Catalytic Mechanism and DNA Substrate Recognition of *Escherichia coli* MutY Protein*

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*Escherichia coli* MutY protein cleaves A/G- or A/7,8-dihydro-8-oxo-guanine (A/GO)-containing DNA on the A-strand by N-glycosylase and apurinic/apyrimidinicendonuclease or lyase activities. In this paper, we show that MutY can be trapped in a stable covalent enzyme-DNA intermediate in the presence of sodium borohydride, a new finding that supports the grouping of MutY in that class of DNA glycosylases that possess concomitant apurinic/apyrimidinic lyase activity. To potentially help determine the substrate recognition site of MutY, mutant proteins were constructed. MutY proteins with a Gly116→Ala (G116A) or Asp (G116D) mutation had reduced binding affinities for both A/G- and A/GO-containing DNA substrates. The catalytic parameters, however, were differentially affected. While A/G- and A/GO-containing DNA were cleaved by MutY with specificity constants ($k_{cat}/K_m$) of 10 and 3.3 min$^{-1}$µm$^{-3}$, respectively, MutY(G116D) cleaved these DNAs 2,300- and 9-fold less efficiently. The catalytic activities of MutY(G116A) with A/G- and A/GO-containing DNA were about the same as that of wild-type MutY. Both MutY(G116A) and MutY(G116D) could be trapped in covalent intermediates with A/GO-containing DNA, but with lower efficiencies than the wild-type enzyme in the presence of sodium borohydride. MutY(G116A) also formed a covalent intermediate with A/GO-containing DNA, but MutY(G116D) did not. Since Gly116 of MutY lies in a region that is highly conserved among several DNA glycosylases, it is likely this conserved region is in the proximity of the substrate binding and/or catalytic sites.

Oxidative stress and metabolic processes that produce reactive oxygen species have been implicated in aging and a number of diseases including cancer (1, 2). The frequency of oxidative damage to DNA has been estimated at 10$^4$ lesions/cell/day in humans (3). Organisms have developed many different defense mechanisms to repair oxidative damage to DNA. The 7,8-dihydro-8-oxo-guanine (GO)$^3$ lesion is one of the most stable products of oxidative damage to DNA known. In *Escherichia coli*, the proteins MutY, MutM, and MutT are involved in defending against the mutagenic effects of GO lesions (4, 5). The MutT protein specifically eliminates 8-oxo-dGTP from the nucleotide pool (6–8). The MutM protein (Fpg protein) removes both ring-opened purine lesions and mutagenic GO adducts from DNA (9, 10). MutM removes GO lesions efficiently from C/GO mispairs but poorly from A/GO mispairs (10). When C/GO is not repaired by MutM, adenines are frequently incorporated opposite GO bases during DNA replication (11, 12). A second round of replication through this mismatch subsequently leads to a G/C to T/A transversion (12–15). The putative role of the MutY pathway in *E. coli* is the removal of adenines misincorporated opposite GO or G following DNA replication (4, 16, 17). This is consistent with the phenotype of *E. coli mutY* (or *micA*) mutants, which have higher mutation rates for G/C to T/A transversions than the wild-type strains (18, 19).

The short patch MutY repair pathway specifically repairs A/GO and A/G to C/G, respectively, and corrects A/C to G/C at a much lower rate (16, 19–24). The MutY protein is a 39-kDa iron-sulfur protein (24–26). Tsai-Wu et al. (24) have shown that MutY has both DNA glycosylase and apurinic/apyrimidic (AP) endonuclease/lyase activities, although the AP endonuclease/lyase activity could not be detected by Au et al. (27) in their MutY preparation. The DNA glycosylase activity removes the adenine bases from the mismatched bases (16, 27), and the AP endonuclease/lyase activity cleaves the first phosphodiester bond 3’ to the AP site (24, 28). In this paper we confirm that the AP endonuclease/lyase activity is intrinsic to MutY. MutY functions via an imino covalent enzyme-DNA intermediate.

Structural studies have demonstrated that A/G mispairs can adopt several conformations depending on their neighboring sequences (29–33). The common features of MutY substrates (A/G, A/GO, A/C, and A/GO) suggest that N-1 of adenine may be protonated and/or that guanine is in the syn configuration (22). Recently, we have shown that MutY binds to mismatch-containing DNA with an affinity ranked in the order A/GO $\gg$ A/G $> A/C \gg C/G$ (17). Although MutY binds much tighter to A/GO-containing DNA (17), kinetic studies have shown that MutY cleaves A/G-containing DNA about 3-fold more efficiently than it does A/GO-containing DNA.

The amino acid sequences of MutY are highly homologous to another iron-sulfur protein in *E. coli*, endonuclease III (24–26). Endonuclease III repairs thymine glycol and oxidized pyrimidines in DNA by N-glycosylase and AP lyase activities (34, 35). Residues 113–119 of MutY (Ala-Leu-Pro-Gly-Val-Gly-Arg) are completely identical to residues 113–119 of endonuclease III. It has been suggested that this region of endonuclease III is involved in substrate recognition (36). To test the putative role of this conserved region in MutY, we constructed two mutant MutY proteins with a Gly116→Ala or Asp mutation, respectively. Analysis of the MutY amino acid sequence reveals that residues 108–127 (FEEVAALPGVRSTAGAILS) have the potential to form a λ-Cro-like structure with a helix-turn-helix motif (37). The presence of Gly116 of MutY is strongly favored at

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*This work was supported by NIGMS, National Institutes of Health, Public Health Service Grant GM 35132. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: GO, 7,8-dihydro-8-oxo-guanine; AP, apurinic/apyrimidinic; AO, 7,8-dihydro-8-oxo-adenine; N, nebularine; Z, 7-deaza-adenosine.
this position in a helix-turn-helix motif. Whereas a Gly to Ala alteration is conserved in terms of side chain structure but would substantially destabilize a helix-turn-helix motif, a Gly to Asp mutation would completely change side chain properties and also reduce the stability of any helix-turn-helix (37). The binding and catalytic activities of these mutants with A/G- and A/GO-containing DNA were compared with those of wild-type MutY protein. Both mutant MutY proteins were substantially reduced in their ability to bind to A/G and A/GO mispairs. The combined glycosylase/AP lyase activity of mutant MutY-(G116D) with A/G-containing DNA was more severely affected than it was with A/GO-containing DNA. These results suggest that this conserved region is in the proximity of the substrate binding and/or catalytic sites.

EXPERIMENTAL PROCEDURES

Bacteria—E. coli PR70 (Su lacZ X74 galU galK Sm' micA68: Tn10Kan) and GBE791 lacI-4000 Lac(-) lacZp5000 (Lac -) strC-300: Tn10) IN (rrn-1r-rrnE1) were obtained from M. S. Fox and G. Barak, respectively. The micA68: Tn10Kan marker was transferred from PR70 to GBE791 by P1 transduction using P1 lysogen cmr clr100 according to standard procedures (38). The transductants were selected for resistance to tetracycline and kanamycin. One transductant (GBE943) (39) had a mutation rate similar to PR70 when tested on rifampicin plates.

Site-directed Mutagenesis and Expression—The method of Kunkel et al. (39) from the Bio-Rad mutagenesis kit was utilized to alter the MutY gene. A 1.2-kilobase EcoRI fragment containing the mutY gene was cloned from pJTW4-21 (26) into the EcoRI site of M13MP18 for mutagenesis. Oligonucleotide (5'-GGGAGAGCATCCGCGAGTCG-3') was used to construct the Gly116-Asp (G116D) mutation (underlined T is mutated nucleotide). The first mutated gene was engineered as described previously (40) for the generation of an AatII restriction site and then further confirmed by DNA sequencing. Oligonucleotide (5'-CGCCCCAGGCCGGCGATGC-3') was used to construct the Gly116-Ala (G116A) mutation (underlined G is the mutated nucleotides) from the G116A mutant gene. The G116A mutant gene was first screened for the loss of an AatII restriction site and then further confirmed by DNA sequencing. The 1.2-kilobase EcoRI fragment containing the mutated mutY gene was inserted into the EcoRI site of the expression vector pKZK223-3 (40) to generate pM6116 and pMGA116. The mutY genes in plasmids pM6116, pMGA116, and pJTW10-12 (containing the wild-type mutY gene) are under the control of the lac promoter. E. coli strain GBE943 harboring the plasmid pJTW10-12, pM6116, or pMGA116 was grown at 37 °C. The expression of mutant MutY was induced at an A590 of 0.6 by the addition of isopropyl b-D-thiogalactoside to a final concentration of 1 mM to the culture. The cells were harvested 3 h later.

MutY Protein—The purification of homogeneous MutY protein from an overproducing E. coli JM109 harboring pJTW10-12 has been described previously (28). The wild-type enzyme used in this study had a specific activity of about 13 x 10^4 units/mg, where 1 unit of endonuclease activity is defined as that resulting in cleavage of 0.018 fmol of paired DNA in 10 min at 37 °C. Iron analysis (41) of purified MutY showed that it contained 3.8 iron atoms/monomer.

Mutant MutY(G116D) and MutY(G116A) were purified from about 40 g of E. coli GBE943 cells harboring overproduction plasmids pM6116 and pMGA116, respectively, in a similar manner as with wild-type enzyme. Binding of A/GO-containing 20-mer DNA was assayed during the purification of the mutant MutY(G116D) enzyme, and cleavage of A/GO-containing 20-mer DNA was assayed during the purification of the mutant MutY(G116A) enzyme. E. coli strain GBE943 (mutY) was chosen as host, since it expressed more plasmid-coded protein than did strain PR70 (data not shown). As judged on a 12% SDS-polyacrylamide gel, both proteins were purified to >99% homogeneity (data not shown). Iron analysis of the mutant MutY(G116D) and MutY(G116A) proteins by a chemical method (41) yielded 3.6 and 3.9 atoms of iron/monomer enzyme, respectively.

Iron Analysis—Iron analysis was performed according to the method of Kennedy et al. (41). Briefly, about 25–50 μg of MutY were diluted to 200 μl with water and mixed with 0.2 ml of reagent A (1.35 g of SDS dissolved in 30 ml of water and then mixed with 0.45 ml of saturated iron-free sodium acetate). After adding 0.2 ml of reagent B (270 mg of ascorbic acid and 9 mg of sodium metabisulfite dissolved in 5.6 ml of water and then mixed with 0.4 ml of saturated iron-free sodium acetate) for 15 min at 30 °C, 10 μl of reagent C (18 mg/ml Feronene) was added to the reaction. The absorption at 593 nm was measured. Iron atomic absorption spectrophotometry (AAS) containing 50–1000 ng of iron were used to construct a standard curve.

Oligonucleotide Substrates—The sequence of 19-mer oligonucleotide duplexes used were as follows,

| Sequence 1 |
|------------|
| 5'-GGGAGAGCATCCGCGAGTCG-3' |
| 3'-CGCCCCAGGCCGGCGATGC-5' |

where X represents adenine (A), cytosine (C), nebularin (N), inosine (I), or 7-deaza-adenosine (Z) and Y represents guanine (G), 7,8-dihydro-8-oxoguanine (GO), or C. The synthesis and annealing of the oligonucleotides were as described previously (42). The annealed duplexes were radiolabeled at the 3'-end of the upper strand with Klenow fragment of DNA polymerase I for 30 min at 25 °C in the presence of [α-32P]dCTP (50 μCi, 3,000 Ci/μmol), 20 μM dTTP, and 20 μM dGTP (43). The resulting blunt-ended duplex DNA were 20 base pairs in length. The reaction mixture was then passed through a G-25 Quick-Spin column (Boehringer Mannheim).

MutY Binding Assay—The binding of MutY to various oligonucleotides was assayed by gel retardation. The standard reaction mixture contained 20 mM Tris-HCl (pH 7.6), 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 2.9% glycerol, 20 ng of poly[d(dC-dC)], and 1.8 fmol of labeled 20-base pair oligonucleotide in a total volume of 20 μl. MutY protein, diluted into dithiothreitol containing 25 mM potassium phosphate (pH 7.4), 1.5 mM dithiothreitol, 0.1 mM EDTA, 50 mM KCl, 200 μg/ml bovine serum albumin, and 50% glycerol, was added to the reaction mixture and incubated at 37 °C for 30 min. Protein-DNA complexes were analyzed on 8% polyacrylamide gels in 50 mM Tris borate (pH 8.3) and 1 mM EDTA. The apparent dissociation constants (Kd values) of mutant MutY and DNA containing A/G or A/GO mismatches were determined using a range of protein concentrations as described previously (17).

MutY Cleavage Assay—The cleavage activity of MutY, which is the combined action of the glycosylase and AP lyase activities, was assayed as described previously (28). 3'-labeled 20-base pair oligonucleotides (1.8 fmol) were incubated with various concentrations of MutY protein as described in the binding assay, except that poly[d(dC-dC)] was omitted from the reaction in a total volume of 10 μl. The reaction products were fractionated on 14% polyacrylamide DNA sequencing gels. Kinetic analyses were performed using a concentration range of 20-mer DNAs with fixed protein concentrations. Following autoradiography, bands corresponding to cleavage products and intact DNA were excised from the gel and quantified by liquid scintillation counting. Km and Vmax values were obtained using a computer-fitted curve generated by the Enzfit program (44).

Formation of MutY-DNA Covalent Complex—Reactions were carried out as described in the MutY endonuclease assay except that the reactions were performed in the presence of 100 mM NaCl or 100 mM NaBH4. A NaBH4 stock solution (1 M) was freshly prepared immediately prior to use. After incubation at 37 °C for 30 min, the products were separated on a 12 or 15% polyacrylamide gel in the presence of SDS (SDS-polyacrylamide gel electrophoresis) according to Laemmli (45), and the gel was dried and autoradiographed.

RESULTS

MutY-DNA Covalent Complex—As shown in Fig. 1, MutY cleaved a 20-mer oligonucleotide containing an A/G mismatch in a dose-dependent manner. The addition of piperidine did not significantly increase the extent of cleavage, suggesting that the AP endonuclease/lyase activity is not rate-limiting to product formation. The cleavage activity of MutY was inhibited by the presence of NaBH4 (Fig. 1, lanes 14–19). The formation of a stable protein-DNA complex in the presence of NaBH4 was demonstrated by analyzing the products on an SDS-polyacrylamide gel (Fig. 2). At enzyme:DNA molar ratios ranging from 40 (Fig. 2, lane 3) to 0.5 (Fig. 2, lane 7), gel-shifted bands were detected. About 50% of A/G-containing DNA was trapped in a covalent complex at an enzyme:DNA molar ratio of 40. There was more observed complex formation for A/G-containing DNA than for A/GO-containing DNA at an enzyme:DNA molar ratio of 40 (Fig. 2, A and B, lane 3 and 20). At lower enzyme:DNA molar ratios, the reactivity of MutY with both DNA substrates was about the same.

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Fig. 1. Cleavage of A/G-containing oligonucleotide by E. coli MutY is inhibited by NaBH₄. Oligonucleotide substrates (3′-end-labeled 20-mer, 1.8 fmol) were incubated with MutY in the presence of 0.1 M NaCl or NaBH₄, followed by (•) or not followed by (○) piperidine (Pip.) treatment. Nicking products were electrophoresed in a 14% polyacrylamide sequencing gel followed by autoradiography. Lane 1 is the DNA substrate alone. Lanes 2–7 show results from reactions containing decreasing amounts of MutY (72, 18, 4.5, 1.8, 0.9, and 0.45 fmol from lane 2 to lane 7, respectively). Lanes 8–13 are similar to lanes 2–7 except that the reactions were followed by piperidine treatment. Lanes 14–19 are similar to lanes 2–7 except that the reactions also contained 0.1 M NaBH₄. The positions of intact oligonucleotide (I) and nicking product (N) are indicated by arrows.

Fig. 2. Formation of covalent complexes of MutY and A/G or A/GO-containing DNA in the presence of NaBH₄. Oligonucleotide substrates (3′-end-labeled 20-mer, 1.8 fmol) containing A/G (A) or A/GO (B) mismatches were incubated with MutY in the presence of 0.1 M NaCl or NaBH₄. The products after heating at 90°C for 2 min were electrophoresed in a 12% SDS-polyacrylamide gel followed by autoradiography. Lane 1 is the DNA substrate alone. Lane 2 shows the result from a reaction containing 72 fmol of MutY in the presence of 0.1 M NaCl. Lanes 3–5 show results from reactions containing decreasing amounts of MutY (72, 18, 4.5, 1.8, 0.9, and 0.45 fmol from lane 3 to lane 8, respectively) in the presence of 0.1 M NaBH₄. The positions of free oligonucleotide (F) and covalent complex (C) are indicated by arrows. The unincorporated [α-32P]dCTP migrated very close to the free oligonucleotide.

As shown in Fig. 3, purified mutant MutY(G116A) formed very few complexes with A/G-containing 20-mer DNA. As the enzyme concentrations increased, the amount of complex did not increase. Because short oligonucleotides were used in these assays, the cleavage products could be observed in a non-denaturing gel (Fig. 3). The cleavage product of MutY(G116A) was completely dissociated from the enzyme at higher enzyme concentrations, while a substantial fraction of DNA remained bound to wild-type MutY. The bound oligonucleotide with wild-type MutY contained a substantial fraction of the total cleavage products, indicating that wild-type MutY remained bound to DNA even after the actions of glycosylase and AP lyase. The cleavage activity of MutY(G116A) with A/G-containing DNA was further demonstrated to be about the same as that of the wild-type enzyme by analyzing the cleavage products on denaturing gels (data not shown). As for A/G-containing DNA, MutY(G116A) had reduced binding affinity for A/G-containing DNA but showed similar cleavage activity as compared with wild-type MutY (data not shown).

Binding Affinity of Mutant MutY(G116D) for Different Mismatches—In initial studies, we observed that MutY(G116D) did not bind and cleave A/G-containing DNA (data not shown). Further analysis showed that this mutant protein had a different affinity for A/G- and A/GO-containing DNA. Detailed studies were then performed to determine the dissociation constants and kinetic parameters of MutY(G116D) for A/G- and A/GO-containing DNA. MutY(G116D), at a concentration of 2.9 nM (enzyme:DNA molar ratio of 32), could bind A/GO-containing DNA but could not bind A/G-, N/G-, I/G-, A/C-, Z/G-, or C/G-containing DNA in a gel mobility shift assay (Fig. 4). The mobility of the complex of MutY(G116D) and A/GO-containing DNA (Fig. 4, lane 7) was the same as that of wild-type MutY and A/GO-containing DNA (Fig. 4, lane 2), which was slightly faster than the complex of MutY and A/G-containing DNA (Fig. 4, lane 1). Even at a concentration of 72 nM (enzyme:DNA molar ratio of 800), little complex of MutY(G116D) and A/G-containing DNA was seen (Fig. 5, lane 5). The apparent dissociation constants (Kᵅ values) of MutY and DNAs containing A/GO and A/G mismatches were determined using a range of protein concentrations with a fixed DNA concentration (Table I). The apparent Kᵅ values of MutY(G116D) and DNAs containing A/GO and A/G mismatches were 1.9 nM and 300 nM, respectively, while the apparent Kᵅ values of wild-type MutY and A/G- and A/GO-containing DNAs were 0.066 nM and 5.3 nM, respectively (17). The apparent Kᵅ values of MutY(G116D) protein and A/GO-containing DNA and A/G-containing DNA were 29- and 56-fold, respectively, higher than that of the wild-type MutY protein. The binding affinity of the mutant MutY(G116D) for A/GO-containing DNA was similar to the binding affinity of wild-type MutY for homoduplex DNA (apparent Kᵅ = 320–370 nM) (17).
Cleavage of A/G- and A/GO-containing DNA by Mutant MutY (G116D) Protein—Sequencing gel analysis revealed that MutY (G116D), at a concentration of 5.8 nM (enzyme:DNA molar ratio of 32), could nick an A/G-containing substrate less efficiently than could wild-type MutY at 6 nM (Fig. 6, compare lanes 7 and 8). MutY (G116D) could not nick A/G-containing DNA at 5.8 nM but did so efficiently at 144 nM (enzyme:DNA molar ratio of 800 (Fig. 6, lanes 3 and 5). Adding piperdine to the reaction followed by an additional incubation for 30 min at 90 °C did not increase the quantity of nicking product (data not shown), indicating that the mutant protein had both glycosylase and AP endonuclease activities.

In gel retardation assay, the band migrating faster than free A/G-containing DNA was the dissociated 9-mer cleavage product (Fig. 5, lanes 2, 4, and 5), indicating that MutY cleaved the A/G-containing DNA substrate and dissociated from it. There was more dissociation observed in reactions of mutant MutY (Fig. 5, lane 5) than in those of wild-type MutY (Fig. 5, lane 4) with A/G-containing DNA; thus, MutY (G116D) at 72 nM could cleave A/G-containing 20-mer DNA but did not remain bound to the substrate. This cleaved DNA band was not observed, however, in binding assays of wild-type or mutant MutY and A/G-containing DNA, even at high protein concentrations (Fig. 5, lanes 7–10).

Kinetic Parameters of MutY and Mutant MutY(G116D)—The efficiencies of cleavage by MutY and mutant MutY (G116D) of duplex oligonucleotides containing A/G or A/GO were compared (Table I). MutY cleaved A/G-containing DNA about 3-fold more efficiently than it did A/GO-containing DNA, with specificity constants (kcat/Km) of 10 and 3.3 min⁻¹ μM⁻¹, respectively. Mutant MutY (G116D) cleaved A/G- and A/GO-DNA with specificity constants (kcat/Km) of 0.0044 and 0.38 min⁻¹ μM⁻¹, respectively. Thus, the specificity constant of mutant MutY (G116D) and A/G-containing DNA was 2,300-fold lower than that of the wild-type enzyme, but the specificity constant of mutant MutY (G116D) and A/GO-containing DNA was only 9-fold lower than that of the wild-type enzyme.

Formation of Enzyme-DNA Covalent Complexes of MutY(G116A) and MutY(G116D)—To correlate the activity of cleavage with the efficiency of covalent DNA-enzyme intermediate formation, both MutY (G116A) and MutY (G116D) were incubated with A/G- and A/GO-containing DNA in the presence of sodium borohydride. As shown in Fig. 7, MutY (G116A) and wild-type enzyme could both readily form covalent complexes with A/G-containing DNA (lanes 2 and 4); MutY (G116D), however, was unable to form covalent complexes with A/GO-containing DNA at an enzyme-DNA molar ratio of 800 (lane 3), a condition under which substantial cleavage could occur (Fig. 6, lane 5). Both MutY (G116A) and MutY (G116D) could be trapped in covalent intermediates with A/GO-containing DNA in the presence of sodium borohydride but with lower efficiencies than with the wild-type enzyme.

DISCUSSION

The MutY protein has been shown to bind and incise A/G-, A/C-, N/G-, A/GO-, and A/AO-containing DNA (16, 17, 22, 27). Cleavage is achieved by both DNA glycosylase and AP endonuclease/lyase activities (24). The cleavage of the AP site by MutY has not been demonstrated to occur either by endonuclease or lyase mechanism. An AP endonuclease catalyzes strand cleavage via hydrolysis of the phosphodiester bond, while an AP lyase activity cleaves the 3′ phosphate group of the AP sugar residue via β-elimination. Imino enzyme-DNA intermediates are characteristic of a group of glycosylase/AP lyases including T4 endonuclease V, Micrococcus luteus UV endonuclease, E. coli endonuclease III, and E. coli Fpg (MutM) (46–48). The ability to isolate a stable covalent enzyme-DNA intermediate in the presence of sodium borohydride suggests that MutY belongs to that class of DNA glycosylases that possess concomitant AP lyase activity. These enzymes use an imino group as the nucleophile, resulting in an enzyme-DNA intermediate (46). A 25-kDa proteolyzed MutY fragment can also form covalent intermediates with A/G- and A/GO-containing DNA that are smaller in size than complexes with intact DNA substrates (3′-end-labeled 20-mer, 1.8 fmol) were assayed for binding at 37 °C for 30 min with 60 fmol of wild-type MutY (lanes 1 and 2) and 58 fmol of MutY (G116D) (lanes 3–9). Oligonucleotides containing each of the following mismatches were used: A/G (lane 1), A/GO (lane 2), A/G (lane 3), N/G (lane 4), Z/G (lane 5), V/G (lane 6), A/GO (lane 7), A/C (lane 8), and C/G (lane 9). Assay products were fractionated in an 8% polyacrylamide gel followed by autoradiography. The protein-bound and free DNA are indicated by arrows. Note the complex of MutY/A/GO-20 migrates faster than the complex of MutY/A/G-containing DNA.

Table 1

| MutY          | DNA | Kd (nM) | Km (nM) | Vmax (pM/min) | kcat (min⁻¹) | kcat/Km (min⁻¹ μM⁻¹) |
|---------------|-----|---------|---------|---------------|---------------|----------------------|
| Wild type     | A/G | 5.3 ± 0.5 | 2.6 ± 0.3 | 21 ± 2 | 0.026 ± 0.002 | 10 ± 2 |
|               | A/GO| 0.066 ± 0.013 | 6.5 ± 1.9 | 17 ± 2 | 0.021 ± 0.002 | 3.3 ± 1.0 |
| G116D         | A/G | 300 ± 80 | 150 ± 20 | 100 ± 10 | 0.00067 ± 0.00007 | 0.0044 ± 0.0007 |
|               | A/GO| 1.9 ± 0.3 | 32 ± 6  | 120 ± 10 | 0.012 ± 0.001 | 0.38 ± 0.08 |

Fig. 4. Binding of mismatch-containing oligonucleotides by mutant MutY (G116D). DNA substrates (3′-end-labeled 20-mer, 1.8 fmol) were assayed for binding at 37 °C for 30 min with 60 fmol of wild-type MutY (lanes 1 and 2) and 58 fmol of MutY (G116D) (lanes 3–9). Oligonucleotides containing each of the following mismatches were used: A/G (lane 1), A/GO (lane 2), A/G (lane 3), N/G (lane 4), Z/G (lane 5), V/G (lane 6), A/GO (lane 7), A/C (lane 8), and C/G (lane 9). Assay products were fractionated in an 8% polyacrylamide gel followed by autoradiography. The protein-bound and free DNA are indicated by arrows. Note the complex of MutY/A/GO-20 migrates faster than the complex of MutY/A/G-containing DNA.
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MutY. These results strongly suggest that MutY possesses AP lyase activity. E. coli MutY and endonuclease III are highly homologous (24–26); however, while lysine 120 of E. coli endonuclease III has been suggested to be necessary for the formation of the enzyme-substrate intermediate (49), MutY has a serine residue at this position. It will be interesting to see which amino acid of MutY is involved in the nucleophilic attack upon the C-1 carbon of the sugar of adenine.

Our results show that a MutY with a Gly116 to Ala or Asp mutation is substantially reduced in its ability to bind to DNA substrates. Gly116 of endonuclease III, and 113–119) that is completely conserved among MutY, E. coli MutY sequence.

MutY(G116D) has variably reduced binding to DNA containing different mismatches (Table I). The binding affinity of MutY(G116D) for A/G-containing DNA is similar to the binding affinity of wild-type MutY for homoduplex DNA. The apparent Kd of MutY(G116D) protein and A/G-containing DNA is slightly lower than that of the wild-type MutY and A/G-containing DNA, suggesting that one possible motif of MutY involved in A/G binding is not affected by the Gly to Asp substitution. Gly116 may be also in the proximity of catalytic site, because the kcat values of MutY(G116D) on A/G- and A/GO-containing DNA were decreased.

MutY(G116D) has variably reduced binding to DNA containing different mismatches (Table I). The binding affinity of MutY(G116D) for A/G-containing DNA is similar to the binding affinity of wild-type MutY for homoduplex DNA. The apparent Kd of MutY(G116D) protein and A/G-containing DNA is slightly lower than that of the wild-type MutY and A/G-containing DNA, suggesting that one possible motif of MutY involved in A/G binding is not affected by the Gly to Asp mutation. Recently, we have shown that the removal of a 14-kDa C-terminal region of MutY substantially reduces its binding of A/G- but not A/GO-containing DNA, a result consistent with the differential binding of MutY(G116D) with A/G- and A/GO-containing DNA.

The glycosylase/AP lyase activity of MutY(G116A) is comparable with that of the wild-type enzyme (Fig. 3). Thus, this Gly to Ala alteration does not affect the catalytic activity of MutY, although the imino intermediate of MutY(G116A) and A/G-containing DNA is less easily trapped than the wild-type enzyme and the same substrate. The catalytic activities of MutY(G116D) with A/G- and A/GO-containing DNA are reduced to different extents. The extremely weak catalytic activity...
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ity of MutY(G116D) with A/G-containing DNA is partly attributable to its weakened affinity for this DNA substrate (increased \( K_d \)). The binding affinity of MutY(G116D) for A/G-containing DNA is indistinguishable from that for homoduplexes, suggesting that this mutant protein recognizes A/G-mismatched sites through nonspecific binding. Although the binding of MutY(G116D) to A/G-containing DNA is reduced 29-fold compared with wild-type enzyme, the affinity of the mutant protein for A/G-containing DNA is still 150-fold higher than that for homoduplex DNA. Thus, MutY(G116D) is still able to specifically recognize and cleave an A/G-containing DNA. The mutant protein recognizes A/G-containing DNA, independent of any DNA mismatched sites through nonspecific binding. Although the binding of MutY(G116D) to A/G-containing DNA is reduced 29-fold compared with wild-type enzyme, the affinity of the mutant protein for A/G-containing DNA is still 150-fold higher than that for homoduplex DNA. Thus, MutY(G116D) is still able to specifically recognize and cleave an A/G-containing DNA. The mutant protein recognizes A/G-containing DNA, independent of any DNA mismatched sites through nonspecific binding.

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A-Lien Lu, David S. Yuen and Jason Cillo

*J. Biol. Chem.* 1996, 271:24138-24143.
doi: 10.1074/jbc.271.39.24138

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