MutY DNA Glycosylase: Base Release and Intermediate Complex Formation†

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ABSTRACT: MutY protein, a DNA glycosylase found in *Escherichia coli*, recognizes dA:dG, dA:8-oxodG, and dA:dC mismatches in duplex DNA, excising the adenine moiety. We have investigated the mechanism of action of MutY, addressing several points of disagreement raised by previous studies of this enzyme. MutY forms a covalent intermediate with its DNA substrate but does not catalyze strand cleavage. The covalent intermediate has a half-life of approximately 2.6 h, 2 orders of magnitude greater than the half-life of Schiff bases formed when *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III react with their respective substrates. The covalent complex between MutY and its DNA substrate involves Lys-142; however, the position of this residue in the presumptive active site differs from that of catalytic residues involved in Schiff base formation associated with endonuclease III and related DNA glycosylases/AP lyases. MutY converts DNA duplexes containing the dA:8-oxodG mispair to a product containing an abasic site; heat-induced cleavage of this product may account for the several reports in the literature that ascribe AP lyase activity to MutY. The MutY−DNA intermediate complex is highly stable and hinders access by Fpg to DNA, thereby avoiding a double-strand break. Cross-linking of MutY to DNA may play an important role in the regulation of base excision repair.

MutY is associated with an increase in G:C→T:A transversions in *Escherichia coli* (*1*) and is identical to *micA*, a gene required for short-patch correction of A:G and A:C mismatches in bacteriophage λ heteroduplexes (*2*). MutY protein originally was described as a DNA glycosylase that recognized dA:dG and dA:dC mispairs, excising the mispaired adenine moiety from duplex DNA (*3*, *4*). Following the report that dAMP is incorporated opposite 8-oxoguanine during DNA synthesis (*5*), MutY was shown to act on duplexes containing the dA:8-oxodG[1] mispair (*6*). Genetic studies suggest that MutY participates in the repair of 8-oxoguanine-containing lesions in cells (*6*−*8*). MutY preferentially binds and cleaves dA:8-oxodG (*9*−*11*), supporting the proposal that MutY, MutM (Fpg), and MutT are part of a damage avoidance system that protects bacteria from oxidative DNA damage (*12*, *13*). A repair activity cross-reacting with antibodies against *E. coli* MutY was found in calf thymus and HeLa cells (*14*, *15*). A structural homologue of *E. coli* MutY has been cloned from human cells; this protein has not yet been characterized (*16*).

MutY consists of a large (26 kDa) and a small (13 kDa) domain; these domains can be separated following cleavage with trypsin (*17*) or thermolysin (*18*). The 26 kDa domain displays strong sequence homology with endonuclease III of *E. coli* (*19*) and is fully proficient in its catalytic function (*17*, *18*, *20*). Removal of the 13 kDa domain affects the action of MutY on certain substrates, resulting in the loss of preference for A:8-oxoG over A:G mispairs (*18*, *20*). A role for the small domain has been suggested in substrate specificity and, perhaps, in interactions with other proteins.

The large domain of MutY contains a $[4Fe\text{-}4S]^{2+}$ iron−sulfur cluster (*19*) similar to that found in Endo III for which a structural rather than a catalytic role has been proposed (*21*, *22*). Other structural features shared by MutY and Endo III include the helix-hairpin-helix (HhH) and G/P...D loop motifs (*22*, *23*). These motifs define the Endo III superfamily of DNA-binding proteins which includes *Methanobacterium thermoformicicum* T:G mismatch glycosylase (Tdg), eukaryotic 8-oxoguanine-DNA glycosylase/lyase (Ogg1), *Micrococcus luteus* pyrimidine dimer glycosylase/lyase (Pdg), prokaryotic and eukaryotic 3-methyladenine-DNA glycosylases (*E. coli* AlkA, yeast MAG), and several structurally related proteins not involved in DNA repair (*22*, *23*).

DNA glycosylases in the Endo III superfamily can be divided in two groups, based on the amino acid corresponding to lysine 120 of Endo III. Endo III, Ogg1, and Pdg contain this residue and demonstrate strong AP-lyase activity (*24*−*26*), exercised through the formation of a Schiff base intermediate involving lysine 120 and C1′ of the deoxyribose (abasic site) moiety of the DNA substrate (*27*−*29*). Members of the Endo III superfamily lacking lysine at this position (AlkA, MAG, and Tdg) are classified as monofunctional DNA glycosylases (*28*, *30*, *31*). Based on these considerations, MutY should be a monofunctional glycosylase as it contains serine in place of Lys-120 (*28*). While several groups failed to detect AP lyase activity in MutY (*4*, *6*, *11*, *12*, *28*), others reported this activity to be present (*3*, *9*, *10*, *17*, *18*, *32*). Moreover, Schiff base formation, established

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[1] Abbreviations: 8-oxoG, 7,8-dihydro-8-oxoguanine; 8-oxodG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; AP, apurinic/apyrimidinic site; BSA, bovine serum albumin; Endo III, endonuclease III; HhH, helix-hairpin-helix; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin.

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by $NaBH_4$- or $NaBH_3CN$-induced trapping of MutY with its substrate oligonucleotide, has been reported by some observers (*10, 18*) but not by others (*11, 28*).

The present study was designed to clarify whether MutY contains intrinsic AP lyase activity and to study cross-linking of this protein to an oligonucleotide substrate. Our observations and earlier apparently conflicting reports can be reconciled by a model in which MutY forms a covalent complex with its substrate but does not catalyze β-elimination. This complex has a long half-life, differing in this respect from complexes formed by most other DNA glycosylases/lyases and suggesting a biological role for this complex in the regulation of base excision repair.

MATERIALS AND METHODS

Proteins. MutY, Fpg, and mOgg1 proteins were purified as described (*11, 33, 34*). *E. coli* Endo III was a gift from R. Cunningham (SUNY Albany). Uracil-DNA glycosylase was purchased from Life Technologies, and T4 polynucleotide kinase from New England Biolabs.

Oligonucleotides. The sequences used in this study were as follows:

5′-CTCTCCCTTCXCTCCTTTCCTCT-3′
(X = A, U, or 8-oxoguanine)

5′-AGAGGAAAGGAGYGAAGGGAGAG-3′
(Y = 8-oxoguanine, C, or thymine glycol)

Oligonucleotides were synthesized on an Applied Biosystems model 394 DNA synthesizer and purified as described (*33*). Oligonucleotides containing 8-oxoguanine were prepared by solid-phase synthesis from 8-oxodG phosphoramidite (*35*). Substrates containing a single abasic site were generated by treating duplex dU-containing oligonucleotides with excess uracil-DNA glycosylase for 30 min; for certain experiments, these reaction mixtures were used directly. Oligonucleotides containing a single thymine glycol residue were prepared by treating dT-containing oligonucleotides with OsO_4 (*36*). ^{32}P-Labeling at the 5′-terminus was performed according to the manufacturer's instructions using [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase. After labeling, oligonucleotides were desalted using NenSorb 20 nucleic acid purification cartridges (NEN Research Products).

Thermal Cleavage of Duplex Oligonucleotide Substrates. The reaction mixture, containing 50 nM DNA and 50 nM protein, was incubated at 37 °C for varying periods of time in a buffer containing 50 mM Tris-HCl, pH 7.5; 100 mM KCl; 5 mM EDTA, and 0.1 mg/mL BSA. Aliquots (10 μL) were treated as follows: if heating was not required, 5 μL of formamide loading buffer was added and the reaction tube was frozen in dry ice. In other experiments, 5 μL of formamide loading buffer was added and the reaction tube was heated at 95 °C for various times. For samples treated with alkali, an aliquot was mixed with 10 μL of 20% piperidine, heated at 95 °C for 30 min, dried under vacuum and dissolved in 15 μL of formamide loading buffer diluted 1:3 with water. Following this treatment, an aliquot (5 μL) was subjected to PAGE in 20% polyacrylamide/8 M urea. Radioactive bands were quantified using a Molecular Dynamics PhosphorImager system.

Cross-Linking of Oligonucleotide Substrates to MutY. For initial trials, a 10 μL reaction mixture containing 50 nM DNA and 300 nM protein was incubated for 30 min at 37 °C in a buffer containing 25 mM sodium phosphate, pH 6.8; 1 mM EDTA; 0.1 mg/mL BSA, and either 200 mM NaCl or 100 mM NaCl with 100 mM $NaBH_4$. SDS loading buffer (10 μL) or formamide loading buffer (5 μL) was added, and the reaction was terminated by heating for 5 min at 95 °C. A 5 μL aliquot was loaded onto 12% discontinuous SDS−PAGE or 8% polyacrylamide/8 M urea PAGE.

Determination of Half-Life for the Covalent Complex. The reaction mixture, including 50 nM duplex oligonucleotide, 25 mM sodium phosphate, pH 6.4, 0.1 mg/mL BSA, and 5 μM MutY or other enzyme in a total volume of 10 μL, was incubated for varying times (10 s to 10 h) at 37 °C; $NaBH_4$ was then added to a final concentration of 50 mM. The reaction was allowed to proceed for 2 min, then terminated by adding 10 μL of SDS loading buffer and heating for 5 min at 95 °C. Products were analyzed by 12% discontinuous SDS−PAGE. After plotting the initial velocities of cross-link formation versus the time of preincubation, the half-life of the covalent complex was calculated as $t_{1/2} = \ln 2/k$, where k was determined by fitting experimental data from the descending part of the curve to the equation $E_r = a + (1 - a)e^{-kt}$ using a Jandel SigmaPlot v3.0 nonlinear fit routine (E_r, relative cross-linking efficiency, the ratio between cross-linking at a given time point and maximal cross-linking; a, a parameter for correction used with a kinetically irreversible Schiff base; k, first-order rate coefficient; t, time).

Fpg Cleavage of AP:8-oxodG-Containing Substrate. The reaction mixture included 50 nM duplex oligonucleotide, 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM EDTA, and varying amounts (2−430 nM) of Fpg protein. The reaction mixture was preincubated for 2 min at room temperature with BSA or different amounts of MutY, Fpg was added, and the reaction was allowed to proceed for 2 min at 37 °C. Formamide loading buffer (5 μL) was added and the reaction terminated by heating for 1 min at 95 °C. Aliquots (5 μL) were analyzed by 20% polyacrylamide/8 M urea PAGE.

Preparative Cross-Linking of MutY to Substrate. The reaction mixture (total volume, 100 mL) included 50 nM duplex oligonucleotide substrate containing a dA:8-oxodG mispair; 200 nM MutY; 25 mM sodium phosphate, pH 6.4, and 50 mM $NaBH_4$. Buffer and substrate were prewarmed at 37 °C, MutY was then added, mixed rapidly by swirling the flask, then $NaBH_4$ was added from the freshly prepared 2.5 M stock solution. This order of mixing minimized protein denaturation and improved the yield. The reaction was allowed to proceed for 4 h at 37 °C, then overnight at 4 °C. The solution was filtered through a 0.22 μm Millex-GV membrane (Millipore) and loaded onto a Poros HQ 4.6/50 column (PepSeptive Biosystems) equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, and 1 mM EDTA) containing 120 mM NaCl. The column was washed with 5 mL of the same buffer and eluted with 40 mL of a 120 mM to 990 mM NaCl gradient in buffer A. UV absorption of the eluate was monitored at 254 nm. Aliquots removed from the peak fractions were precipitated with deoxycholate−trichloroacetic acid (*37*), subjected to discontinuous SDS−PAGE, and analyzed by Coomassie Blue staining. The presence of MutY in the shifted band was confirmed by Edman sequencing.

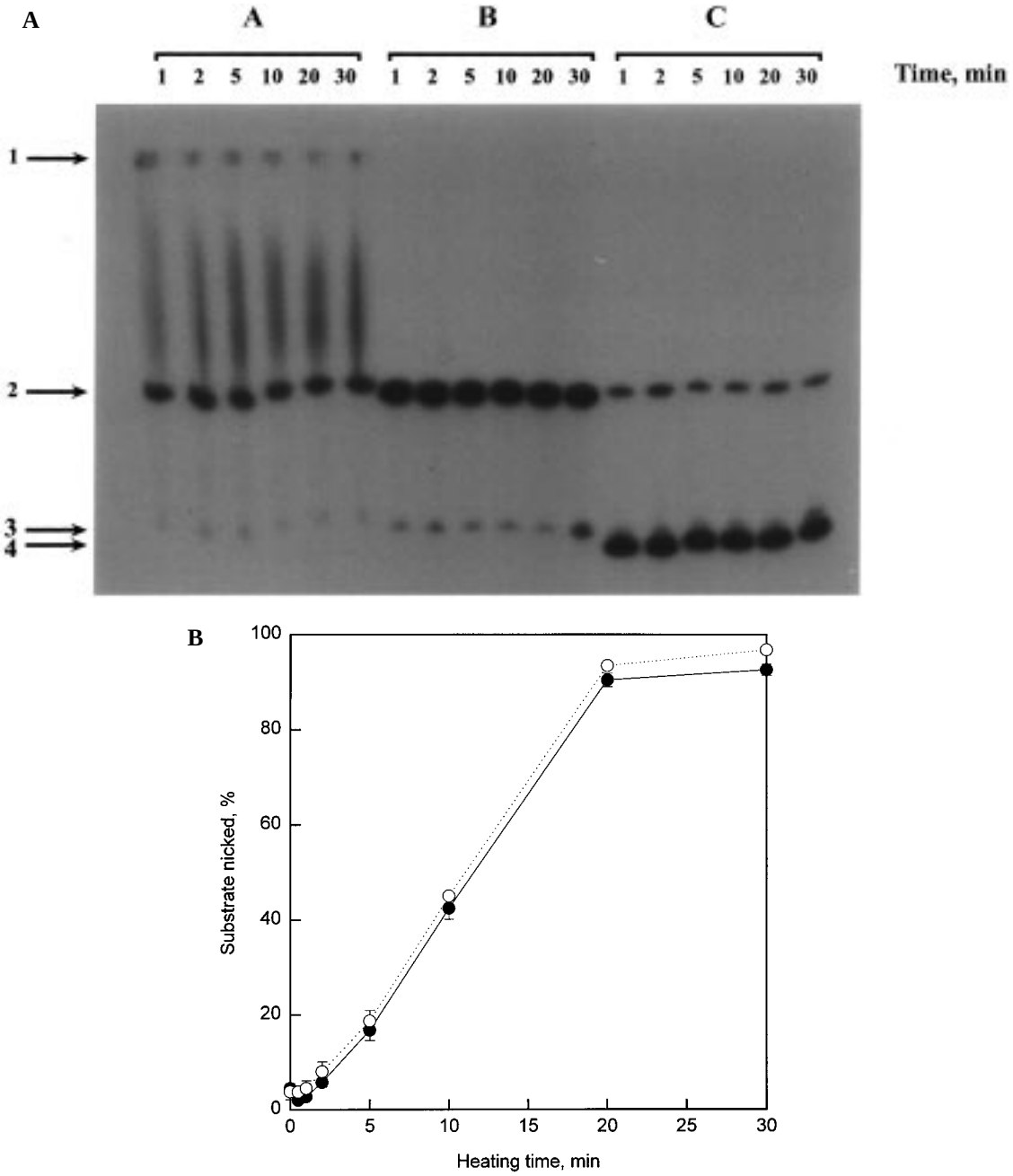

FIGURE 1: Nature of the products generated by MutY. (Panel A) Products generated from MutY-treated duplex DNA under different conditions of heating. All reaction mixtures were incubated for 0−30 min at 37 °C. (A) Formamide loading buffer was added and the reaction was terminated by freezing in a dry ice bath. (B) Cormamide loading buffer was added and the reaction mixture was heated for 5 min at 95 °C. (C) An equal volume of 20% piperidine was added and the reaction was heated for 30 min at 95 °C, dried under vacuum and dissolved in diluted formamide loading buffer. For details, see Materials and Methods. Arrows indicate position of 1, wells; 2, uncleaved oligonucleotide; 3, product of β-elimination; and 4, product of δ-elimination. (Panel B) Thermal cleavage of duplex oligonucleotides containing an abasic site. Reaction mixtures were incubated for 30 min at 37 °C (1 unit of uracil-DNA glycosylase with DNA containing dU:8-oxodG) or for 5 min at 37° (4.7 μg of MutY with DNA containing dA:8-oxodG). Formamide loading buffer was added and aliquots of each reaction were heated at 95 °C for varying periods of time. Open circles represent the dU:8-oxodG-containing duplex treated with uracil-DNA glycosylase; filled circles represent the dA:8-oxodG-containing duplex treated with MutY.

Tryptic Digestion of the Cross-Linked Complex. Fractions containing MutY/oligonucleotide cross-link obtained from four separate reactions were pooled and dialyzed overnight against water. A_{280} was measured. The solution was dried in a SpeedVac, dissolved in 10 M urea and heated for 15 min at 95 °C. Water, Tris-HCl, pH 7.5, and trypsin were added, bringing the final volume to 200 μL. The final concentrations of reactants were 2 M urea, 20 mM Tris-HCl, pH 7.5, and trypsin:MutY 1:5 w/w, assuming all A_{280} is due to the presence of MutY protein. The mixture was incubated at 37 °C for 24 h with the same amount of trypsin added at 6 and 12 h, diluted to 2 mL, loaded onto a Centricon-3 concentration device (Amicon), and concentrated at 4 °C to approximately 250 μL. The dilution and concentration procedure was repeated twice more and the desalted solution dried in a SpeedVac. The pellet was dissolved in 10 μL of 10 M urea, heated for 10 min at 95 °C and loaded onto a 20% polyacrylamide/8 M urea gel. Bands containing DNA were localized by UV shadowing, excised from the gel, extracted twice for 2 h and once

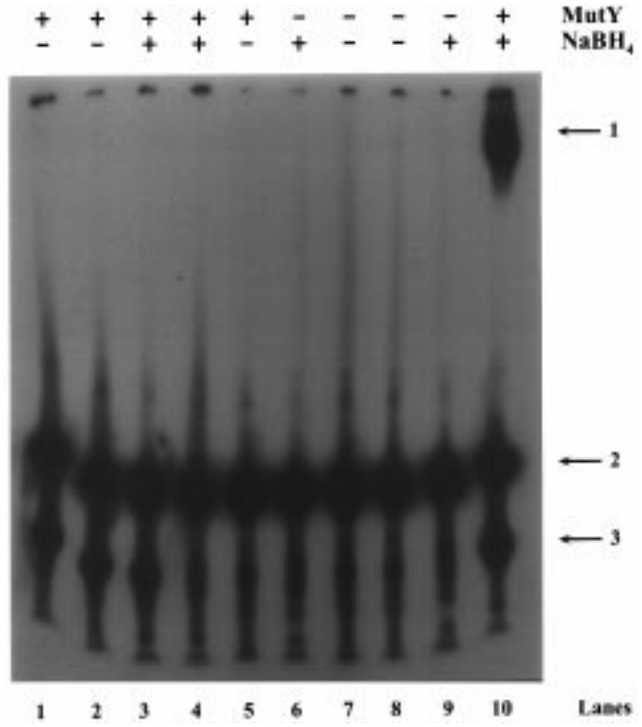

FIGURE 2: Sodium borohydride mediated cross-linking of MutY to DNA. Reaction mixtures were incubated for 30 min at 37 °C under conditions indicated in the figure and contained either 300 nM MutY (lanes 1−5, 10) or 10 μg of BSA (lanes 6−9) and either 100 mM $NaBH_4$ (lanes 3, 4, 6, 9, and 10) or 100 mM NaCl (lanes 1, 2, 5, 7, and 8). Several reactions (lanes 4, 5, and 7) were heated for 30 min at 95 °C prior to addition of substrate and $NaBH_4$ or NaCl; others (lanes 2, 3, and 6) were preincubated for 30 min at 37 °C in the presence of $NaBH_4$ or NaCl prior to addition of substrate. The reaction products were separated on 8% polyacrylamide/8 M urea PAGE. The position of bands corresponding to the reduced covalent complex (arrow 1); intact substrate (arrow 2) and cleaved substrate (arrow 3) are indicated.

overnight with 10 mM Tris HCl, pH 7.5, and 0.1 mM EDTA buffer, then filtered through a 0.22 μm membrane (Ultrafree-CL filtering device, Millipore).

Edman Sequencing. Peptides derived from eight reaction mixtures were combined; the volume was reduced to approximately 200 μL, the solution was applied to a PVDF sequencing membrane using ProSorb cartridges (Perkin-Elmer) and the membrane was washed with 800 μL of water. Sequence analysis was performed using an Applied Biosystems 475A Protein Sequencer and standard Edman degradation chemistry.

RESULTS

Heat Requirement for Substrate Cleavage by MutY. The effect of heating under various experimental conditions is shown in Figure 1, panel A. Substrate was not cleaved in unheated samples (lanes A). Heating without piperidine (lanes B) generated a product with the mobility expected for the product of β-elimination (arrow 3); a very small amount of material migrated as a product of δ-elimination (arrow 4). After heating with piperidine (lanes C), the primary cleavage product reflected δ-elimination; some β-elimination product remained. Similar results were obtained when 25 mM sodium phosphate buffer was used in place of Tris-HCl.

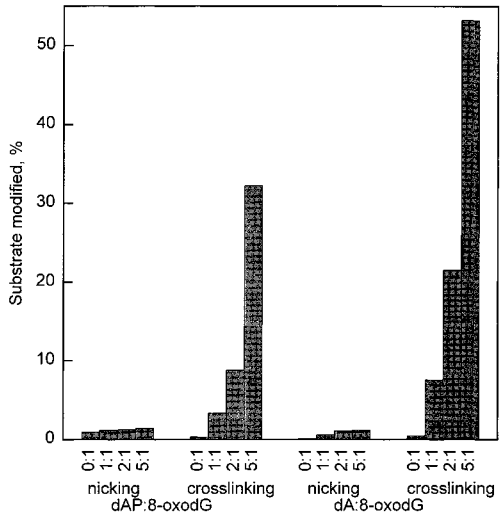

FIGURE 3: MutY-dependent nicking and cross-linking of DNA substrates containing an abasic site or dA opposite 8-oxodG. Reaction mixtures (10 μL) were incubated for 5 min at 37 °C in a buffer containing 25 mM sodium phosphate, pH 6.4, 1 mM EDTA, 0.1 mg/mL BSA, and 50 mM $NaBH_4$ or NaCl. The substrate:MutY ratio was varied as indicated on the abscissa. Nicking was measured after addition of formamide loading buffer and heating for 1 min at 95 °C. Products were resolved by 20% polyacrylamide/8 M urea PAGE. Cross-linking was measured after addition of SDS loading buffer and heating for 5 min at 95 °C; products were resolved by 12% discontinuous PAGE.

When the product resulting from the action of MutY on a dA:8-oxodG-containing duplex was heated for varying periods of time, the time course of cleavage was identical to that of the product obtained following similar treatment of a dU:8-oxodG-containing duplex with uracil-DNA glycosylase (Figure 1, panel B). In both cases, the thermal reaction generated a product with a mobility corresponding to that of the β-elimination marker (data not shown). These data suggest that the action of MutY on duplex DNA results in formation of an abasic site and that nicking does not occur at the site of damage.

Cross-Linking of an Oligonucleotide Substrate to MutY. Cross-linking between MutY and a dA:8-oxodG-containing duplex was examined under varying experimental conditions (Figure 2). When the oligonucleotide was treated with a 6-fold excess of enzyme in the presence of 100 mM $NaBH_4$, approximately 15% of the substrate was trapped as a covalent intermediate (lane 10). Controls were performed to ensure that physical trapping was not caused by heat denaturation of the protein under reducing conditions. After heating or preincubation (30 min) with $NaBH_4$, neither BSA nor MutY formed a cross-link with DNA (lanes 3−7). Cross-link formation was enzyme-dependent; however, the efficiency of this reaction is not as great as with *E. coli* Fpg protein, which cross-links more than 90% of substrate under optimal conditions (*33*). To optimize the reaction, pH was varied from 6.4 to 8.0, MutY:substrate molar ratio from 1:2 to 10:1, temperature from 0 to 37 °C, concentration of $NaBH_4$ from 50 to 100 mM, and concentration of NaCl from 0 to 200 mM. Using an enzyme:substrate molar ratio of 4:1 and 50 mM $NaBH_4$ with no additional salts, up to 60% of the oligonucleotide can be cross-linked to MutY at pH 6.4 (data not shown).

Cross-linking and cleavage reactions were compared under similar conditions (Figure 3). The extent of cross-linking

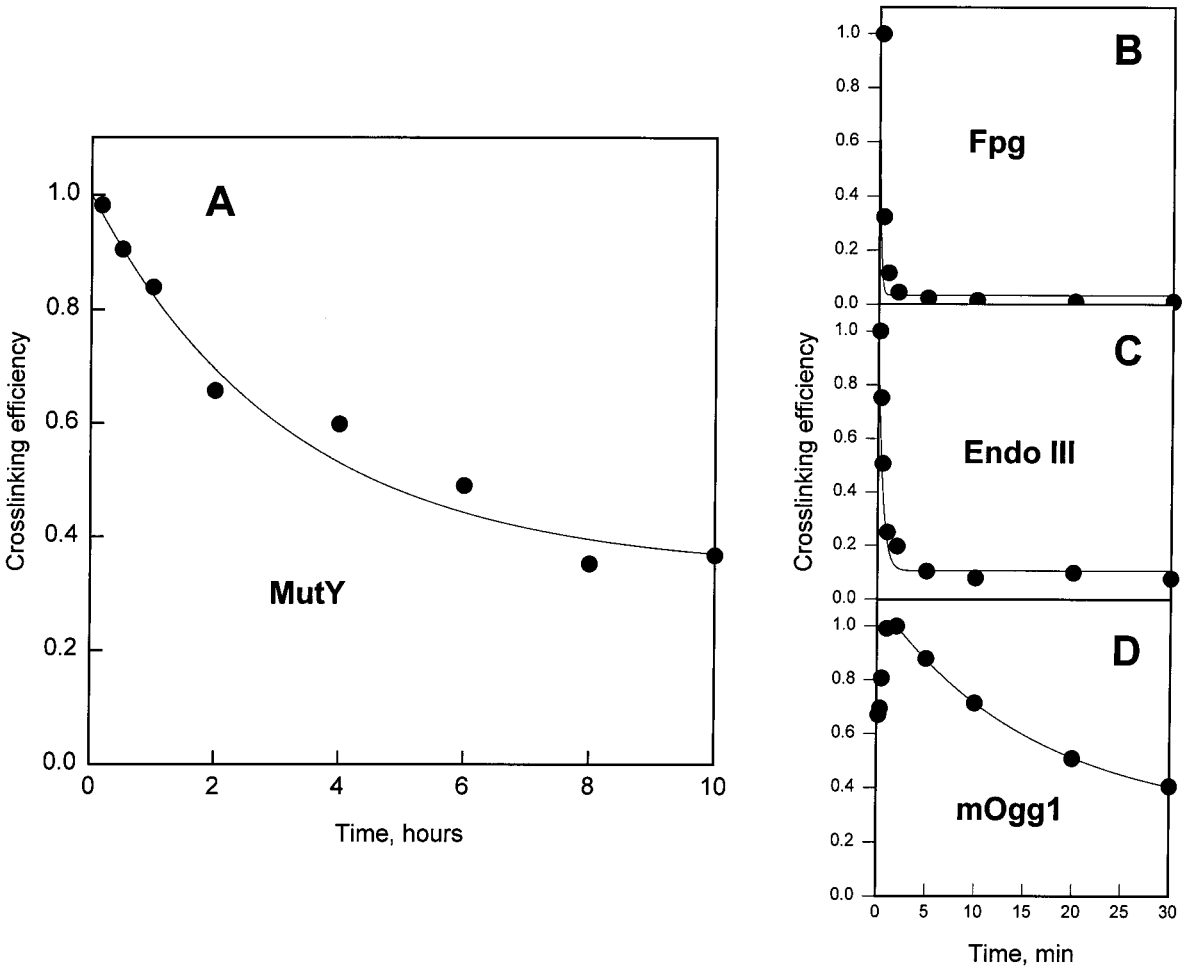

FIGURE 4: Time course for decay of covalent reaction intermediates. Reaction mixtures were incubated at 37 °C for the time indicated, then treated for 2 min with 50 mM $NaBH_4$. After heating for 5 min at 95°, products were analyzed by 12% discontinuous SDS−PAGE. Cross-linking efficiency at the time of maximal cross-linking was assigned a value of 1, cross-linking efficiencies at other time points are plotted as a fraction of this value. Curves were established by fitting experimental data to the equation $E_r = a + (1 - a)e^{-kt}$, as described in Materials and Methods. (Panel A) MutY and duplex oligodeoxynucleotide containing dA:8-oxodG. (Panel B) Fpg and duplex oligodeoxynucleotide containing 8-oxodG:dC. (Panel C) Endo III and duplex oligodeoxynucleotide containing thymine glycol:dA. (Panel D) mOgg1 and duplex oligodeoxynucleotide containing 8-oxodG:dC.

was dependent on the concentration of MutY and is significantly higher than strand cleavage. Residual nicking also was concentration-dependent; however, this reaction is inefficient compared to cross-linking, suggesting that the primary reaction product formed between MutY and its substrate DNA is a covalent complex.

Site of MutY Cross-Linking to DNA. To determine the amino acid residue involved in cross-link formation, the covalent complex was purified by anion-exchange chromatography and digested with trypsin. The complex was more resistant to tryptic cleavage than the comparable covalent complex produced using Fpg. For example, digestion with 4% (w/w) trypsin for 1 h converted all cross-linked material in an Fpg−oligonucleotide complex into one major and one minor peptide (*33*). In contrast, reacting cross-linked MutY/oligonucleotide for 24 h under similar conditions with 20% (w/w) trypsin generated three oligonucleotide-tagged fragments in approximately similar amounts. Peptides cross-linked to oligonucleotides were gel-purified and subjected to Edman degradation. The peptide fragment with the highest mobility on PAGE was identified as the tryptic digest product, His-133−Arg-143. No PTH derivative for lysine 142 or any other amino acid derivative was observed at this position. N-Termini of bands with lower gel mobility did not correspond to those expected from a tryptic cleavage site. The intermediate and lowest gel mobility species observed in this experiment were identified as Ser-129−Arg-143 and Ser-127−Arg-143, respectively. No PTH derivative was observed for Lys-142. In both cases, PTH-lysine was missing at position 132 and the derivatives observed were attributed to amino acids other than lysine. All three peptides yielded robust sequences through Arg-143, after which no PTH derivatives were detected.

Stability of MutY−Substrate Complex. Processing of substrate by MutY does not fit a logarithmic time course model (see Figure 1, panel A). To estimate the turnover rate of this enzyme, we determined the rate of decay of the covalent complex. $NaBH_4$ was added to a reaction mixture containing MutY in 100-fold excess over substrate and the initial rate of cross-linking was measured. The covalent complex decayed exponentially (Figure 4, panel A) with a half-life of approximately 2.6 h. After reacting with their preferred substrates, the Fpg complex decayed with a half-life of 30 s, the *E.coli* Endo III complex, 33 s, and the mouse Ogg1 complex, 22 min (Figure 4, panels B−D). Unlike covalent complexes formed between Fpg or Endo III and

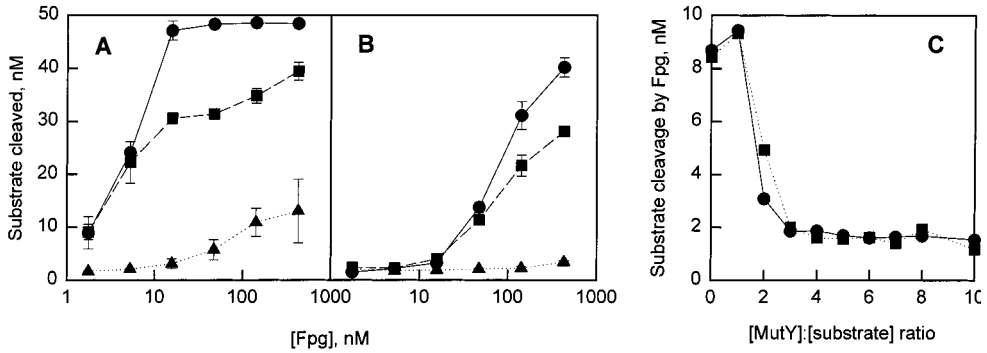

FIGURE 5: Blockage by MutY of cleavage by Fpg of a DNA containing an abasic site opposite 8-oxodG. (Panels A and B) A duplex oligodeoxynucleotide containing dU:8-oxodG, labeled on both strands, was treated with uracil-DNA glycosylase to produce a duplex substrate containing an abasic site positioned opposite 8-oxodG. Reaction mixtures containing this substrate were preincubated for 2 min with 0, 50, or 500 nM MutY; Fpg (2−430 nM) was added and the reaction mixture was incubated for 2 min at 37 °C. After heating for 1 min at 95 °C, products were analyzed by 20% polyacrylamide/8 M urea PAGE: (circles) without MutY; (squares) 50 nM MutY; (triangles) 500 nM MutY. Panel A represents cleavage at the abasic site, Panel B cleavage at 8-oxodG. (Panel C) Reaction mixtures (50 nM substrate) were incubated with varying amounts of MutY, Fpg was added (final concentration, 2 nM, to detect cleavage at the abasic site, and 50 nM to detect cleavage at the 8-oxodG site): (circles) cleavage at 8-oxodG; (squares) cleavage at the abasic site.

DNA, the concentration curve of the MutY complex did not fall to zero over time, indicating that approximately 30% of the protein is kinetically irreversibly bound to DNA.

Interference of MutY with Fpg Cleavage. We explored whether MutY could affect the action of DNA repair enzymes that act on AP:8-oxodG mispairs. For example, Fpg excises 8-oxodG and cleaves abasic sites in DNA. Cleavage of the 8-oxodG-containing strand of an 8-oxodG:AP-containing duplex coupled with AP lyase activity could lead to a double-strand break. A duplex containing an AP:8-oxodG pair was labeled with ^{32}P on both strands and treated with Fpg in the presence of varying amounts of MutY. Oligonucleotides used in this experiment differ by 10% in molecular mass; the site of modification is 2 bases from the central position. Thus, intact strands and products of β- and δ-elimination can be resolved by 20% PAGE in a single experiment. The experiment is shown in Figure 5; panels A and B reveal that MutY blocks cleavage by Fpg on either strand. The lack of Fpg activity on 8-oxodG-containing DNA cannot be attributed to formation of a single-strand break at an abasic site due to the combined action of MutY and heat since β-elimination at an abasic site was insignificant over the full range of MutY and Fpg concentrations tested (data not shown). A 2-fold excess of MutY over substrate was sufficient to block cleavage by Fpg (panel C). Gel mobility shift analysis indicates that MutY does not bind Fpg in the presence or absence of oligonucleotides (data not shown). We conclude that MutY interferes with binding of Fpg to DNA, hindering access of this protein to both the nascent abasic site and 8-oxodG residues.

Comparison of MutY Preparations from Different Sources. Striking differences in the reported enzymatic properties of MutY protein led us to compare side by side MutY proteins purified in three different laboratories (*10*, *11*, *17*) for their abilities to nick a DNA duplex at the site of a dA:8-oxodG mispair and to form covalent cross-links with this substrate. Results of this experiment are presented in Figure 6. All three samples behaved identically, showing cross-linking but no DNA nicking activity. Thus, previously reported differences in the properties of MutY cannot be attributed to intrinsic differences in the purified enzyme preparations employed.

DISCUSSION

DNA Nicking by MutY Requires Heating. Heating is commonly employed to stop enzymatic reactions and to denature DNA prior to loading samples on polyacrylamide gels. During thermal treatment at neutral pH, abasic sites undergo syn β-elimination, leaving a trans α,β-unsaturated aldose fragment at the 3′-terminus (*38*). Endo III and bacteriophage T4 endonuclease V have AP lyase activity; they employ similar stereochemistry and generate the same reaction products as thermal decomposition (*39*, *40*). We investigated the possibility that different heating protocols might have contributed to apparent discrepancies in reports of AP lyase activity in MutY.

Under carefully controlled conditions and in the absence of heating, little cleavage is observed. The polyacrylamide gels used in our experiments were prerun for 3−4 h, then operated at relatively low voltage (30−40 V/cm) to prevent generation of heat. We regularly observe "tailing" of an intact band and the failure of some material to enter a 20% polyacrylamide gel (Figure 1, panel A; Figure 6, panel A); these phenomena are associated with a labile high-molecular-weight complex. Following thermal treatment, "tailing" disappeared, and cleaved products accumulate. Longer gel runs revealed doublet bands (data not shown) characteristic of imine addition to an α,β-unsaturated aldehyde in Tris-containing buffers (*40*). At neutral pH, the mobility of both components of the doublet were lower than that of a Fpg- or hot piperidine-treated abasic site, indicating that δ-elimination did not occur. The mobility of a MutY-treated, heated double-stranded oligonucleotide containing a dA:8-oxodG pair under denaturing conditions was the same as that of an uracil-DNA glycosylase-treated and heated single-stranded oligonucleotide containing a dU residue or of a double-stranded oligonucleotide containing a dU:8-oxodG pair treated in a similar fashion. The time course for thermal strand cleavage of these oligonucleotides was essentially identical. We therefore conclude that the reaction of MutY with a duplex oligodeoxynucleotide substrate releases the base, leaving an abasic site and an intact phosphodiester backbone.

MutY is Cross-Linked to DNA by Sodium Borohydride. All DNA glycosylases/AP lyases investigated to date can

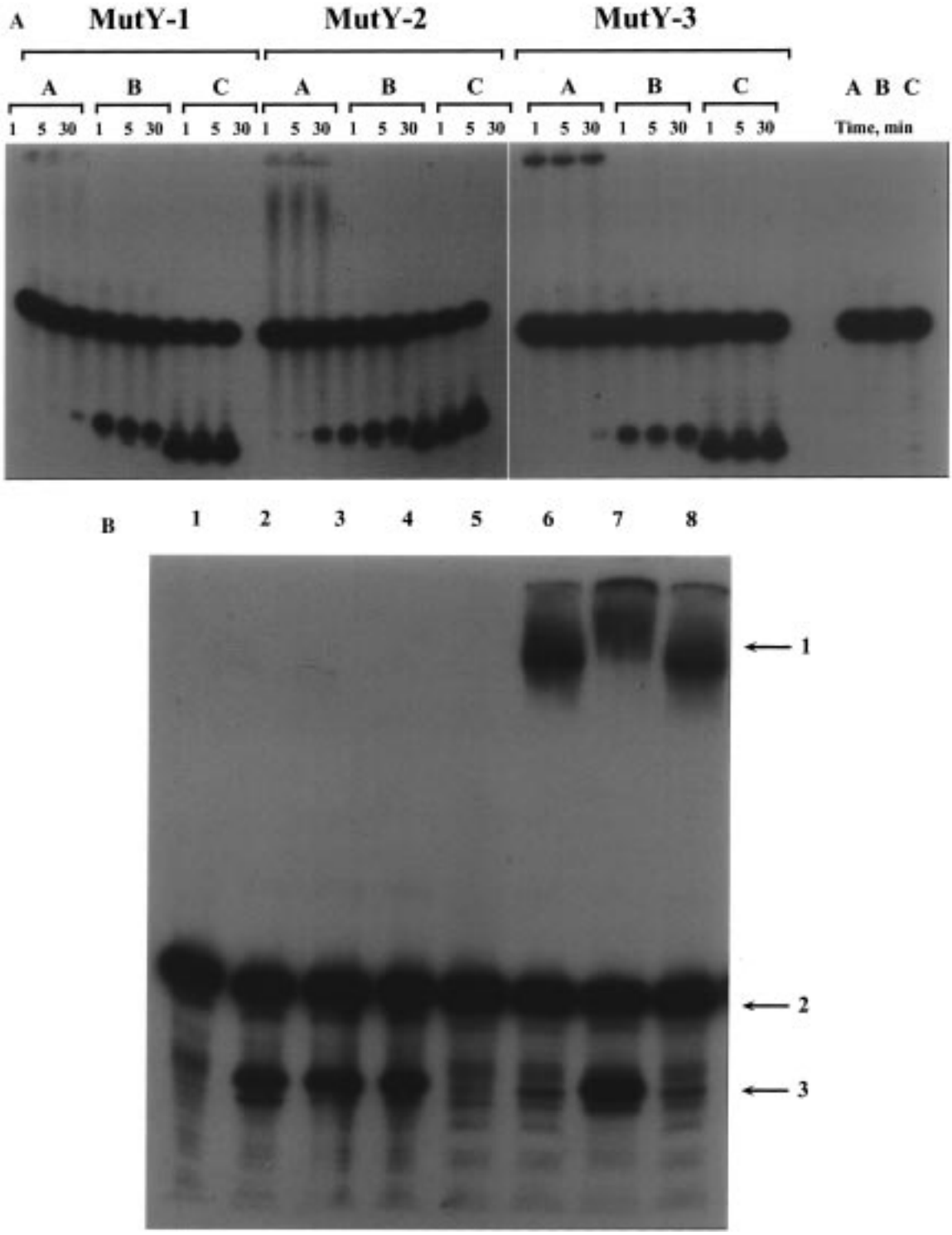

FIGURE 6: Properties of three different preparations of MutY protein. (Panel A) Temperature dependence of strand cleavage. Reaction mixtures were incubated for 1, 5, or 30 min at 37 °C. (A) Formamide loading buffer was added and the reaction stopped by freezing in a dry ice bath. (B) Formamide loading buffer was added and the reaction mixture heated for 5 min at 95 °C. (C) An equal volume of 20% piperidine was added, the reaction mixture was heated for 30 min at 95 °C, dried under vacuum and dissolved in formamide loading buffer as described in Materials and Methods. (Panel B) Cross-linking to a dA:8-oxodG-containing duplex. Reaction mixtures containing 300 nM MutY (lanes 2−4 and 6−8) or 10 μg BSA (lanes 1 and 5), and either 100 mM NaCl (lanes 1−4) or $NaBH_4$ (lanes 5−8) were incubated for 30 min at 37 °C. Products were resolved on 8% polyacrylamide/8 M urea PAGE. Arrows indicate migration of reduced covalent complex intact substrate and cleaved substrate (see Figure 2).

be cross-linked to their substrates by treating with $NaBH_4$ or $NaBH_3CN$ (*28*), reflecting formation of a covalent imino (Schiff base) intermediate (*27*). In the case of MutY, cross-linking was noted in some reports (*10*, *20*) but not in others (*11*, *28*).

On the basis of its primary sequence, MutY is grouped with the Endo III family of DNA glycosylases/AP lyases, characterized by the presence of helix-hairpin-helix (HhH) and G/P...D loop motifs (*22*, *23*). Members of this family have a lysine residue at the origin of the second helix in the

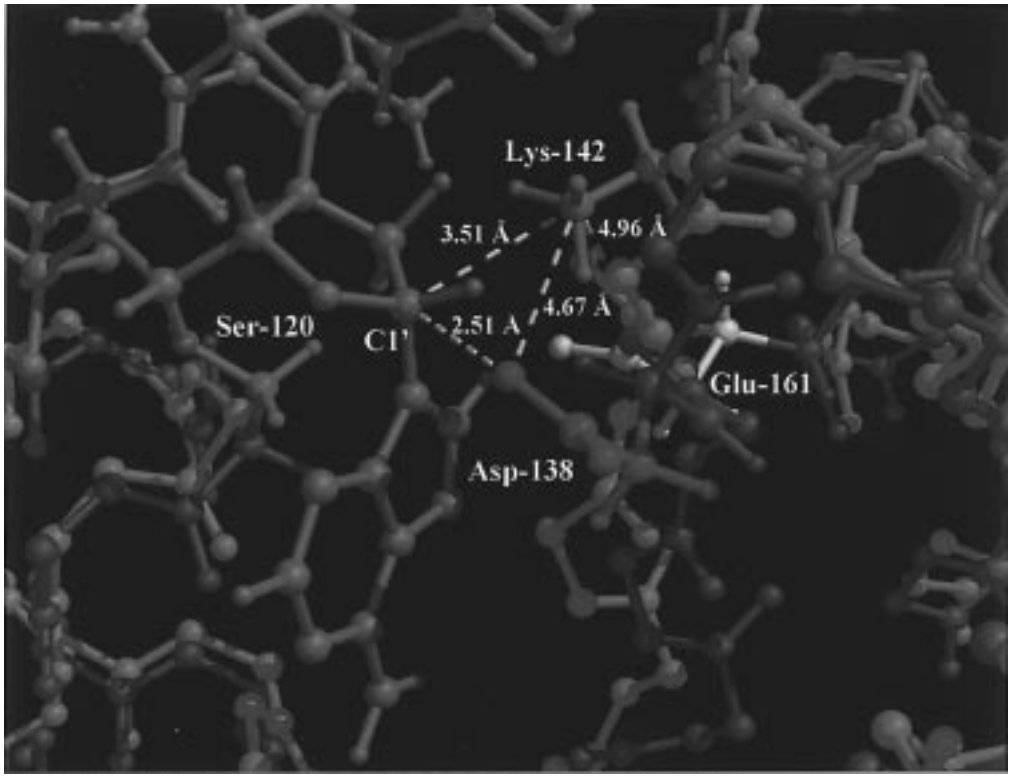

FIGURE 7: Molecular model of a deoxyadenosine residue in an active center of MutY. The peptide backbones of MutY and Endo III have been superimposed; the position of Ser-120, Asp-138, and Lys-142 of MutY are shown. Using the atomic coordinates for Endo III (PDB access code 2ABK), amino acids at positions 84−153 of Endo III were exchanged for corresponding residues in MutY. A 500 step energy minimization was conducted using X-PLOR v3.851 (*47*). Then a 3 base pair sequence of the DNA substrate containing an everted adenosine residue was positioned near the pocket, and a 1000 step minimization was performed. MidasPlus (UCSF Computer Graphics Laboratory) was used to visualize the structure. Magenta color indicates Endo III peptide backbone, blue indicates MutY peptide backbone. Ser-120 and Lys-142 are shown in cyan, Asp-138 in green, and Glu-161 in yellow.

HhH motif (Lys-120 for Endo III, Lys-241 for yeast Ogg1, and Lys-135 for Pdg); these residues are postulated to form a covalent imino intermediate with C1′ of a deoxyribose moiety in DNA (*23*, *27*, *29*). MutY contains serine in place of lysine at this position and therefore is expected to act as a monofunctional glycosylase.

Contrary to predictions for the monofunctional glycosylase model (*28*), cross-linking of MutY to DNA containing a dA:8-oxodG mispair was easily accomplished, albeit less efficiently than with other DNA glycosylases/AP lyases. Initially, conditions used for cross-linking were similar to those used in our studies of Fpg protein (*33*); modifications were required to achieve maximal cross-linking efficiency. The efficiency of the cross-linking reaction increases with decreasing pH, despite the fact that $NaBH_4$ is less stable in acidic solutions, while the efficiency of the MutY-catalyzed reaction increased with increasing pH (data not shown). Moreover, MutY can be cross-linked to a substrate containing a natural abasic site positioned opposite 8-oxodG with only about 2-fold less efficiency than to a substrate containing a dA:8-oxodG mispair. Cross-linking to the abasic site did not cause strand cleavage, as indicated by the amount of $NaBH_4$-trapped and nicked products observed (Figure 3). We conclude from these studies that formation of a covalent complex between MutY and the substrate is not sufficient to promote β-elimination.

Lysine 142 Is Involved in MutY Cross-Linking to DNA. The three-dimensional structure of Endo III (*22*) shows 66% sequence similarity and 23% identity to MutY (*19*). Several nucleophilic residues are in the vicinity of the putative active site of MutY (Arg-119, Asn-140, Lys-142, and Arg-143) and therefore capable of reacting at C1′ of the modified base. We have determined the sequence of three peptides obtained from trypsin cleavage of the MutY/DNA cross-link: His-133−Arg-143 and two putative products of degradation of Ser-120−Arg-143. The latter are observed during prolonged incubation at high trypsin:MutY ratio and appear to be formed by residual unspecific protease activities in the trypsin preparation. The PTH derivative of Lys-142 was missing in all three peptides, strongly suggesting that this residue is involved in Schiff base formation. In the longer peptides, the PTH derivative of Lys-132 also was missing. However, while a PTH derivative was not observed for Lys-142, proline, alanine, glutamine, or glutamic acid were possible PTH derivatives at position 132.

Homology modeling studies of the large domain of MutY, based on its strong sequence similarity to Endo III, suggest nearly perfect overlap of peptide backbones and significant overlap of side chains in the three-dimensional structure (*17*). Using coordinates for Endo III, amino acid residues in MutY were substituted for the corresponding amino acids of Endo III. This model enzyme, together with a 3 base pair DNA fragment in which the adenine target base is flipped out of the helix (*11*, *41*), was subjected to energy minimization. Lys-142 was found in the vicinity of the putative active site of MutY while Lys-132 was distant from this region (Figure 7). Thus, modeling supports the proposal that a Schiff base is formed with Lys-142. Both Lys-142 of MutY and Lys-

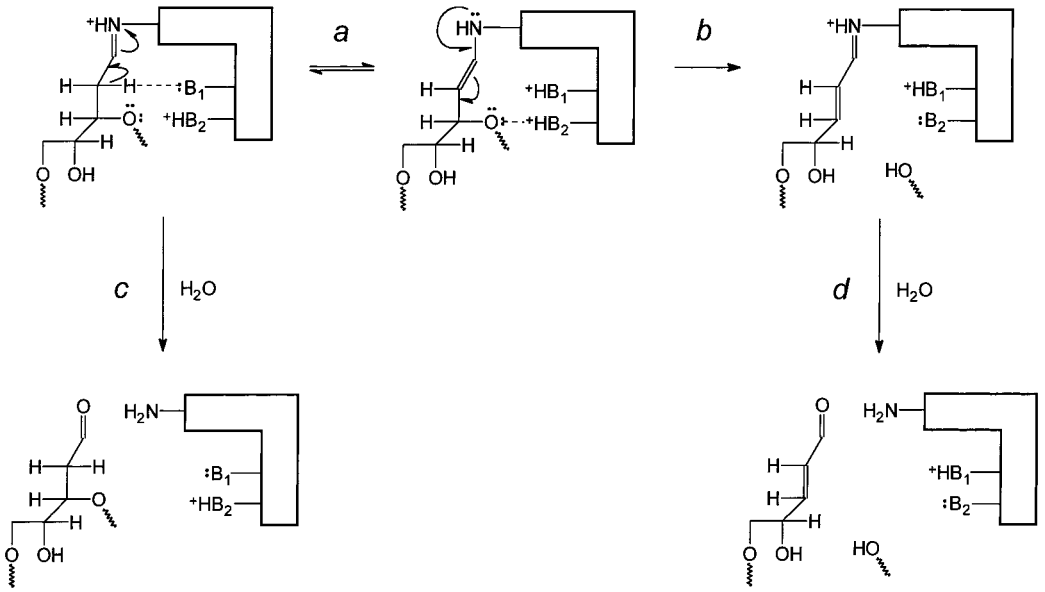

FIGURE 8: General scheme for β-elimination reaction after Schiff base formation. When a Schiff base forms between an amino group of the enzyme and C1′ of the deoxyribose moiety, a deprotonated basic group of the enzyme (B_1) induces abstraction of a 2′-*pro-S* proton (reaction *a*). To break the phosphodiester backbone, a protonated group (B_2) must transfer a proton to an oxygen at C3′ (reaction *b*). If B_1 or B_2 is lacking, the Schiff base may be hydrolyzed (reaction *c*), leaving an open-ring abasic site. If B_1 and B_2 are present and β-elimination occurs, the Schiff base is hydrolyzed, leaving a 3′-terminal α,β-unsaturated aldehyde (reaction *d*). B_1 and B_2 may be the same moiety, otherwise a recycling step must be added to restore their respective protonation states.

120 of Endo III are positioned to engage in an S_N2 displacement at C1′. These residues must be deprotonated in order to perform nucleophilic attack. On the basis of the model, we propose that either Asp-138 or Glu-162, which are positioned 4.25 and 4.67 Å, respectively, from the ϵN of Lys-142 (Figure 7) in the model enzyme, may activate the lysine residue. The apparent absence of Lys-132 in certain sequences is probably an artifact of the chemical degradation reaction.

Several steps are involved in β-elimination reactions, including Schiff base formation, abstraction of the C2′ proton (Figure 8, reaction a) and protonation of oxygen at C3′ (Figure 8, reaction b). These reactions compete with hydrolysis of the Schiff base (Figure 8, reaction c), which leaves an aldehydic abasic site in DNA and an intact phosphodiester backbone (*27*). MutY may lack a critical amino acid residue (B_1 or B_2) required for one of these steps and, therefore, cannot catalyze efficient β-elimination even if a Schiff base is formed. Identification of all intermediates and complexes formed in the reactions of MutY with DNA will be required to unequivocally establish the position of blockage and the nature of the amino acids involved.

Our experiments do not distinguish between two possible mechanisms of base excision: nucleophilic attack at C1′ by Lys-142 and attack at this position by an activated water molecule. It has been proposed that Asp-138, conserved among members of Endo III family, could activate a water molecule (*22*, *23*, *42*); we suggest that Asp-138 might instead activate Lys-142. However, even if Schiff base formation through Lys-142 is not mechanistically involved in base excision, formation of the complex may play an important biological role, as discussed below.

The MutY-DNA Complex Is Long-Lived. $NaBH_4$-induced trapping of a covalent complex between a DNA-binding protein and DNA containing an abasic site has been observed for DNA glycosylases/AP lyases that attack C1′ of deoxyribose and for proteins such as HIV-1 integrase that presumably react with DNA by other mechanisms (*43*). In the integrase reaction, unspecific Schiff base formation was attributed to the close proximity of the aldehyde function of the open ring form of deoxyribose and an amino group of the protein. The covalent complex formed between MutY and its duplex DNA substrate proved extremely stable, exhibiting a half-life of 2.6 h. By comparison, covalent intermediates formed by glycosylases/AP lyases, such as Fpg and Endo III, demonstrated a half-life of about 30 s while mouse 8-oxoguanine-DNA glycosylase had a half-life of about 20 min. Another DNA repair protein, a T/G-specific glycosylase from *M. thermoformicicum*, was shown by gel mobility shift assay to have a residency time in excess of 4 h (R. Cunningham, personal communication).

$NaBH_4$-induced cross-linking between a DNA repair enzyme and its substrate is regarded as an indicator of Schiff base formation (*27*, *28*, *44*, *45*). In general, demonstration of cross-linking is not sufficient to establish a β-elimination reaction, since, as noted above, a non-AP lyase can be cross-linked to a preformed abasic site by means of a fortuitously placed amino group (*43*). Special caution must be used when suggesting Schiff base formation for enzymes that remain in contact with their substrate for extended periods of time. An additional indication that a reaction proceeds via Schiff base formation is inhibition by cyanide (*27*, *45*); this experiment could not be conducted with MutY since the enzyme is irreversibly inactivated by cyanide (D.O.Z., and A.P.G., unpublished material). Thus, while the long residency time of MutY on DNA favors the hypothesis that a covalent bond is formed between the enzyme and substrate, we cannot exclude the possibility that the complex binds tightly through noncovalent interactions and that $NaBH_4$ promotes conformational changes that bring the amino function of Lys-142 close to the aldehyde group of deoxyribose where it can facilitate Schiff base formation.

Potential Role of Tight Complex Formation. Following MutY-catalyzed cleavage of the glycosidic bond in DNA, an abasic site is formed opposite 8-oxodG or dG. DNA containing 8-oxodG is a primary substrate for Fpg protein, an enzyme that also acts on abasic sites. If Fpg cleaves the 8-oxodG-containing strand, a nick or single-base gap would appear opposite the abasic site, potentially leading to a double-strand break or miscoding during DNA repair. When presented with this substrate, Fpg cleaves preferentially at the abasic site; however, significant cleavage also occurs at 8-oxodG (Figure 5). By remaining bound to the intermediate, MutY prevents cleavage by Fpg and, possibly, interactions with other DNA repair enzymes that cleave at sites of oxidative damage. MutY also may interact with other components of the repair machinery (AP endonuclease, DNA polymerase, DNA ligase), attracting these proteins to the site of damage, as proposed in a study of human AP endonuclease (*46*).

Comparison with Results of Others. To ensure that our results were not unique to the enzyme preparation used in our experiments (MutY-1), we compared the ability of MutY prepared in our and two other laboratories to nick DNA and to form cross-links with substrate following treatment with $NaBH_4$. The purification scheme for MutY-2 (*17*) was different from that used in the present paper. The purification procedure for MutY-3 (*3*) was similar but not identical to our own. We found the three enzyme preparations indistinguishable with respect to the thermal dependence of the cleavage reaction, although MutY-2 was somewhat more active in DNA nicking after prolonged incubation. The plateau phase of the base excision reaction was reached within 1 min, as judged by treatment with piperidine (Figure 6). The only difference in the ability of the three preparations of MutY to cross-link substrate was a slightly lower cross-linking activity for MutY-2 and slightly greater tendency to form abasic sites or nick DNA in the presence of $NaBH_4$. These differences may be attributed to the lower concentration of protein in the MutY-2 sample, which introduced significant amounts of storage buffer components in the reaction mixture. The presence of these components may have increased the stability of MutY or slightly modified the reaction chemistry. The high concentration of salt and glycerol in the storage buffer is consistent with the slower migration of the covalent complex formed by MutY-2 (Figure 6, panel B, lane 7). These experiments suggest that differences reported in the literature for the properties of MutY are not intrinsic to the protein and are most probably due to nonuniform reaction conditions, including variations in the heating regime and suboptimal conditions used for the cross-linking reaction.

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