Insight into the Roles of Tyrosine 82 and Glycine 253 in the *Escherichia coli* Adenine Glycosylase MutY†

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ABSTRACT: The oxidation product of 2′-deoxyguanosine, 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG), produces G:C to T:A transversion mutations. The *Escherichia coli* base excision repair glycosylase MutY plays an important role in preventing OG-associated mutations by removing adenines misincorporated opposite OG lesions during DNA replication. Recently, biallelic mutations in the human MutY homologue (hMYH) have been correlated with the development of colorectal cancer. The two most common mutations correspond to two single amino acid substitutions in the hMYH protein: Y165C and G382D [Al-Tassan, N., et al. (2002) *Nat. Genet. 30*, 227−232]. Previously, our laboratory analyzed the adenine glycosylase activity of the homologous variant *E. coli* MutY enzymes, Y82C and G253D [Chmiel, N. H., et al. (2003) *J. Mol. Biol. 327*, 431−443]. This work demonstrated that both variants have a reduced adenine glycosylase activity and affinity for substrate analogues compared to wild-type MutY. Recent structural work on *Bacillus stearothermophilus* MutY bound to an OG:A mismatch-containing duplex indicates that both residues aid in recognition of OG [Fromme, J. C., et al. (2004) *Nature 427*, 652−656]. To determine the extent with which Tyr 82 and Gly 253 contribute to catalysis of adenine removal by *E. coli* MutY, we made a series of additional modifications in these residues, namely, Y82F, Y82L, and G253A. When the substrate analogue 2′-deoxy-2′-fluoroadenosine (FA) in duplex paired with G or OG is used, both Y82F and G253A showed reduced binding affinity, and G253A was unable to discriminate between OG and G when paired with FA. Additionally, compromised glycosylase activity of Y82F, Y82C, and G253A MutY was observed using the nonoptimal G:A substrate, or at low reaction temperatures. Interestingly, adenine removal from an OG:A-containing DNA substrate by Y82C MutY was also shown to be extremely sensitive to the NaCl concentration. The most surprising result was the remarkably similar activity of Y82L MutY to the WT enzyme under all conditions examined, indicating that a leucine residue may effectively replace tyrosine for intercalation at the OG:A mismatch. The results contained herein provide further insight regarding the intricate roles of Tyr 82 and Gly 253 in the OG recognition and adenine excision functions of MutY.

DNA damage can arise through various sources including ionizing radiation, alkylating agents, reactive oxygen species, and replication errors (*1*). The integrity of DNA is preserved by a variety of DNA repair pathways (*2*). Indeed, DNA damage and its inefficient repair are important contributors to aging and cancer (*3*). The repair of individual base lesions is accomplished primarily through the process of base excision repair (BER).[1] BER is initiated by a DNA glycosylase, which hydrolyzes the *N*-glycosidic bond of the aberrant base, resulting in the formation of an abasic (AP) site (*4*). AP endonucleases then act to cleave the DNA strand 5′ to the AP site, followed by sugar removal by phosphodiesterases and strand synthesis by DNA polymerases. DNA ligases complete the repair by closing the gap in the DNA strand (*2*, *5*, *6*).

One of the most common and well-studied DNA lesions formed under conditions of oxidative stress is 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG) (*1*, *7*, *8*). The problem with OG is that polymerases often misinsert 2′-deoxyadenosine (A) across from the OG lesion (*9*) to form an OG:A mismatch that, if left unrepaired, results in a G:C to T:A transversion mutation upon subsequent replication events (*6*, *7*, *10*). In *Escherichia coli*, the "GO" repair pathway that includes three enzymes, MutT, MutM, and MutY, prevents

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[1] Abbreviations: A, 2′-deoxyadenosine; Abs, absorbance; AP, apurinic/apyrimidinic; APC, adenomatous polyposis coli; BER, base excision repair; BSA, bovine serum albumin; BsMY, *Bacillus stearothermophilus* MutY; C, 2′-deoxycytidine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMSA, eletrophoretic mobility shift assay; endo III, *Escherichia coli* endonuclease III; FA, 2′-deoxy-2′-fluoroadenosine; FPLC, fast protein liquid chromatography; Fpg, *Escherichia coli* formamidopyrimidine-DNA glycosylase; G, 2′-deoxyguanosine; HhH, helix-hairpin-helix; hMYH, human MutY homologue; HPLC, high-performance liquid chromatography; IPTG, isopropyl-*β*-D-thiogalactoside; MTO, multiple-turnover; OG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; ORF, open reading frame; MutY, *E. coli* MutY; PMSF, phenylmethyl-sulfonyl fluoride; STO, single-turnover; T, thymidine; TBE, tris−borate−EDTA; Tris, tris(hydroxymethyl) aminomethane; UDG, uracil-DNA glycosylase; WT, wild-type.

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mutagenicity associated with OG (*11*, *12*). MutT hydrolyzes dOGTP, removing it from the nucleotide pool and preventing incorporation of this damaged nucleotide into DNA by polymerases during replication (*13*, *14*). MutM, also known as Fpg, is a BER glycosylase that excises the OG lesion from OG:C base pairs, while the MutY glycosylase removes adenine from OG:A mispairs. The subsequent enzymes in the BER pathway then act sequentially to restore the appropriate G:C base pair (*6*). Functionally similar enzymes mediating OG repair have been identified in other organisms, including mice and humans (*15*−*18*).

E. coli MutY is a monofunctional DNA glycosylase capable of removing adenine paired with OG, G, and, to a lesser extent, C, from duplex DNA in vitro (*11*, *19*). Pre-steady-state and single-turnover kinetics of this enzyme have been performed extensively by our laboratory (*20*, *21*) and others (*22*, *23*). This work has uncovered that *E. coli* MutY displays a biphasic profile for product formation under conditions of substrate excess due to slow release of the DNA product. Similar behavior has also been observed with other DNA glycosylases, including alkyl adenine glycosylase (AAG) (*24*), the human OG glycosylase (hOGG1) (*25*), and the human G:T mismatch specific glycosylase (MED1) (*26*). Our laboratory also demonstrated that *E. coli* MutY prefers to bind DNA containing OG paired with the substrate analogue 2′-deoxy-2′-fluoroadenosine (FA) with 10-fold greater affinity than G (*27*). The tighter binding to the OG-containing mispair, coupled with increased rate of adenine removal from an OG:A versus a G:A mispair, is consistent with genetic experiments (*28*) that have indicated that OG:A is likely to be the most important substrate for MutY in vivo.

Relatively recent work has provided a direct link between mutations in the human MutY homologue (*hMYH*) gene and colorectal cancer (*29*). In 2002, a British family was studied in which three out of seven siblings were afflicted with symptoms characteristic of Familial Adenomatous Polyposis coli, or FAP, a common form of colorectal cancer (*29*). FAP is usually associated with inherited mutations in the adenomatous polyposis coli (*APC*) gene. Sequencing of the *APC* open reading frame (ORF) demonstrated no inherited defect in *APC*; however, inactivating somatic mutations were observed in the *APC* gene from tumors of afflicted family members. A striking feature of the *APC* mutation spectrum was the large preponderance of G:C to T:A transversions. Since this type of mutation is associated with OG, this suggested that faulty repair of OG may be responsible for the *APC* mutations. The genes of the human homologues of enzymes in the GO pathway were sequenced, revealing two germline mutations in *hMYH* that result in amino acid substitutions in the hMYH protein, Y165C and G382D (*29*). The two altered positions, Tyr 165 and Gly 382, are found to be highly conserved in MutY enzymes across phylogeny (*18*, *30*).

Extensive quantitative in vitro analysis of the *E. coli* enzymes corresponding to the hMYH variants Y165C and G382D (Y82C and G253D, respectively) was previously performed by our laboratory (*31*). This work demonstrated that both mutated enzymes were kinetically compromised compared to WT MutY, particularly Y82C MutY. Moreover, a significant decrease in binding affinity and loss of preference for OG:FA was observed with both Y82C and G253D when compared to WT MutY. The information on the *E. coli* variants supported the hypothesis that the presence of the analogous hMYH variants could result in significantly reduced repair of OG:A mismatches in the *APC* gene and, therefore, provide for mutagenesis and inactivation of APC. Subsequent genetic studies have found that mutations in *hMYH* are more generally correlated with the multiple colorectal adenoma phenotype (*32*−*36*), and this new condition is often referred to as MYH-associated polyposis (MAP). Though the Y165C and G382D variants appear to be the most prevalent, dozens of other *hMYH* mutations have since been identified in colorectal cancer patients (*29*, *34*, *35*, *37*−*40*).

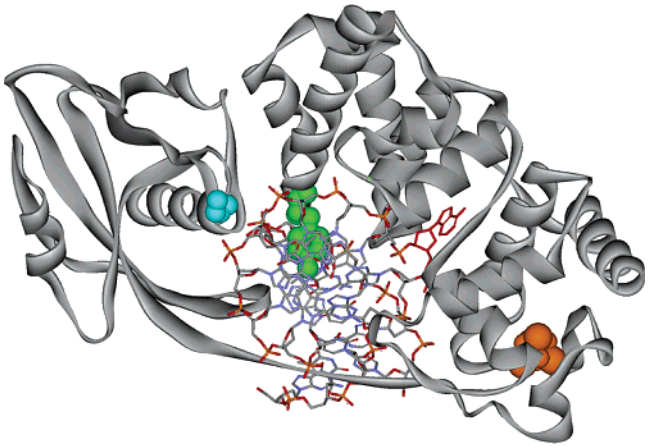

FIGURE 1: Positions of Tyr 82 and Gly 253 (corresponding to Tyr 88 and Gly 260) in the cocrystal structure of MutY from *B. stearothermophilus* viewed down the bent DNA helical axis. Tyr 88 (green) is located near the active site cleft in the N-terminal domain of the enzyme and intercalates into the DNA 5′ to the estranged OG. Gly 260 (blue) initiates a tight turn within the C-terminal domain. The flipped adenine is shown in red and the [4Fe-4S] cluster in orange. Adapted from ref *48*.

The crystal structure of the N-terminal catalytic domain of *E. coli* MutY solved in the presence of a free adenine base (*41*), together with fluorescence studies, suggested that the adenine base is flipped out of the DNA duplex into the active site pocket (*42*, *43*). Indeed, this type of base-flipping mechanism is a common theme of BER glycosylases in order to gain access to the *N*-glycosidic bond (*44*−*47*). On the basis of the location of Tyr 82 of *E. coli* MutY in the pseudo-helix-hairpin-helix (HhH) motif, it was suspected to be involved in intercalation into the DNA duplex, stabilizing the protein−DNA complex (*31*). The effect of the cysteine replacement for Tyr 82 on the adenine glycosylase activity and affinity for the substrate analogue duplex was consistent with the proposed role of Tyr 82 intercalation. More recently, the cocrystal structure of MutY from *Bacillus stearothermophilus* (BsMY) in complex with a duplex DNA substrate further illuminated the roles of amino acids Tyr 82 and Gly 253 (Figure 1) (*48*). Tyr 88, the amino acid corresponding to Tyr 82 in *E. coli* MutY, was found to be intercalated into the DNA at the nucleotide 5′ to the estranged and intrahelical OG base. The hydroxyl of the tyrosine side chain hydrogen-bonds to an adjacent serine side chain hydroxyl, which in turn hydrogen-bonds to the NH7 of the OG base (*48*).

The role of Gly 253, located in the C-terminal domain of MutY, was less clear prior to elucidation of the cocrystal structure (*48*). Previous work had established that the C-terminal domain is related in sequence (*49*) and structure to the d(OGTP) hydrolase MutT (*50*). Analysis of adenine

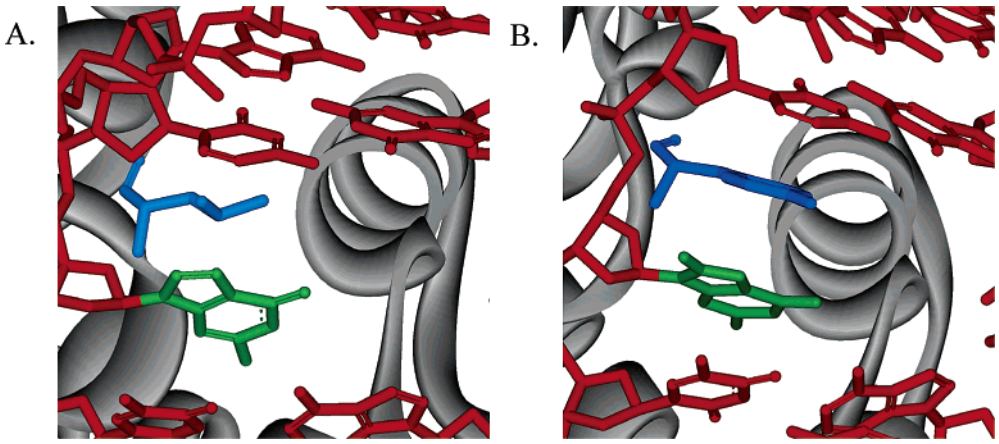

FIGURE 2: Cocrystal structure of (A) *E. coli* endonuclease III and (B) *B. stearothermophilus* MutY complexed with DNA. The Leu 82 (A) or Tyr 88 (B) residue is shown in blue intercalating 5′ to the orphaned base (guanine in A or OG in B) shown in green. In B, the OG base shown is flipped into the *syn* conformation upon DNA binding and adenine extrusion. Adapted from refs *48* and *51*.

removal activity and binding affinities to substrate analogues revealed that the absence of the C-terminal domain results in reduction of glycosylase activity and loss of specificity for OG (*21*, *49*). On the basis of the observed loss of specificity for binding OG over G opposite FA and the rather subtle decrease in glycosylase activity of G253DMutY, we suggested that the presence of glycine at the 253 position may be important for providing the flexibility needed for OG recognition and coordination with the N-terminal domain (*31*). Consistent with this proposal, in the cocrystal structure, Gly 260 in BsMY, corresponding to the Gly 253 in *E. coli* MutY, was revealed to initiate a tight turn in which backbone amides hydrogen-bond to the phosphodiester backbone 5′ to the OG (*48*).

The proposed roles for Tyr 82 and Gly 253 may be further elaborated to elucidate the relative contributions to catalysis by additional residue alterations. For this reason, three additional MutY variants, Y82F, Y82L, and G253A, were examined herein. Replacing tyrosine with phenylalanine was chosen as a conservative mutation that would eliminate only the hydroxyl group. In the structure of *B. stearothermophilus* endonuclease III (endo III) borohydride-trapped to its product, a leucine residue is intercalated at the analogous position of the tyrosine residue in MutY (Figure 2) (*51*). Though highly homologous to MutY, endo III recognizes and removes oxidized thymines, such as thymine glycol (*6*). Thus, we examined Y82L to determine if the choice of tyrosine rather than leucine may be related to the different substrates of the two enzymes. Finally, mutation of glycine to alanine at position 253 was chosen as a conservative mutation, and sequence alignment with MutY demonstrates that an alanine residue in MutT corresponds to Gly 253 in MutY (*49*, *50*). The functional ramifications of the amino acid variations, in addition to further evaluation of the catalytic deficiencies of Y82C and G253D MutY, were assessed by detailed analysis of the processing of OG:A- and G:A-containing DNA substrate duplexes, as well as analysis of binding affinity using substrate analogue-containing duplexes.

MATERIALS AND METHODS

General Materials and Instrumentation. For these experiments, the JM101 *mutY*$^{-}$ *E. coli* cell strain was used, which has been described previously (*52*). The expression plasmid used was pKKYEco, which was kindly provided to us by Drs. M. Michaels and J. H. Miller (UCLA) (*11*). Standard protocols were followed for DNA manipulation (*53*). Standard 2′-deoxy phosphoramidites were purchased from Perkin-Elmer, while the 2′-deoxy-8-oxoG phosphoramidite was purchased from Glen Research. DNA oligonucleotides were synthesized according to the manufacturer's protocol on an Applied Biosystems model 392 DNA/RNA synthesizer. Oligonucleotides used for mutagenesis via PCR were purified using oligonucleotide purification cartridges (OPC) from Perkin-Elmer according to the protocol provided by the manufacturer. All other oligonucleotides were purified via HPLC on a Beckman Gold Nouveau system with a C18 RCM column from Waters. PCR was performed on a GeneAmp PCR system 2400 from Perkin-Elmer. Radiolabeling was done using [γ-^{32}P]ATP purchased from ICN with T4 polynucleotide kinase from New England BioLabs. Labeled oligonucleotides were purified using ProbeQuant G-50 spin columns from Amersham Pharmacia according to the manufacturer's protocol. Storage phosphor autoradiography was performed on either a Molecular Dynamics Storm 840 or Typhoon 9400 phosphorimager system. Data analysis was done using ImageQuaNT 5.0 and GraFit 5 software. Additional enzymes used in this study were purchased from either New England BioLabs or Roche. All other chemicals, buffers, and reagents used for these experiments were purchased from VWR, Fisher, or Sigma.

Enzyme Cloning. A Quik-Change "megaprimer"-based mutagenesis kit (Stratagene) was used for site-directed mutagensis according to the manufacturer's protocol. The necessary codon changes which encode for mutated enzymes Y82L, Y82F, and G253A were incorporated into the *mutY* gene using the following primers: Y82L-1, 5′-GCGCGCGGGCGTA**CAA**GCCAAGCCCGGTCC-3′; Y82L-2, 5′-GGACCGGGCTTGGC**TTG**TACGCCCGCGCGC-3′; Y82F-1, 5′-GCGCGCGGGCGTA**AAA**GCCAAGCCCGGTCC-3′; Y82F-2, 5′-GGACCGGGCTTGGC**TTT**TACGCCCGCGCGC-3′; G253A-1, 5′-CCGCCCCACAA**TGC**GCTCGGCGGACGCTGC-3′; and G253A-2, 5′-GCAGCGTCCGCCGAGC**GCA**TTGTGGGGCGG-3′.

The altered sequence for mutagenesis to the appropriate amino acid is in bold and underlined. Primers for mutagenesis

of the Y82C and G253D MutY enzymes have been previously reported (*31*). Plasmid DNA was isolated from XL-1 blue *E. coli* cells using a Wizard Plus DNA purification kit (Promega) according to the protocol provided by the manufacturer. The presence of the correct mutations, as well as the integrity of the entire *mutY* gene was confirmed by DNA sequencing by the DNA sequencing core facility located at the University of Utah Medical School, Salt Lake City, UT.

Substrate Oligonucleotide Preparation. The following oligonucleotide duplex was used for these experiments: 5′-CGATCATGGAGCCAC**X**AGCTCCCGTTACAG-3′, where **X** = OG or G; and 5′-CTGTAACGGGAGCT**Y**GTGGCTC-CATGATCG-3′, where **Y** = A or FA. For all experiments, the Y-containing DNA strand was radiolabeled to produce ^{32}P-5′-DNA. For glycosylase activity assays, additional nonradioactive A-containing DNA was added to the labeled strand to allow 5% labeled DNA, which was then annealed to the complement (added at 20% excess) by heating to 90 °C for 5 min and allowing to cool to 25 °C slowly overnight in annealing buffer (20 mM Tris-HCl, pH 7.6, 10 mM EDTA, and 150 mM NaCl). For the binding affinity experiments, an equimolar amount of the complement was added to 100% radiolabeled FA-containing DNA and the duplex was annealed as described above.

Enzyme Overexpression and Purification. Overexpression and purification of MutY mutants used in this study were done in a manner identical to that reported previously for WT MutY, as well as for Y82C and G253D MutY (*20*, *21*, *31*). Briefly, plasmids containing either a wild-type copy of the *mutY* gene or altered *mutY* gene to produce the Y82F, Y82L, or G253A mutated protein products were transformed into JM101 *mutY*$^-$ *E. coli* cells. These plasmids were named pKKYEco, pKKYY82L, pKKYY82F, and pKKYG253A, respectively. Cells were grown in 8 L of LB growth media containing 15 μg/mL tetracycline and 100 μg/mL ampicillin antibiotics at 37 °C until an OD_{600} of 0.9 was obtained. Expression of MutY protein was induced by adding IPTG to a final concentration of 1 mM and incubating the cell cultures at 30 °C for 3 h. Cells were harvested by centrifugation and resuspended in 5 mL buffer A (50 mM Tris-HCl, pH 8, 2 mM EDTA, 5 mM DTT, and 5% (v/v) glycerol) per 1 g cells. PMSF was added to a concentration of 5 mM, and cells were stored at −80 °C for future use.

For protein purification, cells were lysed by sonication at 70% power (Branson Sonifier 250) and cell debris pelleted by centrifugation. Streptomycin sulfate was added to the supernatant to precipitate nucleic acids, and the proteins were precipitated using a 40% solution of ammonium sulfate. Following centrifugation, the protein pellet was resuspended in buffer B (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, and 5% (v/v) glycerol). Purification was performed on a BioRad Biologic FPLC system using a HiPrep 26/10 desalting column (Pharmacia), followed by an EconoPak HiS column (BioRad), and finally on a HiTrap heparin column (Pharmacia). MutY eluted from the heparin column at approximately 50% buffer B containing 1M NaCl. Fractions that eluted from the heparin column with an Abs_{280}/Abs_{410} ratio of between 6 and 7 were pooled. The total concentration of protein was determined by the method of Bradford (*54*) and the active site concentration as reported previously (*20*). All enzyme concentrations listed in this manuscript are the active concentrations. The protein was then diluted to 50% with glycerol and stored in liquid nitrogen.

General Adenine Glycosylase Activity Assays and Determination of the Activation Energy. Adenine glycosylase activity assays of WT, Y82L, Y82F, and G253A *E. coli* MutY in these experiments were done according to the protocol previously published by our laboratory (*20*). Briefly, the active site concentration was determined using 20 nM OG:A-containing duplex, incubated with 2−12 nM total protein concentration in assay buffer (20 mM Tris-HCl, pH 7.6, 10 mM EDTA, 0.1 mg/mL BSA, and 30 mM NaCl) at 37 °C. Aliquots were removed at various times (between 15 s and 60 min), quenched in a final concentration of 0.2 M NaOH to invoke strand scission, and incubated at 90 °C for 5 min. An equal volume of formamide loading dye (80% formamide, 0.025% xylene cyanol, and 0.025% bromophenol blue in TBE buffer) was added to each aliquot. The samples were run on a 15% denaturing polyacrylamide gel in 1× TBE at 1600 V for 2 h. The separation of the 14-nucleotide DNA product from the 30-nucleotide substrate was visualized using autoradiography by exposure to a storage phosphor screen overnight. Multiple-turnover (MTO) experiments were performed in the same fashion, except enzyme concentrations were adjusted to allow for 10−20% product formation during the burst phase of the experiment, usually falling between 2 and 4 nM active enzyme. For single-turnover (STO) experiments, the active enzyme concentration used was 30−40 nM, and the reactions were otherwise performed analogously to the active site determination and multiple-turnover experiments outlined above. Single-turnover experiments were also performed with an OG:A-containing DNA duplex at 25 and 2 °C in addition to 37 °C identically to the glycosylase assays described above. For determination of the activation energy for the adenine glycosylase activity of MutY, rate constants (k_2) at 37, 25, and 2 °C were used to create an Arrhenius plot. The slope of the resulting line provided the activation energy (E_a) of the reaction.

For reactions under single-turnover conditions in which the glycosylase reaction was too fast to measure manually ($k_2 > 2$ min^{-1}), a rapid-quench flow instrument (Kintek) was used. Slight modifications to the manual glycosylase activity assay were made to accommodate use of this instrument. A final active enzyme concentration of 75 nM was preincubated in the presence of 12.5 nM duplex DNA containing a centrally located G:C pair at positions X and Y described above. Preincubation of the enzyme with nonspecific DNA is necessary to stabilize the enzyme during the time course of the experiment; indeed, loss of activity is observed with MutY in the absence of DNA when stored at or above 4 °C after several minutes. The enzyme was mixed with the OG:A-containing DNA duplex (20 nM), and samples were removed at various time points between 0.2 s and 5 min and quenched in a final concentration of 0.2 M NaOH. The presence of the nonspecific DNA duplex does not affect the observed rates of processing of OG:A duplexes.

Fitting of the data was performed using GraFit software with the appropriate equations, and rate constants k_2 and k_3 were determined as described previously by our laboratory (*20*). In all cases, the data are reported as the average of at least three separate experiments, and the error is reported as

Table 1: Pre-Steady State Kinetic Parameters of the Reaction of WT and Mutanted MutY Enzymes with OG:A and G:A substrates at 37 °C

	rates of product release (k_3, min^{-1})		
enzyme	OG:A	G:A	% activity[a]
WT MutY[b]	0.004 ± 0.002	0.03 ± 0.01	31
Y82C[c]	0.017 ± 0.001	ND[d]	53
Y82F	0.003 ± 0.001	ND[d]	34
Y82L	0.004 ± 0.002	0.012 ± 0.008	39
G253D[c]	0.002 ± 0.001	0.02 ± 0.01	80
G253A	0.010 ± 0.001	0.028 ± 0.003	30

[a] The percentage of active enzyme is relative to total protein concentration. [b] Similar to rate constants reported previously (*20*). [c] Rate constants previously published (*31*). [d] A value could not be determined due to the lack of biphasic behavior with this enzyme with a G:A-containing DNA substrate.

the standard deviation of the sample set.

Binding Experiments. Electrophoretic mobility shift assays (EMSA) were performed as described previously (*21*, *27*, *31*, *55*). Briefly, serial dilutions of *E. coli* WT, Y82L, Y82F, and G253A MutY were made in dilution buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 20% glycerol) to a concentration range between 2000 and 0.05 nM at 4 °C. Twenty pM G:FA or 10 pM OG:FA-containing DNA duplexes were incubated with increasing concentrations of enzyme for 30 min at 25 °C. Bound versus unbound DNA was resolved using electrophoresis on 8% nondenaturing polyacrylamide gel (29:1 acrylamide/bis) at 4 °C in 0.5× TBE buffer at 120 V for 2 h. Gels were dried and exposed to a storage phosphor screen for at least 24 h. Analysis of the gel was performed as described previously (*27*, *31*, *55*). Due to the presence of a supershifted band at high enzyme concentration (data not shown), the reported K_d is a combination of the K_d representing binding to the specific lesion and the nonspecific binding of the enzyme to DNA. Therefore, the K_d values reported herein can be considered an apparent K_d and are reported to demonstrate binding of mutated enzymes relative to WT MutY.

RESULTS

Adenine Glycosylase Activity of Y82F, Y82L, and G253A MutY. Our approach for analyzing the adenine removal activity of WT *E. coli* MutY, as well as several mutated MutY enzymes, with OG:A- and G:A-containing DNA duplexes has been reported previously (*20*, *31*). Under conditions in which [DNA] > [MutY], *E. coli* MutY displays a biphasic profile for product formation with both OG:A- and G:A-containing substrates. An exponential "burst" of product formation is observed, followed by a slow linear steady-state portion attributed to slow release of MutY from the DNA product. Under analogous conditions, Y82L, Y82F, and G253A MutY exhibited similar biphasic behavior. Our laboratory also previously observed similar behavior with Y82C and G253D MutY (*31*). The amplitude of the burst phase is directly related to the concentration of active sites and established that the percent active sites for all of the mutated enzymes was in a range consistent with that observed with WT MutY (20−80% active enzyme compared to total protein concentration) (*20*, *27*, *31*, *55*−*58*) (Table 1). Thus, based on the similarity in the behavior of the mutated enzymes, we have used the same kinetic scheme and approach previously reported with WT MutY (*20*) (Scheme 1). Appropriate fitting of data allows for determination of the rate constants, k_2 and k_3, that are related to steps including chemistry of adenine excision and product release, respectively (*20*, *22*). Although both rate constants k_2 and k_3 can be determined under multiple-turnover conditions, k_2 is more accurately obtained through single-turnover experiments, in which [DNA] < [MutY].

Scheme 1: Minimal Kinetic Scheme for the Adenine Glycosylase Activity of *E. coli* MutY (*20*)

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

Under single-turnover conditions, with an OG:A-containing DNA substrate at 37 °C, no significant difference was observed between WT MutY and Y82L, Y82F, and G253A MutY in terms of the rate of adenine excision (k_2) (Table 2). We often have observed a lack of sensitivity of the reaction with the OG:A substrate under these conditions with mutated MutY enzymes (*29*, *31*, *56*, *59*). However, a more drastic reduction in the rate of adenine removal from a G:A-containing DNA duplex was observed with all of the mutated MutY enzymes compared to the reaction with the OG:A-containing substrate. The rate of adenine removal (k_2) of WT MutY with the G:A substrate at 37 °C is approximately 10-fold slower than with the OG:A substrate (*31*). The most dramatic decrease was with Y82F MutY, in which the rate of adenine removal from a G:A mispair was so slow that the reaction did not achieve completion within the time-course monitored (90 min). Therefore, based on an estimated upper limit of the rate constant k_2 of $<0.02\ min^{-1}$, processing of G:A relative to OG:A is reduced 70-fold with Y82F. The magnitude of the reduced activity is similar to that estimated for the same reaction with Y82C MutY (*31*). In contrast, the k_2 value of Y82L MutY with the G:A substrate was found to be only slightly slower than WT MutY ($0.8 \pm 0.1\ min^{-1}$). On the basis of these data, it appears that variation at position 82 in MutY leads to different consequences in terms of efficiency of adenine excision depending on the nature of the amino acid substitution. On the other hand, the rate of adenine removal from G:A by G253A MutY was similar ($k_2 = 0.10 \pm 0.01\ min^{-1}$) to that observed for G253D MutY ($k_2 = 0.19 \pm 0.04\ min^{-1}$). These results suggest that any amino acid substitution at position 253 in MutY will invoke at least a similar effect on adenine removal.

By fitting the production curves under multiple-turnover conditions (*20*), the rate constant describing the rate of turnover, k_3, is determined from the slope of the linear phase (Table 1). With an OG:A-containing DNA duplex, the rate of turnover (k_3) for Y82F and Y82L MutY was found to be within error of WT MutY ($0.004 \pm 0.002\ min^{-1}$). However, for the reaction of Y82F with the G:A-containing DNA duplex, a biphasic kinetic profile is not observed, similar to what was seen for the Y82C MutY (*31*). This observation is consistent with the significant decrease in the chemistry step of the glycosylase reaction such that the rate of adenine removal (k_2) likely becomes similar to the rate of product release (k_3). In such cases, the rate constant k_3 may not be easily determined from these experiments. However, in the reaction with a G:A-containing substrate under multiple-turnover conditions, Y82L behaved similarly to the WT enzyme, with measured rate constants k_3 of 0.012 ± 0.008

Table 2: Rate Constants (k_2, min^{-1}) and Activation Energy of WT and Mutant MutY Enzymes for Glycosylase Activity under Single-Turnover Conditions[a]

enzyme	G:A	OG:A (150 mM)[b]	OG:A (37 °C)	OG:A (25 °C)	OG:A (2 °C)	E_a (kJ/mol)[c]
WT MutY	1.4 ± 0.3[d]	12 ± 3	17 ± 4[d]	5.7 ± 0.3	0.7 ± 0.1	64.2
Y82C	<0.017[e]	0.19 ± 0.04	5 ± 2[e]	1.2 ± 0.1	<0.0006[e]	189.5
Y82F	<0.02	14 ± 3	9 ± 1	2.8 ± 0.1	0.12 ± 0.05	88.3
Y82L	0.8 ± 0.1	22 ± 2	17 ± 5	6 ± 2	0.6 ± 0.1	67.8
G253D	0.19 ± 0.04[f]	11 ± 3	15 ± 5[e]	2.9 ± 0.2	0.26 ± 0.05[e]	80.4
G253A	0.10 ± 0.01	2.7 ± 0.4	12 ± 1	1.9 ± 0.7	0.03 ± 0.01	121.5

[a] Rate constants greater than 2 min^{-1} were determined using a rapid-quench instrument, while slower values were determined using a manual assay as described in Materials and Methods. [b] Refers to buffer concentration of NaCl, rate constants determined at 37 °C. [c] The activation energy for adenine excision was calculated experimentally using rate constants k_2. [d] Similar to rate constant reported previously (*20*). [e] Rate constants previously published (*31*). [f] Similar to rate constant reported previously (*31*).

min^{-1} and 0.03 ± 0.01 min^{-1}, respectively. Analysis of G253A MutY with the OG:A-containing DNA substrate resulted in a k_3 value of 0.010 ± 0.001 min^{-1}, which is slightly outside of the error of the k_3 for WT MutY with this substrate. With the G:A-containing DNA substrate, G253A gave a k_3 value identical to the k_3 reported for WT MutY (0.028 ± 0.003 min^{-1} and 0.03 ± 0.01 min^{-1}, respectively).

The Effects of Temperature and Buffer Salt Concentration on Adenine Excision with OG:A Substrates by WT and Mutant MutY Enzymes. To uncover potentially subtle differences in the catalytic behavior of Y82L, Y82F, and G253A relative to WT MutY, the rate of adenine excision was assessed under single-turnover conditions with the three mutated enzymes with an OG:A-containing DNA duplex at 25 and 2 °C and compared to the analogous experiments with the WT enzyme (Table 2). Monitoring the rate of reaction at reduced temperatures would allow for calculation of the activation energy required for catalysis to take place and thereby reveal potential deficiencies of the mutated enzymes in one or more steps involved in the adenine excision process. At 25 °C, variants Y82F and G253A MutY display a 2-fold (2.8 ± 0.1 min^{-1}) and 3-fold (1.9 ± 0.7 min^{-1}) reduction in rate of adenine removal, respectively, compared to WT MutY (5.7 ± 0.3 min^{-1}). When the temperature is lowered to 2 °C, a greater effect is observed, with the k_2 values dropping to 0.12 ± 0.05 min^{-1} and 0.03 ± 0.01 min^{-1} for Y82F and G253A, respectively, representing a 6-fold and 23-fold reduction in rate compared to WT MutY (0.7 ± 0.1 min^{-1}). These results are consistent with our previous findings with Y82C and G253D, which showed that, as the temperature of the reaction decreased to 2 °C, the catalytic rate of adenine removal by these mutated enzymes decreased more rapidly than WT MutY (*31*). For G253D MutY operating on the OG:A substrate at this temperature, the k_2 was determined to be 0.26 ± 0.05 min^{-1}, which represents a 3-fold reduction in rate compared to the WT enzyme. Moreover, the observed rate for adenine removal with the OG:A substrate for Y82C at 2 °C was extremely slow and did not reach completion in the 90 min time-course of the experiment (k_2(est) < 0.0006 min^{-1}). The temperature dependence of the adenine excision reaction with G253A was similar to that observed for G253D (Table 2). Interestingly, k_2 values for Y82L at each of the three temperatures were within the error of those determined for WT MutY. These results indicate that, while a phenylalanine is not able to replace tyrosine at position 82 in MutY, leucine is able to competently perform the role of tyrosine at this position.

By monitoring the rate of a reaction as a function of temperature, the activation energy (E_a) for the reaction process can be determined using the Arrhenius equation (Table 2). Therefore, the activation energy associated with the excision of an adenine base from duplex DNA by MutY was found by fitting of a plot of $\ln k_2$ versus $1/T$. The E_a determined for WT MutY was 64.2 kJ/mol. Notably, this is similar to the activation energy determined for the removal of uracil by the human uracil-DNA glycosylase, which was found to be 50.6 kJ/mol (*60*). Comparison of the activation energy for WT MutY with the mutated enzymes Y82L, Y82F, and G253A, as well as Y82C and G253D, provides insight into the thermodynamics of the reaction of each MutY enzyme with an OG:A-containing DNA substrate. Y82C MutY showed the largest increase in activation energy compared to WT MutY, with an E_a value of 189.5 kJ/mol. This represents a 3-fold increase compared to the analogous value measured for WT MutY. Y82F, G253D, and G253A MutY displayed E_a values intermediate between Y82C and WT MutY, the values of which were 88.3, 80.4, and 121.5 kJ/mol, respectively. The activation energy of 67.8 kJ/mol for Y82L MutY is nearly identical to the value for WT MutY.

We also examined the effect of increasing the buffer NaCl concentration to 150 mM on the rate of adenine removal from the OG:A-containing DNA duplex by WT MutY and mutant enzymes (Table 2) in order to determine the contribution of nonspecific versus specific binding events in the adenine removal activity (*61*). Under these conditions, the observed rates with most of the mutated enzymes followed trends similar to those observed at the lower salt concentration of 30 mM NaCl. Only the reaction with Y82C MutY was shown to be sensitive to the increased NaCl concentration, with the rate of adenine removal (k_2) reduced approximately 25-fold, from 5 ± 2 min^{-1} at 30 mM NaCl to 0.19 ± 0.04 min^{-1} at 150 mM NaCl. Additionally, the k_2 rate for Y82C at 150 mM NaCl was reduced 60-fold compared to WT MutY, with a k_2 of 12 ± 3 min^{-1} at 150 mM NaCl. All other MutY enzymes tested, including WT MutY, displayed no significant reduction in the rate of adenine removal from an OG:A lesion as a result of the increased NaCl concentration (Table 2).

Dissociation Constant Measurements (K_d) Using Electrophoretic Mobility Shift Assays (EMSA) with a Substrate Analogue. We have previously exploited the use of noncleavable 2′-deoxyadenosine analogues, such as 2′-deoxy-2′-fluoroadenosine (FA), incorporated opposite OG and G in duplex DNA, to evaluate features of substrate recognition by MutY without complications of enzymatic base removal (*21*, *27*, *31*, *55*, *56*). We previously demonstrated by

Table 3: Dissociation Constants (K_d, nM) Determined for WT and Mutant MutY Enzymes with a Substrate Analogue at 25 °C

enzyme	OG:FA	G:FA
WT MutY[a]	0.8 ± 0.4	7 ± 3
Y82C[a]	50 ± 6	60 ± 7
Y82F	16 ± 1	28 ± 5
Y82L	1.1 ± 0.5	11 ± 3
G253D[b]	20 ± 6	20 ± 7
G253A	23 ± 8	23 ± 9

[a] Similar to dissociation constant reported previously (*27*). [b] Dissociation constant previously published (*31*).

Table 4: Summary of Intercalating Residues of Various DNA Glycosylase Enzymes of the Base Excision Repair Superfamily

enzyme	intercalator	ref
E. coli endo III	Leu 82	51
E. coli AlkA	Leu 125	66
human AAG	Tyr 162	63
E. coli UDG	Leu 191	67
human UDG	Leu 272	64
E. coli MutY	Tyr 82	48
E. coli Nei	Tyr 71	70
E. coli Fpg	Phe 110	77

measuring relevant dissociation constants (K_d) using electrophoretic mobility shift assays (EMSA) that WT MutY binds with high affinity (nM) to the FA-containing duplexes with a marked preference for OG over G (*27*). We also showed that both Y82C and G253D MutY exhibit reduced affinity for these analogue-containing duplexes (*31*). In fact, the preference for OG as indicated by a higher affinity for OG:FA over G:FA observed with WT MutY was absent with both the Y82C and G253D variants, indicating that these amino acid substitutions hamper the ability of the modified enzymes to recognize OG.

Similar experiments were performed with Y82L, Y82F, and G253A MutY enzymes to determine the effects of amino acid substitution at these positions on the damage recognition properties of MutY (Table 3). Analogous to what was observed with G253D MutY, the affinity of G253A was reduced in magnitude and similar with both OG:FA and G:FA duplexes with K_d values of 23 ± 8 nM and 23 ± 9 nM, respectively. Y82F MutY was able to restore some of the affinity lost with the variant Y82C, but not completely. In addition, unlike Y82C, Y82F MutY exhibited an approximately 2-fold greater affinity for OG:FA (16 ± 1 nM) over G:FA (28 ± 5 nM). This is in contrast to WT MutY, which prefers OG:FA with about a 10-fold preference. In addition, binding constants (K_d) are higher for Y82F MutY compared to WT, but lower than for Y82C MutY. The measured binding affinities for Y82L MutY were similar to those determined for WT MutY.

DISCUSSION

The finding that variations in hMYH are correlated with colorectal cancer has put this BER glycosylase in the spotlight. The two most common variants found in hMYH that are associated with MYH-associated polyposis are Y165C and G382D. In this study, the roles of the corresponding residues Tyr 82 and Gly 253 in the glycosylase function of *E. coli* MutY were further elaborated by analysis of a series of "unnatural" amino acid variants. Analysis of the adenine glycosylase activity and binding affinity with substrate analogues of Y82L, Y82F, and Y82C MutY and comparison to the WT enzyme have indicated the importance of a bulky amino acid, such as tyrosine or leucine, at position 82. In contrast, replacement of Gly 253 with alanine or aspartic acid gave similar results, with less dramatic effects than replacements of Tyr 82, suggesting that only glycine at this position will provide for WT activity.

A role for aromatic residues in DNA binding proteins is commonly observed; in fact, the human alkyl adenine glycosylase (AAG) has been shown through X-ray crystallography to possess a tyrosine side chain intercalated into the duplex, replacing the flipped-out ethenoadenine base (*62*, *63*). Inspection of X-ray crystal structures suggests that leucine and tyrosine generally play intercalation roles (Table 4) in the context of the BER glycosylases, with phenylalanine being more involved in base-stacking roles, such as in π-stacking interactions with an extruded base (*64*, *65*). Several examples of enzymes with such intercalator residues are listed in Table 4 and include glycosylases *E. coli* endo III, UDG, Fpg, and AlkA, as well the human homologues of UDG and AlkA (AAG) (*62*−*64*, *66*−*68*). These intercalating amino acids have been suggested to assist in both lesion recognition and the base flipping process, as well as in promoting formation of and stabilizing distorted conformations that precede base excision (*24*, *51*, *64*, *69*, *70*). In the structure of inactive BsMY bound to an OG:A mismatch (*48*), the tyrosine residue was found to be intercalated at the OG rather than stacking with the flipped-out adenine. Indeed, this tyrosine is positioned analogously to the leucine residue in the endo III−DNA structure (*51*) (Figure 2).

Under single-turnover conditions at 37 °C, Y82C (*31*), Y82L, and Y82F MutY displayed only slightly reduced rates of adenine removal compared to the WT enzyme. Since MutY is extremely efficient at processing the OG:A lesion, subtle changes in the rate of adenine excision by a mutated enzyme may not be detected under these assay conditions. When the temperature of the reaction is lowered, differences in the activation energy barrier for catalysis between mutated enzymes are magnified such that more significant differences in adenine excision rates are detected. Indeed, Y82F MutY displayed a greater sensitivity to temperature changes on the rate of adenine excision than WT MutY, indicating that this mutated enzyme is catalytically compromised relative to the WT enzyme. In fact, the activation energy (E_a) determined experimentally for Y82F (88.3 kJ/mol) was increased compared to WT MutY (64.2 kJ/mol). However, the E_a value calculated for Y82C was found to be the largest of all of the mutated enzymes (189.5 kJ/mol), representing a 3-fold increase compared to WT MutY. This illustrates that Tyr 82 plays an important role in forming and stabilizing a distorted conformation that facilitates adenine excision. The replacement of tyrosine with cysteine makes it more difficult to access this conformation needed for base excision.

The importance of this tyrosine in promoting adenine excision is also underscored by the sensitivity of Y82C MutY to the NaCl concentration of the buffer. When large, bulky amino acids are used as replacements for the tyrosine, such as phenylalanine and leucine, adenine is removed from duplex DNA at a similar rate at both the low and high NaCl concentrations. However, if a cysteine is substituted in place of tyrosine at this position, a reduction in the rate of adenine

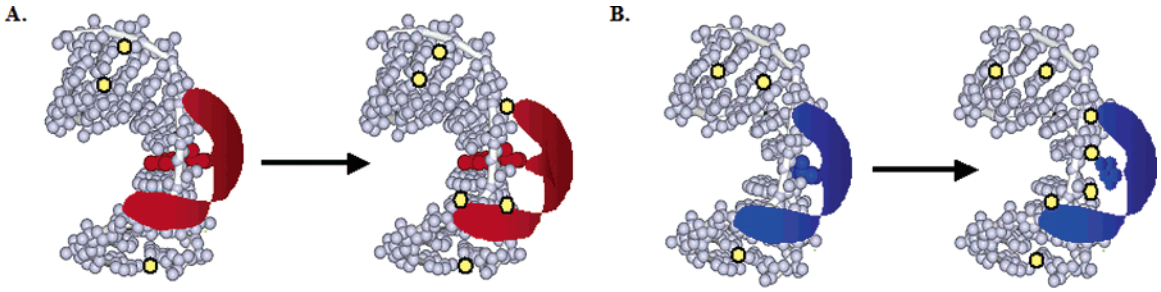

FIGURE 3: The effect of increasing salt on the DNA binding interactions by MutY. Increasing the buffer salt concentration shows no effect on the rate of adenine excision in the WT MutY enzyme (A) due to DNA−protein complex stabilization by both the Tyr 82 intercalation and electrostatic interactions between positively charged residues and backbone phosphates. When Tyr 82 has been replaced with cysteine (B), electrostatic interactions are not sufficient to stabilize the DNA−protein complex in the absence of a strong intercalator at position 82 at high buffer salt concentrations. Therefore, with Y82C, the adenine excision efficiency is reduced more relative to WR MutY. Salt cations are depicted as yellow circles, DNA in gray, WT MutY in red, and Y82C MutY in blue. Model is based on an X-ray cocrystal structure (*48*) with elements of the protein removed for clarity.

removal is observed that is considerably magnified at the higher salt concentration (60-fold) relative to the low salt conditions (3-fold). We hypothesize that the DNA bending that is associated with formation of the catalytically competent DNA−MutY intermediate is facilitated through electrostatic and hydrogen-bonding interactions of DNA backbone phosphates with amino acid side chains and backbone amides, as well as intercalation of the amino acid side chain at position 82 (Figure 3). Substitution at this position with a small side chain, such as cysteine, eliminates this DNA distorting and stabilizing intercalative interaction. Thus, in the case of Y82C MutY, DNA binding events critical for catalysis rely primarily on electrostatic interactions. In vitro, at low salt conditions of 30 mM, electrostatic interactions between the DNA phosphates and MutY may be sufficient to access the catalytically competent MutY-substrate DNA conformation. However, when the NaCl concentration is increased to 150 mM, the sodium cations will more effectively compete for the phosphate-binding sites, inhibiting DNA binding and distortion by MutY, consequently reducing the observed rate of adenine excision (Figure 3). WT, Y82F, and Y82L MutY are less sensitive to the increased salt concentration, since these phosphate contacts may not be as critical in the presence of an intercalating amino acid.

Perhaps the most interesting aspect of this work is the observation that all rate constants for Y82L are nearly exactly like WT MutY under all of the conditions examined and with both OG:A and G:A substrates. In addition, dissociation constants with both OG:FA and G:FA duplexes are nearly identical to those for WT MutY. These results were surprising, since a leucine residue at position 82 in *E. coli* MutY would not provide the aromatic group thought to be important for intercalation nor the hydroxyl group thought to be important for hydrogen bonding to OG. One possibility is that the hydrogen-bonding network involving the Tyr-OH observed in the BsMY−DNA structure may not be as critical as the presence of a bulky residue. The hydrophobic Leu residue may be bulky enough to penetrate the helix and promote DNA bending and distortion that precedes adenine flipping, and this may be more important than stabilization provided via π-stacking and hydrogen-bonding. One could argue that the fact that the related enzyme endo III utilizes a leucine instead of tyrosine suggests that leucine is a reasonable replacement for tyrosine. In the endo III−DNA structure, the side chain of the leucine residue is wedged in from the minor groove 5′ to the orphaned G residue opposite the lesion site and is also in van der Waals contact with this G. Recent work has indicated that G and OG have local dipoles with opposite directions due to the charge inversion at position 7 and 8 (*71*). Though these factors suggested an importance of tyrosine π-stacking, the similarity in enzyme activity of Y82L and WT MutY argue that the steric factors dominate. Indeed, the prevalence of leucine and tyrosine side chains in this type of helix-intercalating interaction shows these residues are ideally suited for this role.

The WT-like activity of Y82L is even more surprising based on the compromised activity of Y82F MutY. The competency of Y82F MutY to recognize and repair OG:A and G:A lesions in DNA falls somewhere between the efficiency of WT and Y82C MutY enzymes. Interestingly, in terms of adenine excision from OG:A mispairs, the activity of Y82F is more similar to the WT enzyme. However, in the adenine excision with G:A mispairs, Y82F is more similar to Y82C. In the binding experiments with the FA analogue, the specificity of Y82F for OG over G is only 2-fold, compared to 10-fold with the WT enzyme. The obvious difference between tyrosine and phenylalanine of the hydroxyl group suggests an importance for the hydrogen-bonding network involving the tyrosine hydroxyl group; however, the efficient activity of Y82L argues against this idea. One possibility is that the electronic properties of the phenylalanine may hinder efficient insertion and stacking into the helix needed to initiate catalysis. Steric and electronic factors may be more important than loss of the hydrogen-bonding network to OG. With the optimal OG:A substrate, compensatory effects of many interactions facilitating catalysis may allow for efficient catalysis by Y82F. However, at lower temperatures with the OG:A substrate or with the G:A substrate, the loss of the important features of tyrosine are magnified in the activity of Y82F. In addition, the binding to the analogue-containing duplexes points to importance of tyrosine in recognition of OG, since specificity for OG over G is severely compromised with Y82F.

An alternative explanation for the observed activity of Y82L is that the leucine side chain provides sufficient space to allow for an ordered water molecule to fill the role of the hydroxyl group of Tyr 82 in hydrogen bonding and orienting

the serine hydroxyl for hydrogen-bonding with NH-7 of the OG lesion. This hypothesis could be validated by solving the structure of Y88L BsMY in complex with OG:A-containing DNA. It is also possible that the superior activity of Y82L over Y82F is related to their difference in size such that a phenylalanine at this position does not allow for the presence of such an ordered water molecule to replace the role of the tyrosine hydroxyl. The use of water-mediated interactions that allow for alternative recognition is commonly seen in DNA protein recognition (*72*). A recent example illustrated the recruitment of water-mediated interactions in the recognition of FapydG rather than OG by Fpg (*73*).

Another interesting feature of the BsMY−DNA structure (*48*) was that OG was found to be stacked within the helix, but in the *anti* conformation. This suggests a significant conformational change, since OG paired with A in the DNA double helix would be in the *syn* conformation. Previous fluorescence studies using 2-aminopurine flanking OG or A indicated changes in fluorescence associated with OG occur first, followed by changes associated with A (*43*). This was interpreted as MutY flipping of OG followed by flipping of A. In light of the structural data, this suggests that the OG-related fluorescence changes may be associated with the change of OG from the *syn* to *anti* conformation, and this 180° swiveling motion would require a sufficient amount of space. Thus, the size of leucine and tyrosine may be optimal in promoting and facilitating these conformational changes of the OG that are associated with the DNA bending event. Indeed, the alteration of the OG conformation is expected to result in a steric clash with adenine and, thus, has been suggested to provide an important driving force for adenine extrusion (*48*).

The location of Gly 260 in the BsMY−DNA structure (corresponding to Gly 253 in *E. coli* MutY) in a tight turn region that contacts the phosphate backbone 5′ to the OG suggests that the conformational flexibility provided by glycine may be important at this position. Consistent with this idea, the adenine excision activity of G253D and G253A was nearly identical with both OG:A and G:A substrates, under all conditions that we examined. Consistently, the rates of adenine excision for G253A are less than those for G253D, indicating that the deleterious activity of G253D is not related to the presence of the charged Asp residue but rather the deviation from glycine. This is also reflected in the activation energies determined from the temperature dependence of 80.4 and 121.5 kJ/mol for G253D and G253A MutY, respectively. Binding analysis of G253A MutY demonstrated that this variant was unable to distinguish OG:FA from G:FA and bound both duplexes with equal affinity. In fact, the binding affinities for G253A MutY matched the K_d values obtained previously for G253D MutY. These results suggest that only glycine can be present at position 253 for the most efficient activity of MutY, and we hypothesize that any other amino acid will render the enzyme less effective in processing OG:A lesions in DNA. The dramatic effect of replacements of Gly 253 on the ability to recognize OG indicates that the interaction of the turn region with the two phosphates 5′ to OG are a key feature to indirectly recognizing OG. Perhaps these interactions play an important role in distorting the backbone allowing for alterations of the OG base conformation. Once the OG position is altered, hydrogen bonds within the active site may help to capture the OG in the *anti* conformation, which then drives adenine flipping (*48*).

The process of damage recognition and excision by DNA glycosylases has been described as a multistep process involving a "pinch, push, pull, plug" type of mechanism (*23*, *42*, *74*). Recent works on hOGG1 (*71*) and *E. coli* UDG (*75*) have revealed metastable destacked DNA intermediates in the base-flipping process. In this multistep process, encounter of a specific DNA lesion by the protein is followed by bending and distortion of the DNA double helix, and this process has been referred to as the "pinch". The pinch, in conjunction with the "push" of the appropriate base into the active site of the enzyme, has been demonstrated to be facilitated by intercalation of amino acid side chains into the DNA helix, acting as a "plug" to fill the position of the flipped-out base. The role of Tyr 82 seems to fall in line with providing the "pinch", in which the tyrosine side chain inserts itself between bases 5′ to the OG lesion, opening and unwinding the helix, and therefore facilitating a bend in the DNA duplex (*48*). It is unclear at this point if Tyr 82 may be also involved in the "push" step, which would entail participation in extrusion of the adenine base from the helix. The rotation of OG from *anti* to *syn* may participate in the "push" step to extrude the adenine, and the interaction of Tyr 82 with OG may facilitate this process. Notably, similar effects on glycosylase activity and binding affinity were observed in Y162S and Y162F mutants of the human alkyl adenine glycosylase (AAG). In AAG, the role of the intercalating tyrosine in providing the "push" and "plug" is more clear since it replaces the extruded damaged base within the helix (*24*). Tyr 82 in MutY does not play the classic role of the "plug" by taking the place of the flipped-out base but likely imparts stabilization to the distorted and base-flipped DNA conformation. The hydrogen-bonding of the backbone amides in the turn region that contains Gly 253 with the phosphodiester backbone may also participate in the "pinch" role of MutY. Indeed, this pinching motion, coupled with opening of the helix provided by Tyr 82, may aid in the rotation of OG around the *N*-glycosidic bond.

The presence of a bulky amino acid at position 82 is also important for the high affinity that MutY displays for the OG:AP product. MutY, as well as other DNA glycosylases, has been shown to exhibit a slow rate of product release, which is thought to be a mechanism of protection from strand breaks at the highly labile AP sites (*6*). Indeed, it has been hypothesized that the glycosylase remains bound to its product until another enzyme or complex can be recruited to the site for subsequent repair activities (*76*). Under multiple-turnover conditions, Y82C MutY releases the OG:AP DNA product 4-fold faster than WT MutY. On the basis of these data, we previously suggested that higher turnover by Y82C, and by association Y165C hMYH, may also lead to increased mutagenesis in vivo (*31*). In this study, Y82F and Y82L MutY are shown to release the OG:AP and G:AP DNA product at the same rate as WT MutY. This suggests that only a dramatic change to position 82, such as replacement with cysteine, affects the affinity of MutY for its product DNA.

This work has provided insight into the complexity of the ensemble of interactions that lead to faithful recognition of OG:A mismatches and removal of the misincorporated adenines by MutY. Indeed, the importance of recognition of only adenine opposite OG demands that the enzyme use a careful and multistep checking process. On the basis of the finding of the missense variants at the corresponding positions of Tyr 82 and Gly 253 in hMYH from colorectal cancer patients, the importance of these two residues in the activity of MutY has been revealed. This work illustrates an important synergism between understanding the basic properties of the enzyme and understanding its contribution to prevention of human cancer. Indeed, the detailed understanding of the roles of these two amino acids provided previously and herein will allow for more accurate prediction of the potential consequences of other missense variants in hMYH. Analogously, other missense variations found in hMYH will highlight important regions of MYH and MutY that are involved in mediating OG:A repair and will likely provide new and interesting insight into the properties of MutY/MYH, as well as BER glycosylases in general.

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