Reaction Intermediates in the Catalytic Mechanism of Escherichia coli MutY DNA Glycosylase*

Received for publication, April 8, 2004, and in revised form, August 19, 2004
Published, JBC Papers in Press, August 23, 2004, DOI 10.1074/jbc.M403944200


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The Escherichia coli adenine DNA glycosylase, MutY, plays an important role in the maintenance of genomic stability by catalyzing the removal of adenine opposite 8-oxo-7,8-dihydroguanine or guanine in duplex DNA. Although the x-ray crystal structure of the catalytic domain of MutY revealed a mechanism for catalysis of the glycosyl bond, it appeared that several opportunity-cally positioned lysine side chains could participate in a secondary β-elimination reaction. In this investigation, it is established via site-directed mutagenesis and the determination of a 1.35-Å structure of MutY in complex with adenine that the abasic site (apurinic/apyrimidinic) lyase activity is alternatively regulated by two lysines, Lys142 and Lys20. Analyses of the crystallographic structure also suggest a role for Glu61 in the apurinic/apyrimidinic lyase chemistry. The β-elimination reaction is structurally and chemically uncoupled from the initial glycosyl bond scission, indicating that this reaction occurs as a consequence of active site plasticity and slow dissociation of the product complex. MutY with either the K142A or K20A mutation still catalyzes β and β-δ elimination reactions, and both mutants can be trapped as covalent enzyme-DNA intermediates by chemical reduction. The trapping was observed to occur both pre- and post-phosphodiester bond scission, establishing that both of these intermediates have significant half-lives. Thus, the final spectrum of DNA products generated reflects the outcome of a delicate balance of closely related equilibrium constants.

The atomic coordinates and structure factors (codes 1WEF, 1WEG, and 1WEI) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://wws.rcsb.org/).

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base excision repair (BER) pathway (reviewed in Refs. 1–3). The elucidation of structure-activity relationships for DNA glycosylases and glycosylase/abasic site (AP) lyases has been facilitated both by solving the crystal structures and cocystal complexes and analyses of chemical modification and trapping experiments (4–17). These investigations have been successful in both localizing the active site pocket of these enzymes and identifying the key amino acids that participate in the catalytic events that lead to the excision of the inappropriate base. Determination of the chemical steps in the catalytic mechanism of DNA glycosylases is fundamental for understanding how organisms maintain their genome, despite the inevitable damage caused by exogenous and endogenous agents.

DNA glycosylases with an associated AP lyase activity (β-elimination) can be distinguished from DNA glycosylases without such activity, based on the identity of the nucleophile that attacks C1 of the deoxyribose sugar and whether the reaction proceeds through a covalent DNA-enzyme intermediate. DNA glycosylases that also catalyze a β-elimination reaction utilize a primary or secondary amine in the active site, whereas monofunctional glycosylases cleave the glyosyl bond via either the activation of a water molecule or a S3′1 attack (reviewed in Refs. 1 and 18). When a primary or secondary amine is utilized as the nucleophile, a Schiff base intermediate is formed that can undergo β-elimination, resulting in the cleavage of the phosphodiester bond (5). The identity of the amino acid residue involved in the formation of the Schiff base intermediate can be elucidated by reduction with NaBH4 or NaCNBH3, resulting in the formation of a covalently trapped enzyme-DNA complex that can undergo peptide sequencing. This technique has been widely used as a diagnostic tool to differentiate glycosylases from glycosylase/AP lyases.

In recent years, several reports have probed the catalytic mechanism of MutY (12, 16, 19–28); however, this mechanism and an accounting of the roles of catalytic residues have not been formalized. A straightforward interpretation of the complex catalytic mechanism of MutY has remained a challenge due to observations that the enzyme does not demonstrate standard Michaelis-Menten kinetics, with very slow enzyme turnover and apparent redundancies in potential catalytic residues (16, 19, 22, 24, 29). Although there is a consensus on the glycosylase base excision activity of MutY, the AP lyase activity of MutY has remained poorly defined. Biochemical evidence has been presented that argues for MutY being a monofunctional glycosylase (19–21), whereas other data are consistent

1 The abbreviations used are: BER, base-excision repair; AP, apurinic/apyrimidinic; 8-oxoG, 7,8-dihydro-8-oxoguanine.
with the enzyme being a glycosylase with a concomitant AP lyase activity (30, 31).

Although the latter data suggested an intrinsic AP lyase activity, initial attempts to trap the lyase reaction with NaBH₄ were unsuccessful (6). Insight into the identity of specific catalytic residues came from the determination of the crystal structure of the 26-kDa catalytic domain of MutY (cMutY, residues Met¹–Lys²²⁵) complexed with adenine (12). Examination of this structure suggested a reasonable hypothesis to explain and rationalize all prior data. The architecture of the 26-kDa domain of MutY revealed a structure consisting of two subdomains: a 4α-helical, 4Fe-4S cluster domain connected to a 6α-helical bundle containing a helix-hairpin-helix motif (Fig. 1A). Between these subdomains is a deep cleft that binds adenine; the glycosylase active site is composed of two acidic residues Glu³⁷ and Asp¹³₈ (shown as the D138N mutant) that are located on opposite sides of the cleft (Fig. 1B). It was hypothesized that these residues have side chains positioned to both protonate N7 of adenine and activate a water molecule for nucleophilic attack at C1’ of the corresponding deoxyribose, a standard glycosylase reaction mechanism. Recently, a catalytically inactive cocrystal complex was solved of a full-length thermophilic homolog of MutY from Bacillus steatorrhophilus with DNA containing an A:8-oxoG mismatch (17). In this structure, the DNA was bent 55°, and the adenine was fully extruded from the helix and occupied roughly the same position as determined in the previous structure. The reaction mechanism inferred from this structure, suggests that the essential aspartic acid residue stabilizes the positively charged oxocarbonium intermediate in the base cleavage reaction (17).

The experimental observation that gave the clue to the source of the lyase reaction came from data that demonstrated that the cMutY bound DNAs containing abasic site analogs with ~1 nM binding constants. With the enzyme dissociation being very slow, it was hypothesized that a lysine residue (Lys¹⁴₅) would be available for initiating the lyase chemistry through the formation of a Schiff base intermediate (12). Thus, the glycosylase and AP lyase reactions were postulated to be uncoupled, and the lyase chemistry occurred only as a consequence of an opportunistically positioned lysine residue. These hypotheses provided reasonable explanations for why NaBH₄-induced trapping of MutY was inefficient, since following the glycosylase reaction, the strong reducing agent NaBH₄ will reduce the transiently ring-opened deoxyribose, rendering it unreactive for further β-elimination chemistry. Experimental verification of this hypothesis appeared concomitantly with the first crystal structure in which conditions were established for the trapping of a covalent complex between MutY and an A-G mismatch containing DNA with the linkage through Lys¹⁴₂ (16, 19–23, 32). However, these conclusions do not rule out the possibility that lysines other than Lys¹⁴₂ could also participate in catalyzing the β-elimination reaction, and none of these data reveal whether the reaction occurs at a pre- or postincision complex. The investigations presented here provide insights into the underlying structural chemistry of the glycosylase and lyase activities of MutY.

**EXPERIMENTAL PROCEDURES**

**Materials**—All DNA substrates were synthesized by standard phosphoramidite chemistry on a 394 DNA/RNA synthesizer (Applied Biosystems). 8-oxo-7,8-dihydro-2′-deoxyguanosine phosphoramidite was purchased from Glen Research. DNA sequencing was performed using an ABI Prism 310 Genetic Analyzer (PerkinElmer Life Sciences). All DNA syntheses and sequence analyses were performed by the NIEHS Center (Molecular Biology Core, Dr. Thomas G. Wood, Director, University of Texas Medical Branch). The plasmid pKK223-3 (derivative of pKK223-3; Amersham Biosciences) containing the mutY gene was gifted from Drs. J. H. Miller and M. L. Michaels (UCLA). Adenine, NaCNBH₄, and NaCNBH₄ were purchased from Sigma. T4 polynucleotide kinase was purchased from New England Biolabs, Inc., and [γ²³⁵P]ATP was obtained from PerkinElmer Life Sciences.

**Site-directed Mutagenesis**—In vitro site-directed mutagenesis was performed by polymerase chain reaction using the QuikChangeTM site-directed mutagenesis kit (Stratagene). Synthetic oligonucleotides were used to create the desired changes in the mutY gene were synthesized and purified by gel electrophoresis. Following mutagenesis, the sequence of the entire mutY gene or the region coding for the catalytic domain of MutY (cMutY) was verified by DNA sequence analyses. Amino acids Glu³⁷ and Asp¹³₈ were independently changed to cysteines in MutY. In addition, E37S, D138N, K142A, and K20A mutations were engineered independently in cMutY.

**Protein Purification**—Wild type MutY, cMutY, and the mutant forms of these were expressed in Escherichia coli strain CC104mutY. MutY and cMutY were purified by sequential affinity chromatography (Q-Sepharose, SP-Sepharose, and single-stranded DNA cellulose) as previously described (31, 33). Similar procedures were adopted for purifying the mutant forms of MutY and cMutY.

**DNA Substrates**—Heteroduplex DNAs used in enzymatic assays are listed in Table I. The oligodeoxynucleotides were purified by electrophoresis through a 15% polyacrylamide gel containing 7M urea.

**Adenine Glycosylase and AP Lyase Assay**—To monitor adenine excision and AP lyase reactions, the DNA reactions were analyzed by single-stranded DNA cellulose) as previously described (31, 33). Similar procedures were adopted for purifying the mutant forms of MutY and cMutY.

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**Guanine Glycosylase Assays**—Duplex DNAs containing G opposite 8-oxoG were used to assay additional mismatch recognition and glycosylase activity (Table I, sequence 1). The strand containing the mismatched adenine (Table I, sequence 1) was radiolabeled at the 5′-end and annealed to the complementary strand containing guanine or 8-oxoG opposite the adenine. The DNA substrate (0.5 nM) was reacted with the enzyme (200 nm) in a reaction buffer containing 25 mM sodium phosphate (pH 6.8), 1 mM EDTA, 50 mM NaCl, and 100 μg/ml bovine serum albumin. The 20-μl reaction mixture was incubated at 37 °C for various times. Aliquots of these reactions were further incubated for 15 min, at 90 °C with piperidine. The reactions were terminated with an equal volume of formamide buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue) and heated at 90 °C for 4 min prior to separating the DNA reaction products through 15% polyacrylamide gels containing 7 M urea. The substrates and products of the incision reactions were analyzed by PhosphorImager analyses using ImageQuant Software (Amersham Biosciences).

**Guanine Glycosylase Assays**—Duplex DNAs containing G opposite 8-oxoG were used to assay additional mismatch recognition and glycosylase activity (Table I, sequence 1). The strand containing the mismatched guanine was radiolabeled at the 5′-end with [γ²³⁵P]ATP. Two sets of experiments were conducted in which guanine excision was monitored as a function of time or increasing concentrations of MutY. To monitor the kinetics of guanine excision, MutY (400 nM) was incubated with the DNA substrate (2 nM) in a buffer containing 25 mM sodium phosphate (pH 7.0), 1 mM EDTA, 50 mM NaCl, and 100 μg/ml bovine serum albumin. Aliquots were withdrawn at specific times (0, 1, 5, 15, 30, 60, and 300 min) and treated with piperidine for 15 min at 90 °C. Quantitation of guanine glycosylase activity with increasing enzyme concentrations was performed using conditions described above except with different concentrations of MutY (0, 1, 2, 5, 10, 25, 50, and 100 nM). The mismatch cleavage reaction was allowed to proceed for 12 h at 37 °C, prior to the addition of the piperidine. Incubations with piperidine continued for another 15 min before they were terminated and quantitated as described above.

**Characterization of Schiff Base Intermediates**—DNAs containing a single A-G or A:8-oxoG mismatch (Table I, sequences 1–3) were used in the following assay. The strand containing the mismatched adenine was 5′-end-labeled with [γ²³⁵P]ATP. The concentration of each enzyme (MutY, cMutY, cMutY-K142A, and cMutY-K20A) was 200 nM. Substrate DNA (5 nM) and the corresponding protein were incubated in a buffer containing 25 mM HEPES (pH 7.4), 1 mM EDTA, 50 mM NaCl, and 100 μg/ml bovine serum albumin, in the presence of 50 mM

| Sequence | 5′- *TACGAAATCTCTGCTTGCGACGCATGT-3′ | 3′- AAGTCCAGAAGGGCCTGCTGAGCA-5′ |
| Sequence | 5′- *TACGAAATCTCTGCTTGCGACGCATGT-3′ | 3′- AAGTCCAGAAGGGCCTGCTGAGCA-5′ |
| Sequence | 5′- *GACTGACGCGTT-3′ | 3′- CCTACACCCGGA-5′ |

**Table I**

**Sequences of synthetic oligonucleotides used as substrate DNAs, where X represents adenine or guanine and Y represents guanine or 8-oxoguanine.**
NaBH₄, or 25 mM NaCNBH₃. The 20-μl reaction volume was incubated at 37 °C for 60 min. The reactions were terminated with an equal volume of SDS buffer (50 mM Tris-HCl (pH 6.8), 10 mM dithiothreitol, 2% SDS, 10% glycerol, 0.1% bromphenol blue) and boiled at 100 °C for 5 min. The covalently trapped protein-DNA complexes were resolved through 15% polyacrylamide gels and analyzed by PhosphorImager analyses.

Crystallization reactions were also performed in which the strand containing the mismatched adenine was 3'-end-labeled with [32P]cytidine 5'-triphosphate and terminal transferase. DNA substrates were incubated with cMutY or cMutY-K20A in the presence of 50 mM NaBH₄. The buffer components, reaction conditions, and analyses of the covalently trapped protein-DNA complexes were similar to that described above for the 5'-end-labeled DNA substrates.

Crystallization and Structure Determination of K20A, K142A cMutY, and K20A cMutY-Adenine Complex—Crystals of K20A cMutY were obtained from a solution containing 50 mM Tris-HCl (pH 8.2), 500 mM NaCl, 100 mM MgSO₄, 1.2 M Li₂SO₄, and 10% ethylene glycol. For the K20A cMutY-adenine complex, the mother liquor was saturated with adenine. K142A cMutY was crystallized using a solution containing 100 mM Tris-HCl (pH 8.0), 45% saturated (NH₄)₂SO₄, 500 mM adenine. K142A cMutY was crystallized using a solution containing 50 mM Tris-HCl (pH 8.2), 500 mM NaCl, 100 mM MgSO₄, 1.2 M Li₂SO₄, and 10% ethylene glycol. To obtain suitable crystals, initial crystals were used for microseeding. Data for K20A and K20A-adenine complex were collected on a Rigaku RU200 rotating anode generator and trapped protein-DNA complexes were identical within experimental error to those described above for the 5'-end-labeled DNA substrates.

Crystallographic diffraction data collection and refinement

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RESULTS

The Fold and Active Site of MutY and Experimental Rationale

The three new enzyme structures (K20A, K142A, and K20A with adenine) of the 26-kDa catalytic domain of MutY (cMutY) that are reported herein, closely match the x-ray crystal structures of cMutY with either adenine or imidazole bound in the base specificity pocket that were previously solved (Fig. 1A). This experimentally defined preservation of the folds and two-domain structures for these cMutY mutant enzymes suggests that biochemical investigations of these proteins should reflect the functional roles of the mutated residues rather than changes due to misfolding. These new structures and the original structural data are furthermore consistent with localizing the active site of MutY at the interdomain cleft region and suggest putative amino acid residues that contribute to the glycosylase and lyase chemistry of MutY (Fig. 1B) (12). The recent cocrystal structure of a thermophilic homolog of full-length MutY has also confirmed the general positioning of the extrahelical adenine (17). It was hypothesized that Asp138 was the acidic residue that activates a water molecule for nucleophilic attack on the C1 carbon of the deoxyribose sugar, facilitating the release of the mismatched adenine. Glu172 was hypothesized to protonate N7 of adenine during the nucleophilic displacement reaction. The presence of lysine activity in MutY and the ability of MutY to form Schiff base intermediates led to the hypothesis that an amine group from a side chain of a lysine in the vicinity of the trapped adenine could catalyze the β-elimination reaction. Further analyses of the adenine specificity pocket identified the e-amino group of Lys142 as a candidate residue for the formation of the Schiff base intermediate. Williams and David (21) demonstrated that although various lysine mutants of MutY (K16A, K132A, K142A, K157G, and K158A) could nick DNA containing an A:8-oxoG mismatch, only K142A was diminished in the formation of a covalent intermediate in the presence of NaBH₄. Additionally, we hypothesized that K20 located within the interdomain loop may modulate the reaction mechanism of MutY (Fig. 1B). The above hypotheses were tested by altering the putative catalytic residues by site-directed mutagenesis and analyzing the wild type and mutant active sites by x-ray crystallography and biochemical enzymology.

Crystal Structures of cMutY-K20A and K142A

The detailed atomic structures of cMutY K20A, K142A, and K20A with adenine are identical within experimental error to the cMutY D138N structure reported earlier (12) except for informative local changes and interactions at the mutation site noted here. The crystal structures of cMutY-K20A and cMutY-K142A were determined to 1.9 and 1.8 Å, respectively (Table II, Fig. 2) to provide accurate positions for the mutated residue and its environment. In the wild type structure of cMutY, Lys28 is located on a loop that connects the two subdomains of the [4Fe-4S] cluster and six-helix barrel domain (Fig. 1). A hook, consisting of Lys28 or both Lys28 and Lys58 connects to the [4Fe-4S] domain at the Glu172 main chain through a water molecule, drawing the loop near the two subdomains (Fig. 2A). In D138N and K142A mutant structures, Lys28 twists up 90°...
toward the surface of the protein, whereas Arg19 turns in the opposite direction into the catalytic pocket (data not shown). The K20A substitution disturbs neither the vicinity of residue 20 nor the active site but is expected to increase flexibility due to the removal of Lys20-forming hydrogen bonds and destabilization of the linker-domain interactions (Fig. 2A). The three-dimensional conservation of the catalytic domain (12) was also used to solve the crystal structure of cMutY in complex with adenine at 1.35 Å by molecular replacement, indicating that the K20A mutant retains the active site features suitable for adenine binding. In this very high resolution structure, the adenine position and interactions are extremely well defined at the atomic level, giving confidence in the detailed assessment of the active site. Adenine was found in the proposed active site of cMutY-K20A, as was seen for the structure of the active site cMutY-D138N mutant (12) (Fig. 2C). Adenine binds between hydrophobic residues Leu40 and Met185, at the domain interface of cMutY, by forming hydrogen bonds with Glu37 at the N6 and N7 positions and Gln182 at the N1 and N6 positions. Asp138 is 4.9 Å away from the adenine N9 position. In the crystal structure of cMutY, the side chains of Lys142 and Glu161 share a water molecule with the main chain oxygen of Pro155 (Fig. 2B). Glu161 also interacts with another water molecule that is also flanked by the Pro155 main chain and Lys158. Lys142 is located in the neighborhood of Lys157 and Lys158, and these three lysines contribute to the positive charge at the edge of the groove. Whereas the side chain of Glu161 is well defined in wild type and the K20A and D138N mutants, its density is poorly defined in the cMutY-K142A structure, due to a loss in the hydrogen bonding network that orients Glu161. Glu161 also behaves differently in the adenine-bound and free forms of cMutY-K20A (Fig. 2D). In the presence of adenine, the side chain of Glu161 rotates away, resulting in a loss of its interaction through a water molecule with Lys142. This may be due to an environmental effect of adenine and suggests that Lys142 and its vicinity including a possible active site residue, Glu161, is more flexible in its position and interactions than previously shown. These new observations and results are nevertheless consistent with the cMutY-D138N adenine complex and its free form where Glu161 is flipped in toward the adenine bound in the active site compared with the free form as shown here for the high resolution K20A-adenine complex (see Fig. 2D).

**Modulation of MutY Activity**

**Active Site Lysine Residues**—The data presented in Fig. 3 show the results of cleavage assays using duplex DNAs containing either an A:G (Fig. 3A) or an A:8-oxoG (Fig. 3B) mismatch treated with no enzyme (lane 1), with cMutY (lanes 2 and 3), or cMutY containing the single amino acid substitution of K142A (lanes 4 and 5) or cMutY-K20A (lanes 6 and 7). All enzymes retained both adenine glycosylase and AP lyase activities. As has been previously reported, cMutY exhibited significantly increased activity on the A:G mismatch relative to the A:8-oxoG substrate (compare lanes 2–7 between A and B). In addition to the intersubstrate differences, DNAs reacted with cMutY and the K142A mutant also exhibited a significant increase in the percentage of product that was observed after piperidine treatment. These data suggest that the glycosylase reaction was partially uncoupled from the AP lyase step and that often glycosyl bond scission was observed without phosphodiester bond scission. The products generated from reactions with the A:G substrates were a mixture of β- and δ-elimination products, whereas the A:8-oxoG substrates generated almost exclusively β-elimination products.

Using these same enzymes, kinetic analyses of the glycosylase and glycosylase/AP lyase reactions were performed to assess the magnitude of the differences observed with or without
secondary piperidine treatment (Fig. 4). In the cases of cMutY and the K142A mutant, it is evident that only approximately half of the glycosylase reactions continue through the /H9252- or /H9254-elimination reaction (Fig. 4, A and B, respectively). In contrast to what was observed for cMutY and the K142A mutant, the activity of the K20A mutant (Fig. 3, lanes 6 and 7), although being marginally diminished, was approximately equal using DNA substrates containing either A:G or A:8-oxoG mismatches. Additionally, the K20A mutant yielded a higher proportion of 8-elimination product relative to the other enzymes (Fig. 3, lane 6). Quantitation of the amount of products generated by cMutY-K20A, in the presence and absence of piperidine, revealed that the amounts of nicked products were almost equal (Fig. 3, lanes 6 and 7). This catalytic behavior of the K20A mutant was observed with substrate DNAs containing either A:G or A:8-oxoG mismatches (Fig. 3, A and B). Kinetic analyses of adenine excision and strand cleavage for the K20A mutant verified these observations (Fig. 4C). These data imply that the rates of adenine excision and phosphodiester bond cleavage were approximately equal and may both occur prior to enzyme dissociation. An overall comparison of these biochemical data in concert with the new mutant enzyme structural

FIG. 2. Detailed structural comparisons of the cMutY wild type and mutant enzymes. The wild type cMutY is shown in orange, K20A in blue, D138N in yellow, and K142A in green. A, conservation of the active site in the K20A mutant. B, contribution of Lys142 to the ordering of the Pro155-Gly156-Lys157-Lys158-Glu159 loop through a water. In the K142A mutants, Glu161, shown in a box, was disordered. C, the K20A mutant with adenine bound. Adenine is shown in pale green. D, adenine binding changes the position of the Glu161 side chain.

FIG. 3. DNA glycosylase and AP lyase activities of cMutY and selected lysine mutants. A, substrate DNA with A:G mismatch. B, substrate DNA with A:8-oxoG mismatch. DNA (0.5 nM) was incubated with 200 nM enzyme at 37 °C for 60 min. Lane 1, DNA substrate; lanes 2 and 3, cMutY-WT; lanes 4 and 5, cMutY-K142A; lanes 6 and 7, cMutY-K20A. Reactions were performed in duplicate, and one set of reactions (lanes 3, 5, and 7) was incubated with piperidine for 15 min at 90 °C. uncleaved DNA substrate (S) and the products (P) resulting from 8- and 8-elimination are shown.
results suggest that the presence of Lys^{20} in the vicinity of the active site modulates the lyase activity of Lys^{142} and that Lys^{20} can substitute for Lys^{142} in the lyase reaction.

**Active Site Acidic Residues**—Having established that at least two lysine residues can catalyze the AP lyase reaction, we investigated our previous hypothesis that the side chains of Glu^{37} and Asp^{138} participate in the glycosylase reaction. Site-directed mutagenesis was employed to independently engineer E37C, E37S, D138C, and D138N mutations in cMutY. The cysteine mutants were also constructed in the full-length MutY. To assess the mismatch cleavage efficiency of the mutants, wild type and mutant proteins were reacted with DNA containing an A:G or A:8-oxoG mismatch (Fig. 5). Relative to full-length MutY (lane 2) and cMutY (lane 3), proteins containing mutations at either Glu^{37} or Asp^{138} severely hampered the ability of the enzyme to release the mismatched adenine (lanes 4–9). One of the mutants, D138C, in cMutY retained low levels of adenine glycosylase activity on the A:G and even lower incision on the A:8-oxoG mismatch (lane 7 in A and B, respectively). In order to measure the total glycosylase activity, all samples were incubated with piperidine. The glycosylase activity was less in DNA containing an A:8-oxoG mismatch when compared with DNA containing an A:G mismatch (Fig. 5, compare A and B). These glycosylase assays demonstrated the critical role of Glu^{37} and Asp^{138} in the cleavage of the N-glycosyl bond.

**Guanine Glycosylase Activity**

Although the function of MutY has been generally associated with A:G and A:8-oxoG adenine DNA glycosylase activity, full-length MutY also possesses guanine glycosylase activity when G is paired with 8-oxoG (Fig. 6). However, the level of activity was significantly diminished compared with its adenine glycosylase activity. Kinetic assays showed that only about 12% of the substrate could be incised (Fig. 6, A and B). As the rate of guanine excision was linear up to 5 h, a different experiment was designed to allow the reaction to proceed for 12 h at different enzyme concentrations. Results from these experiments showed that MutY was able to maximally excise only 18% of the guanines from the substrate DNA (Fig. 6, C and D). Activity assays showed that E37C and D138C mutations completely inhibited the ability of MutY to release guanine from DNA containing a G:8-oxoG mismatch (Fig. 6E). In control experiments, neither MutY nor cMutY was able to release guanine from DNA containing a G:G mismatch (data not shown). In addition, deletion of the C-terminal domain of MutY abolished the ability of MutY to excise guanine from DNA containing G:8-oxoG mismatch (data not shown).

**Formation of Multiple Covalent DNA-MutY Complexes**

DNA glycosylases with lyase activity that form a Schiff base intermediate in the catalytic pathway can be irreversibly cross-linked to their substrates in the presence of a strong reducing agent (5, 7). Full-length MutY, cMutY, cMutY-K142A, and cMutY-K20A enzymes were incubated with DNA substrates containing A:G or A:8-oxoG mismatches in the presence of NaBH₄ (Fig. 7A). The results indicated that the removal of the amine group from residue 142 prevented the formation of a covalent enzyme-substrate complex (Fig. 7A, lanes 3 and 7). The K20A mutation diminished the ability of the enzyme to generate the covalent intermediates when compared with the wild type MutY and cMutY (Fig. 7A, lanes 4 and 8). However, when the same experiment was performed using a mild reducing agent, NaCNBH₃, the K142A mutant was trapped as efficiently as the wild type or the K20A mutant (Fig. 7B).

In the NaBH₄ reduction experiments described above, it was evident that more than one protein-DNA covalent complex was formed. To help determine the identity of these complexes and to shed light on the reaction mechanism, additional trapping experiments were designed with DNA substrates of different lengths: 30, 17, and 12 base pairs (Table I, sequences 1–3). The 30-bp DNA substrate contained an A:G or A:8-oxoG mismatch, whereas others contained only an A:G mismatch. All DNA substrates were labeled at the 5'-end with [γ−32P]ATP.

MutY and cMutY were able to form covalent complexes with all of the DNA substrates (Fig. 8A). However, the ability of
cMutY-K20A to form the covalent complex was limited (Fig. 8A, lanes 6 and 8). The results indicated that up to three different protein-DNA covalent complexes could be formed (Fig. 8A, lanes 2 and 5). One of the complexes formed between MutY and the 30-mer DNA (Fig. 8A, lane 2) aligned with a complex formed between MutY and 17-mer DNA (Fig. 8A, lane 3). This result was also observed with cMutY (Fig. 8A, lanes 5 and 7). The nicked DNA product derived from the 30-pp substrate DNA (Table I, sequence 1) is approximately the same length as the uncleaved 17-bp duplex DNA. This suggests that the specific protein-DNA complex arising from the 30-base pair substrate DNA was cleaved and aligns with the 17-bp duplex that is intact. These results indicated that the Schiff base intermediate could be trapped in both a precleaved and postcleaved covalent DNA complex.

In order to establish that MutY can form a covalent complex with precleaved substrate DNA and the postcleaved product DNA, additional analyses were performed with substrate DNAs containing an A:G mismatch labeled at the 3′-end of the mismatched adenine-containing strand. In this set of experiments, there should be only one protein-DNA complex that represents the precleaved substrate DNA covalently attached to MutY. Indeed, these data showed that only one reaction intermediate could be trapped with NaBH₄ (Fig. 8B). MutY and

FIG. 6. Guanine glycosylase activity of MutY. A, kinetic analyses. MutY (400 nM) was incubated with 2 nM substrate DNA containing a G:8-oxoG mismatch. The reaction mixture was incubated at 37 °C, and aliquots were withdrawn at specific time points (1, 5, 15, 30, 60, and 300 min, lanes 2–7, respectively). Piperidine was added to these aliquots, and incubation continued at 85 °C for 15 min. Lane 1 contains DNA substrate only. The reaction products were separated by electrophoresis in a 15% polyacrylamide gel containing 7 M urea and analyzed by PhosphorImager analysis. B, quantitation of the kinetics of guanine glycosylase activity of MutY. C, guanine glycosylase activity as a function of enzyme concentration. Different amounts of MutY (1, 2, 5, 10, 25, 50, and 100 nM, lanes 2–8, respectively) were incubated with DNA (2 nM) containing a G:8-oxoG mismatch. Lane 1 contains DNA substrate only. Reaction mixtures were incubated at 37 °C for 12 h, followed by incubation with piperidine at 85 °C for 15 min. The reaction products were separated and analyzed as described above. D, quantitation of MutY incision of DNA containing a G:8-oxoG mismatch. E, substrate DNA with G:8-oxoG mismatch (2 nM) was incubated with 200 nM enzyme at 37 °C for 60 min. Incubation continued for an additional 15 min at 90 °C with piperidine. Lane 1, DNA substrate; lane 2, MutY-WT; lane 3, MutY-D138C; lane 4, MutY-E37C. Reaction products (P) were separated from the uncleaved substrate (S) and analyzed as described above.
DISCUSSION

Previous investigations into the sequential activities of MutY on A:G or A:8-oxoG mismatch-containing DNAs have not completely captured the complexities of these reactions. The combination of x-ray crystallographic analyses and detailed enzymatic studies using native and mutant enzymes have now established that MutY initially functions as a prototypical DNA glycosylase, a class of enzymes that are currently understood to generally protonate the leaving base and activate a water molecule to serve in the net nucleophilic displacement reaction. The complexities and controversies regarding the MutY catalytic mechanism are associated with its ability (or lack thereof) to catalyze phosphodiester bond scission, the residues responsible for producing the covalent Schiff base intermediate, and the relationship (if any) of the Schiff base to the glycosylase step. The current investigations, coupled with many prior studies, suggest that the slow dissociation of the enzyme from the newly created abasic site gives sufficient time for one or more opportunistically positioned e-amino groups of lysine residues near the active site to form covalent intermediates (Figs. 1 and 2). In this study, we have established the key role that the two conserved acidic residues (Glu37 and Asp138) play in the glycosylase reaction and have shown that substitution of alanine at either Lys4142 or Lys20 affects the formation of covalent intermediates with the abasic site, albeit with different efficiencies.

Fig. 9 depicts our current understanding of the reaction coordinate for MutY, with A describing the glycosylase portion of the reaction and B outlining the complexities of the abasic site lyase reaction. Step 1 represents a composite of the initial interactions between MutY and DNA, in which the enzyme probes for the presence of mismatched bases, ultimately recognizing an A:G, A:C, or A:8-oxoG mismatch or possibly a G:8-oxoG mismatch. Specific binding is achieved through flipping the adenine nucleotide into an active site cleft within the catalytic domain of MutY. In the case of full-length MutY, it has been suggested that the 8-oxoG nucleotide is also flipped to an extrahelical position on the p13 domain, which has structural homology with the 8-oxo-dGTPase, MutT (25, 38, 39), although no evidence for double flipping was observed in the co-crystal complex (17). When specific binding has occurred, it is postulated that in step 2, there is a coupled protonation of N7 adenine and a nucleophilic attack by an activated water molecule at the C1' of the extrahelical deoxyribose utilizing Glu37 and Asp138 (12). The result is cleavage of the glycosyl bond, yielding an abasic site. This step constitutes the culmination of the glycosylase reaction, which is the biologically essential activity for limiting mutagenesis due to dAMP incorporation opposite 8-oxoG or G in template DNAs (16, 21).

In addition to establishing the key roles of both Glu37 and Asp138 in the glycosylase reaction for A:G mismatches, it was demonstrated that, following glycosyl bond scission, both cMutY and cMutY-K142A do not always proceed through the β-elimination reaction, suggesting enzyme dissociation prior to additional chemistry. This was evidenced in kinetic analyses in which piperidine treatment resulted in an approximate doubling of incised product, a result that is interpreted to mean that ~50% of glycosyl bond scissions were not followed by β-elimination. However, we cannot rule out that some of these encounters did not proceed to the ring-opened form of the sugar and form a carbinolamine intermediate (steps 4 and 5). It is predicted that heat treatment will reverse the carbinolamine intermediate to yield free enzyme and an abasic site. In contrast to cMutY and cMutY-K142A, the cMutY-K20A mutant proceeds with a very high efficiency to the β- and δ-elimination products, since secondary treatment with piperidine did not increase the percentage of cleaved DNA product. These data also reaffirmed that cMutY and cMutY K142A have enhanced rates of incision on A:G versus A:8-oxoG mismatches. Again, in contrast, cMutY-K20A differs in that there was not a significant difference in the kinetics of incision of the two mismatched
substrates. We conclude from these data that the removal of the lysine side chain from position 20 facilitates the accessibility of Lys142 to initiate the lyase reaction on the abasic site. Once the glycosylase step is complete, the probability of the reactions proceeding to \( \beta \)- or \( \delta \)-elimination products will depend on multiple equilibrium constants. Specifically, several reaction intermediates that arise from the lyase step are predicted to occur, which can be isolated as stable chemical species in the presence of a reducing agent (Fig. 8 B). This scheme was developed (in part) based on additional work from our laboratory in which peptide-catalyzed \( \beta \)-elimination gave rise to pre- and postincision complexes (40). For the MutY enzyme-catalyzed reactions described in the current investigation, the reaction steps are expected to proceed through the following: sugar ring closed/open equilibrium (step 4); formation of the reversible (nonreducible) carbinolamine intermediate (step 5); conversion to the Schiff base intermediate (step 6); general base abstraction of the 2'-H, leading to a covalent enzyme-DNA intermediate containing a single-stranded break (step 7); a water-mediated dissociation of the covalent intermediate yielding a 3'-ring-opened \( \alpha,\beta \)-unsaturated aldehyde, a 5'-phosphate, and free enzyme (step 8); or catalysis of a \( \delta \)-elimination reaction, yielding both 5'- and 3'-phosphates with a single nucleotide gap and enzyme (step 9). If reactions are carried out in the presence of strong reducing agents such as NaBH4, the following intermediates can be reduced: the ring-opened AP site to yield a reduced AP site (step 10) and the precission (step 11) and postincision (step 12) Schiff base intermediates, yielding stable MutY-DNA cross-links.

The data described herein demonstrate that on AP sites (in the absence of reducing agents), MutY, cMutY, cMutY-K142A, and cMutY-K20A all favor catalyzing the full \( \beta,\delta \)-elimination reaction approximately two-thirds of the time while catalyzing only \( \beta \)-elimination in the remaining one-third of the events. However, similar reactions using A:8-oxoG mismatches did not generally proceed through the \( \delta \)-elimination step but were terminated following \( \beta \)-elimination. When reactions were carried out in the presence of NaBH4, for most of the enzymes examined, the AP site was simply reduced, suggesting that the reaction could not have proceeded to the carbinolamine intermediate (step 5). In the case of cMutY-K142A, in which the domain including Lys157 and Lys158 could be more flexible, there was no formation of a covalent intermediate, suggesting that reduction of the ring-opened sugar was significantly faster than formation of the Schiff base intermediate. However, when these reactions were carried out in the presence of a milder reducing agent, NaCNBH3, in which the half-life of the nonreduced sugar is ~6 h, cMutY-K142A could be trapped as effectively as cMutY or cMutY K20A. These data reinforce that Lys20 is capable of catalyzing \( \beta \)- and \( \delta \)-elimination reactions, but with much slower kinetics than Lys142.

Using the cMutY or cMutY-K20A and either mismatched DNA substrate, there was an almost equal partitioning be-
between NaBH₄ trapping the Schiff base intermediate pre- and postincision; these data suggest that the rate of abstraction of the 2’ H is roughly equal to NaBH₄ reduction of the intermediate.

The crystal structures provide a molecular basis for the observed mutant biochemistry. Lys142 is about 12 Å from the target bond position, whereas Lys20 is about 13 Å away but not as well positioned (Fig. 1B). Whereas the crystal structures have implied the MutY two-domain architecture might be flexible due to the relatively small interdomain interface and the linkage of the domains by loop regions, there has not been direct evidence for such implied conformational changes. Here, combination of mutant crystal structures with the defined lyase reactions in the Lys20 and Lys142 mutants provides robust experimental evidence for more flexibility of the two MutY domains and their linker region, where Lys20 is positioned better than has been seen in any of the experimental structures. This newly defined flexibility is further supported by the role of Lys20 in forming hydrogen bonds expected to stabilize the linker-domain interactions (Fig. 2A).

The K20A mutant results provide a test of the idea that flexibility is important to the lyase activity. Removing the Lys20 side chain in the K20A mutant should enhance the existing flexibility of the MutY two-domain architecture. Our analyses predict this increased flexibility would make the lyase activity more efficient. This structure also suggests a possible fraction of the time plasticity that is needed for the lyase reaction to occur. These arguments imply that Lys20 helps to stabilize the positioning and architectural arrangement of the linkers and domains. However, a lysine side chain is not a rigid brace, so mutating Lys20 should simply be increasing the existing flexibility and thus the lyase activity.

Overall, these data suggest that there is a delicate balance of equilibrium constants that ultimately determine the final spectrum of products that one might observe between MutY and its substrates in vitro. Clearly, subtle changes in buffer conditions, salt concentrations, sequence contexts, and a variety of other parameters could influence the final products. Thus, it is not surprising that interlaboratory differences have been reported concerning the lyase activity of MutY. However, it is clear that there is sufficient plasticity in the active site of MutY to allow for recognition and incision of other bases, such as was demonstrated for the removal of guanine when mispaired with 8-oxoG. Additionally, this plasticity allows for at least two opportunistically positioned lysine residues to catalyze incision of the phosphodiester backbone.

Due to the slow release of DNA product by MutY following the adenine glycosylase reaction, it may be the case that β- and δ-elimination reactions follow the glycosylase step a significant fraction of the time in vivo. If this is the case, the base excision repair pathway can be completed without the action of an AP endonuclease (41). In this study, it was demonstrated that following the action of a β,δ-elimination catalyst, polynucleotide kinase could generate a 3’-hydroxyl, suitable for polymerization. However, others have shown that interactions between MutY and AP endonuclease enhance the rate of MutY product release from an A-G substrate following the glycosylase reaction (42). Thus, it is presently unclear whether MutY-catalyzed DNA incision has a significant influence on repair processes in vivo. Ultimately, it is expected that the spectrum of products resulting from MutY catalysis in vivo will depend on the considerations discussed here as well as protein-protein interactions between MutY and other proteins in the BER pathway.

Acknowledgments—Synthesis of oligonucleotides and DNA sequencing was performed by the Recombinant DNA Laboratory, Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston. We thank the staffs for use of the facilities for x-ray diffraction data collection at the Stanford Synchrotron Radiation Laboratory and the Lawrence Berkeley Lab Advanced Light Source and thank Brian R. Chapados for data collection at Advanced Light Source.
Reaction Intermediates in the Catalytic Mechanism of *Escherichia coli* MutY DNA Glycosylase

doi: 10.1074/jbc.M403944200 originally published online August 23, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403944200

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