The Active Site of the *Escherichia coli* MutY DNA Adenine Glycosylase *

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*Escherichia coli* MutY is an adenine DNA glycosylase active on DNA substrates containing A/G, A/C, or A/8-oxoG mismatches. Although MutY can form a covalent intermediate with its DNA substrates, its possession of 3′ apurinic lyase activity is controversial. To study the reaction mechanism of MutY, the conserved Asp-138 was mutated to Asn and the reactivity of this mutant MutY protein determined. The glycosylase activity was completely abolished in the D138N MutY mutant. The D138N mutant and wild-type MutY protein also possessed different DNA binding activities with various mismatches. Several lysine residues were identified in the proximity of the active site by analyzing the imino-covalent MutY-DNA intermediate. Mutation of Lys-157 and Lys-158 both individually and combined, had no effect on MutY activities but the K142A mutant protein was unable to form Schiff base intermediates with DNA substrates. However, the MutY K142A mutant could still bind DNA substrates and had adenine glycosylase activity. Surprisingly, the K142A mutant MutY, but not the wild-type enzyme, could promote a β/δ-elimination on apurinic DNA substrates. Our results suggest that Asp-138 acts as a general base to deprotonate either the ε-amino group of Lys-142 or to activate a water molecule and the resulting apurinic DNA then reacts with Lys-142 to form the Schiff base intermediate with DNA. With the K142A mutant, Asp-138 activates a water molecule to attack the C1′ of the adenosine; the resulting apurinic DNA is cleaved through β/δ-elimination without Schiff base formation.

In *Escherichia coli*, the proteins MutY, MutM, and MutT are involved in defending against the mutagenic effects of 7,8-dihydro-8-oxo-guanine (8-oxoG or GO) lesions, the most stable products known due to oxidative damage to DNA (1, 2). The MutT protein specifically eliminates 8-oxo-dGTP from the nucleotide pool (3–5). The MutM protein (Fpg protein) removes both ring-opened purine lesions and mutagenic GO adducts from DNA (6, 7). MutM removes GO lesions efficiently from C/G mispairs but poorly from A/GO mispairs (7). When C/GO is not repaired by MutM, adenines are frequently incorporated opposite GO bases during DNA replication (8, 9). A second round of replication through this mismatch subsequently leads to a G-C to T-A transversion (9–12). A role of the MutY pathway in *E. coli* is the removal of adenines misincorporated opposite GO or G following DNA replication (1, 13, 14). 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 wild-type strains (15, 16). Similar repair pathways involved in the defense against oxidized guanines are found in higher organisms. MutY homologous activity was found in calf thymus and HeLa cell extracts (17, 18). The amino acid sequence of human MutY homolog shows about 40% identity to *E. coli* MutY protein, but its encoded protein has not yet been characterized (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 (13, 16, 20–24). The MutY protein is a 39-kDa iron-sulfur protein (24–26) with its N-terminal domain sharing structural similarity with endonuclease III (endo III) and AlkA (24–28). This includes the helix-hairpin-helix (HhH) and Gly/Pro...Asp loop motifs. Endonuclease III repairs thymine glycol and oxidized pyrimidines in DNA (29, 30) and AlkA repairs methylated purines (31, 32). The C-terminal domain of MutY plays an important role in the recognition of GO lesions (33), and AlkA has an additional N-terminal domain of unknown function (31, 32).

DNA glycosylases in the endo III superfamily can be divided into two groups (34, 35). Bifunctional DNA glycosylases, including endo III and 8-oxoG glycosylase, use the conserved lysine (Lys-120 in endo III and Lys-249 in hOGG1) to form a Schiff base intermediate and also possess strong AP lyase activity (27, 36–38). Monofunctional glycosylases such as AlkA lack the conserved lysine and AP lyase activity (31, 32). Because MutY has a serine residue at this conserved position, it was originally grouped as a monofunctional glycosylase (39). While several groups failed to detect 3′ apurinic/apyrimidinic (AP) lyase activity in their MutY preparations (13, 22, 39–41), our laboratory and others have reported that MutY has a weak AP lyase activity (14, 24, 33, 42–45). MutY can also cleave DNA containing an unmodified AP site (43). Other supportive evidence for MutY AP lyase activity is that MutY can form a covalent Schiff base intermediate with its DNA substrates (33, 43, 44, 46, 47). Trapping the covalent protein-DNA complex with sodium borohydride has been used as a diagnostic tool for bifunctional glycosylase/AP lyases (35, 38, 39). Thus, MutY represents a unique group of glycosylases because it does not have the conserved lysine but can form a Schiff base intermediate with its DNA substrates. In this paper, we have investigated the reaction mechanism of MutY.

We show that Asp-138 can act as a general base to activate a nucleophile. The glycosylase and trapping activities were completely abolished in the D138N MutY protein but the DNA binding activity of this mutant protein was not drastically different from that of the wild-type enzyme. The N-terminal domains of MutY, residues 1–226 (M25) (33) and residues

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1 The abbreviations used are: GO (8-oxoG), 7,8-dihydro-8-oxo-guanine; AP, apurinic/apyrimidinic; M25, the N-terminal 25-kDa MutY domain; UDG, *E. coli* uracil DNA glycosylase; endo III, *E. coli* endonuclease III; OGG1, 8-oxoG glycosylase; HPLC, high performance liquid chromatography; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction.

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1–225 (p26) (43, 45) have been shown to retain catalytic activity. We isolated the imino-covalent M25-DNA intermediate and identified several lysine residues (Lys-142, Lys-157, and Lys-158) of MutY in the proximity of the active site. Mutation of Lys-157 and Lys-158 both individually and combined, had no effect on MutY activities. Mutagenesis of Lys-142 to Ala confirmed that Lys-142 could form a Schiff base with DNA. However, K142A MutY mutant still had adenosine glycosylase activity. Moreover, the K142A MutY mutant is different from the wild-type enzyme by its possession of a βββ-elimination activity on DNA containing an AP/8-oxoG. Possible reaction mechanisms of MutY are discussed.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Bacteria**—The entire MutY gene in pJTW10–12 (24) was amplified by PCR with two primers: Chang 222 (5′-GGCAGGCA-TATGCAAGCTCGCAATTTTC-3′) and Chang 90 (5′-GGCGGAGGAT-CCCTAAACGGCGACGTCG-3′). Similarly, the N-terminal domain of the mutY gene corresponding to Met-1 to Gin-226 (M25) was PCR-amplified from pJTW10–12 by primers Chang 222 and Chang 223 (5′-GGCGGAGGATCCCTATTGGCGGGCGACGTCG-3′). The resulting clones pMYW-1 for the entire mutY gene and pJ16–146-13 for Met-1 to Gin-226 were confirmed by DNA sequencing. The mutY gene and its derivatives in the plasmid pET11a were under the control of the T7 promoter. The expression host of the mutY and its derivatives in the plasmid pET11a was E. coli, which was induced at an 1.1 ratio and used as templates for another PCR reaction containing Chang 222 and Chang 90 primers. The resulting PCR products were cloned into pET11a as described above. All the oligonucleotides used are listed in Table I. Mutants K157Q, K157A/K158A, and K142A were first screened for the generation of the MalE, Sol, and XhoI restriction sites, respectively, then confirmed by DNA sequencing.

**Expression and Purification**—E. coli cells PR70/DE3 harboring the expression plasmid containing MutY, MutY mutants, or M25 were grown in LB broth containing 50 μg/ml ampicillin at 30 °C. The expression of MutY and its derivatives were induced at an A600 of 0.6 by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM to the culture at 23 °C. The cells were harvested 16 h later.

Mutant MutY proteins were quickly checked for activity in the crude cell extracts from 50-ml cultures. Cells were centrifuged in a SS34 rotor at 10,000 rpm for 15 min. The cell pellets were resuspended in 4 ml of 20 mM potassium phosphate (pH 7.4), 0.5 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol and sonicated for 6 cycles of 10 s, followed by 5 s of rest each cycle. After centrifuging at 10,000 rpm in a SS34 rotor for 40 min at 4 °C, the supernatant was quickly frozen in small aliquots at −80 °C. Alternatively, cell extracts from 1 liter of E. coli cells were prepared as described previously (50).

M25 domain and MutY mutant proteins (D138N and K142A) were purified from approximately 40 g of E. coli PR70/DE3 cells harboring the respective overproduction plasmids, similar to the method used with wild-type enzyme (24). Nicking of A/G-containing 20-mer DNA was assayed during the purification of the M25 domain. Binding of A/G-containing 20-mer DNA was assayed during the purification of the MutY(D138N) and MutY(K142A) enzymes. As judged on a 12% SDS-polyacrylamide gel, all proteins were purified to >99% homogeneity (data not shown). The purification of homogeneous MutY protein from an overproducing E. coli JM109 strain, harboring pJTW10–12, has been described previously (24).

**Oligonucleotide Substrates and Enzymes**—Oligonucleotides of 19-mer containing base mismatches were labeled as described by Lu et al. (14). E. coli uracil DNA glycosylase (UDG) was purchased from Life Technologies, Inc. DNA substrates containing U/G or U/O (300 fmol) were fully converted to AP/G or AP/O by treating with 1.5 units of UDG at 37 °C for 1 h in MutY buffer (20 mM Tris-HCl, pH 7.6, 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 2.9% glycerol). MutY Binding, Cleavage, and Trapping Assay—The MutY activity assay with labeled oligonucleotide substrates were performed as described by Lu et al. (44) with some modifications. MutY enzyme was diluted with diluent (20 mM potassium phosphate, pH 7.4, 50 mM KCl, 1.5 mM dithiothreitol, 0.1 mM EDTA, 200 μg/ml bovine serum albumin, and 50% glycerol) before use. MutY binding and cleavage (glycosylase) buffer contains 20 mM Tris-HCl, pH 7.6, 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 2.9% glycerol. MutY trapping buffer contains 20 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 1 mM EDTA, and 2.9% glycerol. For the glycosylase assays, unless specified in reactions, samples were treated at 37 °C for 1 min, cooled on ice, and loaded onto 14% 7 M urea sequencing gels.

**Formation of Large Scale Enzyme-DNA Covalent Complex**—A covalent complex of M25 with A/G-containing 19-mer DNA was formed in a reaction containing 1.8 nmol of DNA and 1.8 nmol of M25 in 180 μl of
cleft reaction buffer in the presence of 0.1 \text{m} \text{NaBH}_4, \text{A NaBH}_4 stock solution was freshly prepared and was added immediately after M25 was added. After incubation at 37 °C for 30 min, 5× dye buffer (0.5 \text{ m} sucrose, 15% SDS, 312.5 \text{ mM} \text{Tris}-\text{HCl}, pH 6.9, 10 \text{ mM} \text{EDTA}, 5% \beta\text{-mercaptoethanol}, and 0.0025% bromphenol blue) was added to the samples, which were heated at 90 °C for 2 min and separated on a 12% polyacrylamide gel in the presence of SDS (SDS-PAGE) according to Laemmli (51). The SDS-polyacrylamide gel used was cast 1–3 days prior to gelation. Electrophoresis was performed using a Trans-Blot cell. The membrane was pre-wet in 100% methanol followed by transfer to a PVDF membrane (Millipore) was performed using a Trans-Blot cell. The final enzyme concentrations were as follows: 0.5 \text{nM to 0.18 \text{nM}} (lane 1), 25 \text{nM (lane 2), 10 \text{nM (lane 3), 5 \text{nM (lane 4), 2.5 \text{nM (lane 5), and 0.5 \text{nM (lane 6). Arrows indicate the positions of intact oligonucleotide (I) and the nicked product (N).}

Mutant MutY(D138N) Is Defective in Glycosylase Activity—As shown in Fig. 1, purified MutY(D138N) mutant was inactive in the glycosylase assay with both A/G- and A/GO-containing 20-mer DNA even at an enzyme concentration of 25 \text{nM} with an enzyme/DNA molar ratio of 278. However, in the control reactions, wild-type MutY protein cleaved both A/G- and A/GO-containing DNA. The trapping assay of MutY (D138N) in the presence of sodium borohydride indicated that this mutant did not form any covalent-complexes with A/G- and A/GO-containing DNA (data not shown). Thus, these results confirmed that MutY(D138N) was defective in glycosylase activity.

Binding Affinity of the MutY(D138N) Mutant for Different Mismatches—In initial studies with crude extracts, we observed that MutY(D138N) could bind A/G- and A/GO-containing DNA (data not shown). Thus, the binding of A/GO-containing DNA was used to monitor the activity during the enzyme purification. Further studies were then performed to determine the dissociation constants of MutY(D138N) with A/G- and A/GO-containing DNA substrates. DNA substrates were incubated with various concentrations of MutY in binding assays, and experiments were repeated at least three times. The apparent dissociation constants (K_d) of MutY(D138N) and DNAs containing A/GO and A/G mismatches were determined using a range of protein concentrations with a fixed DNA concentration (Table II). The apparent K_d values of MutY(D138N) and DNAs containing A/GO and A/G mismatches were 0.10 and 24 \text{nM}, respectively, while the apparent K_d values of wild-type MutY and A/GO- and A/G-containing DNAs were 0.066 and 5.3 \text{nM}, respectively (14). The apparent K_d values of the MutY(D138N) with A/GO- and A/GO-containing DNA were 1.5- and 4.5-fold, respectively, higher than that of the wild-type MutY protein. Despite the fact that MutY(D138N) is defective in glycosylase activity, it is capable of binding to both A/G- and A/GO-containing DNA substrates although with slightly lower affinity than the wild-type.

The binding of MutY(D138N) with DNA substrates containing other mismatches was also tested. The apparent dissociation constants of the mutant protein were compared with those of the wild-type MutY (Table II). The binding affinities of MutY(D138N) with these substrates were not significantly different from that of the wild-type MutY. MutY(D138N) has 5–6-fold weaker binding to A/G-, Z/G-, A/2AP-, and C/GO-containing DNA (Z, 7-deaza-adenosine; 2AP, 2-aminopurine), but has affinity to A/GO, A/C, A/I, N/G, and C/GO substrates (I, inosine; N, nebularine) similar to that of wild-type MutY.

Determination of the MutY Residue Responsible for the Covalent Complex Formation Detected by Peptide Sequencing—The amino acid sequences of E. coli MutY and endo III are highly homologous (25, 26). However, although lysine 120 of E. coli endo III has been suggested to be necessary for the formation of the enzyme-substrate intermediate (37), MutY has a serine residue at this position but can form Schiff base intermediate quite efficiently (33, 43, 44, 46, 47). To determine which amino acid residue in MutY is involved in the nucleophilic attack upon the C1‘ carbon of the sugar of adenosine, the covalent complexes of MutY with DNA and its catalytically active domain (M25) with DNA were separated from the free protein by SDS-PAGE and stained with Coomassie Blue. This experiment was performed differently from other published results that used labeled DNA and monitored the extent of bound substrates (33, 43, 44, 46, 47). As expected, Fig. 2 shows that the protein-DNA complexes displayed slower mobility than the free proteins. The molecular masses of the MutY-DNA and M25-DNA complexes were estimated to be 45 and 31 kDa,
respective. About 25% of M25 and MutY could be trapped with A/G-containing 20-mer DNA substrate at an enzyme/DNA molar ratio of one in the presence of sodium borohydride. The DNA-bound and free M25 from a large scale reaction were transfereed to a PVDF membrane, digested with trypsin, and analyzed by HPLC. The chromatograms (Fig. 3) showed that three peptide peaks (peaks 35, 50, and 89) were reduced and four peptide peaks (peaks 22–24, 25, 27, and 92) were increased in the DNA-bound sample as compared with free protein. Edman degradation of peak 50 from the free protein unfortunately revealed no sequence information. The sequence of peak 35 of free M25 was determined to be HFILDGNYK (residues 133–142). Based on the absorption at 204, 277, and 292 nm, peaks 27 and 92 of the DNA-bound M25 sample were expected to be Trp- and Tyr-containing peptides, respectively. Peak 22/24 from DNA-bound M25 sample had unusual UV absorption and was determined to have the sequence CYAVSGWPGK (residues 148–157) (Fig. 4). Mass spectrometric analysis of 22/24 M25B-PT identified a peak that corresponded to the size of residues 148–157 but not to residues 148–158. According to our initial model, based on the endo III structure and the recently solved x-ray structure of the MutY N-terminal domain with adenine bound in the active site, ε-NH₂ groups of these Lys residues are all located in the proximity of the adenine active site pocket (28) (Fig. 5). Specifically, the ε-NH₂ of Lys-142 is positioned in the same position as Lys-120 of endo III. Although the data obtained are not definitive, they implicate that a closely spaced Lys-142, Lys-157, or Lys-158 is a likely candidate to provide the primary amine involved in forming a Schiff base with DNA.

Lys-142 of MutY Forms a Schiff Base with DNA—Based on the peptide sequencing of DNA-bound M25, residues Lys-142, Lys-157, or Lys-158 could be involved in the Schiff base formation. Thus, K142A, K157Q, K158A, and K157QK158A MutY mutants were constructed by site-directed mutagenesis. These mutant proteins were expressed in mutY mutant cells, and cell extracts were assayed for A/G and A/GO glycosylase, trapping, and binding activities. The expression of these mutant proteins was comparable to the wild type, except the K157Q mutant protein was less soluble than the others (data not shown). Mutation of Lys-157 and Lys-158 both individually and combined, had no effect on the MutY activities. However, the K142A mutant protein was unable to form the Schiff base intermediates with DNA substrates at an enzyme/DNA molar ratio of 40 (Fig. 6). At much higher enzyme/DNA molar ratios, a very small amount (<0.5%) of covalent complex could be observed with the K142A mutant protein (data not shown). Thus, ε-NH₂ of Lys-142 is the amine used in forming the Schiff base with DNA. However, all of these mutant proteins retained their DNA binding activity (Fig. 7). K142A Mutant MutY Has DNA Glycosylase Activity—To test whether K142A retained the glycosylase activity of native MutY, three reactions were carried out for the cleavage assays. In the first reaction, the samples were directly loaded onto the sequencing gel without heating, samples in the second reaction were heated at 90 °C for 2 min before loading to the gel, and samples in the third reaction were treated with 1 M piperidine for 30 min (this condition promotes β-elimination) after the MutY reaction as well as being heated at 90 °C for 2 min before loading onto the gel. As shown in Fig. 8, both MutY and K142A mutant could cleave 3'-labeled A/G-containing DNA. It ap-
peared that the K142A mutant was even more active than the wild-type MutY. Both the K142A mutant and the wild-type MutY had similar kinetics of glycosylase activity in time-course studies (data not shown). Heating the samples at 90 °C for 2 min (lanes 3 and 6) enhanced the cleavage activities. However, further treatment of the products with piperidine at 90 °C (lanes 4 and 7) did not significantly increase the extent of cleavage by both MutY and K142A proteins. Thus, both the K142A mutant and wild-type MutY possess the adenine glycosylase activity.

To address whether the products in the heated lanes are derived by heat-promoted β-elimination or AP lyase activity, we analyzed the reaction products of monofunctional UDG (36). The AP/GO-containing DNA in the presence of NaBH₄ was assayed for binding with 3.6 nM purified MutY (lane 2) or extracts of MutY mutants (lanes 3–14) at 37 °C for 30 min. Lanes labeled with H used 100 ng of DNA alone. Lanes labeled with L used 20 ng of cell extracts prepared as described (50): lane 1, K142A; lane 2, wild-type MutY; lane 3, vector pET11a; lane 4, K157Q; lane 5, K158A; lane 6, K157A/K158A double mutant. Lane 7 contained 3.6 nM purified MutY. The products after heating at 90 °C for 2 min were electrophoresed on a 12% SDS-PAGE gel. The gel was dried and autoradiographed. The positions of free DNA (F) and covalent complex (C) are indicated.

FIG. 4. Some trypsin peptides of E. coli MutY. The residue numbers of lysines (K, bold) and arginines (R, circled) were indicated underneath. The sequences of peak 35 of M25FR-PT and peak 22/24 of M25B-PT were marked by boxes.

FIG. 5. The x-ray structure of MutY catalytic core (28). The side chains of Asp-138, Lys-142, Lys-157, Lys-158, and Ser-120 are shown in sticks and balls. Ser-120 is located in the helix-hairpin-helix domain conserved in the endo III superfamily. The iron-sulfur cluster located to the upper right of the figure is represented as large spheres.

FIG. 6. Formation of covalent complexes of MutY mutants with A/GO-containing DNA in the presence of NaBH₄. Oligonucleotide (3'-end-labeled 20-mer, 1.8 fmol) containing an A/GO mismatch was incubated with MutY or cell extracts containing expressed MutY mutant proteins in the presence of NaBH₄. Reactions were carried out in 20 μl of trapping buffer at 37 °C for 30 min. Lanes 1–6 used 100 ng of cell extracts prepared as described (50): lane 1, K142A; lane 2, wild-type MutY; lane 3, vector pET11a; lane 4, K157Q; lane 5, K158A; lane 6, K157A/K158A double mutant. Lane 7 contained 3.6 nM purified MutY. The products after heating at 90 °C for 2 min were electrophoresed on a 12% SDS-PAGE gel. The gel was dried and autoradiographed. The positions of free DNA (F) and covalent complex (C) are indicated.

FIG. 7. Binding of the A/GO-containing oligonucleotide by MutY mutants. A/GO-containing 20-mer oligonucleotide was assayed for binding with 3.6 nM purified MutY (lane 2) or extracts of MutY mutants (lanes 3–14) at 37 °C for 30 min. Lane 1 represents DNA alone. Lanes labeled with H used 100 ng and lanes labeled with L used 20 ng of cell extracts prepared as described (50): Lanes 3 and 4, vector pET11a; lanes 5 and 6, wild-type MutY; lanes 7 and 8, K142A; lanes 9 and 10, K157Q; lanes 11 and 12, K158A; and lanes 13 and 14, K157A/K158A double mutant. The products were analyzed on an 8% native gel. Arrows indicate the positions of MutY-DNA complex (B) and free DNA (F).
promoted β-elimination, it did not contribute to all the cleavage observed in the MutY reaction. It appears that heat is required to release the MutY protein from the cleaved products, as some retarded and smeared DNA, migrating slower than the intact DNA, was detected in non-heated samples (data not shown).

**K142A Mutant MutY Can Cleave AP-containing DNA**—If MutY truly possesses AP lyase activity, it should be able to cleave a phosphodiester bond at an AP site. 5'-Labeled AP-containing DNA was reacted with K142A and MutY. To minimize heat-promoted β-elimination, after cleavage reactions, samples were kept in a low salt concentration and one third of the formamide dye. The sequencing gel was run at 1200 V at 32 °C. As compared with the dilution buffer alone (Fig. 9, lanes 9 and 10), MutY did contain a weak AP lyase activity (Fig. 9, lanes 11 and 12). Quantitation of the PhosphorImager images indicated about 5–10% of AP-DNA was cleaved by MutY after background subtraction. The MutY AP lyase activity was consistently observed higher than the background and increased when the enzyme concentration increased (data not shown).

The AP lyase activity is about 20% of that of MutY glycosylase (compare lanes 3 and 4 of Fig. 8 to lanes 11 and 12 of Fig. 9).

When AP-GO-DNA was cleaved with the K142A protein, there was a new band (marked with P in Fig. 9, lanes 13 and 14) migrating to the same position as that in the piperidine-treated sample (Fig. 9, lane 7). This product with a 3'-phosphate appeared even without heating at 90 °C for 2 min. Thus, the K142A mutant MutY is different from the wild-type enzyme by its possession of a β/β'-elimination activity on DNA containing an AP/GO. This surprising result supports the idea that the K142A mutant MutY protein incises both 3' and 5' of an AP site, although it does not form the Schiff base intermediate (Fig. 10).

**Binding Affinity of the MutY(K142A) Mutant for DNA Sub-
Does MutY Have AP Lyase Activity?—MutY has been classified as both a monofunctional and a bifunctional enzyme. While several groups failed to detect AP lyase activity in their MutY preparations (13, 22, 39–41), our laboratory and others have reported that MutY has a weak AP lyase activity (14, 24, 33, 42–45). The covalent complex formation of MutY or M25 with A/G-containing DNA is quite efficient (Fig. 2). About 25% (sometimes up to 50%) of enzyme can be trapped with DNA substrates containing an unmodified AP site but with less efficiency (47).2 The Schiff base formation is characteristic of MutY glycosylase activity. Taken together with our previous results that MutY can cleave more than 60% of A/G-containing DNA and that this mutant gains a new AP lyase activity and that this AP lyase activity was about 20% of the MutY glycosylase activity. Taken together with our previous results that MutY can cleave more than 60% of A/G-containing DNA without piperidine treatment (14, 44), we propose that MutY is not a monofunctional glycosylase. Our results are slightly different from that of Manuel and Lloyd (43), who reported that AP lyase activity was active as MutY glycosylase activity. The discrepancy may be caused from heating the DNA samples before loading and/or high temperature of the sequencing gel run at a high voltage.

Zharkov and Grollman (47) attributed the AP lyase activity of MutY, observed, as an artifact of heating at 90°C in Tris-containing buffer and thus concluded that MutY did not cleave the phosphodiester bond. However, a small amount of cleavage was observed in reactions without the heating step. We have reported that MutY nicking product could be detected in a native gel in the binding assays (14) in which samples were never heated above 37°C. To address whether heat can promote β-elimination, the AP/GO-DNA generated by UDG was heated at 90°C for 2 min at two different salt concentrations. At a low salt concentration, no significant cleavage was observed, but, at a high salt concentration, a condition used in our MutY assays, about 15% of the AP site was cleaved. Although heating promoted β-elimination, it did not contribute to all the cleavage observed in the MutY reaction because the cleavage product of AP-DNA by MutY could be detected even without heat.

Williams and David (46) compared the DNA cleavage between MutY and UDG and suggested that MutY is a monofunctional glycosylase. Their assays included a heating step, but limited cleavage was observed with 30-mer substrates and some cleavage was observed with 18-mer DNA in the reactions without NaOH treatment. Zharkov and Grollman (47) compared MutY proteins from three laboratories including ours and found they behaved similarly. Therefore, the conflict reported in the literature for the MutY properties may be due to the length and sequence contents of the DNA substrates as well as reaction conditions used in different laboratories.

Lys-142 Forms the Protein-DNA Covalent Complex.—A central issue in DNA glycosylase/AP lyase action is the identity of the enzyme amine that forms the Schiff base intermediate. Two approaches were taken to identify the active site amine of MutY. One approach is to analyze the imino protein-DNA complex and determine the DNA-bound amino acid by peptide sequencing. The other approach involves site-directed mutagenesis which confirms that Lys-142 can form a Schiff base with DNA. Inspection of the structure of the MutY catalytic domain and of a model for how this domain might bind to DNA (28) suggests that the ε-amine of Lys-142 is close to the catalytic Asp-138 (Fig. 5). Our result is in agreement with this prediction and the data of Zharkov and Grollman (47), who also showed Lys-142 of MutY is involved in cross-linking to DNA.

The HPLC chromatograms in Fig. 3 show that three peptide peaks (peaks 35, 50, and 89) were reduced and four peptide peaks (peaks 22–24, 25, 27, and 92) were increased in the DNA-bound sample as compared with free protein. We have identified the peak 35 containing Lys-142, but unfortunately, the DNA-bound polypeptide was not identified. Originally, peak 22/24 from DNA-bound M25 sample was suspected to bind DNA but it turned out to be a polypeptide (Cys-148–Lys-157) close to Lys-142 (Fig. 4). Thus, Lys-157 appears to be more susceptible to trypsin digestion in the DNA-bound enzyme. This suggests that there are some conformation changes upon substrate binding. With respect to MutY structure, the helix containing Lys-157 and Lys-158 is positioned next to Lys-142 (Fig. 5); thus, a conformational change is likely.

Model for MutY Action.—Based on our results, we propose two possible reaction mechanisms for the wild-type MutY. In one model, Asp-138 activates a water molecule to attack the C1-unsaturated carbon (Fig. 10, step 1) (35). The adenine base is released by this DNA glycosylase activity. The resulting deoxyribose undergoes tautomerization with equilibration favoring the closed ring structure (Fig. 10A, step 2). Lys-142 of MutY is promoted by a general base to attack the C1’ of the opened sugar. This second nucleophilic attack generates a trappable Schiff base with several chemical conversions (Fig. 10A, step 3). Alternatively, the original nucleophilic attack at C1’ involves the ε-amine of Lys-142. Asp-138 deprotonates the ε-amine of Lys-142. This nitrogen nucleophile displaces the mismatched adenine from the DNA leading to a Schiff base formation (Fig. 10A, steps 1’–2’). The Schiff base intermediate of MutY-DNA is quite stable (47). Wild-type MutY can promote weak β-elimination thus strand cleavage occurs through step 4 (Fig. 10A). The products are two fragments: one with a 3’-α, β-unsaturated aldehyde and one with a 5’ phosphate group.

Lys-142 Has a β/δ-Elimination Activity.—The most surprising results from this study are that the MutY K142A mutant still has adenine glycosylase activity on A/G- and A/8-oxoG-containing DNA and that this mutant gains a new δ-elimina-

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2 X.-H. Li, P. Wright, and A.-L. Lu, unpublished results.
tion activity not possessed by wild-type MutY. These properties are different from other bifunctional glycosylase/AP lyases in which mutation of the conserved lysine abolishes catalysis (27, 37, 54). Since K142A has glycosylase activity, the mutant protein must use a water molecule to attack the C1' of the adenosine (Fig. 10B, step 1). When Lys-142 is mutated to Ala, it is possible that a water molecule occupies the position of e-amnine of Lys-142.

The K142A mutant MutY is different from the wild-type enzyme by its possession of a β/β'-elimination activity toward DNA containing an AP/8-oxoG mismatch. In step 3 (Fig. 10B), DNA with an abasic deoxyribose can be cleaved by K142A MutY through β-elimination directly without the Schiff base formation. MutY(K142A) then carries out a δ-elimination and subsequently releases the unsaturated sugar (Fig. 10B, step 4). The final products are a DNA fragment with a 3'-phosphate end, a fragment with a 5'-phosphate group, and an unsaturated sugar. In this regard, the K142A MutY mutant behaves like the Fpg (MutM) protein, incising both 3'- and 5'-phosphates.

The mechanistic switch between K142A and wild-type MutY raises an interesting question about the mechanistic preference of MutY since the biological significance of the Schiff base formation of wild-type MutY is not clear. Because MutY turns over extremely slowly, especially with A/GO substrates (14, 34, 58), the e-amine of Lys-142 may just happen to lie closely to the active site and experience a chance encounter with the AP site (Fig. 10A, step 3). The reaction may be similar to the reported lyase activity of small lysine-containing peptides on abasic sites in DNA (59). Alternatively, the Schiff base formation may play an active role (Fig. 10A, steps 3 and 2). It has been shown that the MutY-DNA covalent complex has a long half-life (47). MutY binding to its substrate in a stable covalent form may prevent Fpg from reacting with the AP/GO-DNA or may recruit other proteins for repair and re-synthesis. In the case of the second possibility, one would expect the K142A mutant protein to have a weaker affinity to AP-DNA than the wild-type enzyme. Our data seem to favor this notion because the dissociation constant of K142A with APGO-containing DNA is 6-fold higher than that of the wild-type MutY.

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