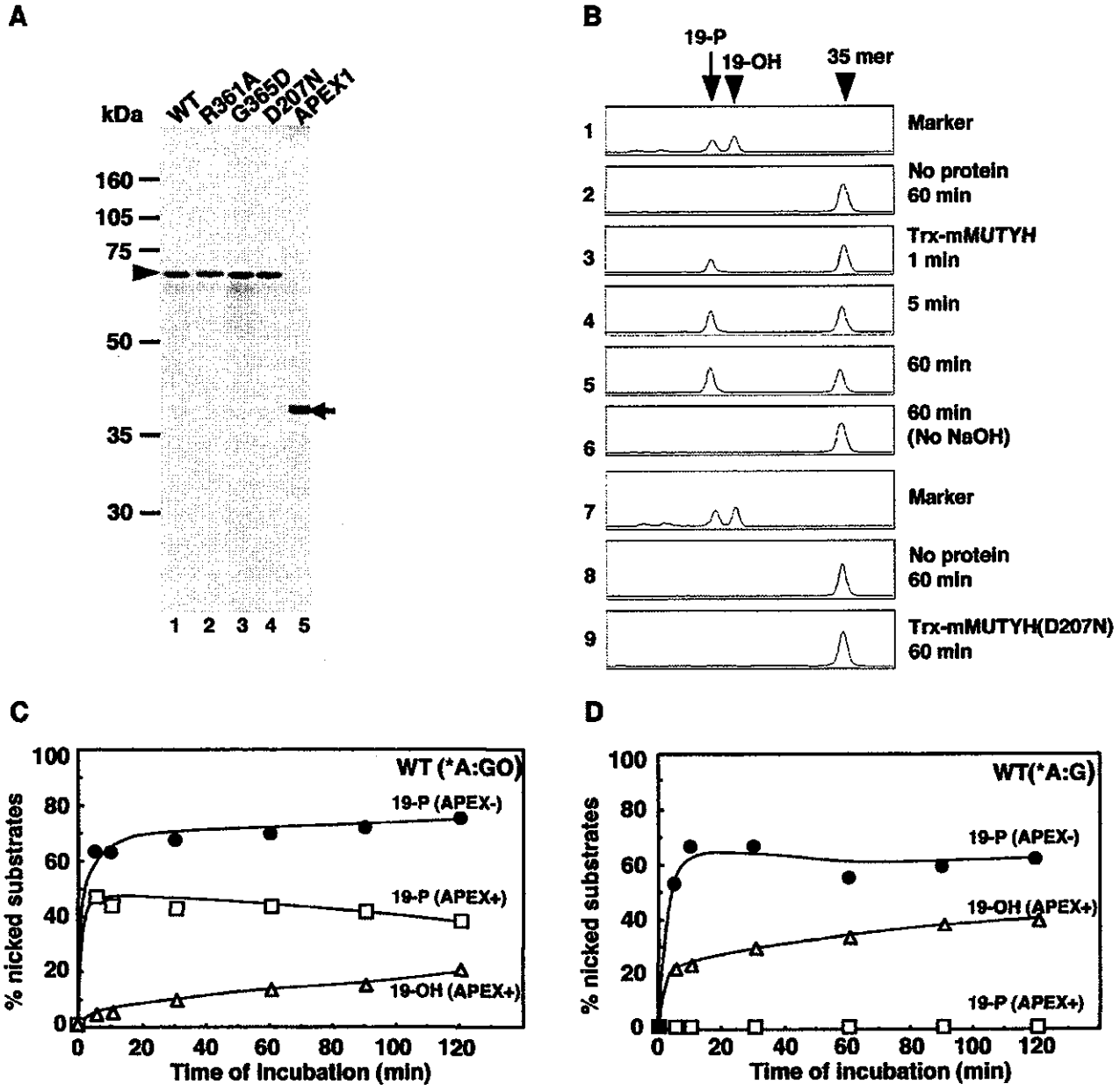



**Figure 3.** MUTYH prevents APEX1 from incising the generated AP site opposite 8-oxoG but not guanine. (A) Purified recombinant proteins, wild-type Trx-mMUTYH (lane 1, WT), Trx-mMUTYH (R361A) (lane 2), Trx-mMUTYH (G365D) (lane 3), Trx-mMUTYH (D207N) (lane 4), and His-mAPEX1 (APEX1) (lane5) were analyzed 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.

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

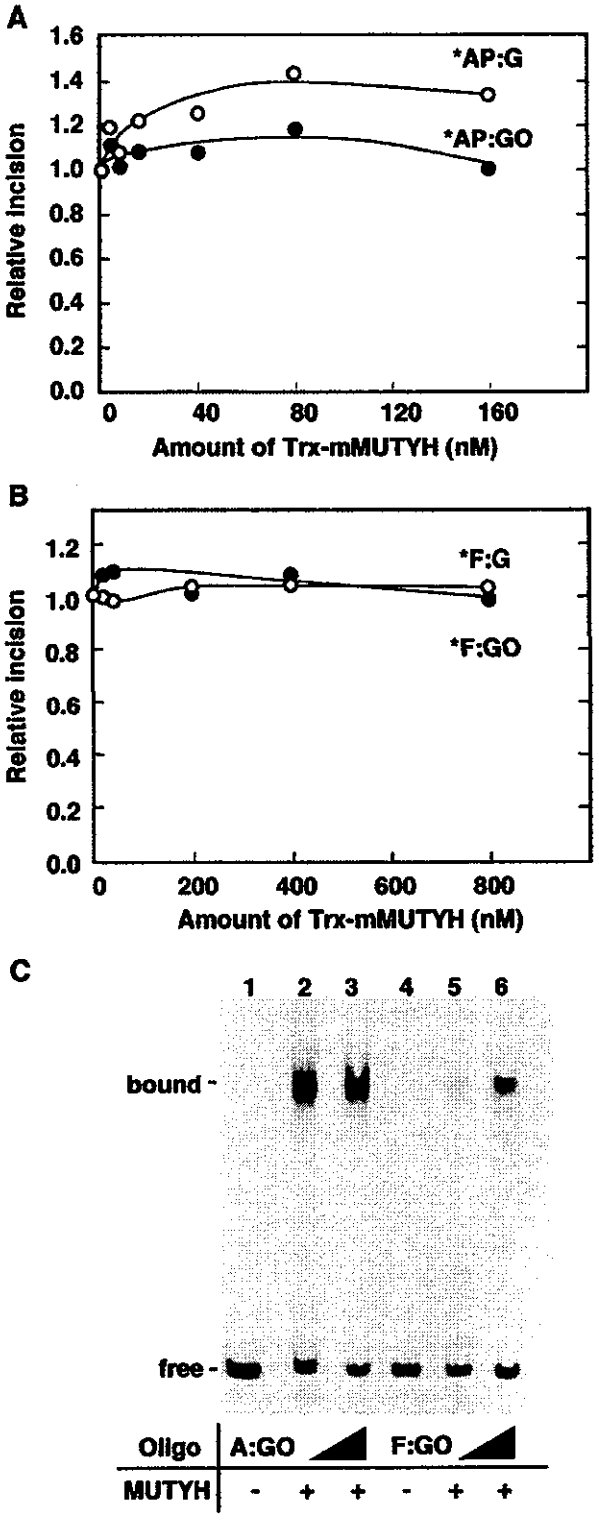



**Figure 4.** MUTYH does not prevent APEX1 from incising the AP sites generated by uracil DNA glycosylase or a synthetic AP site with a reduced affinity. (A) Duplex oligonucleotides (4 nM) containing *AP:G (open circle), or *AP:GO pair (closed circle) were incubated with various concentrations of Trx-mMUYTH (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.

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,

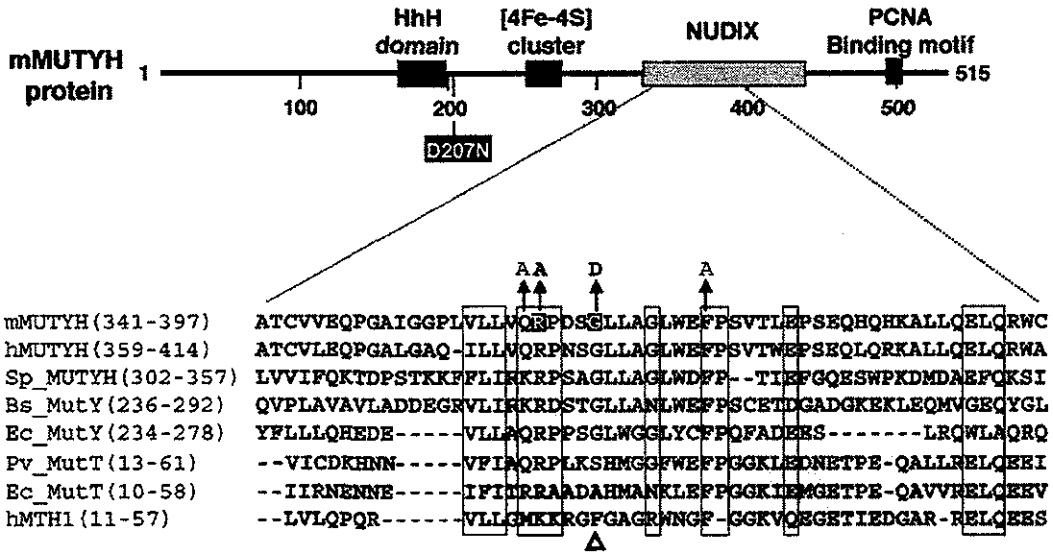
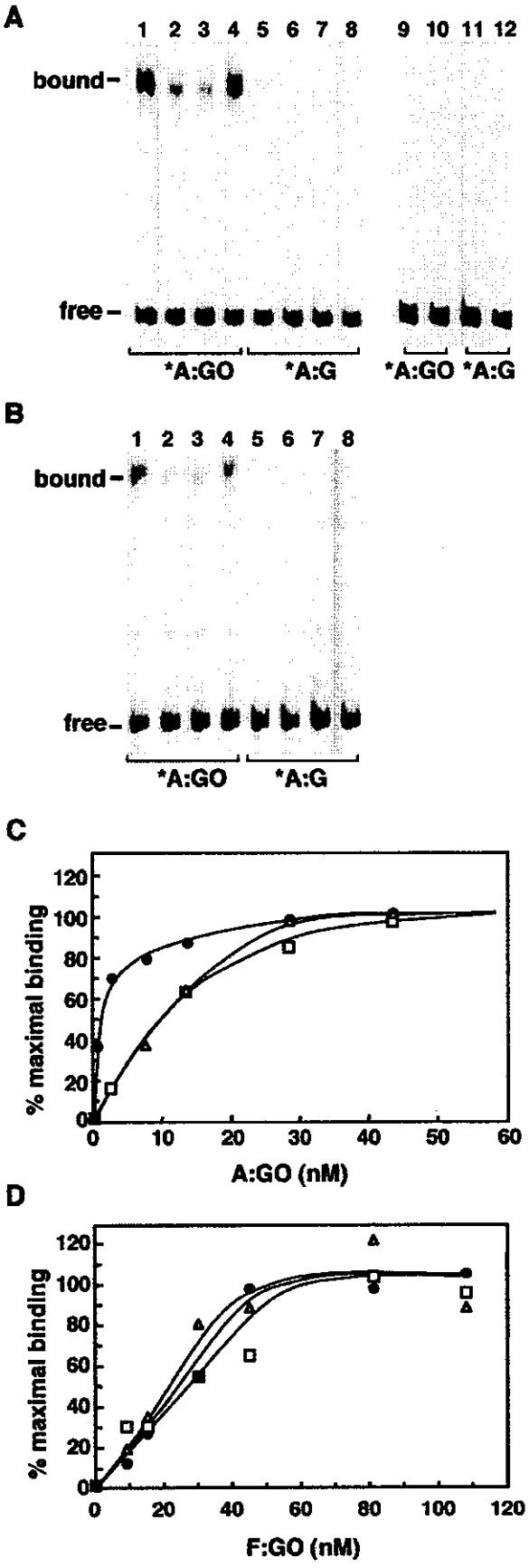



**Figure 5.** NUDIX domains conserved among MutY and MutT homologs. Functional motifs in mMUTYH protein are shown in the top panel. Amino acid sequences of NUDIX domains of various MutY and MutT homologs are aligned (bottom panel). Highly conserved residues are shown in the box. Amino acid residues (Q360, 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.

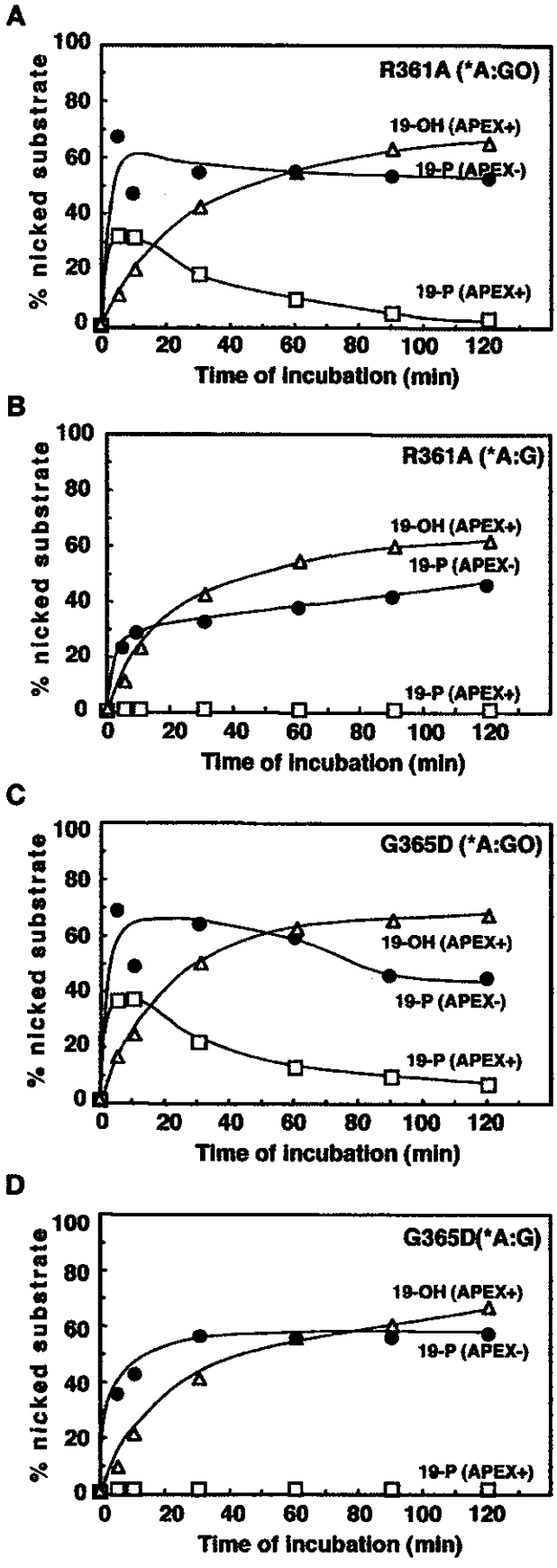
A mutant Trx-mMUTYH with a G365D substitution also exhibited the same effect on the incision of the generated AP site by mAPEX1 as did Trx-mMUTYH(R361A) (Figure 7C and D). The effect of each substitution on the adenine DNA glycosylase activity itself was not apparent towards both *A:GO and *A:G pair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




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 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).

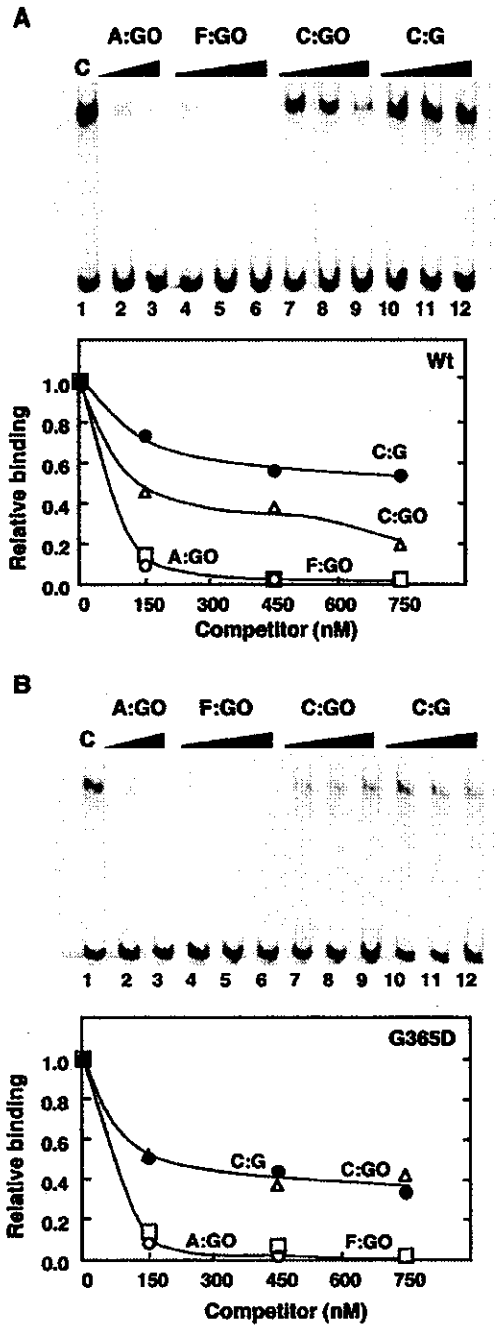



**Figure 8.** MUTYH protein has much less affinity to duplex oligonucleotide containing C:GO pair. (**A**) Duplex oligonucleotides (15 nM) containing *A:GO pair were incubated with 40 nM wild-type Trx-mMUTYH in the presence or absence of various concentrations (150, 450 or 750 nM) of non-labeled oligonucleotides 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).

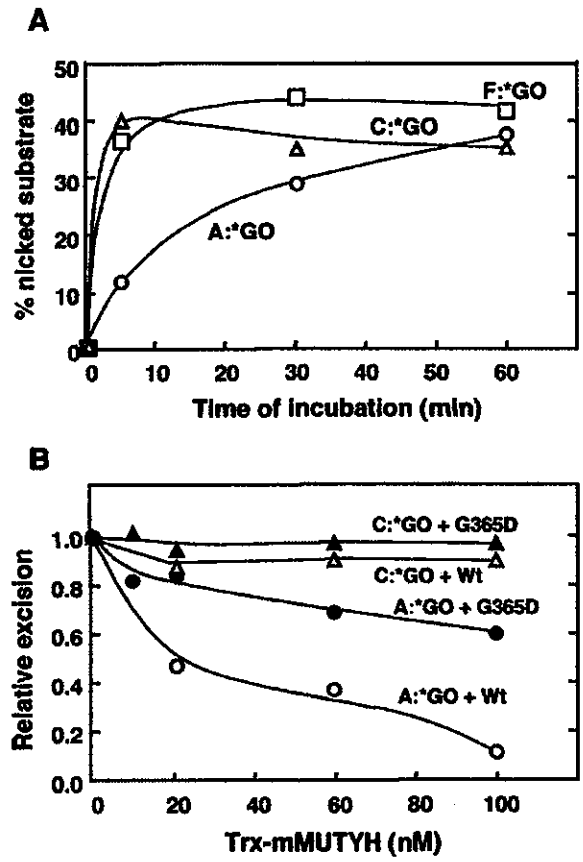



**Figure 9.** The G365D substitution reduced the capability of mMUTYH protein to prevent OGG1 from excising 8-oxoG opposite adenine. (**A**) Duplex oligonucleotides (20 nM) containing A:*GO (circle), C:*GO (triangle), or F:*GO (square), in which the GO-containing strand was labeled with FAM (*), were 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

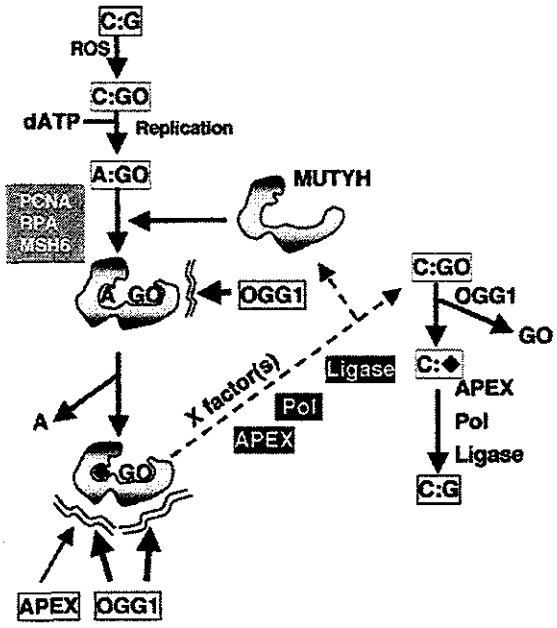



**Figure 10.** MUTYH-initiated BER and MUTYH-product complex. MUTYH protein specifically recognizes an adenine base inserted in the nascent strand, opposite 8-oxoG in the *syn* conformation (A:GO) in the template strand, and PCNA, RPA and MSH6 may facilitate the recognition (15–17). Then, MUTYH may convert 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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# A functional analysis of the DNA glycosylase activity of mouse MUTYH protein excising 2-hydroxyadenine opposite guanine in DNA

**Yasuhiro Ushijima, Yohei Tominaga, Tomofumi Miura, Daisuke Tsuchimoto, Kunihiko Sakumi and Yusaku Nakabeppu***

Division of Neurofunctional Genomics, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan

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## ABSTRACT

**2-Hydroxy-2-deoxyadenosine triphosphate (2-OH-dATP), generated by the oxidation of dATP, can be misincorporated by DNA polymerases opposite guanine in template DNA during DNA replication, thus causing spontaneous mutagenesis. We demonstrated that mouse MUTYH (mMUTYH) has a DNA glycosylase activity excising not only adenine opposite 8-oxoguanine (8-oxoG) but also 2-hydroxyadenine (2-OH-A) opposite guanine, using purified recombinant thioredoxin-mMUTYH fusion protein. mMUTYH formed a stable complex with duplex oligonucleotides containing an adenine: 8-oxoG pair, but the binding of mMUTYH to oligonucleotides containing a 2-OH-A:guanine pair was barely detectable, thus suggesting that mMUTYH recognizes and interacts with these two substrates in a different manner which may reflect the difference in the base excision repair process for each substrate. Mutant mMUTYH with G365D amino acid substitution, corresponding to a G382D germline mutation of human MUTYH found in familial adenomatous polyposis patients, almost completely retained its DNA glycosylase activity excising adenine opposite 8-oxoG; however, it possessed 1.5% of the wild-type activity excising 2-OH-A opposite guanine. Our results imply that the reduced repair capacity of the mutant hMUTYH(G382D), which inefficiently excises 2-OH-A opposite guanine, results in an increased occurrence of somatic G:C to T:A transversion mutations in the *APC* gene as well as tumorigenesis in the colon.**

## INTRODUCTION

Cellular DNA and its precursor nucleotides are 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 has been implicated in various age-related diseases such as cancer and neurodegeneration (1). Among various types of oxidized damage in DNA, 8-oxoguanine (8-oxoG) and 2-hydroxyadenine (2-OH-A), namely the oxidized forms of guanine and adenine, can pair with adenine or guanine in template DNA, respectively, during DNA replication. It is therefore considered to be one of the spontaneous causes of mutagenesis or cell death (2–4). The direct oxidation of DNA by a hydroxyradical has been reported to generate substantial amount of 8-oxoG, but little 2-OH-A. In contrast, 2-OH-A is exclusively generated by the oxidation of dATP in the nucleotide pool (5). The difference in the origins of each oxidized base suggests that 8-oxoG and 2-OH-A cause mutagenesis in a different manner, and therefore cells possess different error avoiding mechanisms for each.

In *Escherichia coli*, 8-oxo-dGTP generated in the nucleotide pool is hydrolyzed to the monophosphate by MutT protein, and 8-oxoG generated by the direct oxidation of guanine in DNA is excised by 8-oxoG DNA glycosylase, MutM (FPG) protein. Furthermore, adenine misincorporated opposite 8-oxoG in the template strand of DNA is removed by adenine DNA glycosylase, MutY protein. As a result of the cooperative action of MutT/MutM/MutY, *E.coli* efficiently protects the occurrence of spontaneous mutations such as A:T to C:G and G:C to T:A transversions, which are caused by 8-oxoG (6–8). Recently, it has been shown that the *Orf135* gene in *E.coli* encodes 2-OH-dATPase and that the occurrence of G:C to T:A transversion in *Orf135*$^-$ mutants increased 2- to 3-fold

*To whom correspondence should be addressed. Tel: +81 92 642 6800; Fax: +81 92 642 6791; Email: yusaku@bioreg.kyushu-u.ac.jp

when compared with wild type, thus suggesting that the oxidation of dATP in the nucleotide pool is one of the spontaneous causes of mutagenesis (9).

In eukaryotes, it was demonstrated that the human homolog of MutT (hMTH1) hydrolyses both 8-oxo-dGTP and 2-OH-dATP, thus preventing the spontaneous mutagenesis and cell death caused by these oxidized nucleotides (10–13). We previously reported that a partially purified human MutY homolog (hMUTYH) protein from Jurkat cells possesses DNA glycosylase activities excising 2-OH-A opposite guanine as well as adenine opposite 8-oxoG (14). *E.coli* MutY protein has also been reported to have a little or no 2-OH-A DNA glycosylase activity (15). It is therefore likely that the excision of 2-OH-A in DNA as well as the sanitization of 2-OH-dATP from the nucleotide pool is accomplished by different mechanisms in prokaryotes and mammals.

Recently, several germline mutations in the human *MUTYH* (*hMUTYH*) gene have been reported in familial adenomatous polyposis (FAP) patients (16,17). These patients have no germline mutation in the *APC* gene, frequently found in the major type of FAP patients, but somatic mutations in the *APC* gene were found in tumor tissue and most of them are G:C to T:A transversion mutations. These observations strongly suggest that a hMUTYH deficiency causes somatic mutations in the *APC* gene, thus resulting in multiple colorectal tumors. *hMUTYH* mutations found in the patients are mainly Y165C and/or G382D amino acid substitutions, and the patients carrying such homozygous or heterozygous mutations are highly susceptible to multiple colorectal tumors. The *E.coli* MutY(Y82C) mutant corresponding to the former markedly reduced the DNA glycosylase activity excising adenine opposite 8-oxoG, while the MutY(G253D) mutant corresponding to the latter exhibited no gross biochemical dysfunction but showed a subtle reduction in the DNA glycosylase activity which is apparent only at 2°C (16). We recently reported that the spontaneous mutation rate of mouse MUTYH-null embryonic stem cells is 2-fold higher than that of wild type and that the mMUTYH(G365D) mutant corresponding to the hMUTYH(G382D) mutant could not suppress the elevated mutation rate at all (18). Moreover, in OGG1-null mice which lack 8-oxoG DNA glycosylase activity, spontaneous lung tumorigenesis and the accumulation of 8-oxoG in DNA were elevated by several fold when compared with wild type, but no tumors in either the gastrointestinal tract or other organs were observed (19). These observations raise the question as to why the G:C to T:A transversion mutation or tumorigenesis occurs so frequently in the colon tissue of patients with germline mutations in the *hMUTYH* gene.

A biochemical analysis of the MUTYH function took an extremely long time to complete because of the difficulty in preparing a functional recombinant MUTYH protein. We recently obtained a purified recombinant thioredoxin-mMUTYH fusion protein, in which more than 90% of the residues were identical to those of hMUTYH (20). In the present study, we performed detailed biochemical analyses of the DNA glycosylases of mMUTYH and demonstrated that mMUTYH has the DNA glycosylase activity excising 2-OH-A opposite guanine in DNA as well as adenine opposite 8-oxoG, and that mMUTYH recognizes and interacts with each substrate in a different manner. In addition, the mMUTYH(G365D) mutant protein almost completely retained its DNA glycosylase activity excising adenine opposite 8-oxoG but possessed 1.5% of the wild-type activity excising 2-OH-A opposite guanine, thus suggesting that the reduced repair capacity of the mutant hMUTYH(G382D) excising 2-OH-A opposite guanine results in an increased occurrence of somatic G:C to T:A transversion mutations in the *APC* gene as well as tumorigenesis in the colon.

**Table 1.** Oligonucleotides used in this study

| Oligonucleotide | Sequence[a] |
|---|---|
| *A | FAM-AGCGGCCATCGATACCGTC*A*ACCTCG-AGGAATTCC |
| *AO | FAM-AGCGGCCATCGATACCGTC*AO*ACCTC-GAGGAATTCC |
| G | GGAATTCCTCGAGGT*G*GACGGTATCGATG-GCCGCT |
| GO | GGAATTCCTCGAGGT*GO* GACGGTATCGAT-GGCCGCT |
| T | GGAATTCCTCGAGGT*T* GACGGTATCGATG-GCCGCT |
| 19-P | FAM-AGCGGCCATCGATACCGTC-phosphate |
| 19-OH | FAM-AGCGGCCATCGATACCGTC-OH |

FAM, 5′ end was labeled with FAM; GO, 8-oxoguanine; AO, 2-hydroxyadenine; Phosphate, the phosphate group was attached to the 3′-hydroxy group; OH, 3′ end was the hydroxy group.
*, base in the FAM-labeled strand.
[a]Italic letters indicate the target base of MUTYH (A, Ao) and its opposite base (G, Go, T).

## MATERIALS AND METHODS

### Oligonucleotides

The oligonucleotides shown in Table 1 were obtained from Greiner Japan and the Hokkaido System Science.

### Preparation of the recombinant mouse MUTYH proteins

To obtain soluble recombinant mMUTYH preparations, fusion proteins with thioredoxin (Trx), Trx-mMUTYH, Trx-mMUTYH(D207N), Trx-mMUTYH(R361A) and Trx-mMUTYH(G365D), were prepared as described previously (20,21).

### Preparation of the MutY protein

The *E.coli* MutY protein was prepared as previously described by Miyako *et al.* (22). Briefly, the expression of *E.coli* MutY was induced by the addition of 1 mM isopropyl β-D-thiogalactoside (Wako Pure Chemicals) to the culture of *E.coli* SURE cells transformed with pU85Y following incubation at 37°C for 6 h. The cells were suspended with buffer T [50 mM Tris–HCl, pH 7.6, 0.1 mM EDTA, 0.5 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)], disrupted by sonication and cell lysates were clarified by centrifugation. The lysates were treated with 25% streptomycin, and proteins precipitated by 40–75% ammonium sulfate were resuspended with buffer T and dialyzed against buffer A (20 mM potassium phosphate, pH 7.4, 0.5 mM DTT, 0.1 mM EDTA and 0.1 mM PMSF) containing 50 mM KCl. The lysates were then applied to a phosphocellulose column and eluted with a linear gradient of KCl (50–500 mM). The elutions were dialyzed against buffer S (50 mM potassium phosphate, pH 7.5, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF and 10% glycerol)

containing 50 mM NaCl and applied to a Hitrap-SP (Amersham Biosciences) column, eluted with a linear gradient of NaCl (50–500 mM). The elutions, dialyzed against buffer S with 50 mM NaCl, were applied to a Hitrap-heparin (Amersham Biosciences) column and eluted with a linear gradient of NaCl (50–500 mM), 50% glycerol was added and stocked at −80°C.

### Preparation of the recombinant mAPEX1 protein

The recombinant mAPEX1 protein was prepared as described previously (21).

### 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 fluorescence dye, 6-carboxy fluorescein-aminohexyl amidite (FAM) was attached to the 5′ end of the strand with a target base or damaged base and duplex oligonucleotide substrates for a nicking assay were prepared as previously described (23). The labeled strand was shown with an asterisk as *A and *AO as summarized in Table 1. A standard nicking assay was performed as previously described (21,23). Briefly, 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 and 100 μg/ml BSA) with either mMUTYH or MutY protein at 37°C for a given time. In the presence of mAPEX1, 0.2 mM $MgCl_2$ was carried over from the buffer B in which mAPEX1 was stored. Next, the reaction mixture was heated in the presence of 0.17 N NaOH at 95°C for 4 min and added to 15 μl of 80% formamide containing 6 μg/ml BlueDextran (Sigma) and 20 mM EDTA. 3 μl of the mixture was fractionated on an 8% Long Ranger denatured gel (24 cm length) containing 7 M urea at 30 W for 2 h. Specifically, the nicked 19mer oligonucleotides labeled with FAM were detected using the model 373 automated DNA sequencer and quantified using the GeneScan 3.1 software package (Perkin Elmer), according to the manufacturer's instructions.

### Gel shift assay

FAM-labeled duplex oligonucleotides (40 nM) were incubated in the reaction buffer with 80 nM Trx-MUTYH at 37°C for 60 min. Next, the reaction products were fractionated on a 4% native polyacrylamide gel (12 cm length) in 0.5× TBE at 4 W for 3 h. The oligonucleotides labeled with FAM were detected using the model 373 automated DNA sequencer and then analyzed using the GeneScan 3.1 software package. The results from one of several independent experiments are presented.

## RESULTS

### Mouse MUTYH protein but not *E.coli* MutY has 2-OH-A DNA glycosylase activity

Duplex oligonucleotides containing adenine opposite 8-oxoG (*A:GO) or 2-OH-A opposite guanine (*AO:G), in which the asterisk indicates a base in the FAM-labeled strand, were incubated with the wild-type mMUTYH protein and the reaction products were fractionated by denatured-gel electrophoresis after alkaline treatment. Abasic sites produced by removal of adenine or 2-OH-A were cleaved by β/δ-elimination catalyzed by alkaline treatment and the cleaved products were detected as FAM-labeled 19mer oligonucleotides with phosphates at the 3′ termini (19-P). When duplex oligonucleotide substrates containing *A:GO or *AO:G pairs were incubated with mMUTYH, about 50% of the labeled strands were detected as cleaved 19mer fragments (19-P) (Figure 1A, lanes 2 and 3), indicating that mMUTYH has DNA glycosylase activity excising 2-OH-A opposite guanine as well as adenine opposite 8-oxoG. From duplex oligonucleotides containing *AO:GO and *A:G pairs, mMUTYH produced the 19-P product as much as from the substrates containing *A:GO or *AO:G pairs (Figure 1A, lanes 4 and 5). No cleaved band was produced from substrates containing the *AO:T pair by

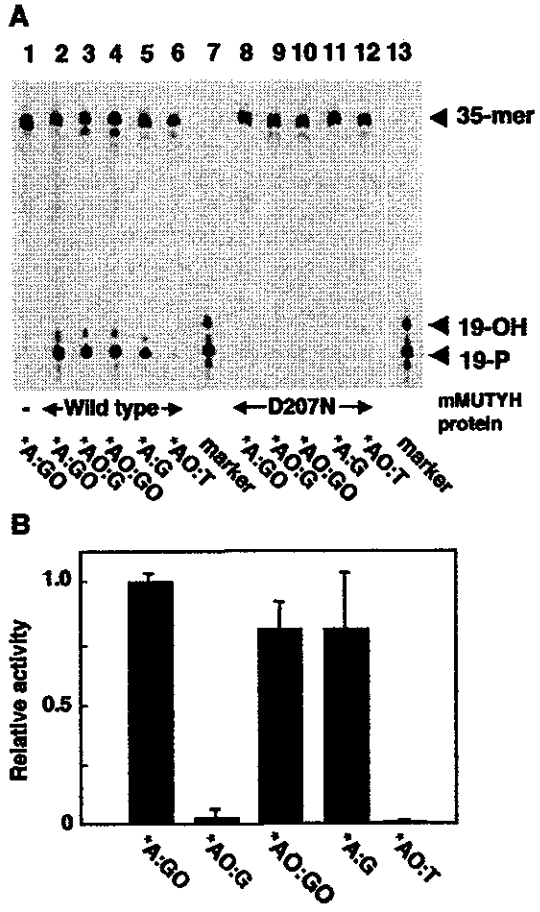


**Figure 1.** MUTYH has a DNA glycosylase activity excising 2-OH-A opposite guanine as well as adenine opposite 8-oxoG, but *E.coli* MutY has a poor activity for excising 2-OH-A opposite guanine. (**A**) 20 nM duplex oligonucleotides containing *A:GO (lanes 1, 2 and 8), *AO:G (lanes 3 and 9), *AO:GO (lanes 4 and 10), *A:G (lanes 5 and 11) or *AO:T pairs (lanes 6 and 12) were incubated with 40 nM wild-type mMUTYH (lanes 2–6) or mutant mMUTYH(D207N) (lanes 8–12) at 37°C for 60 min. The reaction products were treated with NaOH and fractionated. Lane 1, no protein; lanes 7 and 13, marker oligonucleotides, 19-P and 19-OH. (**B**) 20 nM duplex oligonucleotides containing *A:GO, *AO:G, *AO:GO, *A:G or *AO:T pairs were incubated with 50 ng/μl *E.coli* MutY at 37°C for 60 min. The relative amount of the cleaved product for each duplex in comparison with that of the duplex oligonucleotides containing the *A:GO pair is shown as the relative activity, with SEM. *, base in the FAM-labeled strand.

mMUTYH (Figure 1A, lane 6). Mutant mMUTYH(D207N) protein, in which Asp207, an active site residue for the adenine DNA glycosylase, was substituted with Asn, produced no 19-P product from any of the substrates examined (Figure 1A, lanes 8–12).

In Figure 1A, two bands were detected in the reaction products by mMUTYH. The major one was the band corresponding to the 19-P product which was the product of δ-elimination at the abasic site. The other minor band which migrated between 19-P and 19-OH probably corresponds to the β-elimination product with a deoxyribose moiety at the 3′ terminus of the abasic site. In conclusion, both bands were certainly derived from the abasic site produced by the DNA glycosylase activity of mMUTYH.

Next, we examined the DNA glycosylase activity of *E.coli* MutY protein with the same substrates. Duplex substrates containing *A:GO or *A:G pairs were efficiently cleaved by *E.coli* MutY protein with alkaline treatment, while the relative cleavage of the duplex substrates containing *AO:G by MutY was less than 5% level of the substrate with the *A:GO pair (Figure 1B). These data were consistent with those of another report (15). Interestingly, duplex substrates containing the *AO:GO pair were cleaved by MutY as efficiently as were the substrates with the *A:G pair. Again, MutY did not cleave the substrates containing the *AO:T pair at all.

Neither mMUTYH nor *E.coli* MutY protein exhibited any DNA glycosylase activity excising 2-OH-A from the duplex substrates with the *AO:C pair (data not shown).

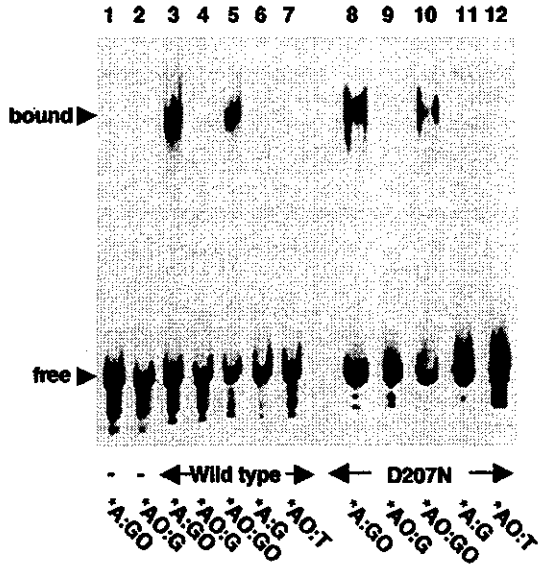


**Figure 2.** MUTYH does not form a stable complex with duplex oligonucleotides containing 2-OH-A opposite guanine. 20 nM duplex oligonucleotides containing *A:GO (lanes 1, 3 and 8), *AO:G (lanes 2, 4 and 9), *AO:GO (lanes 5 and 10), *A:G (lanes 6 and 11) or *AO:T pairs (lanes 7 and 12) were incubated with 80 nM wild-type mMUTYH (lanes 3–7) or mutant mMUTYH(D207N) (lanes 8–12) at 37°C for 60 min. The reaction products were fractionated on a 4% native polyacrylamide gel. Lanes 1 and 2, no protein; free, unbound 35mer duplex; bound, MUTYH-bound duplex. *, base in the FAM-labeled strand.

### mMUTYH protein does not form a stable complex with duplex oligonucleotides containing the 2-OH-A:G pair

We previously reported that mMUTYH forms a stable complex with duplex oligonucleotides containing the adenine: 8-oxoG pair and its reaction product (21). We thus examined the binding of mMUTYH to duplex oligonucleotides containing the 2-OH-A:G pair by a gel mobility shift assay. mMUTYH formed a stable complex with duplex oligonucleotides containing the *A:GO pair (Figure 2, lane 3), as previously reported (21), but no complex was formed with duplex oligonucleotides containing *AO:G, *A:G or *AO:T pairs (Figure 2, lanes 4, 6 and 7). In contrast, mMUTYH formed a substantial amount of complex with duplex oligonucleotides containing the *AO:GO pair (Figure 2, lane 5).

Next, we examined the binding of mutant mMUTYH-(D207N), which completely lacks any DNA glycosylase activity, to each duplex oligonucleotide. This mutant enables us to examine the formation of the mMUTYH–DNA complex without excising either adenine or the 2-OH-A base in the duplex. As shown in Figure 2 (lanes 8–12), essentially similar results were obtained with the mutant mMUTYH as with wild-type mMUTYH. A stable DNA–protein complex was formed between mMUTYH(D207N) and duplex oligonucleotides containing *A:GO, and to a lesser extent *AO:GO, but not *AO:G, *A:G and *AO:T. Neither the wild-type nor the mutant mMUTYH formed a stable complex with duplex oligonucleotides containing *AO:C (data not shown).

These results indicate that mMUTYH protein binds to DNA depending on the opposite base (8-oxoG or G), but not the base excised by itself (A or 2-OH-A).

### mMUTYH protein does not protect the abasic site opposite guanine after excising 2-OH-A

We previously demonstrated that mMUTYH excises adenine opposite 8-oxoG while staying on its own reaction product containing the abasic site opposite 8-oxoG, thus protecting the abasic site from being attacked by AP endonuclease (APEX1) and the 8-oxoG base from being excised by 8-oxoG DNA glycosylase (OGG1) (21). As described above, mMUTYH does not form a stable complex with duplex oligonucleotides containing the 2-OH-A:G pair, thus suggesting that the abasic site produced by the excision of 2-OH-A opposite guanine can be immediately incised by APEX1.

To examine this possibility, we incubated duplex oligonucleotides containing either the *A:GO or the *AO:G pair with mMUTYH and mAPEX1 simultaneously. If mAPEX1 incises the 5′ end of abasic sites generated by mMUTYH, then FAM-labeled 19mer oligonucleotides with hydroxy groups at the 3′ termini (19-OH) can be produced. On the other hand, the intact abasic sites can be detected as FAM-labeled 19mer oligonucleotides with phosphate groups at the 3′ termini (19-P) after δ-elimination by alkaline treatment. As shown in Figure 3A, a major product from duplex oligonucleotides containing the *A:GO pair was 19-P, thus confirming that the abasic sites opposite 8-oxoG generated by mMUTYH remained intact even in the presence of mAPEX1, as previously described (21). In contrast, most of the product from duplex oligonucleotides containing the *AO:G pair was 19-OH, thus indicating that the abasic sites generated through the removal of 2-OH-A opposite guanine by mMUTYH were efficiently incised by mAPEX1. Without alkaline treatment of the

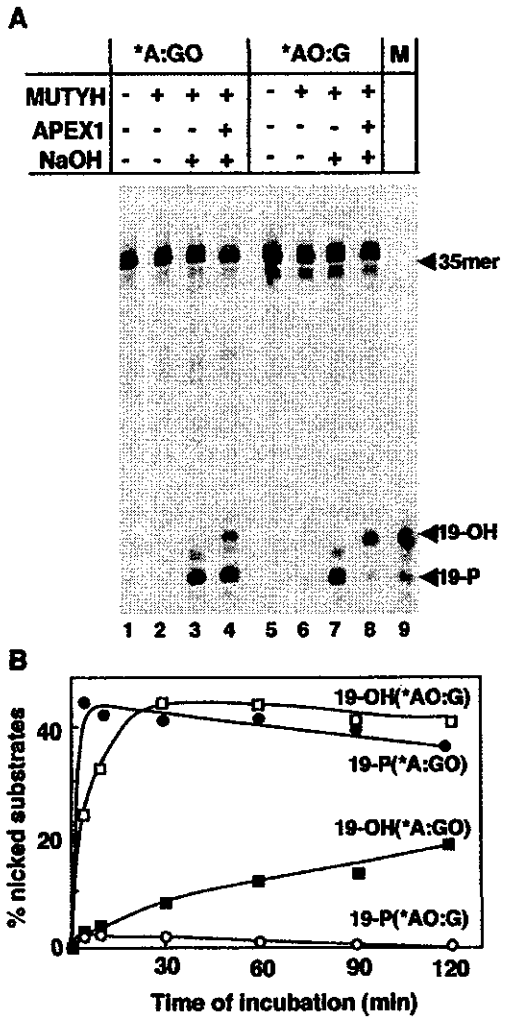


Figure 3. MUTYH does not prevent APEX1 from incising the abasic site generated opposite guanine after excising 2-OH-A. (A) 20 nM duplex oligonucleotides containing *A:GO (lanes 1–4) or *AO:G pairs (lanes 5–8) were incubated with 40 nM mMUTYH (lanes 2–4 and lanes 6–8) and 200 nM mAPEX1 (lanes 4 and 8) at 37°C for 60 min. The reaction products were treated with NaOH (lanes 3, 4, 7 and 8) or without NaOH (lanes 1, 2, 5 and 6) and then fractioned. (B) As in (A), duplex oligonucleotides containing *A:GO or *AO:G pairs were incubated with mMUTYH and mAPEX1 at 37°C. The percentage of nicked substrates for the times noted are plotted. Closed circles, production of 19-P from duplex containing *A:GO; closed squares, production of 19-OH from the duplex containing *A:GO; open circles, production of 19-P from duplex containing *AO:G; open squares, production of 19-OH from duplex containing *AO:G. *, base in the FAM-labeled strand.

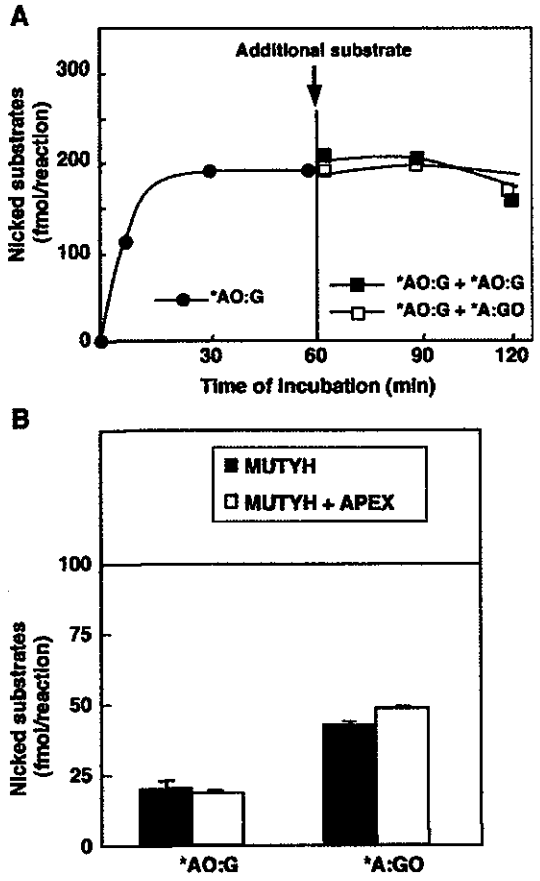


Figure 4. The DNA glycosylase activity of MUTYH excising 2-OH-A opposite guanine does not turn over. (A) 20 nM duplex oligonucleotides containing the *A:GO pair were incubated with 70 nM mMUTYH at 37°C for 60 min (closed circles) and additional duplex oligonucleotides (20 nM) containing the *A:GO pair (closed squares) or the *A:GO pair (open squares) were added to the reaction mixture and were further incubated. The reaction products were treated with NaOH at the time noted and fractionated. The amounts of 19-P products (fmol/reaction) at the times noted are plotted. (B) 40 nM duplex oligonucleotides containing either the *A:GO or *AO:G pairs were incubated with 8 nM mMUTYH with or without 40 nM mAPEX1 at 37°C for 9h. The reaction products were treated with NaOH and fractioned. Amounts of 19-P and 19-OH products (fmol/reaction) are shown with SEM. The amount of mMUTYH in the reaction (100 fmol) is indicated as a horizontal line. Closed bars, reaction with mMUTYH; open bars, reaction with mMUTYH and mAPEX1. *, base in the FAM-labeled strand.

reaction product, neither 19-P nor 19-OH was produced by mMUTYH itself, thus confirming that mMUTYH lacks AP lyase activity (Figure 3A, lanes 2 and 6).

As shown in Figure 3B, more than 60% of the nicked products from the duplex oligonucleotides containing the *A:GO pair was detected as 19-P, even 2 h after incubation with mMUTYH and mAPEX1. On the other hand, from duplex oligonucleotides containing the *AO:G pair, only a little amount of 19-P was produced immediately after the duplex oligonucleotides were incubated with mMUTYH and mAPEX1, and thereafter almost all products were detected as 19-OH, products incised by mAPEX1. As a result, we concluded that mMUTYH does not protect the abasic site opposite guanine generated by the excision of 2-OH-A by itself.

## DNA glycosylase activity of mMUTYH excising 2-OH-A opposite guanine does not turn over

It is likely that the DNA glycosylase activity of mMUTYH excising 2-OH-A opposite guanine may turn over, because the mMUTYH protein does not form a stable complex with its reaction product containing the abasic site opposite guanine. To investigate this hypothesis, duplex oligonucleotides containing the *AO:G pair were incubated with mMUTYH for 60 min (when the reaction reached a plateau level) and then another duplex substrate containing the *AO:G or *A:GO pairs was added to the reaction. As shown in Figure 4A, an

increased production of the 19-P product was not observed at all even after adding either of the substrates.

It has been previously reported that APEX1 enhances the DNA glycosylase activity of MUTYH excising adenine opposite 8-oxoG (24). Therefore, we added mAPEX1 to the reaction in which a large amount of duplex oligonucleotides containing *AO:G or *A:GO pairs was incubated with a limited amount of mMUTYH. As shown in Figure 4B, essentially the same amount of reaction products was generated from the two substrates, in the absence or presence of mAPEX1. The amounts of the cleaved products from both substrates did not exceed those of mMUTYH in the reaction mixture even after 9 h incubation. As a result, we concluded that the DNA glycosylase activity of mMUTYH never turns over regardless of its substrates or the presence of mAPEX1.

### Amino acid substitution in the NUDIX domain of mMUTYH reduces DNA glycosylase activity excising 2-OH-A opposite guanine

To identify the critical amino acid residue for DNA glycosylase activity of mMUTYH excising 2-OH-A opposite guanine, we examined the DNA glycosylase activity of three mutant mMUTYH with an amino acid substitution; G365D corresponding to hMUTYH(G382D) found in FAP patients, R361A in the NUDIX domain conserved among MUTYH, MutY, MutT and MTH1, and D207N as described above. Duplex oligonucleotides containing *A:GO, *AO:G, *AO:GO, *A:G or *AO:T pairs were incubated with wild-type or mutant (G365D, R361A or D207N) mMUTYH for 60 min, and the amounts of the cleaved fragment were measured after alkaline treatment (Figure 5). Wild-type mMUTYH produced an equivalent level of cleaved fragments from all substrates except for oligonucleotides containing the *AO:T pair, from which mMUTYH did not produce any product at all. As expected, mMUTYH(D207N) did not produce any product from all the substrates examined. On the other hand, two mutant proteins with G365D or R361A substitution produced essentially the same levels of cleaved fragments from duplex oligonucleotides containing *A:GO, *AO:GO and to a lesser extent the *A:G pair when compared with wild-type mMUTYH; however, only 15 or 9% of duplex oligonucleotides containing *AO:G were converted to the cleaved products by mMUTYH(G365D) or mMUTYH(R361A) after 60 min incubation, respectively.

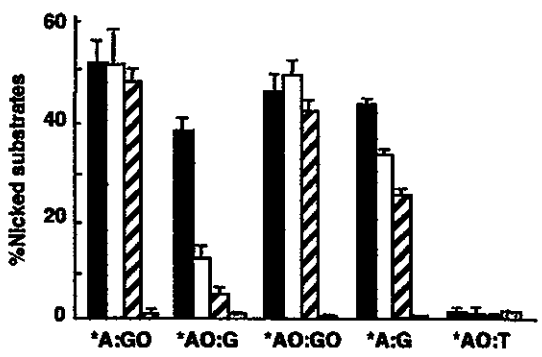


**Figure 5.** Amino acid substitution in the NUDIX domain of MUTYH reduces the DNA glycosylase activity excising 2-OH-A opposite guanine. 20 nM duplex oligonucleotides containing *A:GO, *AO:G, *AO:GO, *A:G or *AO:T pairs were incubated with 40 nM wild-type mMUTYH, mMUTYH(G365D), mMUTYH(R361A) or mMUTYH(D207N) at 37°C for 60 min. The reaction products were then treated with NaOH and fractionated. The percentages of the nicked substrates are shown with SEM. Closed bars, wild-type mMUTYH; open bars, mMUTYH(G365D); hatched bars, mMUTYH(R361A); dotted bars, mMUTYH(D207N). *, base in the FAM-labeled strand.

### The G365D substitution selectively reduces DNA glycosylase activity of mMUTYH excising 2-OH-A opposite guanine

In order to delineate the biochemical dysfunction of the mMUTYH(G365D) mutant, we examined the time course of cleavage for each substrate oligonucleotide by mutant mMUTYH(G365D) and compared the findings with that by wild type (Figure 6). Reactions with substrates containing *A:GO or *AO:GO pairs were carried out at 4°C, because reactions with these substrates reached the plateau level within a minute at 37°C. Reactions with substrates containing *AO:G or *A:G pairs were carried out at 37°C as usual. There was no apparent difference in both the initial velocity and the plateau level of the reaction with the substrates containing *A:GO or *AO:GO pairs between the two mMUTYH (Figure 6A and C). However, mMUTYH(G365D) exhibited significant reductions in both the initial velocity and the plateau level of the reaction with substrates containing *AO:G or *A:G when compared with wild type mMUTYH (Figure 6B and D). We then determined the initial velocities of the glycosylase activity of wild-type mMUTYH and mMUTYH(G365D) with various concentrations of substrates containing *A:GO or *AO:G and plotted them against each concentration of the substrates (Figure 7). With increasing concentrations of the substrate containing *A:GO, mMUTYH(G365D) similarly increased its initial velocity but to slightly less a degree than for wild-type mMUTYH. Their apparent maximal velocities ($V_{max}$) and apparent Michaelis–Menten constant (apparent $K_m$) with the substrate were estimated from the curves shown in Figure 7 (Table 2). The catalytic efficiency ($k_{cat}/K_m$) of mMUTYH(G365D), calculated from these two parameters, was 85% of the level observed in wild-type mMUTYH, thus indicating that the catalytic power of mMUTYH (G365D) excising adenine opposite 8-oxoG was nearly equivalent to that of wild-type mMUTYH.

On the other hand, the initial velocity of mMUTYH (G365D) increased slightly with increasing concentrations of the substrate containing *AO:G, and reached only a 10% level of that observed for the wild-type mMUTYH in the presence of 80 nM of the substrate. As shown in Table 2, the apparent $V_{max}$ of mMUTYH(G365D) with the substrate was only 14% of the level observed in wild-type mMUTYH, and its apparent $K_m$ increased more than 9-fold when compared with that of wild-type mMUTYH. As a result, the catalytic efficiency ($k_{cat}/K_m$) of mMUTYH(G365D) was only about 1.5% of the level observed in wild type. However, the catalytic efficiency of mMUTYH(G365D) with the substrate containing *A:G was 10% of the level observed in the wild-type enzyme (data not shown). As a result, we concluded that the G365D substitution in mMUTYH significantly reduced the DNA glycosylase activity excising 2-OH-A opposite guanine but not that excising adenine opposite 8-oxoG.

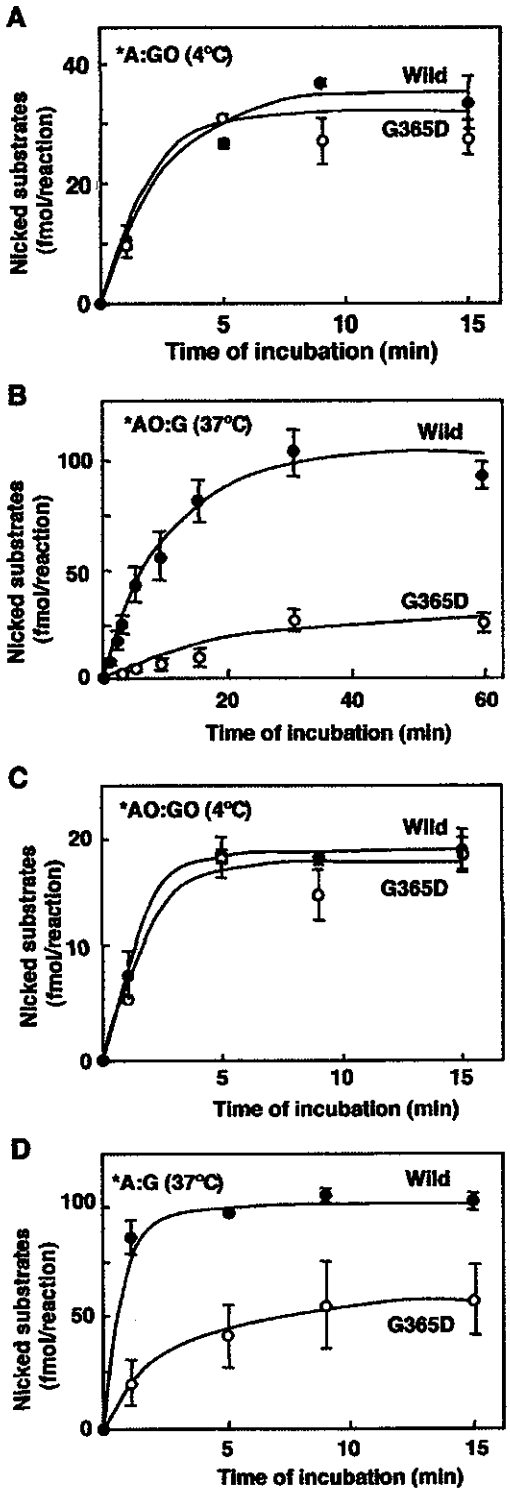


**Figure 6.** Time course of the DNA glycosylase reaction of wild-type mMUTYH and mutant mMUTYH(G365D). Duplex oligonucleotides containing either the *A:GO (A) or *AO:GO pairs (C) were incubated with wild-type mMUTYH (closed circles) or mMUTYH(G365D) (open circles) at 4°C for the time noted, and those containing the *AO:G (B) or *A:G (D) pairs were incubated at 37°C. The concentrations of the oligonucleotides and mMUTYH in the reaction were the same as in Figure 5. The amounts of cleaved 19-P products (fmol/reaction) at the times noted are indicated with SEM. *, base in the FAM-labeled strand.

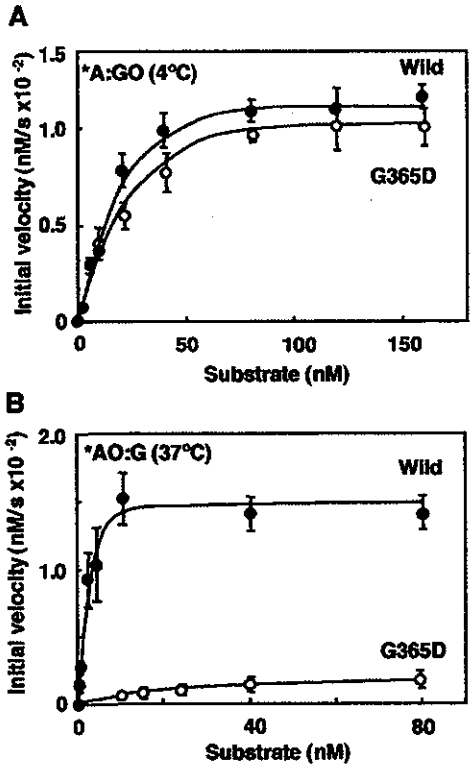


**Figure 7.** Kinetics of the DNA glycosylase reaction of wild-type mMUTYH and mutant mMUTYH(G365D). Various concentrations of duplex oligonucleotides containing *A:GO (A) or *AO:G (B) were incubated with 40 nM wild-type mMUTYH (closed circles) or mutant mMUTYH(G365D) (open circles). The values of the initial velocities were plotted against the concentrations of the substrates. Reactions with duplex oligonucleotides containing the *A:GO pair were performed at 4°C, and those with duplex oligonucleotides containing the *AO:G pair were performed at 37°C as described in the legend for Figure 6. The mean values of the initial velocities were determined by more than three independent experiments and are shown with SEM. *, base in the FAM-labeled strand.

**Table 2.** Kinetic parameters of wild-type mMUTYH and mutant mMUTYH(G365D)

| | Apparent $V_{max}$ (nM $s^{-1}$) × $10^{-2}$ | Apparent $K_m$ (nM) | $k_{cat}/K_m$ ($nM^{-1}s^{-1}$) × $10^{-5}$ |
|---|---|---|---|
| *A:GO (4°C) | | | |
| Wild | 1.12 | 15.37 | 1.82 |
| G365D | 1.02 (0.91) | 16.50 (1.11) | 1.55 (0.85) |
| *AO:G (37°C) | | | |
| Wild | 1.53 | 2.34 | 16.37 |
| G365D | 0.21 (0.14) | 21.95(9.38) | 0.24 (0.015) |

Values of apparent $V_{max}$ and apparent $K_m$ of wild-type mMUTYH and mutant mMUTYH(G365D) for each substrate containing *A:GO and *AO:G pairs were calculated from Figure 7. The values of $k_{cat}$ were calculated as the molecules of the product formed per molecule of MUTYH per second. The values in parentheses are the relative ratios to those of wild-type mMUTYH.
*, base in the FAM-labeled strand.

## DISCUSSION

In the present study, we demonstrated that the recombinant mMUTYH protein has a DNA glycosylase activity excising 2-OH-A opposite guanine as well as adenine opposite 8-oxoG

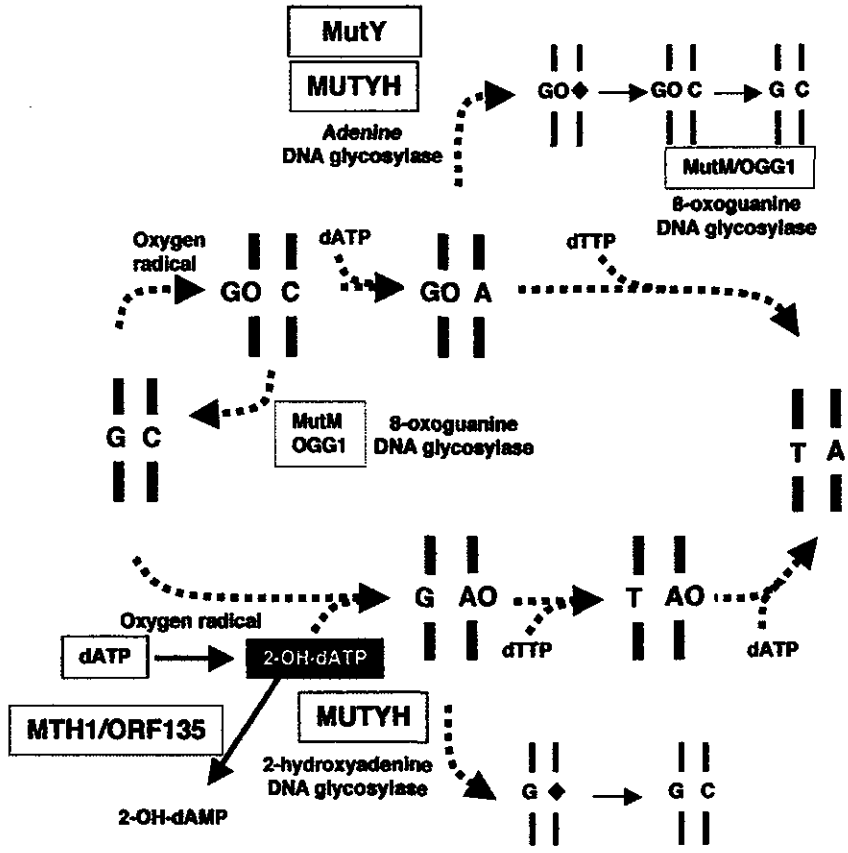


**Figure 8.** The mutagenic pathways initiated 2-OH-dATP or 8-oxoG formation in DNA resulting in G:C to T:A transversion mutation and its protection by MutY and MUTYH. The direct oxidation of guanine in DNA generates 8-oxoG (GO), opposite to which dATP can be misinserted by DNA polymerase during replication, thus producing the 8-oxoG:A pair (GO:A). The 8-oxoG:A pair is converted to a thymine:adenine (T:A) pair during the next round of replication, thus resulting in a G:C to T:A transversion mutation. On the other hand, the oxidized form of dATP, 2-OH-dATP, was misinserted into the nascent strand opposite guanine in the template strand, thus producing a guanine:2-OH-A pair (G:AO). The guanine:2-OH-A pair is converted to a T:A pair after two more rounds of replication, thus resulting in a G:C to T:A transversion mutation. MutY and *MUTYH* excise adenine misinserted into nascent strand opposite 8-oxoG in the template, by their adenine DNA glycosylase activity, and thereafter MutM or OGG1 removes 8-oxoG opposite cytosine, thus avoiding the occurrence of G:C to T:A transversion mutation. In contrast, MUTYH has a 2-OH-A DNA glycosylase activity which is able to excise 2-OH-A opposite guanine, thus avoiding the occurrence of a G:C to T:A transversion mutation. 2-OH-dATP can be hydrolyzed to 2-OH-dAMP by MTH1 in mammals or by ORF135 in *E.coli*. A rhombus indicates an abasic site. *, base in the FAM-labeled strand.

and that residues R361 and G365 were critical for this activity. We previously reported that the fraction containing partially purified hMUTYH from Jurkat cells contains a DNA glycosylase activity which efficiently removes 2-OH-A opposite guanine (14). The present study finally proves that mammalian MUTYH proteins have DNA glycosylase activity excising 2-OH-A opposite guanine.

As shown in Figure 8, a G:C to T:A transversion mutation can be caused not only by the direct oxidation of guanine, generating 8-oxoG in DNA, but also by the misincorporation of 2-OH-dATP, formed by the oxidation of dATP in the nucleotide pool, during replication. The results in this study indicate that MUTYH recognizes both adenine misincorporated opposite 8-oxoG in the template strand and 2-OH-A misincorporated opposite guanine in the template strand and removes these premutagenic bases by its DNA glycosylase activity, thus preventing the occurrence of the G:C to T:A transversion mutation. Moreover, it has been previously reported that *E.coli* MutY hardly removes 2-OH-A opposite guanine (15), and we also confirmed this result with our own preparation of the MutY protein. These results suggest that there is a significant difference in the degree of accumulation of 2-OH-A in the genome of *E.coli* and mammals (25,26).

We previously reported that *E.coli* MutT efficiently hydrolyzes 8-oxo-dGTP but not the oxidized forms of dATP, while the human MutT homolog (hMTH1) hydrolyzes 2-OH-dATP and 8-oxo-dATP as well as 8-oxo-dGTP (11,27). Kamiya *et al.* revealed that the protein coding *Orf135* of *E.coli* hydrolyzes 2-OH-dATP and that disruption of the *Orf135* gene resulted in a 2- to 3-fold increase in the spontaneous occurrence of G:C to T:A and A:T to C:G transversions when compared with the wild type (9). It was also demonstrated that *E.coli* DNA polymerase III inserts 2-OH-dATP opposite guanine or thymine in the template strand (4). It is thus likely that a disruption of the *Orf135* gene increases such spontaneous mutations because MutY can not remove 2-OH-A misincorporated into the genome.

In mammals, it was reported that the occurrence of the A:T to C:G transversion mutation was slightly elevated in MTH1-deficient mice while that of the G:C to T:A transversion was

significantly increased only in mice deficient for both of MSH2 and MTH1(28). A loss of the MTH1 function may cause an accumulation of 8-oxo-dGTP in the nucleotide pool, which increases the incorporation of 8-oxoG opposite adenine in the template strand of DNA during replication, thereby resulting in an increased occurrence of the A:T to C:G transversion. It has been demonstrated that MUTYH interacts with MSH2 and both of them are likely to be responsible for the post-replicative repair of adenine or 2-OH-A misinserted into the nascent strand during replication (29). Thus, we propose that both the accumulation of 2-OH-dATP in the nucleotide pool due to MTH1-deficiency and an inefficient post-replicative repair of 2-OH-A by MUTYH in the absence of MSH2 resulted in a significantly increased occurrence of the G:C to T:A transversion mutation in mice deficient for both of MSH2 and MTH1.

In the present study, we showed that *E.coli* MutY hardly excises 2-OH-A opposite guanine; however, it does efficiently excise 2-OH-A opposite 8-oxoG, thus indicating that MutY can excise 2-OH-A depending on the opposite base. In contrast, mMUTYH efficiently excises 2-OH-A regardless of the opposite base, guanine or 8-oxoG. Furthermore, the mMUTYH(G365D) mutant largely lost the capability to excise 2-OH-A opposite guanine but it did maintain its ability to excise adenine opposite guanine to some degree and also maintained its full activity to excise 2-OH-A opposite 8-oxoG. These observations strongly suggest that mMUTYH, but not *E.coli* MutY, recognizes 2-OH-A and adenine opposite guanine in a different manner. We also found that mMUTYH cannot excise the 2-OH-A paired with thymine. Considering the fact that the generation of 2-OH-A by the oxidation of dATP in the nucleotide pool is significantly higher than that by the direct oxidation of adenine in DNA (5), it seems highly likely that the 2-OH-A:T pair, a product of direct oxidation of the A:T pair in DNA, rarely exists in living cells. Consequently, it is expected that MUTYH has never acquired such a repair capacity.

mMUTYH protein formed a stable complex with duplex oligonucleotides containing the adenine:8-oxoG pair or its reaction product. But such a stable complex was not detected with duplex oligonucleotides containing the 2-OH-A:guanine pair, thus indicating that the mMUTYH protein interacts with duplex oligonucleotides containing adenine:8-oxoG and 2-OH-A:guanine pairs in a different manner. Reflecting these different interactions, duplex oligonucleotides containing 2-OH-A opposite guanine or adenine opposite 8-oxoG were differently processed by APEX1 following excision of 2-OH-A or adenine by MUTYH. The abasic sites generated in the former by MUTYH were immediately incised by APEX1 while most of those in the latter remained intact even in the presence of APEX1.

After the excision of adenine opposite 8-oxoG by MUTYH, the abasic site is likely to be incised by an AP endonuclease, and cytosine must be inserted opposite 8-oxoG during repair DNA synthesis. The remaining 8-oxoG opposite cytosine can be replaced by guanine through OGG1-initiated base excision repair (Figure 8). Since OGG1 efficiently excises the 8-oxoG opposite the abasic site as well as opposite cytosine, the excision of the 8-oxoG opposite the abasic site by OGG1, following the removal of adenine opposite 8-oxoG by MUTYH, generates an abasic site opposite the abasic site, thus causing a loss of genetic information. Moreover, such lesions can be easily converted to double-strand breaks by the AP lyase activity of OGG1 or by other AP lyases or AP endonucleases. As a result, MUTYH tightly binds and protects its own reaction product, the abasic site opposite 8-oxoG in DNA from inappropriate processing by OGG1 or APEX1 (21).

In contrast, after the excision of 2-OH-A opposite guanine by MUTYH, cytosine can be efficiently inserted opposite guanine during repair replication, thus completing the repair process. Since guanine, a normal base, is present opposite the abasic site resulting from the excision of 2-OH-A by MUTYH, MUTYH does not need to bind and protect the abasic site by itself, unlike the abasic site opposite 8-oxoG, otherwise binding of MUTYH may cause a delay in the repair of the abasic site opposite guanine.

The crystal structure of the MutY–DNA complex has revealed that tight hydrogen bonds are formed between MutY and 8-oxoG opposite adenine in DNA, and it was suggested that the presence of guanine opposite adenine in DNA disturbs this hydrogen bonding network, thus destabilizing the complex (30). In the case of 2-OH-A opposite guanine in DNA, it is likely that the guanine residue indeed destabilizes the interaction of MUTYH with its substrates or products, because MUTYH formed a stable complex with duplex oligonucleotides containing 2-OH-A opposite 8-oxoG.

It has been documented that the catalytic activity of MUTYH excising adenine opposite 8-oxoG does not turn over, and this is considered to be probably due to its forming a stable complex with its reaction product. In the present study, we found that the glycosylase activity of mMUTYH excising 2-OH-A opposite guanine also does not turn over at all. We suppose that MUTYH remains on the substrate even after excising 2-OH-A, but the stability of the complex is not strong enough to be detected by a gel mobility shift assay. It was reported that a high concentration of adenine inactivates the adenine DNA glycosylase activity of prokaryote MutY (31), thus raising another possibility for MUTYH turnover. MUTYH may remain bound with the excised adenine or 2-OH-A base and therefore it is inactivated after one round of reaction. Further elucidation is necessary to solve the issue.

An analysis of mutant mMUTYH with an amino acid substitution in the NUDIX domain revealed that R361A or G365D substitution reduces the DNA glycosylase activity of mMUTYH excising 2-OH-A opposite guanine, selectively. Since the G365D substitution corresponds to G382D germline mutation in hMUTYH found in FAP patients or multiple adenomatous polyposis patients (16,17), we carried out a detailed biochemical analysis of mMUTYH(G365D) and found the catalytic power of the mutant mMUTYH excising 2-OH-A opposite guanine to be 1.5% of the level observed in the wild-type enzyme, but the mutant retains equivalent catalytic activity excising adenine or 2-OH-adenine opposite 8-oxoG.

G365D substitution has the greatest influence on the elevation of the $K_m$ value on the substrates containing 2-OH-A opposite guanine, thus indicating that the Gly365 residue is responsible for its affinity to the substrate. According to the crystal structure of the MutY–DNA complex (30), the G260 residue corresponding to G365 in mMUTYH closely interacts

with the phosphate backbone of the 8-oxoG-containing strand, but not directly with 8-oxoG itself or the adenine-containing strand. We assume that mMUTYH(G365D) forms tight hydrogen bonds with 8-oxoG directly through residues other than Asp365 and therefore an excision of adenine or 2-OH-A opposite 8-oxoG may not be affected by this substitution. However, in the case of 2-OH-A opposite guanine, it is expected that electrostatic repulsion is generated between the Asp365 residue and the phosphate backbone of the guanine-containing strand, and that this repulsion may prevent MUTYH and DNA from interacting closely enough for MUTYH to recognize and excise 2-OH-A. R361A substitution is also supposed to reduce the electrostatic interaction between the basic arginine residue and the phosphate backbone in DNA.

Because the DNA glycosylase activity of mMUTYH (G365D) excising 2-OH-A opposite guanine is reduced to 1.5% of the level observed in the wild-type enzyme, it is suggested that the accumulation of 2-OH-A in genomic DNA in the colon may be responsible for the increased somatic G:C to T:A transversion mutations in the *APC* gene, found in the adenomatous polyps of patients with a homozygous hMUTYH(G382D) mutation. A problem still remains as to why patients with mutant MUTYH exhibit a specific elevation of the spontaneous mutation rate in the colon. We reported that OGG1-deficient or MTH1-deficient mice did not exhibit increased tumorigenesis in the gastrointestinal tract (19); however, we recently observed that MUTYH-deficient mice exhibited an increased occurrence of spontaneous adenoma/adenocarcinoma in the small intestine and colon (K. Sakamoto, Y. Tominaga, K. Yamauchi, Y. Nakatsu, K. Sakumi, K. Yoshiyama, A. Asaeda, A. Egashira, S. Kura, T. Yao *et al.*, in preparation), thus suggesting that a common cause(s) exists in both mice and humans which increases the spontaneous mutation rate selectively in the gastrointestinal tract. In patients with hereditary nonpolyposis colorectal cancer, the lack of a mismatch repair protein is known to selectively induce tumorigenesis in the colon (32). In these patients cancer develops without polyp formation, probably because of the significantly increased spontaneous mutation rate due to the loss of a mismatch repair. The mutation rate is higher by two orders of magnitudes than that in MUTYH-deficient patients (28). Since MUTYH and MSH2 interact during the repair process (29), we expect that 2-OH-A may be the common target for the repair by both MUTYH and MSH2, and it may therefore cause tumorigenesis in the gastrointestinal tract if it is not repaired appropriately.

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