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Formation of a Schiff Base Intermediate Is Not Required for the Adenine Glycosylase Activity of *Escherichia coli* MutY†

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ABSTRACT: The *mutY* gene product of *Escherichia coli* is a 39-kDa protein that catalyzes the removal of adenine bases mispaired with 2′-deoxyguanosine and 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG) in DNA. Although adenine removal proceeds via monofunctional glycosylase activity, MutY is able to form covalent adducts with substrate DNA in the presence of borohydride, a trait otherwise known to be associated only with enzymes having bifunctional glycosylase/AP lyase activity. To help identify active site residues involved in the formation of MutY−DNA adducts in the presence of borohydride, a series of site-directed mutant forms of MutY were generated. Our data show that Lys 142 is the primary residue involved in cross-link formation. The absence of Lys 142 results in near elimination of the enzyme−DNA adducts formed relative to wild-type, suggesting that this residue is the primary one involved in forming covalent associations with DNA during MutY catalysis. Importantly, the enzymatic activity and DNA binding of the K142A enzyme is nearly identical to the WT enzyme. This shows that formation of the covalent intermediate is not required for adenine removal by MutY. Furthermore, this suggests that the covalent intermediate is formed by reaction of Lys 142 with the OG/G:(AP site) product, and this may be a consequence of MutY's unusually high affinity for the product of its glycosylase action.

The DNA of every organism is subject to damage from a variety of exogenous and cellular sources (*1*). To help counteract this threat to the genome's informational content, several different DNA repair pathway types have evolved. Crucial to many such pathways is the process of base excision repair (BER),[1] where damaged or inappropriate DNA bases are removed by enzymatic cleavage of the C1′-N glycosyl bond, to yield an apurinic/apyrimidinic (AP) site. A wide variety of BER glycosylases have been characterized (*2*). The action of damage-specific glycosylases in conjunction with AP endonucleases, DNA polymerase, and DNA ligase results in complete restoration of the DNA (*2*). The *Escherichia coli* enzyme MutY is unique among BER glycosylases in catalyzing the removal of a normal adenine base when mispaired with the damaged base, 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG), or G (*3*). The damaged and mismatched base pair, OG:A, arises from errors during DNA replication; therefore, the activity of MutY prevents deleterious G:C → T:A transversion mutations. Indeed, a complex pathway referred to as the GO repair pathway (*3*, *4*), which involves MutY in cooperation with MutM and MutT, has evolved in *E. coli* to prevent mutations caused by OG. MutM (also known as FPG) is an OG glycosylase that removes OG when mispaired with C, while MutT prevents the misincorporation of d(OG)TP into nascent DNA strands by catalyzing its hydrolysis to d(OG)MP (*4*, *5*). Similar pathways have also been uncovered in mammalian systems (*6*−*8*).

Generally, BER enzymes can be classified as either monofunctional glycosylases or bifunctional glycosylase/AP lyases (*2*, *9*). The monofunctional enzymes catalyze C1′-N glycosyl bond cleavage to produce AP sites. Such sites render the phosphodiester backbone subject to strand scission via base-catalyzed β-elimination; therefore, the activity of these enzymes can be monitored by treatment with base and PAGE analysis of the resulting DNA fragments. The bifunctional glycosylase/AP lyases generate AP sites with an associated enzyme-catalyzed β-elimination, providing strand cleavage concomitant with base excision. The bifunctional enzymes employ an active site amine to displace the base, thus forming a transient Schiff base covalent DNA−enzyme intermediate, which can be reduced to form a stable adduct in the presence of sodium borohydride, as shown in Figure 1(*9*). In the case of T4-endonuclease V, for example, the nucleophilic amine

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[1] Abbreviations: AP, apurinic/apyrimidinic; BER, base excision repair; *E. coli*, *Escherichia coli;* EDTA, ethylenediaminetetraacetic acid; F, 2′-deoxyformycin A; HhH, helix−hairpin−helix; OG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TBE, Tris−borate−EDTA; WT, wild-type. Standard abbreviation for mutated forms: K142A, lysine at position 142 substituted with alanine.

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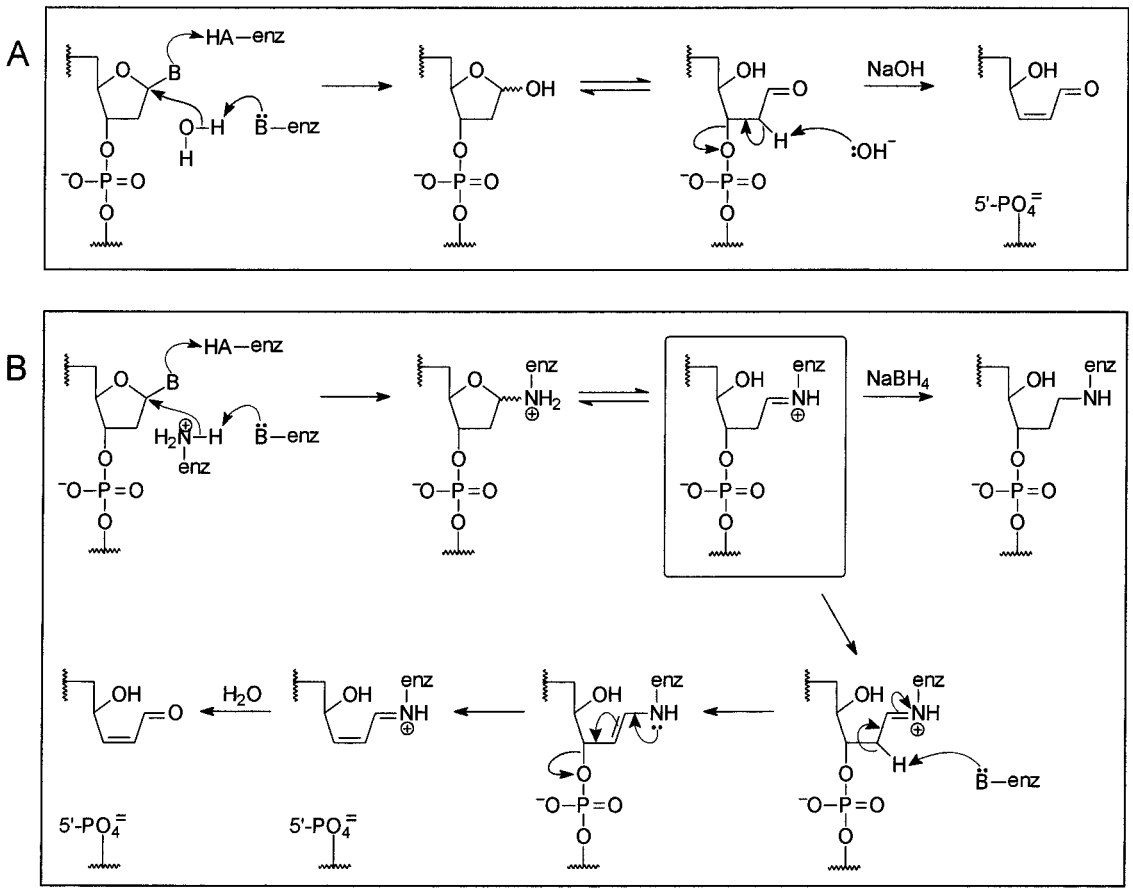

FIGURE 1: Proposed catalytic mechanisms for BER enzymes (*9*). (A) Proposed mechanism for monofunctional glycosylases. Nucleophilic attack at C1′ by an activated water molecule leads to base excision, facilitated by protonation of the base, generating an AP site. The exogenous addition of base leads to 2′ proton abstraction and subsequent DNA strand scission. (B) Proposed mechanism for bifunctional glycosylase/AP lyases. Base excision is brought about by nucleophilic attack at C1′ by an active site amine, forming a Schiff base intermediate that can be reduced by the addition of borohydride to form stable DNA−enzyme adducts. Abstraction of the 2′ proton and subsequent hydrolysis of the covalent intermediate leads to DNA strand scission.

that participates in base displacement and Schiff base formation has been found to be the N-terminus of the protein (*10*). Two experimental techniques are usually performed to distinguish between the two enzyme classes (*9*). The first technique involves determining whether there is a difference in the amount of DNA strand scission in the presence or absence of added base. The second technique involves testing for the intermediacy of a Schiff base formed by an active site amine, by intercepting this intermediate via borohydride reduction to form a stable covalent protein−DNA complex that can be detected by denaturing PAGE.

In the case of MutY, there has been some controversy regarding the activity it harbors since its discovery in 1989 (*11*). Several published works have reported simple glycosylase activity for MutY (*3, 4, 12*−*14*) while there exists an equally impressive number of reports of bifunctional glycosylase/AP lyase activity (*15*−*20*). This is due to the fact that MutY represents a departure from the trends observed with other members of the BER superfamily of glycosylases, since it is a monofunctional glycosylase that can nevertheless form covalent adducts with substrate DNA upon treatment with sodium borohydride (*21, 22*). To help explain this unusual behavior, we previously proposed two possible reaction pathways for MutY (Figure 2). In one model, the original nucleophilic attack at C1′ could be performed by an activated water molecule (or non-amine residue) to form the AP site. The AP site C1′ atom is then subject to a second nucleophilic attack by a nearby amine-containing residue to form the Schiff base intermediate. Alternatively, as shown in Figure 2B, the iminium ion may be initially generated in the base removal step and then subject to hydrolysis to yield the AP site. Both pathways explain how borohydride-mediated cross-linking might occur in a manner dependent upon glycosylase activity, yet disfavoring strand cleavage. Presumably the active site of MutY would lack the required basic residue necessary for catalyzing the β-elimination of the Schiff base intermediate; therefore, significant amounts of strand scission are not observed.

To further clarify the mechanism of MutY, targeted mutagenesis of five lysine residues at positions 16, 132, 142, 157, and 158 was performed for the purpose of locating the residue (or residues) involved in forming transient covalent intermediates with substrate DNA. These candidate side chains present in MutY's active site were replaced with alanine (glycine in the case of Lys 157). Kinetic and DNA binding experiments were performed on the five mutated forms to determine if any of the mutations significantly altered the ability to bind DNA and to remove adenine residues in mispair with OG and G. In addition, experiments using both $NaBH_4$ and $NaCNBH_3$ as reducing agents were conducted to determine any variance in the efficiency of MutY−DNA adduct formation as a function of the absence

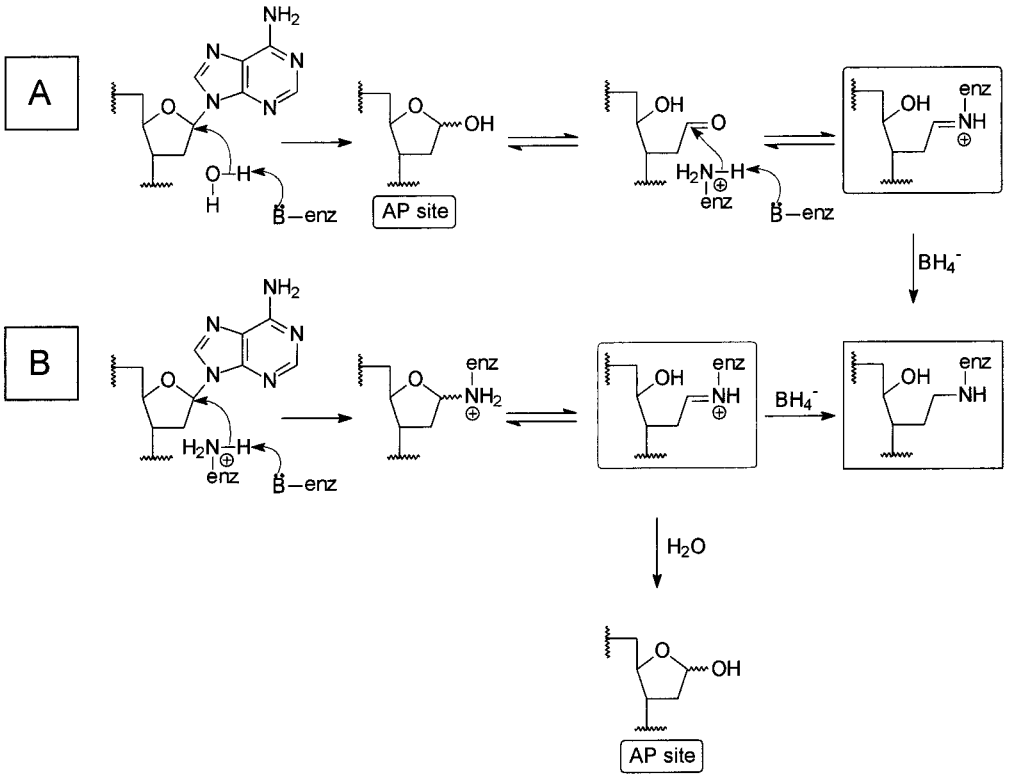

FIGURE 2: Possible active site chemistry for MutY, as outlined earlier (*21*). (A) An abasic site is formed from nucleophilic attack by an activated water molecule. The ring-opened tautomer may be subject to a second attack at C1′ by an active site amine to produce the reducible Schiff base species. (B) Initial attack at C1′ by an active site amine to yield the Schiff base intermediate. The active site of MutY may favor subsequent hydrolysis over β-elimination to produce an AP site without strand scission.

of a specific active site lysine group. These results show that Lys 142 is the primary residue involved in formation of the Schiff base intermediate, since little borohydride-mediated trapping is observed with the K142A mutated form. However, the enzymatic activity and DNA binding of the K142A enzyme as well as the ability to prevent DNA mutations in vivo is essentially identical to the WT enzyme. This indicates that formation of the Schiff base intermediate likely occurs after removal of the mispaired adenine and may be a consequence of MutY's high affinity for the product of its glycosylase activity.

MATERIALS AND METHODS

Materials. The plasmid containing the *mutY* gene, pKKYE-co as well as the *E. coli* strains JM101 *mutY*- and GT100 *mutY-mutM*- were kindly provided by M. Michaels and J. H. Miller (*4*, *23*, *24*). 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. 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. $NaBH_4$ and $NaCNBH_3$ were 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 Site-Directed Mutated Forms of MutY. Site-directed mutagenesis, isolation, and purification of the mutated MutY forms were performed as described (*24*). The percent active enzyme out of total purified MutY protein for each mutated enzyme preparation was determined using standard active site titration methods modified specifically for MutY (*25*). The percent activities were determined using the method of Bradford (Bio-Rad) to assay the total protein concentration in each enzyme preparation (*26*). The values for the various mutated forms were as follows: WT, 37%; K16A, 40%; K132A, 82%; K142A, 24%; K157G, 76%; K158A, 68%. The in vivo activity of the mutated enzymes was determined in a manner analogous to that reported previously using a rifampicin reversion assay (*24*).

Substrate Preparation. Following the automated synthesis and purification of the single-stranded oligonucleotides, duplex mispair-containing substrates were formed by allowing two molar equivalents 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 4 °C overnight. The following 30 base pair sequence was used: (5′-CGATCATGGAGC-CAC**X**AGCTCCCGTTACAG-3′)·(3′-GCTAGTACCTCG-GTG**Y**TCGAGGGCAATGTC-5′)* where X = G or OG and Y = A or F (where F = 2′-deoxyformycin A). The asterisk (*) indicates the position of the ^{32}P label on the duplex substrate.

Dissociation Constant Determination. Equilibrium dissociation constants (K_d) toward DNA duplexes containing a substrate analogue OG:F mispair resistant to catalytic turnover were determined for each mutated form, as de-

scribed previously (*14*). The K_d values have been corrected for active enzyme concentration in each of the mutated form preparations used. In all experiments, the final DNA concentration was no greater than 20 pM.

Glycosylase/AP Lyase Assays. Reactions were conducted similarly to those described previously (*21*). The reactions were performed with DNA concentrations of approximately 10 nM and enzyme concentrations of 800 nM (as determined by Bradford) in Tris-HCl buffer (20 mM, pH 7.6) containing 30 mM NaCl and 10 mM EDTA. Glycosylase assays were also performed using sodium phosphate buffer (25 mM) instead of Tris-HCl at pH values ranging from 5 to 9. It should be noted that the actual DNA concentration may have been lower due to any loss during the post-labeling purification steps. All reactions were performed at 37 °C, and after incubation for 5 min, two separate 5-μL aliquots were taken from each enzyme reaction. 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 cylanol, and 0.025% bromophenol blue in TBE buffer) was subsequently added to all aliquots. Following heat denaturation (2 min), samples were loaded onto 15% denaturing polyacrylamide gels 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 Reduced Enzyme−DNA Intermediates. These experiments were conducted in a manner similar to those previously described (*21*). The reactions were performed with substrate DNA duplex concentration of 10 nM and enzyme concentrations of 300 nM (as determined by the Bradford method) in a sodium phosphate buffer (25 mM, pH 6.8) containing 1 mM EDTA and 0.1 mg/mL BSA. $NaBH_4$ or $NaCNBH_3$ concentrations were kept constant at 90 mM, and NaCl was added to each reaction such that the Na^+ 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. A series of reactions were also performed using the same buffer conditions at pH values ranging from 5 to 9. An equal volume 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 solution was heat denatured prior to loading 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.

RESULTS

Choice of Residues for Site-Directed Mutagenesis. Candidate lysine residues that may participate in Schiff base formation were identified by examination of the X-ray crystallographic structures of MutY's catalytic core (Met 1-Lys 225) with and without bound adenine (*27*). Based upon this information, lysines 132, 142, 157, and 158 were chosen for mutagenesis, as they appeared to be the only groups within a reasonable distance from the presumed location of active site catalysis and therefore the ones having the greatest likelihood of being involved in forming transient covalent associations with substrate DNA. In addition, lysine 16 was targeted for mutagenesis, even though it appears to not be located within the active site cleft to determine if manipulations of lysine residues outside of the active site have any effects on the parameters measured. A rendering of the catalytic N-terminal domain of MutY is shown in Figure 3, with active site lysine side chains highlighted to illustrate their positioning according to the enzyme's crystal structure coordinates.

MutY mutated forms were generated using a standard two-step PCR methodology, the details of which have been described previously (*24*). The isolation and purification in each case required no adjustment of this laboratory's protocol used for wild-type MutY (*14*).

Glycosylase Activity Assays. To assess whether the replacement of the targeted lysine residues resulted in loss of the ability to remove adenine opposite G or OG, the glycosylase activity was monitored under single turnover conditions {[MutY] > [DNA]} in a manner analogous to that previously published (*25*). As shown in Figure 4, each mutated enzyme form was able to process OG:A mispair-containing DNA to a quantitative yield of cleaved product under single turnover conditions. Reactions in each case were complete within 1 min as determined by time-course assays performed under identical conditions (data not shown). Furthermore, full conversion to product was observed only upon base treatment of reaction aliquots. Without base treatment, no increase in DNA strand cleavage was observed relative to reactions with the WT enzyme, where a very small amount (<5%) of cleaved DNA is observed due to the lability of the AP site. These results demonstrate that all five mutated forms behave like wild-type enzyme in their catalytic activity; AP site generation is not followed by enzyme-mediated DNA strand scission. In addition, the adenine glycosylase reaction of all of the mutated enzymes as well as the WT enzyme is complete within 1 min from pH 5 to pH 9 (data not shown), indicating that the Lys substitutions have not caused any major perturbations in the enzymatic activity. Identical results were observed for substrate duplexes containing G:A mispairs as well (data not shown).

Under multiple turnover conditions {[MutY] < [DNA]}, the WT enzyme has been shown to exhibit biphasic behavior with OG:A substrates, which is characterized by an exponential burst of product formation followed by a slow linear steady-state phase (*25*). This slow turnover arises from slow product release, such that product release is a rate-limiting step. Each mutated form displayed similar biphasic behavior toward OG:A substrates (data not shown). Therefore, this mispair substrate was useful in determining percent active enzyme in each case, requiring no alteration of the active site titration method used for the WT enzyme (*25*). The percent active enzyme for the five mutated protein preparations ranged from 24 to 82% and compared well with the range of active site concentrations that we have observed with different preparations of WT MutY.

In Vivo Activity. The ability of MutY to prevent DNA mutations can be evaluated using rifampicin, which blocks bacterial RNA polymerases (*4, 28*). The absence of MutY activity leads to a high mutation frequency, resulting in modification of the rifampicin binding region of RNA polymerase. Such modifications can provide resistance to rifampicin; therefore, the mutation rate can be judged by the

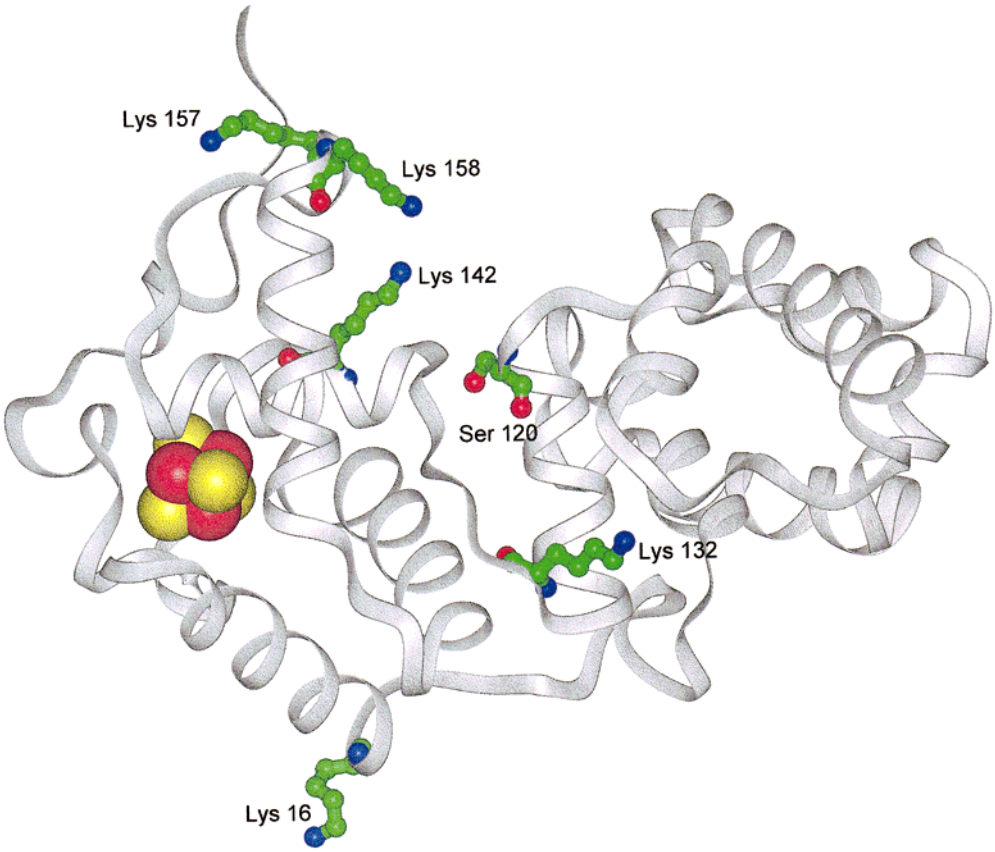

FIGURE 3: X-ray crystallographic structure of MutY generated using coordinates provided by the authors (*27*). The $[4Fe\text{-}4S]^{2+}$ cluster is in space-filling representation, and the active site lysine residues targeted for mutagenesis in this work are highlighted. Additionally, Ser 120 is highlighted since this residue corresponds to the position of a lysine in members of the BER superfamily that are bifunctional glycosylase/lyase enzymes (*32*).

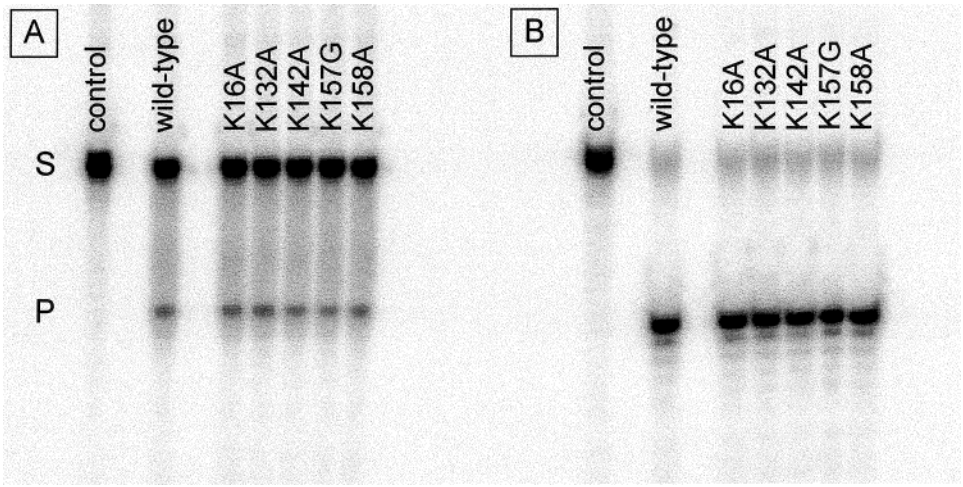

FIGURE 4: Storage phosphor autoradiogram of denaturing polyacrylamide gel upon which 14-nucleotide product DNA strands were separated from the 30-nucleotide substrate strand. Centralized OG:A mispairs in duplex DNA were allowed to react with an approximate 80-fold molar enzyme excess based on the Bradford concentration, in Tris-HCl buffer (20 mM, pH 7.6) containing 30 mM NaCl and 10 mM EDTA. Bands representative of the substrate and product bands are indicated by S and P, respectively. Nonenzyme control lanes are indicated, as are the lanes for different enzyme preparations. (A) Reactions were quenched in dry ice after incubation, with no base treatment. (B) Reactions were quenched by addition of base to a final concentration of 0.1 M NaOH and heated at 90 °C for 5 min before cooling to −78 °C.

number of rifampicin revertant (Rif^r) colonies (4). Using an *E. coli* strain lacking both *mutY* and *mutM* (GT100 *mutY-mutM-*) transformed with a plasmid containing the WT *mutY* gene, the number of Rif^r colonies was small (1 ± 1 per 10^8 cells). In contrast, in analogous experiments performed using a plasmid lacking the *mutY* gene, a large number of Rif^r colonies were observed (1000 ± 100 per 10^8 cells) due to the higher mutation frequency in the absence of MutY. In the corresponding experiments utilizing transformation with a plasmid containing the lysine mutations, small numbers of Rif^r colonies were observed (ranging from 0 to 2 per 10^8 cells). These results are essentially identical to the results

Table 1: Dissociation Constants of the MutY Mutants toward OG:F Mispair-Containing Duplexes

enzyme	K_d (nM) OG:F	enzyme	K_d (nM) OG:F
WT	0.3 ± 0.2	K142A	0.5 ± 0.2
K16A	1.2 ± 0.5	K157G	9.1 ± 2.0
K132A	3.3 ± 0.6	K158A	7.5 ± 1.5

[a] Where F = 2′-deoxyformycin A. K_d values were corrected for percent active enzyme in each preparation.

obtained with the WT enzyme and demonstrate that the series of lysine mutated enzymes retain the ability to suppress DNA mutations in vivo.

Equilibrium Dissociation Constants. Previously published work in our laboratory has shown 2′-deoxyformycin A (F) to be an effective substrate mimic of 2′-deoxyadenosine when base paired with both G and OG in duplex DNA (*14*). Because the C1′-N glycosyl bond has been replaced by a C1′-C glycosyl bond in the case of F, this A analogue is resistant to glycosylase activity. Since MutY binds with high affinity and specificity to OG:F base pairs, the use of F for dissociation constant determinations is quite useful, since the problem of substrate conversion to product can be disregarded.

Dissociation constants of the lysine mutated forms with OG:F mispairs contained within the 30-base pair sequence described above were found to be similar to that for WT MutY, in the case of most mutated forms generated (Table 1). Notable exceptions were K157G and K158A mutated forms that exhibited K_d values of 9.1 and 7.5 nM, respectively. These values are slightly larger than those determined for K16A, K132A, K142A, and wild-type, suggesting that lysines 157 and 158 may be important for electrostatic interactions with DNA. Indeed, their exposed orientation at the edge of the proposed DNA binding groove in the X-ray crystal structure supports this notion (Figure 3) (*27*). Importantly, these dissociation constant values demonstrate the high affinity each mutated form has for the OG:F mispair, and indeed, it is clear that these small changes in binding constant values do not appreciably affect the adenine glycosylase activity of the mutated enzymes under single-turnover conditions (Figure 4).

Formation of Covalent Enzyme−Substrate Adducts by Borohydride Reduction. Results of the borohydride reduction experiments indicate that all mutated forms except the Lys 142 mutant generate covalent enzyme−substrate adducts with similar efficiency as WT MutY, as shown in Figure 5. The amount of covalent complex observed for the K16A, K132A, K157G, and K158A forms was 40%, 64%, 66% and 62%, respectively, which is nearly identical to the amount of MutY-DNA cross-link observed with the WT enzyme (63%). Experiments designed to assay for adduct formation as a function of pH, time, and borohydride concentration all demonstrate the behavior of K16A, K132A, K157G, and K158A to be similar to the wild-type (data not shown). K142A, however, showed a dramatic decrease in the amount of enzyme−DNA cross-links with both OG:A (<2%) and G:A (<2%) mispairs, despite having the ability to bind DNA and to catalyze AP site formation to the same extent as WT MutY. This implies the involvement of Lys 142 in generating a covalent contact with substrate DNA, apparently in a manner mechanistically decoupled from glycosylase activity.

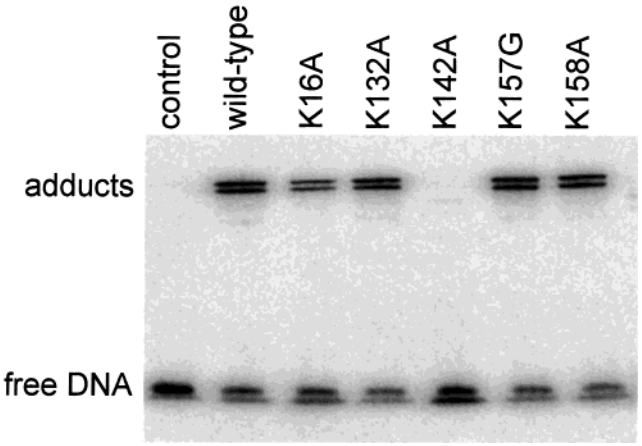

FIGURE 5: Storage phosphor autoradiogram of SDS−PAGE resolution of free OG:A mispair-containing DNA from that covalently cross-linked to enzyme via reduction with sodium borohydride. Adducted and free DNA bands are indicated, as are lanes corresponding to the different enzyme preparations used. The presence of two bands for the protein−DNA complex is possibly due to incomplete denaturation of the DNA duplex with the SDS−PAGE loading buffer.

In addition to sodium borohydride, sodium cyanoborohydride was used to generate enzyme−DNA cross-links under similar conditions (data not shown). Since $NaCNBH_3$ is a milder reducing agent than $NaBH_4$, the percent cross-linked DNA is significantly less with this reagent in each case. Whereas a maximum of 65% of the DNA present in the $NaBH_4$ reactions was irreversibly bound to all enzyme forms except K142A, only 6% was attained in the case of $NaCNBH_3$. Interestingly, in the case of K142A, about 1−2% of the DNA present is covalently bound to the enzyme, regardless of the reducing agent used. The small amount of covalent DNA adducts observed with K142A suggests the ability of another active site group to substitute the cross-linking behavior of Lys 142, albeit to a very small extent.

DISCUSSION

The results reported herein implicate lysine 142 as the primary residue involved in generating covalent adducts with substrate DNA under reducing conditions. Interestingly, replacing lysine for alanine at this position hinders neither substrate binding nor catalysis toward G:A and OG:A mispairs. Therefore, it seems clear that glycosylase catalysis is not dependent upon the formation of stable adducts between DNA and Lys 142. However, cross-link formation is a process dependent upon having active enzyme and substrate DNA in the presence of borohydride. Duplex oligonucleotides containing a normal G:C base pair did not produce covalent adducts (data not shown). Additionally, a catalytically inactive form of MutY (D138N), which binds substrate DNA with high affinity, cannot form covalent adducts in the presence of borohydride, confirming the requirement for catalytic activity in the generation of MutY-DNA cross-links (*29*). In similar experiments with $NaBH_4$, MutY was found to cross-link to product DNA (OG residues opposite AP sites) with only 2-fold less efficiency than with OG:A mispairs (*22*), which suggests that adduct formation may occur after base excision and not during the process of base excision.

The involvement of Lys 142 in generating Schiff base intermediates with substrate DNA is also supported by peptide sequencing data obtained from borohydride-generated MutY−DNA cross-links(22) and structural data obtained by X-ray crystallography (*27*). In work by Zharkov and Grollman, the phenylthiohydantoin (PTH) derivative of Lys 142 was consistently missing from sequencing analysis of borohydride-generated DNA cross-links with three different peptide regions resulting from trypsin proteolysis, implicating this residue in cross-link formation (*22*). The PTH derivative of Lys 132 was also missing, but only in peptide fragments stemming from longer proteolysis incubations. Since this derivative's identification was also consistent with four other possible amino acids, the authors were unable to confirm Lys 132 as an additional Schiff base-forming functionality.

Stable MutY−substrate adducts can be generated using a method alternative to borohydride reduction. $Na_2Ir(Cl)_6$ is a single-electron oxidant that has been shown to selectively oxidize OG in duplex DNA over undamaged nucleobases (*30*). Oxidation of OG by this reagent in the presence of MutY produces stable enzyme−DNA adducts, and such adducts are not formed in the presence of the K142A mutated form (*31*). The chemical nature of the covalent link between MutY and OG:A mispairs that forms upon $Na_2Ir(Cl)_6$ treatment is likely to be different from the one formed upon borohydride reduction but nevertheless supports the idea that Lys 142 lies close to the OG:A mispair upon MutY binding to this substrate.

With its inability to form cross-links with DNA and the fact that the K142A mutant's activity remains unhindered relative to wild-type MutY, more information has been gained regarding MutY's catalytic pathway. In earlier work with WT MutY, two hypothetical pathways were proposed, both of which illustrate how AP site generation might be separated from Schiff base formation with substrate during MutY catalysis (Figure 2) (*21*). This work supports route A, where AP site formation occurs before a transient covalent intermediate with DNA is generated. If a pathway resembling route B were taking place in MutY's active site, one would expect glycosylase activity to be compromised when Lys 142 is replaced with alanine, which is not the case. The data presented herein suggest that the initial nucleophilic attack at C1′ in MutY catalysis is by a non-amine nucleophile and not by an active site lysine. Therefore, even though MutY displays catalytic intermediates characteristic of bifunctional glycosylase/AP lyases, initial AP site generation most likely occurs in a manner consistent with the trend of other known monofunctional DNA repair glycosylases.

This conclusion is supported by recent data acquired for other enzymes, such as *E. coli* endonuclease III, DNA polymerase β, and the human DNA repair OG glycosylase (hOGG1). These three enzymes possess a DNA binding structural motif common to members of the BER superfamily of enzymes known as a helix−hairpin−helix (HhH) domain (*32*). A trend among HhH-containing enzymes that have an associated β-lyase activity is a conserved lysine residue at the N-terminal end of the second (C-terminal) helix in the HhH bundle. Lysine 72 in DNA polymerase β (*33*), Lys 120 in endonuclease III (*34*), and Lys 249 in hOGG1 (*35*) all correspond to this position in the HhH domain. In each case, glycosylase activity was found to be greatly reduced upon removal of the lysine by targeted mutagenesis. The corresponding position 120 in the HhH domain of MutY is a serine residue (*36*). The lysine-rich active site region of MutY is able to form covalent associations with substrate DNA but may require lysine functionality at position 120 to behave as a bifunctional glycosylase/AP lyase. Another enzyme similar to MutY is thymine DNA mismatch glycosylase (TDG), a monofunctional glycosylase from *Methanobacterium thermoformicicum*, which has a tyrosine in the position equivalent to Lys 120 in endonuclease III and Ser 120 in MutY (*37*). Conversion of this tyrosine residue to lysine abolished glycosylase activity for TDG but resulted in AP lyase activity (*37*). This illustrates that conversion of a glycosylase to a bifunctional glycosylase/AP lyase may require more than one amino acid substitution.

It stands to reason that MutY may have evolved to have such high affinity for its product DNA upon consideration of MutY's role in the GO repair pathway (*4*, *25*). Protection of the unstable OG:(AP site) from spontaneous hydrolysis or MutM activity is crucial for the prevention of double strand breaks in the genome. The high affinity of MutY for the OG:(AP site) product may facilitate the formation of the imine intermediate with the abasic site. Subsequently, the tight association with the product may well be further stabilized by the covalent associations made by Lys 142. An important question that remains is the biological relevance for formation of the covalent intermediate in MutY's processing of OG:A mismatches. The observation that the in vivo activity of the K142A enzyme is identical to the WT enzyme suggests that formation of the covalent intermediate is not required for the prevention of DNA mutations; although it should be noted that this in vivo assay may not be sensitive to slight variations of the functional properties of MutY. It is possible that the formation of the covalent complex is a side reaction that occurs in vitro as a consequence of the tight association of MutY with the product OG:(AP site). As discussed herein, we have seen no major alterations in the adenine glycosylase activity of the K142A enzyme; however, a more detailed kinetic investigation is in progress.

MutY is not the only enzyme with such apparent complexities in the active site. The K57G mutated form of *E. coli* MutM and the K72A mutated form of vertebrate DNA polymerase β were both shown to have a decrease in ability to form borohydride-mediated adducts with DNA relative to wild-type. In the case of MutM, catalytic efficiency for OG removal dropped dramatically with the K57G mutation (*38*), yet previous work with this enzyme implicated the N-terminal proline residue as the one responsible for nucleophilic attack at C1′ in base excision (*39*, *40*). In one report of the K72A mutation made in DNA polymerase β, the efficiency of DNA adduct formation with borohydride was 30% that of wild-type in conditions of molar enzyme excess. It was suggested that the significant amount of adducts that formed in the absence of Lys 72 were generated from other neighboring lysine residues in the active site region (*33*).

Even though it seems that Lys 142 is the only group participating in generating covalent associations with substrate DNA, the possibility remains that other active site lysine groups are taking part in MutY's catalytic process, either in covalent or noncovalent associations with intermediate species in the enzyme's reaction mechanism. Therefore, our laboratory is conducting a detailed kinetic analysis of all the

targeted lysine mutated forms to determine if there are any subtle differences in the enzymatic properties relative to wild-type that are not detectable by the experiments outlined here.

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REFERENCES

1. Lindahl, T. (1993) *Nature 362*, 709−715.
2. David, S. S., and Williams, S. D. (1998) *Chem. Rev. 98*, 1221−1261.
3. Michaels, M. L., Tchou, J., Grollman, A. P., and Miller, J. H. (1992) *Biochemistry 31*, 10964−10968.
4. Michaels, M. L., Cruz, C., Grollman, A. P., and Miller, J. H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7022−7025.
5. Tajiri, T., Maki, H., and Sekiguchi, M. (1995) *Mutat. Res. 336*, 257−267.
6. Lu, R., Nash, H. M., and Verdine, G. L. (1997) *Curr. Biol. 7*, 397−407.
7. Slupska, M. M., Baikalov, C., Luther, W. M., Chiang, J.-H., Wei, Y.-F., and Miller, J. H. (1996) *J. Bacteriol. 178*, 3885−3892.
8. McGoldrick, J. P., Yeh, Y.-C., Solomon, M., Essigmann, J. M., and Lu, A.-L. (1995) *Mol. Cell. Biol. 15*, 989−996.
9. Dodson, M. L., Michaels, M. L., and Lloyd, S. R. (1994) *J. Biol. Chem. 269*, 32709−32712.
10. Dodson, M. L., Schrock, R. D., and Lloyd, R. S. (1993) *Biochemistry 32*, 8284−8290.
11. Au, K. G., Clark, S., Miller, J. H., and Modrich, P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8877−8881.
12. Sun, B., Latham, K. A., Dodson, M. L., and Lloyd, R. S. (1995) *J. Biol. Chem. 270*, 19501−19508.
13. Bulychev, N. V., Varaprasad, C. V., Dormán, G., Miller, J. H., Eisenberg, M., Grollman, A. P., and Johnson, F. (1996) *Biochemistry 35*, 13147−13156.
14. Porello, S. L., Williams, S. D., Kuhn, H., Michaels, M. L., and David, S. S. (1996) *J. Am. Chem. Soc. 118*, 10684−10692.
15. Lu, A.-L., Tsai-Wu, J.-J., and Cillo, J. (1995) *J. Biol. Chem. 270*, 23582−23588.
16. Manuel, R. C., Czerwinski, E. W., and Lloyd, R. S. (1996) *J. Biol. Chem. 271*, 16218−16226.
17. Manuel, R. C., and Lloyd, R. S. (1997) *Biochemistry 36*, 11140−11152.
18. Lu, A.-L., Yuen, D. S., and Cillo, J. (1996) *J. Biol. Chem. 271*, 24138−24143.
19. Tsai-Wu, J.-J., Liu, H.-F., and Lu, A.-L. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8779−8783.
20. Gogos, A., Cillo, J., Clarke, N. D., and Lu, A.-L. (1996) *Biochemistry 35*, 16665−16671.
21. Williams, S. D., and David, S. S. (1998) *Nucleic Acids Res. 26*, 5123−5133.
22. Zharkov, D. O., and Grollman, A. P. (1998) *Biochemistry 37*, 12384−12394.
23. Cupples, C. G., and Miller, J. H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5345−5349.
24. Golinelli, M.-P., Chmiel, N. H., and David, S. S. (1999) *Biochemistry 38*, 6997−7007.
25. Porello, S. L., Leyes, A. E., and David, S. S. (1998) *Biochemistry 37*, 14756−14764.
26. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
27. Guan, Y., Manuel, R. C., Arvai, A. S., Parikh, S. S., Mol, C. D., Miller, J. H., Lloyd, R. S., and Tainer, J. A. (1998) *Nat. Struct. Biol. 5*, 1058−1064.
28. Wehrli, W., Knusel, F., Schmid, K., and Staehlin, M. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 667−673.
29. Williams, S. D., and David, S. S. Unpublished results.
30. Muller, J. G., Duarte, V., Hickerson, R. P., and Burrows, C. J. (1998) *Nucleic Acids Res. 26*, 2247−2249.
31. Hickerson, R. P., Chepanoske, C. L., Williams, S. D., David, S. S., and Burrows, C. J. (1999) *J. Am. Chem. Soc. 121*, 9901−9902.
32. Nash, H. M., Bruner, S. D., Schärer, O. D., Kawate, T., Addona, T. A., Spooner, E., Lane, W. S., and Verdine, G. L. (1996) *Curr. Biol. 6*, 1230−1233.
33. Matsumoto, Y., Kim, K., Katz, D. S., and Feng, J.-A. (1998) *Biochemistry 37*, 6456−6464.
34. Thayer, M. M., Ahern, H., Xing, D., Cunningham, R. P., and Tainer, J. A. (1995) *EMBO J. 14*, 4108−4120.
35. Nash, H. M., Lu, R., Lane, W. S., and Verdine, G. L. (1997) *Chem. Biol. 4*, 693−702.
36. Michaels, M. L., Pham, L., Nghiem, Y., Cruz, C., and Miller, J. H. (1990) *Nucleic Acids Res. 18*, 3841−3845.
37. Begley, T. J., and Cunningham, R. P. (1999) *Protein Eng. 12*, 333−340.
38. Sidorkina, O. M., and Laval, J. (1998) *Nucleic Acids Res. 26*, 5351−5357.
39. Tchou, J., and Grollman, A. P. (1995) *J. Biol. Chem. 270*, 11671−11677.
40. Zharkov, D. O., Rieger, R. A., Iden, C., R., and Grollman, A. P. (1997) *J. Biol. Chem. 272*, 5335−5342.

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