A Single Engineered Point Mutation in the Adenine Glycosylase MutY Confers Bifunctional Glycosylase/AP Lyase Activity†

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ABSTRACT: The *E. coli* adenine glycosylase MutY is a member of the base excision repair (BER) superfamily of DNA repair enzymes. MutY plays an important role in preventing mutations caused by 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG) by removing adenine from OG:A base pairs. Some enzymes of the BER superfamily catalyze a strand scission even concomitant with base removal. These bifunctional glycosylase/AP lyases bear a conserved lysine group in the active site region, which is believed to be the species performing the initial nucleophilic attack at C1′ in the catalysis of base removal. Monofunctional glycosylases such as MutY are thought to perform this C1′ nucleophilic displacement by a base-activated water molecule, and, indeed, the conservation of amine functionality positioning has not been observed in protein sequence alignments. Bifunctional glycosylase/AP lyase activity was successfully engineered into MutY by replacing serine 120 with lysine. MutY S120K is capable of catalyzing DNA strand scission at a rate equivalent to that of adenine excision for both G:A and OG:A mispair substrates. The extent of DNA backbone cleavage is independent of treating reaction aliquots with 0.1 M NaOH. Importantly, the replacement of the serine with lysine results in a catalytic rate that is compromised by at least 20-fold. The reduced efficiency in the glycosylase activity is also reflected in a reduced ability of S120K MutY to prevent DNA mutations in vivo. These results illustrate that the mechanisms of action of the two classes of these enzymes are quite similar, such that a single amino acid change is sufficient, in the case of MutY, to convert a monofunctional glycosylase to a bifunctional glycosylase/AP lyase.

Ultraviolet and ionizing radiation, alkylating agents, oxidative stress, and DNA replication errors all pose a threat to the structural integrity of DNA (*1*). The study of DNA repair processes is taking on ever increasing importance as the number of cancer types known to be linked to defects in cellular DNA repair pathways grows (*2*−*4*). Uncovering the catalytic mechanisms by which DNA repair enzymes process substrate DNA damage not only will assist in the development of treatments for certain cancer types but also will aid in the understanding of how organisms ranging from bacteria to humans are able to maintain the integrity of vast amounts of genomic information despite the constant onslaught of DNA-damaging agents.

E. coli MutY is a base excision repair (BER)[1] enzyme involved in the recognition and repair of oxidative damage to DNA (*5*). It is an adenine glycosylase which removes adenine bases mispaired with guanine and 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG) (*6, 7*). MutY is a member of the base excision repair (BER) superfamily of DNA repair enzymes, the members of which share a common helix−hairpin−helix (HhH) motif as well as a highly conserved Asp residue (*5, 8, 9*). The HhH motif has also been observed in a variety of DNA binding enzymes, including enzymes involved in DNA replication and recombination (*10*). Interestingly, BER superfamily members are quite diverse in terms of the types of DNA damage that is recognized and their catalytic activity (*5*). Some are capable of catalyzing a DNA strand cleavage event concomitantly with base excision, and are therefore known as bifunctional glycosylase/apurinic-apyrimidinic (AP) site lyases (*11*). Those which do not have an associated strand cleavage activity, including MutY, are monofunctional glycosylases (*11, 12*). The nature of the species performing initial nucleophilic attack at C1′ to effect base excision is thought to be the central feature which distinguishes bifunctional and monofunctional glycosylase behavior (*11*). As illustrated in Figure 1, monofunctional enzymes are proposed to employ an activated water molecule, whereas the bifunctional glycosylase/AP lyases remove the base with the assistance of the active site amine residue. The participation of the amine residue in the bifunctional enzymes results in formation of an iminium ion intermediate (Schiff base) which is then subject to enzyme-

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[1] Abbreviations: AP, apurinic/apyrimidinic; BER, base excision repair; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; F, 2′-deoxyformycin A; FA, 2′-deoxy-2′-fluoroadenosine; HhH, helix−hairpin−helix; nt, nucleotide; OG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; R, 2′-deoxyaristeromycin; SDS, sodium dodecyl sulfate; TBE, Tris−borate−EDTA; Tris, tris(hydroxymethyl)aminomethane; WT, wild-type; standard abbreviations are used for mutant forms; e.g., S120K is serine at position 120 replaced with lysine.

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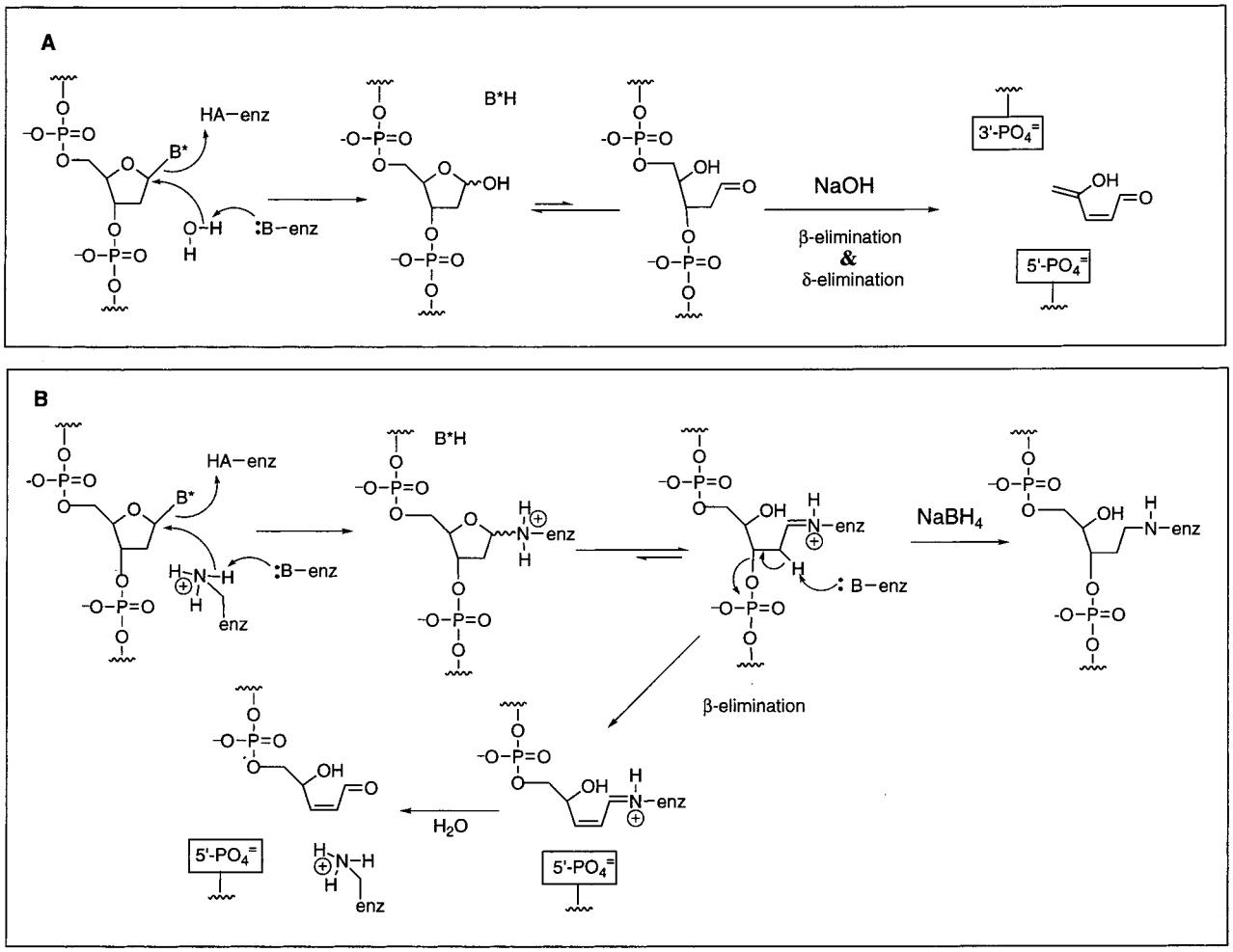

FIGURE 1: Proposed general catalytic mechanism for BER glycosylases (*11*). (A) Catalysis for monofunctional glycosylases is initiated by C1′ nucleophilic attack by an activated water molecule, with protonation of the leaving base, to give a base-labile abasic (AP) site. (B) The scheme for bifunctional glycosylase/AP lyases is initiated by an active site amine functionality performing nucleophilic base displacement directly, to generate an iminium ion covalent enzyme−DNA intermediate. Subsequent abstraction of a 2′ proton facilitates strand scission by β-elimination. The second iminium ion intermediate is then subject to hydrolysis to yield an α,β-unsaturated aldehyde moiety at the 3′ end of the cleaved fragment.

mediated β-elimination to yield strand scission (*11*). Reduction of this intermediate in vitro to produce stable enzyme−DNA adducts has been a means for its detection, and thus has been used as an additional way to identify bifunctional glycosylase/lyase BER enzymes (*11*). The active site chemistry of MutY has recently been shown to be more complex than the simplified schemes of Figure 1. Indeed, MutY is a monofunctional glycosylase capable of forming covalent Schiff base adducts with DNA (*12*). Replacing lysine 142 of MutY with alanine abolishes the ability to observe covalent MutY−DNA complexes via sodium borohydride reduction, indicating the absence of a Schiff base intermediate; however, this replacement does not alter the processing of substrate DNA relative to the wild-type enzyme (*13, 14*). This indicates that formation of the Schiff base intermediate is not required for the adenine glycosylase activity of MutY.

The N-terminal domain of MutY bears high sequence homology to endonuclease III, a bifunctional BER enzyme (*15*). Indeed, endonuclease III and the N-terminal region of MutY are similar in overall structure (*16, 17*). In addition, both enzymes contain a $[Fe_4S_4]^{2+}$ cluster coordinated by four cysteine residues, the spacing of which is unique among iron−sulfur cluster-containing enzymes (*18*). Despite these similarities, however, MutY differs from endonuclease III in terms of both substrate specificity and catalytic activity. Endonuclease III recognizes products of oxidative damage toward pyrimidine residues, and its associated lyase activity causes a DNA strand break 3′ to the AP site, concomitant with base excision (*5*).

A subset of glycosylases within the BER superfamily, including *E. coli* endonuclease III, *M. luteus* UV endonuclease, and *S. cerevisiae* 8-oxo-G DNA glycosylase (yOgg1), bear an associated AP lyase activity, and contain a conserved lysine at the beginning of the second helix in the HhH motif (Figure 2) (*8, 9*). In endonuclease III, this lysine at position 120 has been determined by mutagenesis studies to be crucial for catalytic activity (*18*). There appears to be no functional group conservation at this position in the members of the BER superfamily that are monofunctional glycosylases (Figure 2). The position analogous to that of lysine 120 in endonuclease III is serine 120 in MutY. It was therefore hypothesized that MutY could be converted to a bifunctional glycosylase/AP lyase by replacing serine 120 with lysine[2] (*11*). Herein, a characterization of the functional and kinetic

[2] It was stated in 1994 by Dodson et al. (*11*) that an S120K MutY enzyme did indeed possess bifunctional glycosylase/lyase activity; however, no supporting data were provided or subsequently published.

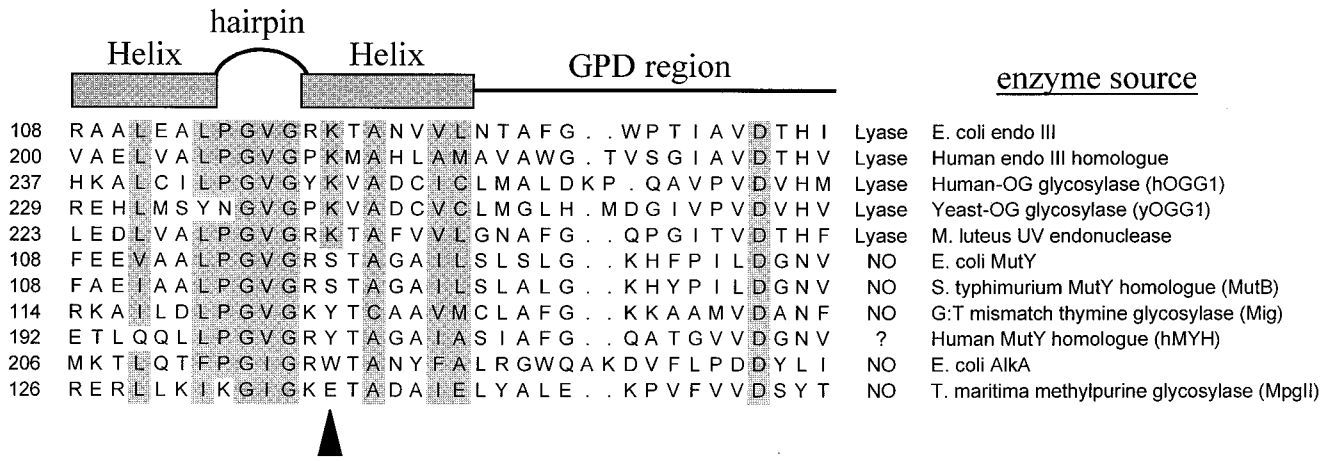

FIGURE 2: Partial sequence alignment of several BER enzymes. Enzyme names and source organisms are given in the right columns, as well as the presence of an associated AP lyase activity. Sequence residue positions are given in the leftmost column. The helix−hairpin−helix (HhH) motif and the glycine/proline-rich plus conserved aspartic acid region (GPD) are indicated above. The dark triangle indicates the positions in all enzymes analogous to that of lysine 120 in endonuclease III. The shaded areas represent similar or identical amino acid functionality shared by the enzymes listed.

properties of S120K MutY is reported, and these properties are compared to those of the WT enzyme. Our results show that the conversion of serine 120 to lysine has bestowed S120K MutY with bifunctional glycosylase/AP lyase activity. Thus, conversion of MutY between the glycosylase and glycosylase/lyase classifications can be achieved by a single point mutation.

MATERIALS AND METHODS

Materials. The plasmid containing the *mutY* gene, pKKYE-co, in addition to *E. coli* strains JM101 *mutY*$^-$ and GT100 *mutY*$^-$ *mutM*$^-$ were kindly provided by M. Michaels and J. H. Miller (*19*−*21*). All substrate 2′-deoxyoligonucleotides were synthesized by standard phosphoramidite chemistry on an Applied Biosystems automated oligonucleotide synthesizer (model 392) as per the manufacturer's protocol. The OG phosphoramidite was purchased from Glen Research. The 2′-deoxyformycin A (F) phosphoramidite was synthesized as described previously (*22*). The 2′-deoxy-2′-fluoroadenosine (FA) phosphoramidite was a generous gift of Dr. Peter Beal and Mr. Greg Kamilar. Oligonucleotides containing 2′-deoxyaristeromycin were provided by Dr. Hiroshi Sugiyama and synthesized as described previously (*23*). Uracil DNA glycosylase (UDG) was purchased from New England Biolabs. All PCR reagents and enzymes were purchased from Boehringer Mannheim. The 5′-end-labeling was performed with T4 polynucleotide kinase purchased from New England Biolabs in the presence of [γ-^{32}P]ATP from Amersham Pharmacia. Labeled oligonucleotides were purified using Probe Quant G-50 spin columns purchased from Amersham Pharmacia. Bradford reagents were purchased from BioRad. $NaBH_4$ was purchased from Aldrich Chemical, and all other buffers and reagents were obtained from either USB or Fisher. Storage phosphor autoradiography was performed using a Molecular Dynamics Storm 840 Phosphor Imager.

Generation of S120K MutY. Site-directed mutagenesis, isolation, and purification of the S120K MutY form were performed as described (*21*). The percent active enzyme out of total purified MutY protein for each mutant enzyme preparation was determined using standard active site titration methods modified specifically for MutY (*7*). The percent activity was determined using the method of Bradford (*24*) to assay the total protein concentration in the S120K preparation, and the final protein preparation was found to contain 46% active enzyme. The percent active enzyme in a double-mutant form, S120K:K142A MutY, was determined to be 34%.

Substrate and Substrate Analogue Duplex Preparation. Following the automated synthesis and purification of single-stranded 2′-deoxyoligonucleotides, duplex mispair-containing substrates were formed by allowing 2 molar equiv of the complement sequence to anneal to the ^{32}P-5′-end-labeled A-containing strand in buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM EDTA, and 150 mM NaCl. Mixtures were heated to 90 °C for 5 min, and allowed to cool slowly to 25 °C overnight. The following 30-base sequence was used: (5′-CGATCATGGAGCCAC**X**AGCTCCCGTTACAG-3′)·(3′-GCTAGTACCTCGGTG**Y**TCGAGGGCAATGTC-5′)* where X = G or OG and Y = A, U, F, R, or FA (where F = 2′-deoxyformycin A, R = 2′-deoxyaristeromycin, and FA = 2′-deoxy-2′-fluoroadenosine). The (*) indicates the position of the ^{32}P label on the duplex substrate.

Glycosylase/AP Lyase Assays. Reactions were conducted under buffer conditions of 20 mM Tris-HCl (pH 7.6), 30 mM NaCl, and 10 mM EDTA. The final DNA concentration was 20 nM, and the final enzyme concentration was kept at 100 nM (based upon active site concentration). Reactions were performed at 37 °C. At time points from 1 min to 4 h, two separate 10 μL aliquots were removed. One aliquot was immediately quenched at −78 °C; the other was exposed to NaOH with a final concentration of 0.1 M, and heated to 90 °C for 5 min before cooling to −78 °C. Denaturing loading dye (80% formamide, 0.025% xylene cyanole, and 0.025% bromophenol blue in TBE buffer) was subsequently added to all aliquots. Following heat denaturation for 1 min, the aliquots were loaded onto 15% polyacrylamide gels containing 8 M urea to separate products from unreacted DNA substrate. Phosphor storage screens were exposed to gels for at least 12 h before image scanning and quantitation.

Assays for AP Lyase Activity toward Abasic Sites. Abasic sites were generated by treating G:U and OG:U mispair-

containing 30 base pair duplexes with uracil DNA glycosylase (UDG) in the presence of the commercially available reaction buffer for UDG [20 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 10 mM DTT]. Maximum DNA concentration was estimated to be 10 nM, owing to some anticipated loss in the purification steps required after 5′-end-labeling of the U-containing strand. After incubation at 37 °C for 30 min, either WT or S120K MutY was added to the reaction at a final active site concentration of 400 nM, and incubation was allowed to progress at 37 °C for an additional 30 min. Reaction volumes were then split, and one fraction was base-treated as described above for the glycosylase/AP lyase assays, while the other did not undergo base treatment. PAGE resolution of the DNA substrates and products was performed also as described above.

Detection of Reduced Enzyme/DNA Intermediates. Final buffer component concentrations in these reactions were 25 mM sodium phosphate (pH 6.8), 1 mM EDTA, and 0.1 mg/mL BSA. Final substrate DNA duplex concentration was 10 nM; final enzyme concentration was 300 nM as determined by the Bradford method. $NaBH_4$ concentration was kept constant at 90 mM. Total reaction volumes were 10 μL each. NaCl was added to each reaction such that the sodium ion concentration was 180 mM, for the purpose of keeping conditions similar to those of previously published experiments (*21*). All reactions were performed at 37 °C for 30 min. An equal volume (10 μL) of SDS−PAGE loading buffer [125 mM Tris-HCl (pH 8.0), 5% sodium dodecyl sulfate, 25% glycerol, 0.025% bromophenol blue] was added to each reaction, and the resulting 20 μL volume was heated to 90 °C for 10 min prior to being loaded onto an 8% polyacrylamide−SDS gel for resolution of free DNA from covalent DNA−enzyme adducts. Phosphor storage screens were exposed to gels for at least 12 h before image scanning and quantitation.

Single- and Multiple-Turnover Kinetic Experiments. S120K MutY kinetic assays were performed as previously described using the same buffer conditions as those for the qualitative glycosylase assays mentioned above (*7*). For the single-turnover assays, the substrate DNA concentration was kept at 40 nM, while the active enzyme concentration was 60 nM. The active enzyme concentration was kept at 3 nM for reactions performed under multiple-turnover conditions, and DNA concentrations were at 20 nM. In each case, the active enzyme concentration was determined by the active site titration with an OG:A substrate and is relative to the total protein concentration determined by Bradford (*24*).

Experiments under single-turnover conditions of S120K and WT MutY with substrate DNA containing OG:AP sites were conducted with a final DNA concentration of 8 nM, and a final active enzyme concentration of 100 nM. The OG:AP site containing substrate was prepared by allowing UDG to react with the OG:U mispair-containing duplexes in the presence of the commercial UDG assay buffer in a small volume (8 μL) at room temperature to ensure thorough excision of the U residues to produce the AP sites. The incubation temperature was raised to 37 °C, MutY assay buffer was added, and volumes were brought to 100 μL upon addition of S120K or WT MutY. Reaction time point aliquots were removed and quenched on dry ice. All samples were subsequently separated by sequencing PAGE, and gel images were quantified by storage phosphor autoradiography. One reaction aliquot was base-treated (0.1 M NaOH) for the purposes of determining the extent of AP site formation by UDG. This, along with control reactions of the UDG-processed DNA unexposed to MutY, served to establish the extent of AP site processing by MutY.

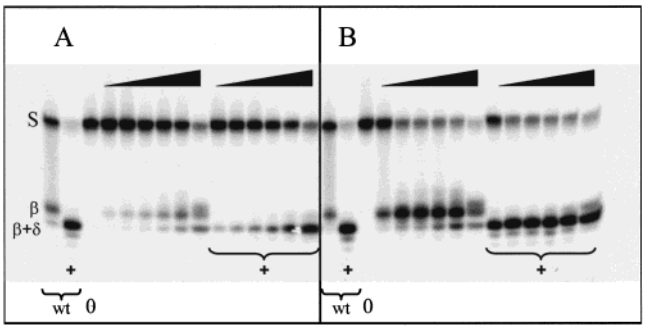

FIGURE 3: Storage phosphor autoradiogram, showing product resolution of a glycosylase/AP lyase assay for S120K MutY. In each case, enzyme concentration was 100 nM based upon active site concentration, and substrate DNA concentration was 20 nM in the case of both G:A (A) and OG:A (B) mispair substrates. Lanes marked with 0 represent controls without enzyme, and black triangles indicate an increase in incubation time from left to right, with 5, 10, 30, 60, 120, and 240 min for G:A, and 1, 5, 10, 30, 60, and 240 min for OG:A. Reactions whose time point aliquots were treated with 0.1 M NaOH are indicated with a plus sign (+). Control reactions with WT MutY at a 60 min incubation time are indicated with "wt". The label "S" indicated the migration of the 30 nt fragment resulting from the substrate while the labels "β" and "$\beta+\delta$" indicate the 14 nt product fragments resulting from β and S-elimination, respectively.

Dissociation Constant Determination. Dissociation constants toward DNA duplexes containing substrate analogue mispairs resistant to catalytic turnover were determined for each mutant form, as described previously (*25*, *26*). K_d values have been corrected for active enzyme concentration. In all experiments, the final DNA concentration was no greater than 10 pM.

RESULTS

Activity of S120K MutY toward OG:A- and G:A-Containing DNA Substrates. Wild-type MutY catalyzes the excision of an adenine base mispaired with OG or G to produce an AP site. Alkali treatment of this DNA product yields phosphodiester backbone cleavage, detectable through standard denaturing polyacrylamide gel electrophoretic separation of cleaved and intact DNA strands. As evident in Figure 3A, S120K MutY is capable of processing G:A mispair-containing DNA to yield a 14 nt strand as the cleavage product of the 30 nt adenine-containing strand, without the addition of base to the reaction. Quantitation of the storage phosphor autoradiogram indicates that the amount of product DNA (14 nt fragment) formed relative to remaining substrate (30 nt strand) does not increase upon the addition of 0.1 M NaOH to reaction aliquots, in contrast to the behavior of WT MutY. Also apparent is the difference in molecular weights of product DNA which arise as a function of base treatment. For the base-treated aliquots, only one major product band is detectable, most likely due to the efficient base abstraction of both protons at the β and δ positions of the deadenylated deoxyribose ring. A band of slightly higher molecular weight is dominant in the non-base-treated reactions of S120K MutY, suggesting that enzyme-mediated strand cleavage

Table 1: Microscopic Kinetic Rate Constants for WT and the S120K MutY Forms with OG:A and GA Substrates

	k_2 (min^{-1})[a]		k_3 (min^{-1})[a]	
enzyme	G:A	OG:A	G:A	OG:A
WT[b]	1.6 ± 0.2	>10	0.027 ± 0.004	0.004 ± 0.002
K142A	1.2 ± 0.1	>8	0.015 ± 0.007	0.004 ± 0.001
S120K[c]	0.03 ± 0.01	0.64 ± 0.13	$<1 \times 10^{-3}$	0.003 ± 0.001
S120K	0.03 ± 0.01	0.69 ± 0.17	$<1 \times 10^{-3}$	0.005 ± 0.002
S120K:K142A[c]	0.03 ± 0.01	0.88 ± 0.25	$<1 \times 10^{-3}$	0.005 ± 0.001
S120K:K142A	0.03 ± 0.01	0.85 ± 0.18	$<1 \times 10^{-3}$	0.004 ± 0.001

[a] Catalytic (k_2) and product release (k_3) rate constants measured at 37 °C under STO and MTO conditions, respectively, as described previously (*7*). [b] WT data were reported previously by Porello et al. (*7*). [c] Reaction aliquots not exposed to base prior to gel loading.

Scheme 1: Minimal Kinetic Scheme for MutY (*7*)[a]

$$\text{MutY} + (\text{DNA})_S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{MutY*}(\text{DNA})_S \xrightarrow{k_2} \text{MutY*}(\text{DNA})_P \xrightarrow{k_3} \text{MutY} + (\text{DNA})_P$$

[a] The catalytic (k_2) step may involve a conformational change involving the protein and/or substrate DNA prior to base excision.

occurs primarily via β-elimination, leaving behind an α,β-unsaturated aldehyde as a remnant of the AP site deoxyribose ring. This band comigrates with the one detectable in reactions with WT MutY. The small amount of cleaved DNA (<5% of total DNA) which arises in the presence of WT enzyme, however, does not increase over extended incubation times (up to 4 h), as it does in the case with S120K MutY.

The S120K MutY enzyme is also able to process OG:A mispair substrates (Figure 3B). The trends observed with G:A substrates are similar to those with OG:A substrates, in that base treatment of reaction aliquots does not increase the amount of cleaved DNA in reaction with S120K MutY. For both OG:A and G:A, it is clear that adenine excision is followed by an enzyme-mediated DNA strand cleavage event at a similar (or faster) rate. In addition, S120K is considerably faster in the reaction toward OG:A mispairs relative to G:A mispairs, observable in the storage phosphor autoradiogram even under conditions of great enzyme molar excess over substrate.

Kinetic Rate Constant Determinations for S120K MutY. A minimal kinetic scheme for WT MutY in reaction with substrate DNA has previously been proposed and discussed in detail (Scheme 1) (*7*). Under conditions where [MutY] < [DNA], MutY has been shown to exhibit biphasic kinetic behavior characterized by an initial exponential burst phase for product formation, followed by a slower linear steady-state phase. The burst behavior under these conditions is indicative of a slow step occurring after the chemistry step. Evidence is accumulating that this slow step is the release of MutY from the DNA product (*7*, *27*). The conversion of OG:A and G:A substrates to product by the S120K enzyme also exhibits a similar kinetic profile, indicating that the same kinetic analysis that has been used with the WT enzyme can be applied to the S120K enzyme. Enzyme turnover (k_3) in the presence of the OG:A substrate, like WT MutY, is extremely low with S120K, permitting the use of active site titration methods employed for the WT enzyme, in determining percent active enzyme for preparations of both the S120K and S120K:K142A MutY forms. These values compared well with the percent active enzyme that is routinely obtained with different preparations of WT MutY.

Rate constants for catalytic steps which include the chemistry of base removal (k_2) and product duplex release (k_3) can be determined by the appropriate fitting of data from single-turnover (STO) and multiple-turnover (MTO) experiments (*7*). Reactions with S120K MutY and the double mutant S120K:K142A form were carried out under conditions similar to those performed for the WT enzyme, and the resulting values at 37 °C for k_2 and k_3 are listed in Table 1. Representative kinetic time courses for the reaction of S120K MutY with both an OG:A- and a G:A-containing duplex under identical single-turnover conditions are shown in Figure 4. The catalytic rate constant for S120K in reaction with a G:A mispair-containing substrate ($k_2 = 0.03 \pm 0.01$ min^{-1}) relative to the rate constant determined for the WT enzyme ($k_2 = 1.6 \pm 0.2$ min^{-1}) (*7*) indicates a dramatic decrease in the efficiency of the enzyme for this substrate. Indeed, the differences in the rate constants represent a 50-fold reduction for the S120K enzyme in the rate for adenine removal from a G:A base pair. The reaction of the WT enzyme with an OG:A-containing substrate is too fast to be measured using a manual method, and therefore a lower limit for $k_2 \geq 10$ min^{-1} was estimated (*7*). In contrast, a k_2 value of 0.69 ± 0.17 min^{-1} for the S120K enzyme in reaction with OG:A substrate was readily determined, since this rate is at least 15-fold slower than with the WT enzyme.

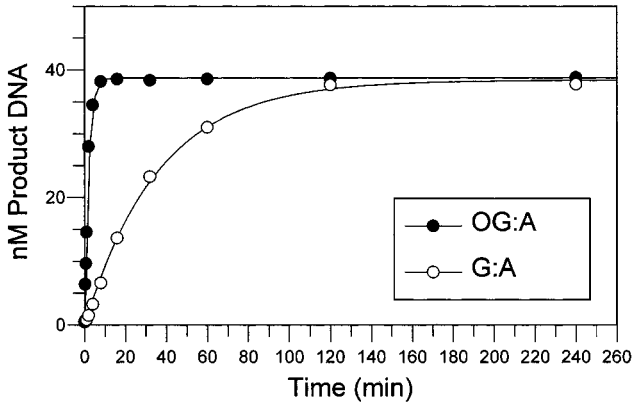

FIGURE 4: Representative plot of product formation as a function of time, under single-turnover conditions with S120K MutY. Final enzyme concentration was 60 nM, as determined by active site titration, and final substrate concentration was 40 nM. Time points taken were at 0 s, 20 s, 40 s, 1 min, 2 min, 4 min, 8 min, 16 min, 32 min, 1 h, 2 h, and 4 h. Non-base-treated aliquots were immediately quenched in dry ice prior to addition of loading buffer and PAGE analysis.

To determine whether S120K MutY was a true bifunctional enzyme in which the β-lyase reaction occurs con-

comitant with adenine base removal, the analogous single-turnover experiments with OG:A and G:A substrates were performed without addition of base to provide strand scission. Thus, in these reactions, strand scission was provided by S120K MutY. Attesting to the bona fide bifunctional activity of S120K MutY, the rate constants determined with both OG:A and G:A substrates (Table 1) did not fluctuate as a function of treating reaction aliquots with 0.1 M NaOH. These results indicate that S120K MutY catalyzes the β-lyase reaction to provide strand scission at a rate equal to that (or greater than that) for adenine removal. Notably, in the analogous reactions with WT MutY, significantly different behavior is observed. In the reaction of the WT enzyme with an OG:A substrate, analysis of the glycosylase reaction by addition of base indicated that the reaction was complete within the first 15 s. In contrast, if the glycosylase and lyase reactions are monitored, less than 5% strand scission is observed after 120 min. Thus, any "lyase" activity of the WT enzyme is considerably slower than its glycosylase activity, while the glycosylase and β-lyase reactions have similar rates with S120K MutY.

Multiple-turnover experiments where [MutY] < [DNA] with S120K MutY were performed in a manner analogous to that previously reported for the WT enzyme (*7*). Appropriate fitting of the data provides both k_2 and k_3 values. The k_2 values determined for S120K from these experiments were similar to those determined from the single-turnover experiments (data not shown). The rate constant value for product release (k_3) for S120K MutY (Table 1) reflects a drop about 2 orders of magnitude below that reported for WT MutY, in the presence of G:A substrate, under multiple-turnover conditions. Indeed, this k_3 value was immeasurably low. In contrast, the k_3 rate constants determined for S120K with the OG:A substrate are similar to those determined for the WT enzyme (Table 1).

Lys 142 has been shown to be the residue responsible for sodium borohydride-mediated cross-links between MutY and substrate DNA (*14*, *28*). Therefore, to remove complications associated with formation of covalent intermediates with this residue, we also prepared the S120K:K142A enzyme. The k_2 and k_3 values determined with this doubly mutated form were similar to those obtained with the S120K enzyme. Since the K142A enzyme has been shown to have enzymatic properties similar to the WT enzyme (Table 1), the dramatic reduction of both k_2 and k_3 values for S120K and S120K:K142A enzymes is due to the replacement of the serine residue at position 120 with a lysine.

Activity of S120K MutY toward AP Site-Containing Substrates. To determine if the AP lyase activity of S120K MutY is mechanistically dependent upon adenine excision or if strand scission can occur at preexisting abasic sites opposite OG, the activity of S120K MutY toward an AP site-containing duplex was evaluated (Figure 5A). Uracil DNA glycosylase (UDG) was utilized as a means to introduce AP sites in duplex 30-mer DNA containing a centralized OG:U mispair. After incubation at 37 °C for 30 min to ensure thorough uracil excision, WT or S120K MutY was added at 40-fold molar excess over substrate DNA, and reactions were kept at 37 °C for an additional 30 min. As illustrated in Figure 5A, OG:U mispair-containing DNA is not a substrate for either WT (lanes 5 and 6) or S120K MutY (lanes 9 and 10), as expected, since no quantifiable difference

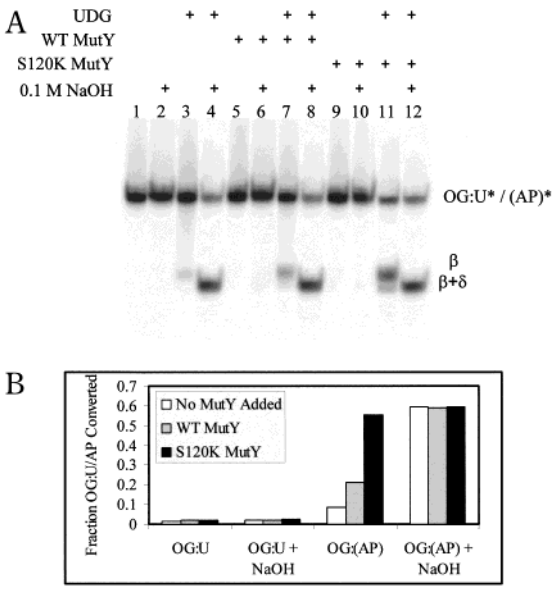

FIGURE 5: (A) Storage phosphor autoradiogram of denaturing polyacrylamide gel showing product resolution of S120K and WT MutY glycosylase/AP lyase activity toward AP sites opposite OG. Reactions were carried out in the presence of OG:U mispair-containing duplex DNA at 10 nM. Lanes 1 and 2 represent no enzyme control reactions. Lanes 3 and 4 are reactions of UDG alone; 5 and 6, WT MutY alone; 9 and 10, S120K MutY alone. Lanes 7 and 8 represent treatment with 40-fold molar WT MutY excess over substrate, after 30 min treatment with UDG, and lanes 11 and 12 represent an analogous treatment with S120K MutY after UDG treatment. Reactions in all even-numbered lanes were treated with 0.1 M NaOH before gel loading. (B) Results of the quantitation of storage phosphor gel image of (A). Fraction of substrate DNA converted to cleaved product is plotted with respect to enzyme reaction with OG:U (lanes 1, 5, and 9), OG:U with subsequent NaOH treatment (lanes 2, 6, and 10), OG:AP site (lanes 3, 7, and 11), and OG:AP site followed by NaOH treatment (lanes 4, 8, and 12).

exists between these lanes and those of the no-enzyme control (lanes 1 and 2). The OG:U substrate is effectively converted to the OG:(AP site) by observation of cleavage at the uracil residue upon exposure of the UDG-treated reaction to base before gel resolution (lane 4). The reaction of UDG with OG:U without base treatment (lane 3) is illustrative of AP site lability in solution, as a very small amount of cleaved DNA fragments is detectable, similar to the case of WT MutY in reaction with OG:A and G:A substrate mispairs. The extent of DNA strand cleavage which takes place with OG:(AP site)-containing DNA after treatment with WT and S120K MutY is shown in lanes 7 and 11, respectively. Only the product bands of S120K MutY show that DNA cleavage has taken place to the same extent as the products resolved in lanes 4, 8, and 12, where reaction aliquots were exposed to base. The results of gel image quantitation are shown in Figure 5B. Similar qualitative results were observed with a G:(AP site)-containing duplex (data not shown). Therefore, S120K MutY has lyase activity toward preestablished AP sites opposite both G and OG, and reactions not treated with base show substrate DNA cleavage to the same extent as reactions exposed to 0.1 M NaOH. As is the case for the glycosylase/AP lyase activity of S120K MutY illustrated in Figure 3, the observed mobility of the DNA fragments (Figure 5A, lane 11) indicates that the major DNA cleavage mechanism for S120K is through β-elimination. In these qualitative experiments, it is apparent that WT MutY is able

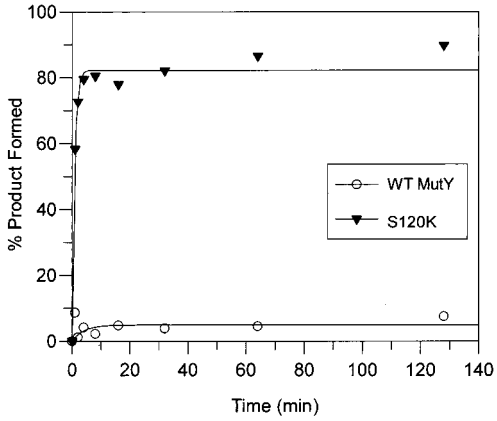

FIGURE 6: Representative plot of product formation as a function of time, under single-turnover conditions with S120K and WT MutY on an OG:AP site substrate at 37 °C. Final active enzyme concentration was 100 nM, and final substrate concentration was 8 nM. Time points taken were at 0 s, 1 min, 2 min, 4 min, 8 min, 16 min, 32 min, 64 min, and 128 min. A base-treated control was used to determine the amount of OG:AP site duplex present after the treatment of the OG:U duplex with UDG. This, along with the control reactions in which MutY was absent, served to establish the percent AP site processing by S120K or WT MutY.

to convert some of the AP site-containing DNA to cleaved product without the exogenous addition of base, but this also holds true for UDG, albeit to a slightly lesser extent. However, it should be noted that these experiments were performed in the commercial buffer for UDG, rather than the conditions typically used for MutY, and this may be providing a slightly higher background lyase reaction. Importantly, both WT MutY and UDG are unable to process AP-site-containing DNA with *complete* conversion to cleaved product unless exogenous base is added. Only S120K MutY is able to do so, regardless of base treatment.

Single-turnover experiments were also performed with S120K and WT MutY using an OG:(AP site)-containing duplex generated from the reaction with UDG on an OG:U-containing duplex. The percent product formed with S120K and WT MutY was normalized using a base-treated sample to determine the amount of AP site present. A representative plot from this experiment is shown in Figure 6. In the case of S120K, the reaction had reached 75% completion at the first time point (1 min), and therefore a lower limit of $k_{lyase} \geq 1.5$ min^{-1} was estimated. Indeed, this rate is comparable to that determined for adenine removal by S120K from an OG:A duplex. In contrast, with WT MutY, the reaction had only proceeded to 6% completion after 130 min.[3] Thus, clearly the lyase activity created by the presence of lysine 120 in S120K MutY is considerably more potent than that of WT MutY.

In Vivo Activity. The activity of S120K MutY in vivo was assayed using a technique described previously (*21*). The assay involves an observation of spontaneous mutations in the rifampicin binding site of *E. coli* RNA polymerases. If DNA repair activity is low, such mutations will render rifampicin less effective as a block to transcription, allowing cell growth in the presence of the drug (*29*). The mutation rate can therefore be determined by the number of *E. coli*

[3] If the rate for this reaction is estimated by extrapolation to completion to the same extent as S120K MutY, this sets an upper limit of $k_{lyase} \leq 6 \times 10^{-4}$ min^{-1} for the WT enzyme.

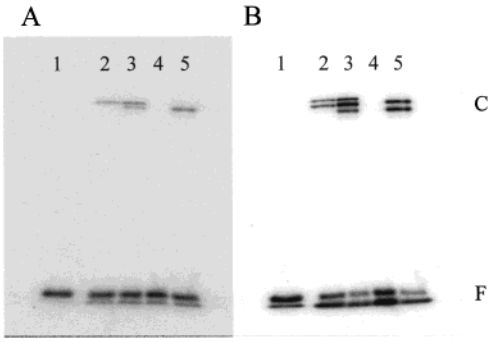

FIGURE 7: Storage phosphor autoradiogram of SDS−PAGE resolution of free duplex DNA from covalently enzyme-bound DNA resulting from borohydride reduction of Schiff base reaction intermediates. Enzyme−DNA adducts (C) as well as free DNA (F) are indicated. Lanes 1, no enzyme control; lanes 2, wild-type MutY; lanes 3, S120K MutY; lanes 4, K142A MutY; and lanes 5, S120K:K142A MutY double mutated form. The OG:A substrate in panel A was 5′-^{32}P-labeled on the OG-containing strand, while the one represented in panel B was 5′-^{32}P-labeled on the A-containing strand. In each case, enzyme concentration was approximately 300 nM, and DNA concentration was 10 nM.

rifampicin revertant (Rif^r) colonies (*19*). In a strain lacking the *mutM* and *mutY* genes (GT100 *mutM*$^-$ *mutY*$^-$), transformed with a plasmid containing the wild-type *mutY* gene, the number of colonies capable of growth on rifampicin-containing media was low (1 ± 1 per 10^8 cells). *E. coli* GT100 cells transformed with plasmid lacking *mutY* produced a high colony count (1000 ± 100 per 10^8 cells). Akin to the case of transformants containing WT *mutY* plasmid, cells transformed with the gene coding for S120K MutY also gave a reduced number of colonies (62 ± 4 per 10^8 cells), indicating an ability of S120K MutY to reduce the DNA mutation frequency, albeit considerably less efficiently than the wild-type enzyme. Since S120K MutY overexpression levels in the cell have no measurable difference from the levels of WT MutY overexpression in the same strain, it is likely that the reduced in vivo activity is reflective of S120K MutY's compromised adenine glycosylase activity.

Sodium Borohydride Trapping of S120K MutY Reaction Intermediates. Sodium borohydride has been widely used as a means of generating stable covalent DNA−enzyme adducts through reduction of the Schiff base intermediate formed with an active site amine residue of bifunctional glycosylase/AP lyases during the process of base excision and strand scission (Figure 1). Although the detection of such stable adducts has been a characteristic of bifunctional BER enzymes, it has been shown that MutY is capable of forming borohydride-dependent cross-links with substrate DNA (*12*, *28*, *30*−*32*). Figure 7 shows the SDS−PAGE resolution of MutY and three mutant forms of this enzyme in the presence of OG:A mispair-containing DNA and sodium borohydride. As shown recently, the K142A MutY enzyme does not form sodium borohydride meditated DNA cross-links (*13*, *14*). Three forms, WT, S120K, and the double mutant S120K:K142A are able to generate covalent adducts with substrate DNA. It is evident from Figure 7 that a lysine group introduced at position 120 in MutY is capable of taking over the role of lysine 142 in the WT enzyme, as shown by the ability of the S120K:K142A double mutant form to generate covalent adducts with substrate DNA. In addition, UDG-generated abasic sites opposite G and OG are also capable

Table 2: Equilibrium Dissociation Constant Values for WT and the S120K MutY Forms with Substrate Analogue Mispair Duplexes

	K_d (nM)[a]				
enzyme	OG:F[b]	OG:R[b]	OG:FA[b]	G:F[b]	G:C
WT	0.28 ± 0.02[c]	0.4 ± 0.3[d]	0.12 ± 0.02[c]	16 ± 4[e]	150 ± 60[e]
S120K	<10 pM	21 ± 3	25 ± 4	230 ± 40	300 ± 90
S120K:K142A	<10 pM	19 ± 5	21 ± 5	160 ± 50	150 ± 20

[a] Dissociation constants were determined by native gel shift assay, with maximum DNA concentration at 10 pM, as described previously in Chepanoske et al. (*25*). [b] Indicates central basepair of 30-bp duplex; consult figure 8 for structures of substrate analogues. [c] Wild-type MutY data are as reported in Chepanoske et al. (*25*). [d] The K_d value determined in this work for the OG:R duplex is slightly higher than the previously reported value of 0.05 ± 0.03 nM (*25*). [e] Wild-type MutY data are as reported in Porello et al. (*26*) and have been corrected using a percent active enzyme concentration of 60%.

of being covalently cross-linked to WT, S120K, and S120K:K142A MutY to produce banding patterns akin to those shown for substrate OG:A and G:A mispair-containing DNA (data not shown). This demonstrates that the process of base excision is not required for Schiff base formation and can occur with a preformed abasic site.

In the SDS−PAGE resolution of MutY−DNA cross-links from free DNA, multiple bands are observed (Figure 7). In Figure 7A, where OG:A duplexes were labeled on the OG-containing strand, a "doublet" band appears only in the reaction with S120K MutY (lane 3). Both WT (lane 2) and the S120K:K142A mutant form (lane 5) produce a DNA adduct band of only one molecular weight each, yet the band produced in the case of the double mutant form is of slightly faster migration than that of WT MutY. In reactions with the OG-containing strand labeled, one would expect the appearance of any high molecular weight bands to be attributed to incomplete denaturation from A-containing strand covalently bound to the protein (Figure 7A). Small variances in the mobility of these shifted bands or the appearance of multiple bands (i.e., in the case of S120K MutY) may therefore be due to the fact that the A-containing strand is subject to cleavage. In the reactions performed with an OG:A duplex where the A-containing strand was labeled, the same general trend was observed and the same rationale can be applied (Figure 7B). In each case, one additional band was present in the lanes analogous to those of Figure 7A. This may be due to visualization of the covalently protein-bound A strand having been completely denatured from the OG-containing strand, a species obviously undetectable in the OG-strand-labeled reactions.

Dissociation Constants of S120K MutY with Substrate Analogue Duplexes. An approach to evaluate the affinity of MutY for substrate-containing DNA is the use of structural mimics of 2′-deoxyadenosine that retain the recognition properties of the substrate and are resistant to the glycosylase action of MutY (*5*, *25*, *26*, *33*). Such analogues allow for the determination of binding affinity without complications associated with the enzymatic reaction (*5*, *26*). Previous work in our laboratory (*25*, *26*) has shown that MutY has a high affinity for the substrate analogues, 2′-fluoro-2′-deoxyadenosine (FA), 2′-deoxyformycin A (F), and 2′-deoxyaristeromycin, in a DNA duplex opposite G or OG, all of which are resistant to the glycosylase activity of MutY (Figure 8). Dissociation constant (K_d) measurements were performed using a native gel retardation assay in a manner analogous to that described previously (*25*). The K_d values determined for S120K and S120K:K142A MutY toward native DNA (the same DNA sequence with a centralized G:C base pair in place of the substrate mispair), and substrate analogue

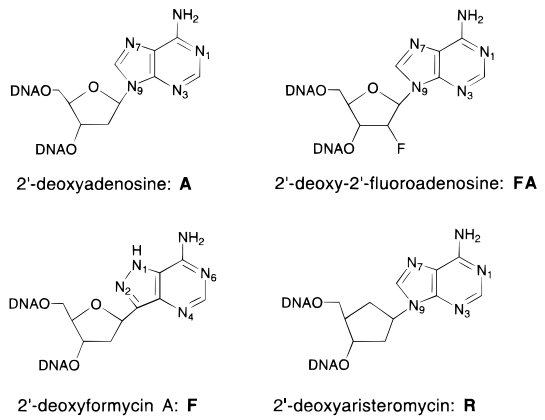

FIGURE 8: Substrate and substrate analogues for MutY. 2′-Deoxyadenosine (A) at top left is the natural substrate for MutY when opposite G or OG. The A analogues, 2′-deoxyaristeromycin, 2′-deoxy-2′-fluoroadenosine, and 2′-deoxyformycin A, mimic structural properties of A, while also providing resistance toward the glycosylase activity of MutY. These analogues were used to assess binding affinity of S120K and S120K:K142A MutY relative to the WT enzyme (Table 2).

duplexes, containing a centralized OG:F, OG:FA, or OG:R mispair, where F = 2′-deoxyformycin A, FA = 2′-deoxy-2′-fluoroadenosine, and R = 2′-deoxyaristeromycin, are listed in Table 2. In terms of dissociation constants toward native DNA, S120K and S120K:K142A MutY have an affinity for nonsubstrate DNA comparable (within error) to that of the WT enzyme. Thus, the introduction of the additional lysine residue in the active site cleft does not significantly alter the nonspecific DNA binding affinity of MutY.

Both S120K and S120K:K142A MutY bind OG:F-containing DNA with an affinity too high to be accurately determined by the native gel retardation method used in this work. Therefore, for both mutant forms, the K_d is estimated to be less than 10 pM. This estimation is based on the observation that at all enzyme concentrations used >90% of the DNA was bound. The lowest enzyme concentration used corresponded to the estimated upper limit of the DNA concentration (10 pM) and therefore serves as an upper limit estimate for the K_d value. The relative affinity toward G:F and OG:R mispairs was also measured, to determine if the tight association between S120K MutY and the OG:F mispair lies primarily with either OG or F. Replacing F with R, which consists of the adenine base, but lacks the deoxyribose ring oxygen heteroatom of 2′-deoxyadenosine, the dissociation constant values for the single and double mutant forms are each approximately 20 nM. A similar trend is seen when F is replaced with FA, which is adenosine where the 2′-hydroxyl functionality has been substituted by a fluorine atom. In comparison to the WT MutY K_d value of 0.4 ± 0.3

nM for the OG:R duplex, the S120K forms bind this substrate analogue with an approximately 50-fold reduced affinity. Similarly, the absence of the 8-keto functionality of OG increases the K_d 10−14-fold toward G:F for the S120K and S120K:K142A mutant forms, relative to WT MutY. A comparison of the K_d values of S120K with OG:F relative to those for G:F and OG:FA duplexes indicates a dramatic decrease in the affinity by replacement of OG with G or F with FA. Clearly, the features of both OG and F contribute toward the unusually high-affinity binding of S120K to the OG:F mispair.

DISCUSSION

Replacing serine 120 with lysine has endowed MutY with bifunctional glycosylase/AP lyase behavior toward both of its primary substrate mispairs, OG:A and G:A. However, in the reaction of S120K with both OG:A and G:A substrates, the kinetic rate constants representing steps which include base excision have been decreased. It is possible that the decreased rates for adenine removal observed with in vitro experiments for S120K also are responsible for the significantly reduced ability of S120K MutY to prevent DNA mutations in vivo. Indeed, it is interesting that in vivo activity is maintained with a mutant form which not only has reduced catalytic rate constants, but whose activity now includes a DNA strand cleavage event for each adenine excised.

The protein sequence alignment (Figure 2) suggests that the presence of a conserved lysine residue may be important for associated AP lyase activity in the glycosylases of the BER superfamily. Indeed, more credence is being lent to this notion as more structural data from these enzymes are published. On the basis of site-directed mutagenesis studies, lysine 120 in *E. coli* endonuclease III has been found to be crucial for catalysis (*18*). More recently, similar studies have been carried out for other BER enzymes, confirming the catalytic importance of lysine group positioning in the HhH domain. A set of elegant experiments demonstrated lysine 249 of the human 8-oxoguanine DNA glycosylase (hOgg1) to be catalytically important (*34*). Replacing this group with cysteine abolished OG glycosylase activity without perturbing substrate recognition. Catalytic activity was successfully restored to the K249C mutant form, by treatment with 2-bromoethylamine. The resulting cysteine group alkylation created a γ-thialysine residue capable of restoring catalytic activity to K249C hOgg1. Lysine 249 of the murine 8-oxoguanine DNA glycosylase (mOgg1) (*4*), lysine 212 of the human endonuclease III homologue (NTH1) (*35*), and lysine 129 of the 8-oxoguanine DNA glycosylase of *Methanococcus jannaschii* (mjOgg) (*36*) were all identified as crucial for catalytic activity, and are positioned analogously to lysine 120 of *E. coli* endonuclease III.

Serine, tyrosine, and tryptophan are common side chains in the monofunctional BER glycosylases present at the conserved lysine position in analogous HhH motifs in bifunctional enzymes (Figure 2). A new member of the BER superfamily, methylpurine DNA glycosylase (MpgII) from *Thermatoga maritima*, has recently been expressed and purified (*37*). It is capable of removing 7-methylguanine and 3-methyladenine from DNA with no associated AP lyase activity. Sequence alignment information places a glutamate residue at position 138, analogous to position 120 in endonuclease III.

The introduction of AP lyase activity into a monofunctional glycosylase of the BER superfamily has recently been reported, using a mutagenesis approach similar to the one employed for this work. *Methanobacterium thermoformicicum* thymine DNA mismatch glycosylase (TDG) is a monofunctional enzyme which removes thymine and uracil residues mispaired with guanine. Tyrosine at position 126 was replaced with lysine, and the authors demonstrated that Y126K TDG is capable of AP lyase activity at preexisting abasic sites opposite G in duplex DNA (*38*). Interestingly, this mutant form lost glycosylase activity toward U:G and T:G mispairs. Since Y126K TDG is still capable of catalyzing AP site strand cleavage in addition to generating covalent adducts with AP site-containing DNA in the presence of borohydride, it was stated that the introduction of a lysine at position 126 caused no significant structural perturbations of the active site. Instead, it was hypothesized that the tyrosine-to-lysine switch may have displaced the necessary activating groups of the active site sufficiently enough to disrupt C1′ nucleophilic attack, and hence base excision.

A similar scenario taking place in the active site of S120K MutY may explain the drop in the value of k_2 for both OG:A and G:A substrates relative to those obtained with WT MutY. This decrease is most significant with the G:A mispair substrate, where complete conversion of substrate DNA to product under single-turnover conditions is seen only at reaction times of at least 2 h at 37 °C. Indeed, the S120K enzyme has a marked preference (20-fold) for the OG:A substrate relative to the G:A substrate. It is possible that the MutY active site environment is more amenable to the introduction of a lysine group than is that of TDG and therefore glycosylase activity is still observed, albeit at a reduced rate. In addition, the ability to form covalent adducts with substrate DNA upon borohydride reduction suggests that the presence of a lysine at position 120 has not affected the gross structure of MutY's active site environment. The decreased catalytic rate constants toward OG:A substrate relative to WT MutY are therefore likely attributable less to impaired substrate recognition, and more to the reduced ability to perform efficient nucleophilic attack at C1′, effecting base excision.

The observation that the measured rate of adenine base removal does not differ when strand scission is provided by an exogenous base or S120K MutY indicates that the β-lyase reaction of S120K MutY is at least as fast as the glycosylase reaction. In addition, measurement of the rate for strand scission at a preformed abasic site opposite OG indicates that the lyase reaction of S120K is somewhat faster than the glycosylase reaction. The correlation between the glycosylase and lyase reactions is highly suggestive of participation of the introduced lysine in the base excision process. However, on the basis of these results, we cannot distinguish between two possible mechanisms (Figure 9). S120K MutY could be a classic bifunctional enzyme wherein the lysine residue is involved in both the base displacement and the β-lyase reaction (Figure 9B). However, it is also possible that another nucleophile (an activated water molecule or another amino acid residue) participates in the base displacement, and subsequent reaction of Lys 120 with the AP site occurs, thereby aiding in catalysis of the β-elimination reaction (Figure 9A). Indeed, based on the similarity of catalytic rate

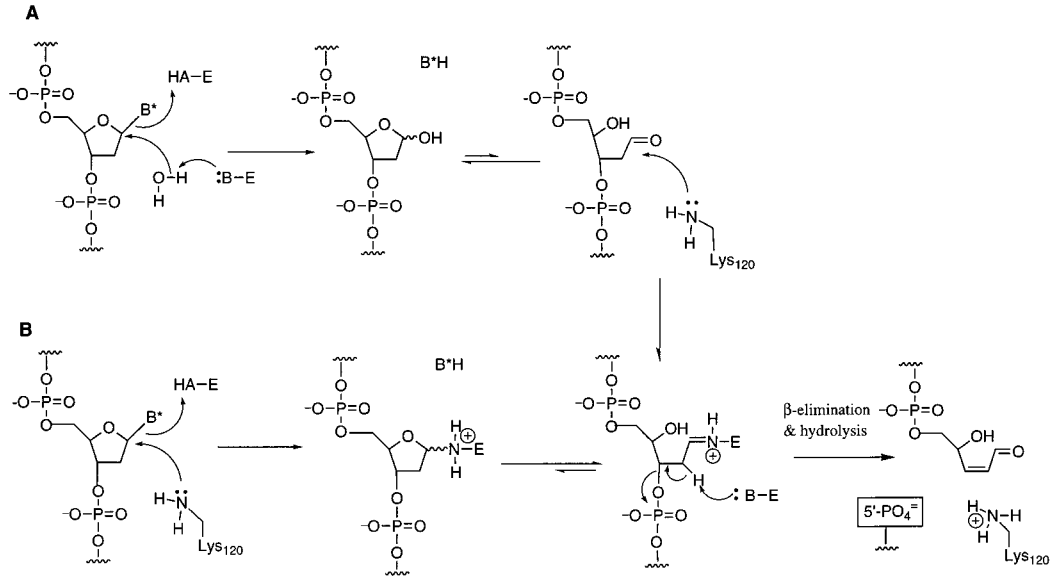

FIGURE 9: Proposed mechanisms for bifunctional glycosylase/β-lyase activity of S120K MutY. (A) Displacement of the adenine base is facilitated by an activated water molecule (or another amino acid) to produce an AP site, which is then subject to reaction with the introduced lysine at postion 120. Enzyme-catalyzed β-elimination of the Schiff base intermediate results in strand scission. (B) In this case, S120K MutY may utilize a mechanism similar to classical bifunctional glycosylases (Figure 1) wherein the introduced lysine 120 serves as the nucleophile in the displacement of the adenine base, as well as facilitating the β-elimination via formation of the Schiff base intermediate. Notably, in the WT enzyme, formation of a Schiff base intermediate with K142 has been detected, though this does not lead to β-elimination as is observed with the introduced lysine in the S120K mutated enzyme. In both mechanisms, additional residues, such as Asp138, may be involved in activating K120 toward reaction with the A or the AP-site.

constants between WT and K142A MutY, we have previously suggested that the covalent intermediates observed via sodium borohydride reduction with WT MutY are due to trapping of a transient intermediate formed by the reaction of MutY with the AP site product. Since AP site-containing DNA can be cross-linked with S120K and S120K:K142A MutY, it is apparent that Lys 120 can react with a AP site, and therefore the glycosylase and lyase reactions could occur as distinct reactions. Presently, the relative importance of the nucleophile in the base displacement process for WT MutY is unknown. Indeed, protonation of the adenine by an acidic residue is likely important in promoting the elimination of the adenine base. Consistent with this is the observation that an E37S MutY enzyme is completely inactive, and this residue had been shown to participate in a hydrogen bond with the N-7 of the adenine base in the X-ray structure of MutY with bound adenine (*17*). Thus, the retention of activity of S120K MutY toward adenine removal may be a result of a mechanism that activates the base for departure, rather than catalyzing nucleophilic displacement. Discerning whether the reaction of WT and S120K proceeds via an S_N1- or S_N2-like mechanism will require a detailed investigation of the mechanism utilizing measurements of kinetic isotope effects.

The presence of multiple bands in SDS−PAGE resolution of sodium borohydride-mediated MutY−DNA cross-links (Figure 5) reveals additional insight as to the nature of the covalent intermediates formed between S120K and WT MutY with substrate DNA. In the case where the OG:A duplex is labeled on the OG-containing strand, two distinct bands are observed with S120K MutY, while only one band is observed with the S120K:K142A mutant form and WT MutY. Since the OG strand is labeled, the observed bands must be due to incomplete denaturation from the covalently bound A strand, and the variances in the mobility of these shifted bands or the appearance of multiple bands (i.e., in the case of S120K MutY) may therefore be due to the fact that the A-containing strand is subject to cleavage. In the reactions performed with A-containing strand-labeled OG:A duplex, an additional band is present likely due to visualization of the single-stranded A strand covalently bound to MutY. These data indicate that the sodium borohydride reduction may intercept either one of the two proposed separate iminium ion intermediates which appear both before and after β-elimination, with the same enzyme-derived amine group covalently bound. It is possible that lysines at positions 120 and 142 are able to compete for the formation of a transient imine species at the AP site, and therefore covalent adduct formation with intact or cleaved DNA is dictated by the specific lysine group which happens to come into sufficient proximity to the AP site first. This notion is supported by the fact that only the slower migrating band of the two bands appears in the case of WT MutY, and only the faster migrating band is apparent in the reaction carried out with the S120K:K142A double mutant.

A contribution to the reduced glycosylase activity of S120K may also be impaired recognition of the substrate as revealed by the reduced binding affinity for the OG:R and OG:FA substrate analogues relative to the WT enzyme. Assuming that the results with the substrate analogues are revealing features important for recognition of the substrate, nonoptimal positioning of the OG:A substrate may translate into reduced rates for adenine removal by S120K. The dramatic decrease in the rate for the processing of G:A mispairs by S120K may attest to the fact that the G:A base pair is structurally less defined than OG:A, and has been observed to adopt a variety of conformations. In contrast, the predominance of the 8-keto tautomer of OG provides a hydrogen bond donor at N-7, allowing for a very stable, well-defined pairing of OG in the syn conformation with A in the anti conformation. Impaired recognition of G:A base-

pairs by S120K MutY is also reflected in the reduced affinity of S120K for the G:F duplex relative to that for the OG:F duplex, and relative to the WT enzyme. The modification of the active site by the introduction of the lysine may be more adversely affecting recognition of G:A base pairs than OG:A base pairs.

A curious consequence of the incorporation of a lysine residue at position 120 in MutY is the extremely tight binding affinity with the OG:F-containing duplex. The relevant dissociation constants indicate that this is a factor of the presence of both OG and the substrate analogue F. This may be due to the unusual structural features of F, namely, the C-glycosidic linkage and the unusual pyrazole ring (Figure 8). For example, the pyrazole ring may be able to serve as an additional locus for hydrogen bonding interactions with the S120K enzyme. Additionally, crystallographic studies of the nucleoside formycin and its derivatives have shown that the C-glycosyl torsion angle was observed to be predominantly in the unusual syn conformation (*39*−*41*). The preference for the syn conformation of F may translate into a less stable double-helical structure which requires the anti conformation of the F in a base pair with OG in the syn conformation. The reduced stability and the unusual conformational properties of the OG:F base pair may provide for more facile recognition of this base pair by S120K, perhaps by facilitating nucleotide flipping of the F and/or the OG.

One of the other unusual consequences of the serine-to-lysine conversion is the dramatic decrease in k_3 values for the S120K and S120K:K142A enzymes with G:A substrates. Indeed, these values were too low to be accurately measured. In contrast, the k_3 values measured with the OG:A substrate are similar to those obtained with the WT enzyme. We have previously suggested that the k_3 rate constants are reflective of the rate of release of MutY from the DNA product, and direct measurements of the MutY−product dissociation rates are consistent with this proposal (*27*, *28*; Porello, S. L., and David, S. S., unpublished results). In the case of S120K and S120K:K142A with G:A substrates, the formation of the covalent Schiff base intermediate during the process of the base removal may slow the release of MutY from the DNA product. In particular, the Schiff base intermediate present after β-elimination (Figure 1B) may be slowly hydrolyzed to regenerate the free enzyme. In the case of OG:A substrates, the presence of OG-specific interactions may be dominating the product release rate. In addition, since the reaction of S120K MutY with the OG:A substrate is considerably faster, the formation of the covalent intermediate and regeneration of the enzyme may not perturb the k_3 value.

The insights gained from these studies of S120K MutY, in combination with those of Y126K TDG, may begin to yield clues about the active site environments of enzymes belonging to the BER superfamily, and to uncover more trends particular to either bifunctional or monofunctional enzymes. In addition, it will be interesting to explore how each damage-specific BER enzyme fits into the entire multienzyme repair pathway of a given organism, and to address the question of why some enzymes are responsible for DNA cleavage at the AP site while others are not, despite the similarity in overall active site geometries. The presence of monofunctional or bifunctional activity in a given BER enzyme is likely to be important for its proper fit in the specific repair pathway in which it participates. Indeed, the high degree of conservation of sequence and AP lyase activity between BER enzymes from different organisms, in conjunction with information about active site catalysis, makes a strong argument against the notion that the existence or absence of an associated AP lyase activity might be of little consequence to the full base excision repair process. Therefore, an intriguing question is how monofunctional and bifunctional activities are placed with regard to the larger scheme of cellular DNA repair addressed by BER. For instance, is there a correlation between bifunctional glycosylase/AP lyases and DNA lesions arising from certain types of stress? Alternatively a correlation may lie more with downstream AP endonuclease activity, in the efficiency of recruitment, or as an aid in AP endonuclease catalysis. If such correlations exist, their discovery would provide significant insight into the role each BER enzyme plays in its particular multienzyme DNA repair pathway.

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