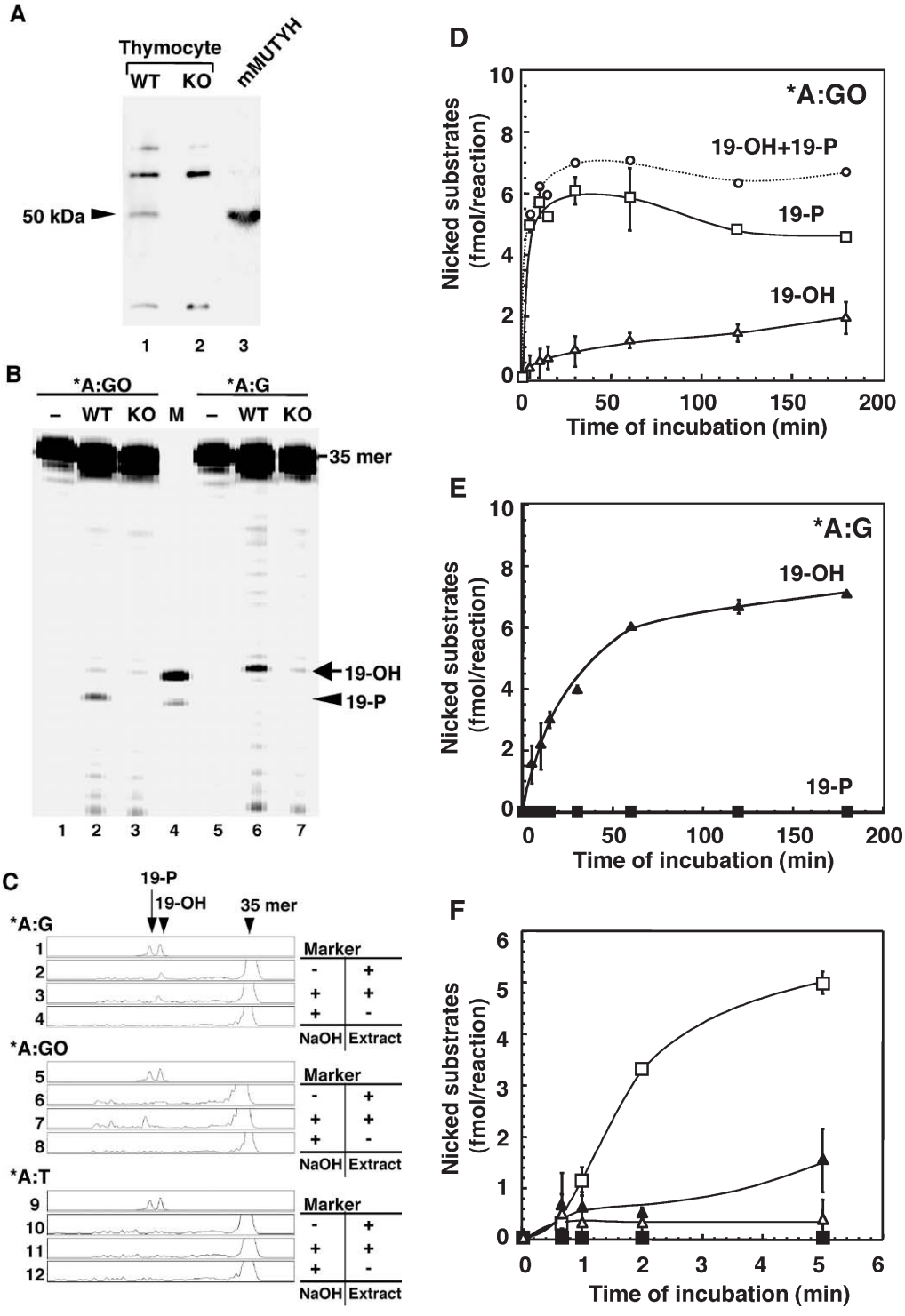


Figure 1. Inefficient incision at an AP site created after excision of adenine opposite 8-oxoguanine by MUTYH in a cell-free extract. (**A**) Thymocyte extracts (30 μg protein per lane) prepared from wild-type (lane 1, WT) and MUTYH-null (lane 2, KO) mice were subjected to western blotting with anti-hMUTYH antibody (25). As a positive control, *in vitro* transcription/translation product (lane 3, mMUTYH) of mouse *Mutyh* cDNA was applied. (**B**) Duplex oligonucleotides (20 nM) containing *A:GO (lanes 1–3) or *A:G (lanes 5–7) were incubated with cell-free extracts (90 μg/12.5 μl reaction) prepared from thymocytes of wild-type (lanes 2, 6, WT) and MUTYH-null (lanes 3, 7, KO) mice for 60 min, and reaction products treated with NaOH were fractionated. Lanes 1, 5, no extract (−); lane 4, marker oligonucleotides (M, 19-OH and 19-P). (**C**) Duplex oligonucleotides containing *A:G (lanes 2–4) *A:GO (lanes 6–8), and *A:T (lanes 10–12) were incubated with (+) or without (−) wild-type cell-free extract as in B, and the reaction mixture treated without (−) or with NaOH (+) was fractionated. The plots obtained by GeneScan are shown. Lanes 1, 5, 9, marker oligonucleotides (19-OH and 19-P). (**D**) Duplex oligonucleotides containing *A:GO were incubated with wild-type cell-free extracts (60 μg/12.5 μl reaction), for the times noted, and reaction products treated with NaOH were fractionated. Open square, production of 19-P; open triangle, production of 19-OH. Open circle, sum of 19-OH and 19-P. (**E**) Duplex oligonucleotides containing *A:G were incubated with wild-type cell-free extracts as in (D). Closed square, production of 19-P; closed triangle, production of 19-OH. (**F**) Preferential action of MUTYH on duplex oligonucleotides containing *A:GO. Results from short-term reactions are shown as in D and E. All data are shown as the means ± S.E.M. of triplicate assays, and the results from one of two independent experiments are presented.

lanes 2, 3, 6, 7), thus indicating that mMUTYH is responsible for the reaction. A fragment co-migrating with a marker oligonucleotide (19-OH) with 3′-OH group corresponds to the incision product by a class II AP endonuclease at the generated AP site after an excision of adenine opposite guanine or 8-oxoG by mMUTYH. Such an incised fragment (19-OH) by AP endonuclease was detected only from duplex oligonucleotides containing *A:G pair but not *A:GO pair (Figure 1B, lanes 2, 6). A fragment co-migrating with a marker oligonucleotide (19-P) with 3′-phosphate, which corresponds to the product cleaved by β/δ-elimination at the generated AP site, was detected in the reaction products from duplex oligonucleotides containing *A:GO pair (Figure 1B, lane 2).

Without NaOH treatment, the 19-OH fragment was also generated from duplex oligonucleotides containing *A:G pair by wild-type extracts (Figure 1C, lane 2), however, neither the 19-P nor the 19-OH fragment was detected in the reaction products from duplex oligonucleotides containing *A:GO pair by wild-type extracts (Figure 1C, lane 6), thus, indicating that wild-type extract excises adenine opposite 8-oxoG but does not incise the generated AP site. No cleaved fragment was detected from the duplex oligonucleotides containing *A:T pair at the corresponding site after incubation with the cell-free extracts, with or without NaOH treatment (Figure 1C, lanes 10, 11).

Addition of 5 mM $MgCl_2$ into the reaction mixture caused non-specific degradation of the substrate (data not shown), probably due to enhanced nuclease activities in the extract. Dialysis of the cell-free extract against the lysis buffer without Mg^{2+}, the activity incising the generated AP site opposite guanine was completely abolished, thus Mg^{2+} or related ions carried over from thymocytes are enough to support the endogenous AP endonuclease activity.

More than two-thirds of the generated AP sites after an excision of adenine opposite 8-oxoG by mMUTYH remained unincised even after 3 h of incubation with the wild-type extract (Figure 1D, open squares), while the rest was slowly incised (Figure 1D, open triangles). In contrast, no unincised AP site was detected in the reaction products from duplex oligonucleotides containing *A:G pair even after a short-term incubation (Figure 1E and F, closed squares). An excision of adenine opposite 8-oxoG by mMUTYH reached a plateau level within 5 min of incubation (Figure 1F, open squares); in contrast, excision of adenine opposite guanine by mMUTYH slowly reached a plateau level after an incubation of >60 min (Figure 1E, closed triangles). These results were also observed in cell-free extracts prepared from splenocytes and ES cells (data not shown).

As a result, mMUTYH more efficiently and rapidly excised adenine opposite 8-oxoG than adenine opposite guanine, thus generating AP sites. The generated AP sites opposite guanine are immediately incised in the cell-free extract, while the AP sites opposite 8-oxoG largely remain unincised, as summarized in Figure 2.

We estimated the content of MUTYH protein in the cell-free extract prepared from wild-type thymocytes to be <10 ng per mg of total protein based on the western blotting findings shown in Figure 1A (lane 1). In the reaction shown in Figure 1D and E, 250 fmol of substrate was incubated with 60 μg of total protein corresponding to 10 fmol or less MUTYH protein, and only 7 fmol of substrates (*A:GO, *A:G) were reacted even after the prolonged incubation, thus indicating that MUTYH exhibits little or no turnover on both substrates with *A:GO and *A:G pairs, as observed for MutY (26).

Recombinant mAPEX1 protein efficiently incises AP sites opposite guanine but not 8-oxoG generated by recombinant mMUTYH protein

To reconstruct the *in vitro* BER reaction initiated by mMUTYH, we prepared recombinant mMUTYH protein fused to thioredoxin (Trx-mMUTYH) and recombinant mAPEX1 with His-tag (His-mAPEX1) (Figure 3A, lanes 1, 5). Purified Trx-mMUTYH possesses an efficient adenine DNA glycosylase activity towards substrates with *A:GO pair with little or no turnover, but not AP lyase activity, since enzymatically incised product was not detected without NaOH treatment at all (Figure 3B, lanes 3–6). These results indicate that Trx-mMUTYH possesses essentially the same catalytic functions as the native MUTYH protein, which also lacks AP lyase activity (22,25).

The incubation of 20 nM substrate with A:GO pair in the presence of 40 nM Trx-mMUTYH resulted in the formation of AP site on ~80% of the substrate, and it is detected as a 19-P fragment after NaOH treatment (Figure 3C, closed circles). In the presence of His-mAPEX1, only a small part of the generated AP site was incised slowly (Figure 3C, open triangles), detected as a 19-OH fragment regardless of NaOH treatment,

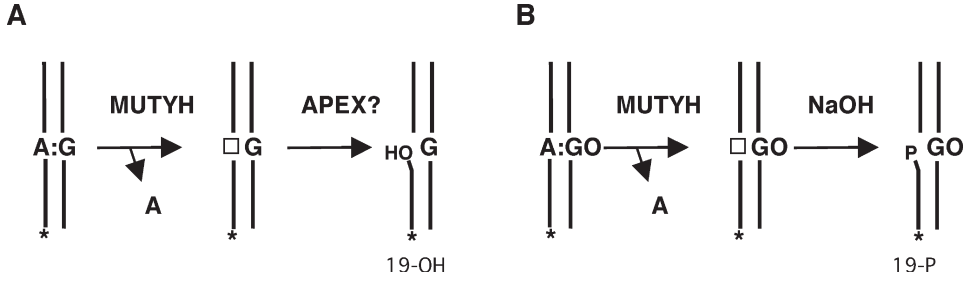


Figure 2. The schematic representation of reaction products generated by MUTYH in the cell-free extracts. mMUTYH excises adenine opposite 8-oxoG or opposite guanine, thus generating an AP site (square). (**A**) The generated AP site opposite guanine is immediately incised by a certain AP enodonuclease (APEX?) in the cell-free extract, and thus the incised fragment with 3′-OH was detected as the 19-OH fragment labeled with FAM(*). (**B**) In contrast, the AP site opposite 8-oxoG remained unincised, and was cleaved by β/δ-elimination catalyzed by NaOH. The cleaved fragment with 3′-phosphate was detected as the 19-P fragment labeled with FAM(*).

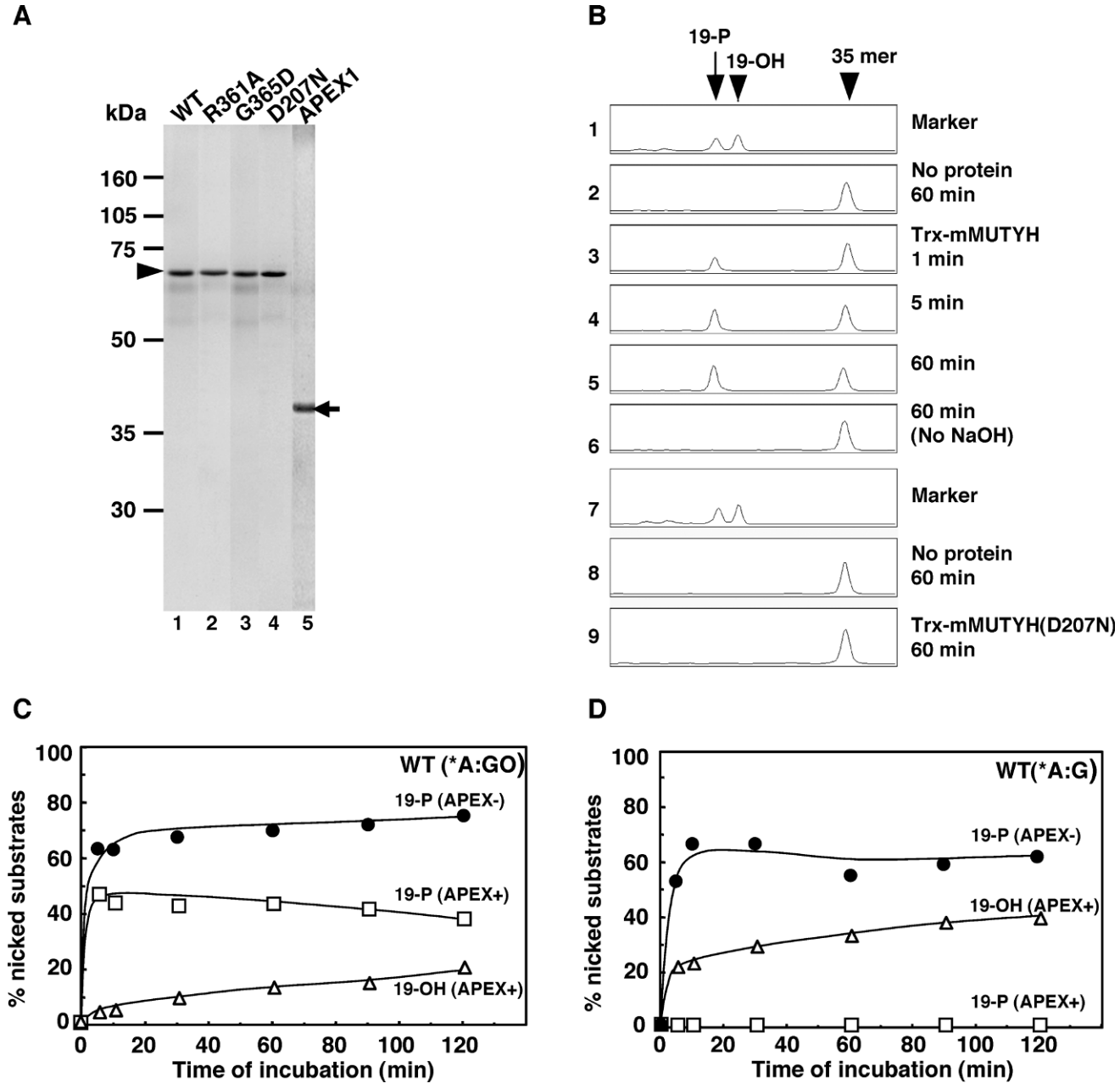


Figure 3. MUTYH prevents APEX1 from incising the generated AP site opposite 8-oxoG but not guanine. (**A**) Purified recombinant proteins, wild-type Trx-mMUTYH (lane 1, WT), Trx-mMUTYH (R361A) (lane 2), Trx-mMUTYH (G365D) (lane 3), Trx-mMUTYH (D207N) (lane 4), and His-mAPEX1 (APEX1) (lane5) were analyzed by SDS–PAGE. An arrow head indicates Trx-mMUTYH proteins, and an arrow indicates His-mAPEX1 protein, respectively. (**B**) Duplex oligonucleotides (20 nM) containing *A:GO was incubated with Trx-mMUTYH (lanes 3–5) and Trx-mMUTYH(D207N) (lane 9) proteins (40 nM), or in the absence of protein (lanes 2, 8, no protein), for the times noted, and the reaction products were fractionated after NaOH treatment. The reaction product of Trx-mMUTYH with 60 min of incubation was also fractionated without NaOH treatment (lane 6, No NaOH). The plots obtained by GeneScan are shown. Lanes 1, 7, marker oligonucleotides (Marker, 19-OH and 19-P). (**C**) Duplex oligonucleotides (20 nM) containing *A:GO were incubated with Trx-mMUYTH (40 nM) in the presence or absence of His-mAPEX1 (200 nM) for the times noted, and reaction products treated with NaOH were fractionated. Open square, production of 19-P in the presence of His-mAPEX1; open triangle, production of 19-OH in the presence of His-mAPEX1; closed circle, production of 19-P in the absence of mAPEX1. (**D**) Duplex oligonucleotides containing *A:G were incubated as in (C). There was no 19-OH detected in the absence of mAPEX1 from both duplex oligonucleotides containing A:GO and A:G.

while two-thirds of the generated AP sites still remained unincised even after 2 h of incubation (Figure 3C, open squares). Since the same results were obtained after 30 min of incubation in the presence of 5 or 10 mM $MgCl_2$ (data not shown), the low concentration of Mg^{2+} (0.2 mM) is not a limiting factor for the poor incision by His-mAPEX1. Previously, Young *et al.* (27) reported that APEX1 enhances excision of adenine opposite 8-oxoG by MUTYH, and we observed such enhancement for an old but not fresh preparation of Trx-MUTYH, indicating that Ref-1 activity of APEX1 might be involved in the enhancement. Furthermore, they also presented data that APEX1 incises only a part of the

generated AP sites opposite 8-oxoG by MUTYH, as we observed in the present study.

In contrast, the substrate with the *A:G pair was completely incised after the excision of adenine by Trx-mMUTYH in the presence of His-mAPEX1, however, the excision of adenine by Trx-mMUTYH in the presence of His-mAPEX1 was apparently less efficient than in the reaction in the absence of His-mAPEX1 (Figure 3D). These results may reflect the APEX1-MUTYH or APEX1-DNA interaction, as previously reported (15–17). Although Pope *et al*. (24) previously reported that AP endonuclease can enhance MutY turnover with the substrate with A:G pair in *E.coli*, we did not observe any enhancement of MUTYH turnover.

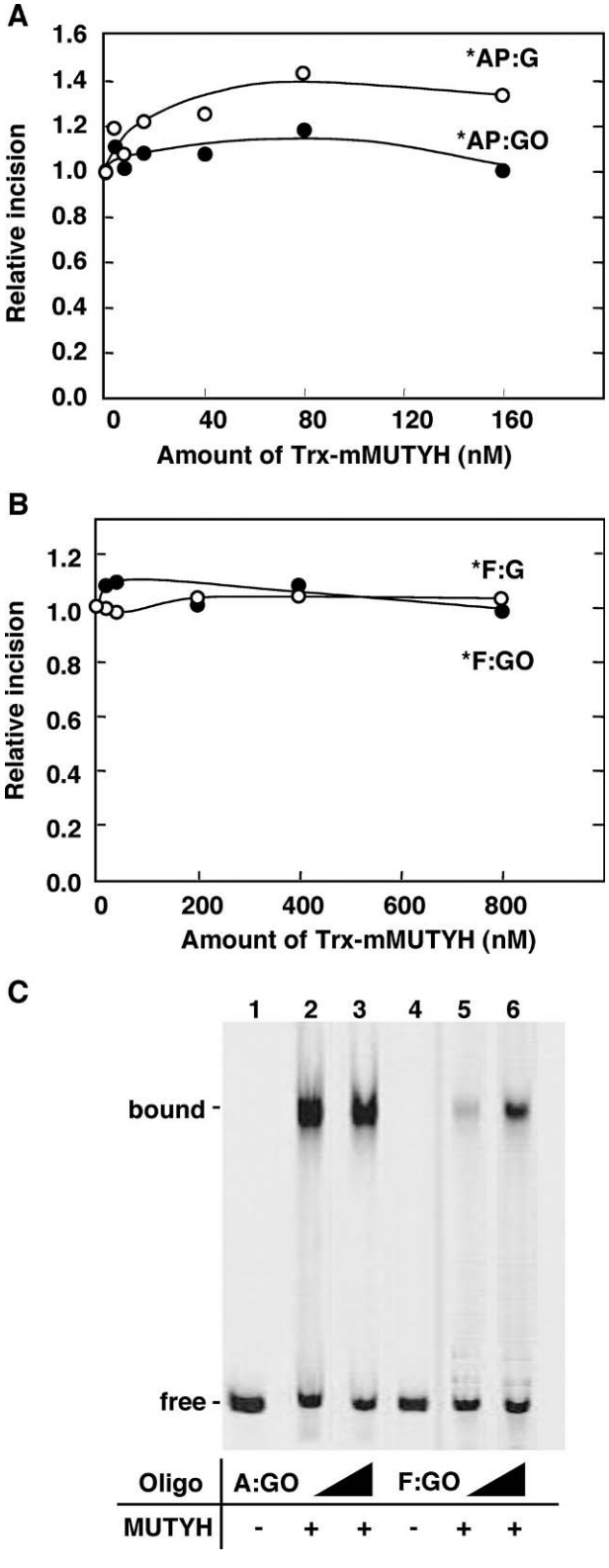


Figure 4. MUTYH does not prevent APEX1 from incising the AP sites generated by uracil DNA glycosylase or a synthetic AP site with a reduced affinity. (**A**) Duplex oligonucleotides (4 nM) containing *AP:G (open circle), or *AP:GO pair (closed circle) were incubated with various concentrations of Trx-mMUYTH (0 to 160 nM) for 30 min, and then were incubated with 40 nM His-mAPEX1 for 10 min, and the reaction products were also fractionated. (**B**) Duplex oligonucleotides (20 nM) containing *F:G (open circle), or *F:GO pair (closed circle) were incubated with various concentrations of Trx-mMUYTH (0–800 nM) at 37°C for 30 min, and then were incubated with 800 nM His-mAPEX1 for 30 min, and reaction products were fractionated. In these conditions, ~50% of the substrate was incised by mAPEX1 without mMUTYH. The relative amount of incised fragment in each reaction to that of the control reaction without mMUTYH is shown as a relative incision. (**C**) Duplex oligonucleotides (lanes 1, 2, 4, 5, 9 nM; lanes 3, 6, 20 nM) with *A:GO pair (lanes 1–3), or *F:GO (lanes 4–6) were incubated with (80 nM, +) or without Trx-mMUTYH (−) at 37°C for 60 min, then the reactions products were fractionated on 4% native polyacrylamide gel.

To examine whether mMUTYH prevents the incision of a pre-existing natural AP site opposite 8-oxoG or guanine by mAPEX1, we prepared duplex oligonucleotides containing uracil opposite 8-oxoG or guanine, and then treated those duplex oligonucleotides with uracil DNA glycosylase. A synthetic AP site analog, tetrahydrofuran (F) was also placed opposite guanine or 8-oxoG (*F:G, *F:GO) in duplex oligonucleotides. Wild-type Trx-mMUTYH failed to prevent His-mAPEX1 from incising the pre-existing AP site (Figure 4A) or its synthetic analog (Figure 4B), either opposite guanine or 8-oxoG.

Next, we examined whether MUTYH forms a complex with duplex oligonucleotides with A:GO or F:GO, using a gel mobility shift assay. Much larger amounts of duplex oligonucleotides with A:GO pair were shifted in the presence of Trx-MUTYH than those with F:GO (Figure 4C). Nicking assay of the reaction mixture revealed that adenine opposite 8-oxoG in the former MUTYH-DNA complex was likely to be mostly excised (data not shown), suggesting that MUTYH tightly binds to its product, duplex oligonucleotides with AP site opposite 8-oxoG, but not duplex oligonucleotides with the pre-exsisting AP sites opposite 8-oxoG. These results suggest that tight binding of MUTYH to its products results in inefficient incision of the generated AP sites by APEX1.

R361A and G365D substitutions reduced the affinity of mMUTYH protein to its product but not the pre-existing AP site opposite 8-oxoG

There are substantial sequence similarities among the C-terminal halves of prokaryotic MutY proteins and mammalian homologs, mMUTYH and hMUTYH (Figure 5), and the C-terminal half of *E.coli* MutY has been reported to determine 8-oxoG specificity and play a crucial role in mutation avoidance (28,29). The C-terminal halves of MutY homologs consist of a NUDIX domain as a major conserved functional domain, and bacterial MutT or hMTH1, which possess phosphohydrolase module in the NUDIX domain and hydrolyze

8-oxo-dGTP, also partly share amino acid sequences with MutY homologs (25,30,31). In hMTH1, it is likely that the amino acid residues outside the phosphohydrolase module are involved in contact with its substrates (32), for example F27A substitution increased its dissociation constant both from 8-oxo-dGTP and 2-OH-dATP 5-fold over wild-type protein. Thus, we introduced substitutions into four conserved residues (Q360, R361, G365, F373) in the region of mMUTYH homologous to the flanking region of the hMTH1 phosphohydrolase module (Figures 3A and 5), in order to obtain mutant Trx-MUTYH proteins with a reduced affinity to its substrate. Among these mutants, we found that mutants with R361A or G365D substitution, but not Q360A or F373A substitution, exhibited efficient incision of duplex oligonucleotides with A:GO pair by mAPEX1 (data not shown), thus these two mutant proteins were purified to homogeneity (Figure 3A, lanes 2, 3). Furthermore, we prepared a mutant Trx-mMUTYH(D207N) protein lacking the adenine DNA glycosylase activity, in which a residue Asp 207 corresponding to the active site residue, Asp138 in MutY protein (33), was substituted with asparagine (Figure 3A. lane 4, 3B, lane 9).

In the absence of mAPEX1, wild-type and D207N mutant Trx-mMUTYH proteins exhibited substantial levels of a shifted band of duplex oligonucleotides with *A:GO (Figure 6A, lanes 1, 4), and only the wild-type preparation exhibited, to a much lesser extent, a shifted band of duplex oligonucleotides with *A:G pairs (Figure 6A, lane 5). While R361A substitution (Figure 6A, lanes 2, 6) markedly reduced such gel shift activity of Trx-mMUTYH protein for duplex oligonucleotides with *A:GO. Similar results were obtained in the presence of His-mAPEX1 (Figure 6B). G365D substitution, which corresponds to a germ-line mutation (G382D) found in patients with autosomal recessive colorectal adenomatous polyposis (13), also exhibited the same effect as the R361A substitution (Figure 6A and B, lanes 3, 7). In the presence of mAPEX1, a shifted band was hardly detected for duplex oligonucleotides with A:G pair even with wild-type mMUTYH (Figure 6B, lanes 5–8).

We next compared the binding affinity of wild-type and mutant Trx-mMUTYH proteins for duplex oligonucleotides with A:GO pair or with F:GO, using a gel mobility shift assay. Since it was hard to achieve the saturated binding with increased concentrations of mutant proteins, we incubated the constant amount of each Trx-mMUTYH protein with increased concentrations of the duplex oligonucleotides, thus determined an apparent dissociation constant (K_d) for each. Wild-type Trx-mMUTYH protein exhibited the lowest K_d value, 2.95 nM, while R361A and G365D exhibited a higher K_d value, 10.8 nM for duplex oligonucleotides with A:GO pair (Figure 6C). In contrast, all three proteins exhibited much higher K_d values (20 to 25 nM) for duplex oligonucleotides with F:GO (Figure 6D), and no apparent difference was observed among the three. Thus, we concluded that R361A and G365D substitutions markedly reduce the affinity of mMUTYH to the duplex oligonucleotides with A:GO pair but not with F:GO.

mAPEX1 efficiently incised AP sites opposite 8-oxoG generated by mutant mMUTYH protein with a reduced affinity to its product

To examine whether the mutant Trx-mMUTYH proteins with a reduced affinity to its product alters the incision of AP sites opposite 8-oxoG in the product by mAPEX1, they were incubated with duplex oligonucleotides with *A:GO or *A:G pair in the presence or absence of mAPEX1. The R361A mutant introduced AP sites into duplex oligonucleotides with *A:GO as efficiently as wild-type Trx-MUTYH (Figure 7A,

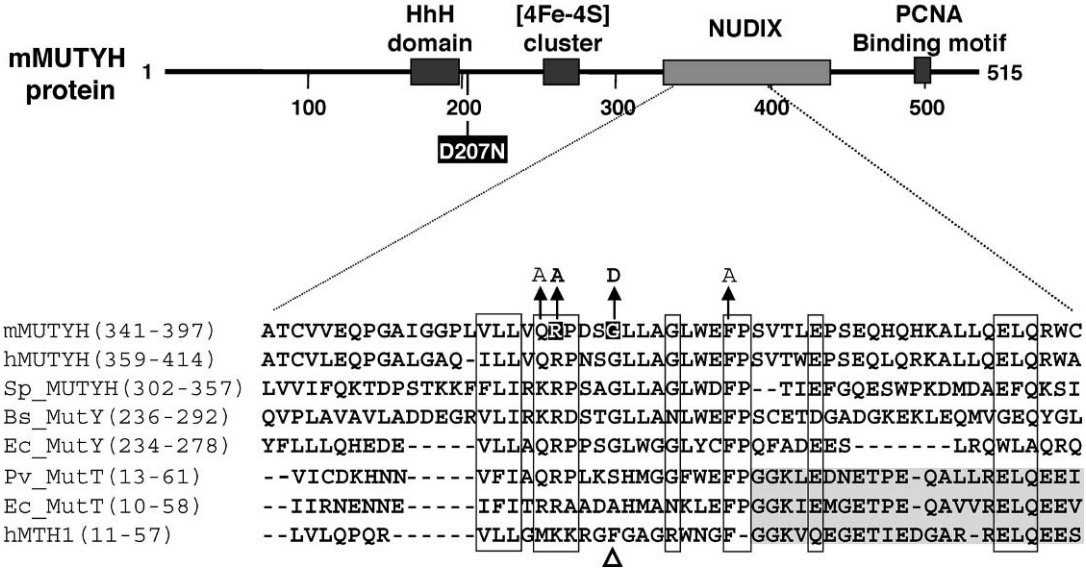
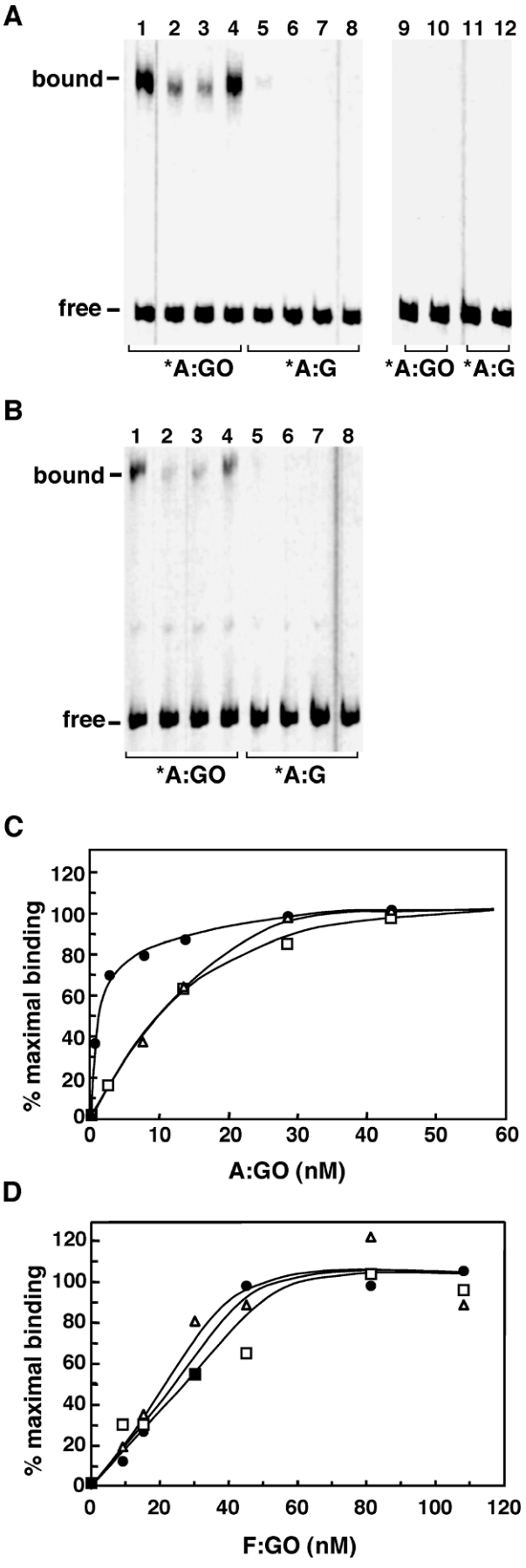


Figure 5. NUDIX domains conserved among MutY and MutT homologs. Functional motifs in mMUTYH protein are shown in the top panel. Amino acid sequences of NUDIX domains of various MutY and MutT homologs are aligned (bottom panel). Highly conserved residues are shown in the box. Amino acid residues (Q360, R361, F373) in mMUTYH were substituted with alanine in the present study, and residue G365 corresponding to G382 in human MUTYH which was reported to be substituted with aspartic acid in patients with autosomal recessive colorectal adenomatous polyposis, was also substituted with aspartic acid. The substitution of F27 shown with a triangle in hMTH1 with alanine increased 5-fold its dissociation constant (K_d) for 8-oxo-dGTP and 2-OH-dATP (32).



closed circles), and the generated AP sites opposite 8-oxoG were efficiently incised by mAPEX1 (Figure 7A, open triangles). After a 2 h incubation of duplex oligonucleotides with *A:GO or *A:G pair in the presence of Trx-mMUTYH (R361A) and His-mAPEX1, >95% of the generated AP sites opposite 8-oxoG as well as opposite guanine were detected as 19-OH fragments (Figure 7A and B, open triangles). Apparently 50% of the AP site generated opposite 8-oxoG was incised by His-mAPEX1 within 20 min.

A mutant Trx-mMUTYH with a G365D substitution also exhibited the same effect on the incision of the generated AP site by mAPEX1 as did Trx-mMUTYH(R361A) (Figure 7C and D). The effect of each substitution on the adenine DNA glycosylase activity itself was not apparent towards both *A:GO and *A:G pair (Figure 7A–D).

As a result, we concluded that the C-terminal domain of MUTYH confers the high affinity of MUTYH protein to its product, and thus APEX1 inefficiently incises the generated AP site opposite 8-oxoG.

G365D substitution reduced the capability of mMUTYH protein to prevent OGG1 from excising 8-oxoG opposite adenine

Binding affinity of Trx-mMUTYH to various duplex oligonucleotides was examined by a competition assay (Figure 8). FAM-labeled duplex oligonucleotides with *A:GO pair (15 nM) were incubated with Trx-mMUTYH or Trx-mMUTYH(G365D) in the presence or absence of various concentrations of non-labeled duplex oligonucleotides containing A:GO, F:GO, C:GO or C:G pair, and then bound fraction of the FAM-labeled duplex was measured. Binding of wild-type Trx-mMUTYH to the FAM-labeled duplex oligonucleotides with *A:GO was efficiently competed by 10-fold excess duplex oligonucleotides (150 nM) with A:GO or F:GO pair (Figure 8A, lanes 1–6). Similar results were obtained with Trx-mMUTYH(G365D) (Figure 8B, lanes 1–6), however, the latter exhibited much less binding to the FAM-labeled duplex with *A:GO pair in the absence of a competitor. There was no apparent difference between extents of the competition by A:GO and F:GO, both for wild type and G365D

Figure 6. R361A and G365D substitutions reduced the affinity of mMUTYH protein to its product. (**A**) Duplex oligonucleotides (20 nM) were incubated with Trx-mMUTYH (40 nM), then the reactions products were fractionated on 4% native polyacrylamide gel. Lanes 1–4, 9, 10, *A:GO; lanes 5–8, 11, 12, *A:G; lanes 1, 5, wild type; lanes 2, 6, G365D; lanes 3, 7, R361A; lanes 4, 8, D207N; lanes 9, 11, Trx; lanes 10, 12, no protein. (**B**). Duplex oligonucleotides (20 nM) were incubated with Trx-mMUTYH (40 nM) and His-mAPEX1 (200 nM), then the reaction products were fractionated on native polyacrylamide gel. Lanes 1–4, *A:GO; lanes 5–8, *A:G; lanes 1, 5, wild type; lanes 2, 6, R361A; lanes 3, 7, G365D; lanes 4, 8, D207N. (**C**) Various concentrations (0–108 nM) of duplex oligonucleotides containing *A:GO were incubated with 80 nM of wild-type Trx-mMUTYH (closed circle), and its derivatives, R361A (open triangle), G365D (open square). The reaction products were fractionated on 4% native polyacrylamide gel. The percent of maximal binding attained with 80 nM or higher concentration of oligonucleotides was plotted against the concentrations of the duplex oligonucleotides. The data obtained with concentrations of oilgonucleotides lower than 60 nM are shown, because the binding was saturated with the higher concentration for all three proteins. (**D**) Various concentrations of duplex oligonucleotides containing *F:GO were incubated with 80 nM of Trx-mMUTYH and its derivatives as in C.

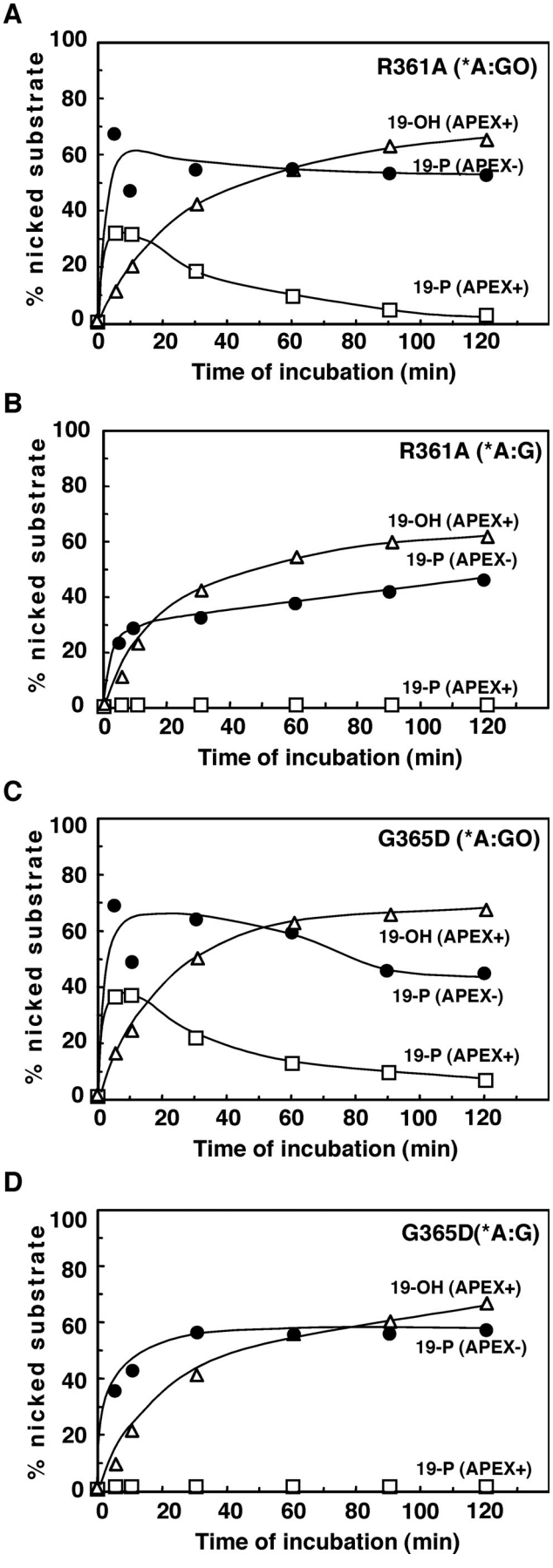


Figure 7. Mutant MUTYH protein with an amino acid substitution at R361 or G365 could not prevent APEX1 from incising the generated AP sites opposite 8-oxoG. (**A**) Duplex oligonucleotides (20 nM) containing an *A:GO were incubated with Trx-mMUTYH(R361A) (40 nM) in the presence (APEX1+) or absence (APEX1−) of His-mAPEX1 (200 nM) for the times noted, and reaction products treated with NaOH were fractionated. Open square, the production of 19-P in the presence of His-mAPEX1; open triangle, the production of 19-OH in the presence of His-mAPEX1; closed circle, the production of 19-P in the absence of His-mAPEX1. (**B**) Duplex oligonucleotides containing an *A:G were incubated with Trx-mMUTYH(R361A) as in (A). (**C**) Duplex oligonucleotides containing *A:GO were incubated with Trx-mMUTYH(G365D) as in (A). (**D**) Duplex oligonucleotides containing *A:G were incubated with Trx-mMUTYH(G365D) as in (A).

mutant (Figure 8A and B, open squares and circles), probably because the concentration of the competitors exceeds their K_d values for F:GO (>20 nM). Binding of Trx-mMUTYH(G365D) to the FAM-labeled duplex with *A:GO was partly competed with 50-fold excess duplex either with C:GO or C:G pair, in a similar extent (Figure 8B, closed circles and open triangles), while the binding of wild-type Trx-mMUTYH was more efficiently competed by the duplex with C:GO than with C:G pair (Figure 8A, closed circles and open triangles). Since Trx-mMUTYH(G365D) exhibited much lower affinity to the duplex with A:GO pair, these results indicate that mMUTYH(G365D) also has a lower affinity to the duplex with C:GO pair than does wild-type mMUTYH.

We next examined whether MUTYH prevents OGG1, which is shown to specifically excise 8-oxoG opposite cytosine (7), from excising 8-oxoG in duplex oligonucleotides or not. Unexpectedly, we found that OGG1 efficiently excised 8-oxoG opposite adenine (A:*GO) (Figure 9A, circles), however, the reaction was slow in comparison to reactions on 8-oxoG opposite either tetrahydrofuran (F:*GO) or cytosine (C:*GO) (Figure 9A, squares and triangles). The excision of 8-oxoG by OGG1 from duplex oligonucleotides containing C:*GO pair was not inhibited after the pre-incubation of the duplex with an increased concentration of wild-type Trx-mMUTYH or Trx-mMUTYH(G365D) (Figure 9B, open and closed triangles). In contrast, the excision of 8-oxoG from duplex oligonucleotides containing A:*GO pair by OGG1 was efficiently inhibited after the pre-incubation of the duplex with wild-type Trx-mMUTYH, and a 50% inhibition was attained in the presence of 20 nM of Trx-mMUTYH, which corresponded to the concentration of the substrate (20 nM), and 90% of substrate was protected in the presence of 100 nM Trx-mMUTYH (Figure 9B, open circles). With increased concentrations of Trx-mMUTYH(G365D) up to 100 nM, a 5-fold higher concentration than the substrate, only 40% of the substrate was protected from an excision of 8-oxoG by OGG1 (Figure 9A, closed circles). We thus concluded that MUTYH prevents OGG1 from excising 8-oxoG opposite adenine or the generated AP site and that G365D substitution apparently impaired the protective function of MUTYH against OGG1.

DISCUSSIONS

In the present study, we found that an AP site generated opposite 8-oxoG by MUTYH remains unincised for a couple hours

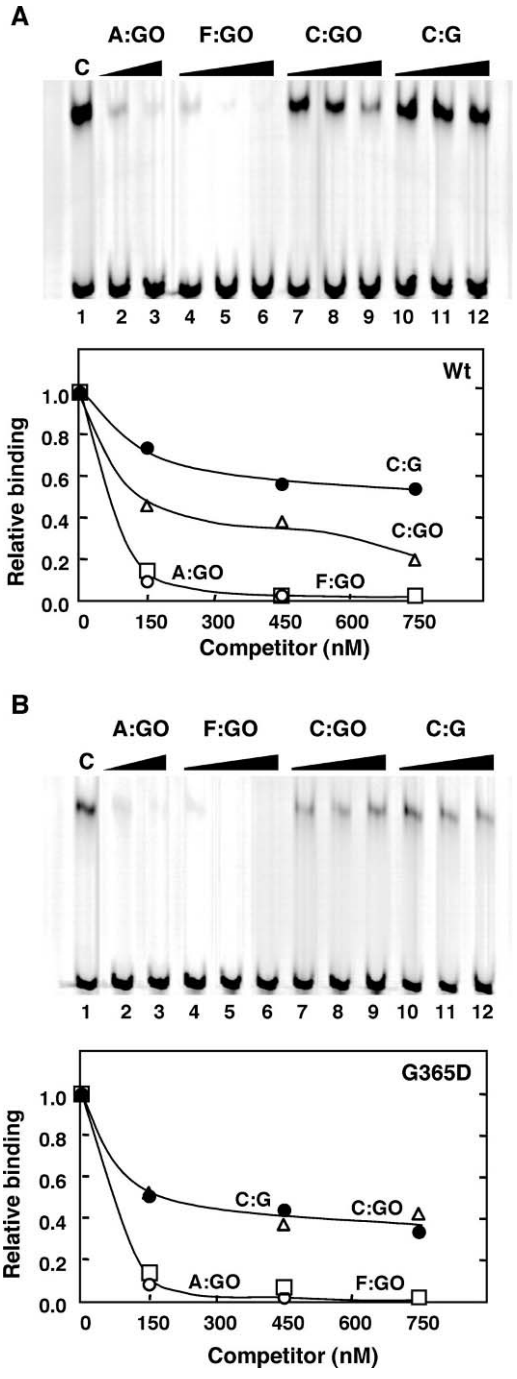


Figure 8. MUTYH protein has much less affinity to duplex oligonucleotide containing C:GO pair. (**A**) Duplex oligonucleotides (15 nM) containing *A:GO pair were incubated with 40 nM wild-type Trx-mMUTYH in the presence or absence of various concentrations (150, 450 or 750 nM) of non-labeled oligonucleotides containing A:GO (lanes 2, 3, circle), F:GO (lanes, 4–6, square), C:GO (lanes 7–9, triangle), or C:G (lanes 10–12, closed circle) pair. The reaction products were fractionated on 4% native polyacrylamide gel (top panels), and the relative binding to that without a competitor was plotted in the lower panels. (**B**) Duplex oligonucleotides (15 nM) containing *A:GO pair were incubated with 40 nM Trx-mMUTYH(G365D) as in (A).

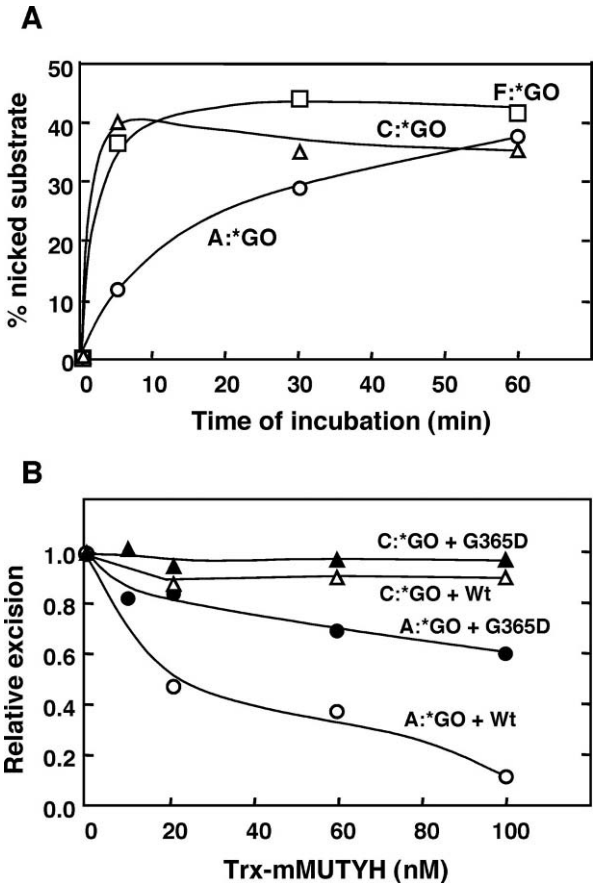


Figure 9. The G365D substitution reduced the capability of mMUTYH protein to prevent OGG1 from excising 8-oxoG opposite adenine. (**A**) Duplex oligonucleotides (20 nM) containing A:*GO (circle), C:*GO (triangle), or F:*GO (square), in which the GO-containing strand was labeled with FAM (*), were incubated with OGG1 (100 nM) for the times noted, and the reaction products treated with NaOH were fractionated. Production of 15-base fragment with phosphate was measured and fraction of the cleaved fragment was plotted. (**B**) Wild-type mMUTYH but not mMUTYH(G365D) prevents OGG1 from excising 8-oxoG opposite adenine. Duplex oligonucleotides (20 nM) containing C:*GO (circle) or A:*GO (triangle) were incubated with various concentrations of wild-type Trx-mMUTYH (open symbol) or Trx-mMUTYH(G365D) (closed symbol) for 30 min, then were incubated with OGG1 (100 nM) for 30 min, and the reaction products treated with NaOH were fractionated. The relative amount of the cleaved fragment to that without MUTYH was plotted as a relative excision.

in cell-free extracts which contain various enzymes involved in the BER pathway, including AP endonucleases and OGG1, thus indicating that the AP site generated opposite 8-oxoG is inefficiently processed by multi-enzyme BER pathway in the cell-free extracts. Using recombinant mMUTYH and mAPEX1 proteins, we demonstrated that mMUTYH protects the generated AP site opposite 8-oxoG by mMUTYH itself. The generated AP site opposite guanine by mMUTYH was not protected at all both in the cell-free extracts and the reconstituted reaction with recombinant mMUTYH and mAPEX1, thus indicating that this protective function of mMUTYH is specific for the AP site opposite 8-oxoG. Interestingly, mMUTYH could not protect either the pre-existing AP site opposite 8-oxoG which was created by uracil DNA glycosylase, or the synthetic AP site analog, tetrahydrofuran opposite 8-oxoG. These results indicate that mMUTYH protects only its own reaction product, the generated AP site opposite 8-oxoG after excision of adenine by its DNA glycosylase activity. We showed that mMUTYH binds duplex oligonucleotides containing tetrahydrofuran opposite 8-oxoG

with a reduced affinity (K_d > 20 nM) in comparison with its binding to duplex oligonucleotides with A: 8-oxoG pair (K_d < 3 nM), thus indicating that a higher affinity is required for the efficient protection of the generated AP site opposite 8-oxoG by mMUTYH. These facts indicate that the binding mode of mMUTYH to its own reaction product, the generated AP site opposite 8-oxoG, must be different from that to the pre-existing AP site opposite 8-oxoG.

8-oxoG paired with cytosine is in an *anti* conformation as is normal guanine opposite cytosine (34), while 8-oxoG opposite adenine is in a *syn* conformation (35). The 8-oxoG opposite the AP site is most likely in the *anti* conformation, because the N-glycosydic bond can freely rotate without base pairing. The binding affinity of wild-type mMUTYH to duplex oligonucleotides containing 8-oxoG is likely to be determined by either the opposite base or the presence of AP site analog, tetrahydrofuran (F), and the order is: A > F ≫ C, thus indicating that MUTYH preferentially recognizes 8-oxoG in the *syn* conformation. Recently, crystal structures of *Bacillus stearothermophilus* MutY–DNA lesion recognition complex, which was stabilized through intermolecular disulphide cross-linking to a Asp114Asn variant of MutY, has been determined, and it was shown that adenine opposite 8-oxoG is flipped out of the DNA helix, while 8-oxoG swivels roughly 180° about its N-glycosidic bond, thus converted to the *anti* conformation (36). In MutY-DNA complexes with F:GO, which mimics the pre-existing AP site opposite 8-oxoG, 8-oxoG is also in the *anti* conformation with or without soaked adenine, and there are some conformational alterations in the active site and the DNA backbone in comparison to the recognition complex (36).

It has been also shown that adenine base is rapidly released from *E.coli* MutY–product complex, which is extremely stable as we observed in mMUTYH–product complex (37). Thus, we may suggest that excision of adenine from the recognition complex by mMUTYH itself may cause some conformational alterations which can stabilize the mMUTYH–product complex. Such conformational alterations may not take place when mMUTYH excises adenine opposite guanine or binds the pre-existing AP site opposite 8-oxoG, probably because of guanine opposite adenine or 8-oxoG opposite AP site are in the *anti* conformation. It is noteworthy that MUTYH does not protect the generated AP site opposite guanine with no turnover on the substrate. Since MUTYH weakly associates with the A:G reaction product as seen in Figure 6A, such weak interaction of MUTYH and its product may be sufficient to prevent its turnover. It is likely that the conformational alterations of the complex induced after excision of adenine by MUTYH itself also supports the interaction.

The residue G260 of the MutY, which correspond to the G365 residue in mMUTYH (see Figure 5), initiates a type II turn in the C-terminal domain and backbone amide nitrogen atoms of this turn form hydrogen bonds with the two phosphates immediately 5′ to the 8-oxoG, suggesting that substitution of this residue G260 with an aspartate results in disruption of the structure of this turn and thus interferes with the ability of MutY to recognize faithfully 8-oxoG in DNA (36). The reduced affinity of mMUTYH(G365D) to duplex oligonucleotides with A:GO supports this hypothesis. While the residue R256 corresponding to the R361 residue in mMUTYH is likely to contact with the residue E267, and both residues reside in the anti-parallel β-sheets holding the turn and their electrostatic interaction may contribute to stabilize the turn (36). Since the residue E267 and its neighboring residues in *B.stearothermophilus* MutY are well conserved in mMUTYH including the residue E372 (see Figure 5), suggesting that mMUTYH also has a similar local structure. Substitution of the residue R361 in mMUTYH with an alanine may disrupt the electrostatic interaction with the E372, and thus destabilizing the turn in the C-terminal domain. Again, destabilization of the turn may results in reduction of the affinity to the strand containing 8-oxoG as we observed in the mMUTYH(R361A) mutant.

It was shown that *E.coli* MutY binds to its own reaction product and protects 8-oxoG from excision by MutM, a functional homolog for OGG1 (28). In the present study, we for the first time, provide biochemical evidence that mMUTYH protects its own product from the excision of 8-oxoG by OGG1 as well as from an incision of the AP site by AP endonucleases. OGG1 preferentially excises 8-oxoG opposite cytosine and AP site, and very slowly excises 8-oxoG opposite adenine. We showed that the pre-incubation of duplex oligonucleotides containing 8-oxoG:A pair with mMUTYH, during which adenine was excised and AP site was generated opposite 8-oxoG, efficiently prevented OGG1 from excising the 8-oxoG opposite AP site that is an efficient substrate for OGG1. Mutant MUTYH with the G365D substitution hardly prevented OGG1 from excising the 8-oxoG opposite adenine or AP site, indicating that the C-terminal domain of mMUTYH plays important role to protect 8-oxoG.

The structures of the prokaryotic MutY-DNA complex also revealed that the six-helix barrel module in the N-terminal catalytic domain directly contacts with 8-oxoG in DNA as well as with the C-terminal domain, thus, allowing MutY to encircle the DNA duplex (36). mMUTYH may also encircle its substrate DNA duplex, and thus can prevent OGG1 or APEX1 from inappropriately processing its substrate and product. If MUTYH could not prevent OGG1 from acting on its products, excision of 8-oxoG opposite the AP site by OGG1 would result in the loss of informative bases on the both strands, and in a double-strand break due to the AP lyase activity of OGG1. Thus, OGG1 is a more hazardous counterpart than APEX1 if MUTYH fails to protect its products (Figure 10).

The generated AP site opposite 8-oxoG by MUTYH has to be processed through the BER pathway in which an incision by AP endonuclease is an essential step (Figure 10). In cell-free extract prepared from mouse thymocytes, the mMUTYH reaction product is fairly stable for >3 h. This fact suggests that a positive mechanism which appears to somehow be inactivated in the cell-free extract, is required to accelerate the progression of BER reaction. It has been reported that BER reaction can be completed in HeLa cell nuclear extract for plasmid DNA containing an A:8-oxoG pair (18), however the efficiency of BER was very low. We examined the effects of ATP or other ribonucleoside triphosphates as well as deoxyribonucleoside triphosphates, and found essentially no effect on the protection or repair process (our unpublished observation).

It was pointed out previously that MUTYH recognizes the adenine misincorporated into the nascent strand opposite 8-oxoG in the template strand during progress of DNA replication, and thus post-replicative BER has been proposed (38–40). Indeed, MUTYH can interact with PCNA, RPA and

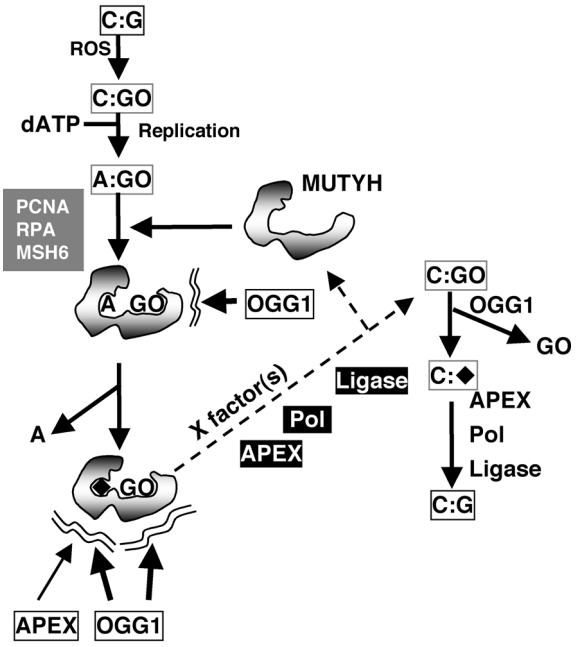


Figure 10. MUTYH-initiated BER and MUTYH-product complex. MUTYH protein specifically recognizes an adenine base inserted in the nascent strand, opposite 8-oxoG in the *syn* conformation (A:GO) in the template strand, and PCNA, RPA and MSH6 may facilitate the recognition (15–17). Then, MUTYH may convert 8-oxoG into the *anti* conformation together with flipping adenine out (36), thus converting the complex into a more stable conformation, and then the adenine is excised by its DNA glycosylase. After the excision of adenine, MUTYH stably remains on its product and prevents OGG1 from excising 8-oxoG opposite the generated AP site or incising the generated AP sites on both strands by its AP lyase activity. Since the MUTYH-product complex is stable enough to prevent APEX1 from incising the generated AP site, some other (X) factors may be required to facilitate the incision of the AP site by AP endonuclease (APEX) which is essential for the repair synthesis to yield cytosine opposite 8-oxoG (C:GO). Thereafter, 8-oxoG opposite cytosine can be repaired by BER initiated by OGG1.

MSH6, which are known to be involved in post-replicative mismatch repair (15–17). It is expected that the MUTYH–product complex should be precisely processed to result in the insertion of cytosine opposite 8-oxoG, which is supposedly processed to a cytosine:guanine pair by a canonical BER pathway initiated by OGG1. For the completion of proper BER reaction form the MUTYH–product complex, some so far unidentified factors, which can support the coordinated actions of AP endonuclease and DNA polymerase or DNA ligase, may also be involved (Figure 10). The MUTYH–product complex generated *in vitro* can be an appropriate starting material to isolate the molecules interacting with or to reconstitute the cell-free repair reaction, and such approaches are now under way.

In patients carrying homozygous G382D mutation in the *MUTYH* gene, which corresponds to the G365D mutation in mMUTYH, multiple colorectal adenoma and adenocarcinoma are often developed with a somatic G to T transversion mutation in *APC* gene (13,41), thus indicating that G382D substitution gives rise to an impaired function of MUTYH. We previously reported that MUTYH-null ES cells exhibited a mutator phenotype and that exogenous expression of wild-type mMUTYH but not mMUTYH(G365D) complemented the mutator phenotype (15). We also showed that there is no detectable adenine DNA glycosylase activity in the cell-free extract prepared from MUTYH-null cells expressing mMUTYH(G365D), which appears to be modified in the cell-free extract (13). In the present study, we showed that the recombinant preparation of mMUTYH(G365D) possesses the adenine DNA glycosylase activity as well as wild-type mMUTYH, therefore we currently could not conclude that mMUTYH(G365D) is totally inactive as DNA glycosylase *in vivo*. However, even if the mutant protein is active *in vivo* as DNA glycosylase, the biochemical defects of mMUTYH(G365D) found in the present study may in part explain the mutator phenotype. mMUTYH(G365D) has a much lower affinity to its own substrate, DNA containing A:8-oxoG pair, and to which OGG1 can also act to excise 8-oxoG. If OGG1 can excise 8-oxoG opposite adenine more efficiently than mMUTYH(G365D) excises adenine opposite 8-oxoG, an uncorrected adenine base were remain to cause G to T transversion mutation.

Further characterizations of the mutant mMUTYH or proteins interacting with the mMUTYH–product complex will hopefully shed some light on the complicated BER pathway as well as on the molecular mechanism for carcinogenesis in the colon.

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