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# Probing the Requirements for Recognition and Catalysis in Fpg and MutY with Nonpolar Adenine Isosteres

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***Abstract:*** The *Escherichia coli* DNA repair enzymes Fpg and MutY are involved in the prevention of mutations resulting from 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG) in DNA. The nonpolar isosteres of 2′-deoxyadenosine, 4-methylbenzimidazole $\beta$-deoxynucleoside (B), and 9-methyl-1*H*-imidazo[4,5-*b*]pyridine $\beta$-deoxynucleoside (Q), were used to examine the importance of hydrogen bonding within the context of DNA repair. Specifically, the rate of base removal under single-turnover conditions by the MutY and Fpg glycosylases from duplexes containing OG:B and OG:Q mismatches, relative to OG:A mismatches, was evalulated. The reaction of Fpg revealed a 5- and 10-fold *increase* in rate of removal of OG from duplexes containing OG:B and OG:Q base pairs, respectively, relative to an OG:A mispair. These results suggest that the lack of the ability to hydrogen bond to the opposite base facilitates removal of OG. In contrast, adenine removal catalyzed by MutY was much more efficient from an OG:A mispair-containing duplex ($k_2 = 12 \pm 2$ min$^{-1}$) compared to the removal of B from an OG:B duplex ($k_{obs} < 0.002$ min$^{-1}$). Surprisingly, MutY was able to catalyze base removal from the OG:Q-containing substrate ($k_2 = 1.2 \pm 0.2$ min$^{-1}$). Importantly, the B and Q analogues are not deleterious to high-affinity DNA binding by MutY. In addition, the B and Q analogues are more susceptible to acid-catalyzed depurination illustrating that the enzyme-catalyzed mechanism is distinct from the nonenzymatic mechanism. Taken together, these results point to the importance of both N7 and N3 in the mechanism of adenine excision catalyzed by MutY.

## Introduction

Reactive oxygen species, ionizing radiation, and environmental pollutants can all lead to cellular DNA damage.[1] A major oxidative product in vivo is 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG).[1,2] During replication, there is a tendency for 2′-deoxyadenosine (A) to be inserted opposite OG to form a relatively stable OG:A mispair (Figure 1).[3] Failure to repair this mispair results in a permanent G:C to T:A transversion mutation in the following round of replication. To prevent mutations associated with oxidative damage to DNA, an assortment of DNA repair mechanisms have evolved. *Escherichia coli* utilizes the “GO” repair pathway which involves three enzymes: MutT, Fpg, and MutY.[4,5] MutT hydrolyzes d(OG)TP to prevent its incorporation into replicating DNA. The Fpg protein removes *OG*[6] from an OG:C base pair, and catalyzes an associated $\beta$- and $\delta$-eliminations at the resulting abasic site. The monofunctional adenine glycosylase MutY cleaves misincorporated adenine from OG:A-containing mispairs to form an apurinic (AP) site.[5,7] Downstream enzymes in the base excision repair (BER) pathway complete restoration of the DNA.[7−9] Eukaryotic functional and sequence homologues of Fpg and MutY have been identified, further underscoring the threat posed by OG to all organisms.[10−15]

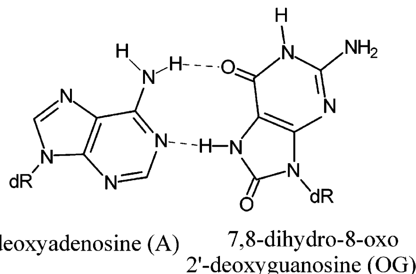


***Figure 1.*** Structure of an $OG_{syn}$:$A_{anti}$ base pair based on structural studies.[3]

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(6) To distinguish the base from the nucleoside the abbreviation is shown in italics, e.g., OG refers to 7,8-dihydro-8-oxo-2′-deoxyguanosine, while *OG* refers to 7,8-dihydro-8-oxoguanine.

(1) Lindahl, T. *Nature* **1993**, *362*, 709−715.
(2) Dizdaroglu, M. *Biochemistry* **1985**, *24*, 4476−4481.
(3) McAuley-Hecht, K. E.; Leonard, G. A.; Gibson, N. J.; Thomson, J. B.; Watson, W. P.; Hunter, W. N.; Brown, T. *Biochemistry* **1994**, *33*, 10266−10270.
(4) Cunningham, R. P. *Mutat. Res* **1997**, *383*, 189−196.
(5) Michaels, M. L.; Tchou, J.; Grollman, A. P.; Miller, J. H. *Biochemistry* **1992**, *31*, 10964−10968.
(7) Michaels, M. L.; Cruz, C.; Grollman, A. P.; Miller, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7022−7025.
(8) Michaels, M. L.; Miller, J. H. *J. Bacteriol.* **1992**, *174*, 6321−6325.
(9) David, S. S.; Williams, S. D. *Chem. Rev.* **1998**, *98*, 1221−1262.
(10) Lu, A.-L.; Fawcett, W. P. *J. Biol. Chem.* **1998**, *273*, 25098−25105.
(11) van der Kemp, P. A.; Thomas, D.; Barbey, R.; de Oliveira, R.; Boiteux, S. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 5197−5202.
(12) Yang, H.; Clendenin, W. M.; Wong, D.; Demple, B.; Slupska, M. M.; Chiang, J. H.; Miller, J. H. *Nucleic Acids Res.* **2001**, *29*, 743−752.
(13) Rosenquist, T. A.; Zharkov, D. O.; Grollman, A. P. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 7429−7434.
(14) Slupska, M. M.; Baikalov, C.; Luther, W. M.; Chiang, J. H.; Wei, Y. F.; Miller, J. H. *J. Bacteriol.* **1996**, *178*, 3885−3892.

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Although Fpg is believed to act chiefly on OG:C-containing substrates, it has also been shown to catalyze the removal of *OG* from OG:T-, OG:G-, and, to a lesser extent, OG:A-containing substrates.[16,17] A variety of oxidized purines, most notably 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) lesions, have been shown to be processed by Fpg.[9] In addition, Fpg has also been shown to remove the OG oxidation products spiroiminodihydantoin and guanidinohydantoin.[16,18]

The MutY glycosylase has been shown to process a variety of substrates in addition to OG:A mismatches.[9] For example, MutY is able to remove adenine in vitro and in vivo from G:A and C:A mispairs.[5,19,20] Binding studies with noncleavable A analogues revealed that MutY has a higher affinity for duplexes containing OG versus G.[21−25] These results support the assertion that duplexes containing OG:A are the preferred substrates for MutY. Additionally, the nature of the processing of substrates by MutY is highly dependent on the base opposite A.[26] Indeed, MutY exhibits a faster intrinsic rate for adenine removal opposite OG compared to G and has a higher affinity for the OG:(AP site) product than the G:(AP site) product.[26] A variety of X-ray crystal structures of Fpg and a truncated form of MutY have been solved which provide important structural backdrops for understanding the enzymatic processes of recognition and catalysis.[27−31]

Elucidation of the required interactions responsible for substrate recognition and catalysis may be facilitated by the use of synthetically derived DNA analogues (Figure 2). Previously, we have shown that MutY exhibits high affinity for the substrate analogues 2′-deoxyformycin A (F),[21] 2′-deoxyaristeromycin (R),[22] and 2′-deoxy-2′-fluoroadenosine (FA).[22] By altering the *N*-glycosidic bond or ribose sugar, duplexes containing F, R, or FA opposite OG or G have revealed features of mismatch recognition by MutY without complications associated with catalysis.[32−34] The benefit of these types of analogues is that base removal is impeded without major modification of potential important recognition elements of the base.

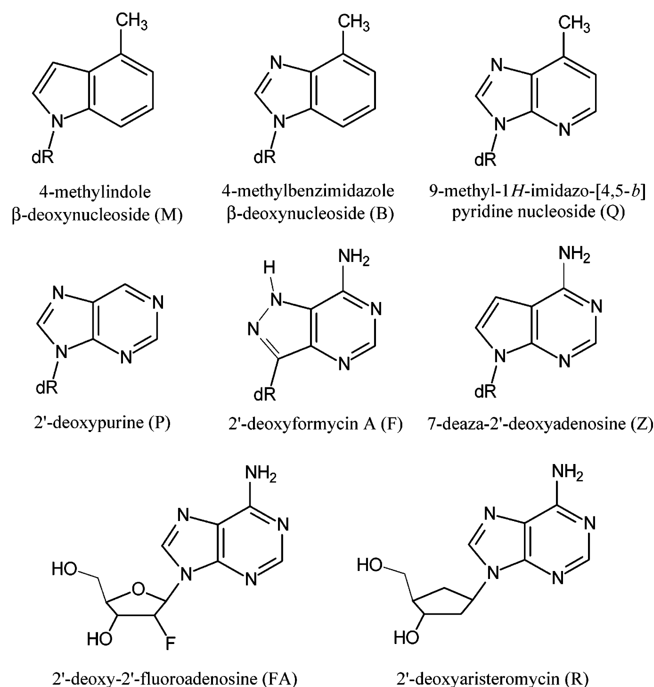


***Figure 2.*** Structures of 2′-deoxyadenosine analogues used to study MutY.

Nonpolar isosteric substrate analogues are specifically designed to examine the effects of hydrogen bonding within protein−substrate complexes without altering the steric properties. Indeed, hydrophobic isosteres of A, 4-methylbenzimidazole $\beta$-deoxynucleoside (B) (Figure 2), and T, 2,4-difluorotoluene $\beta$-deoxynucleoside (DF), have been successfully incorporated into replicating DNA as dNTPs.[35] In addition, a number of DNA polymerases correctly copy template strands containing these base analogues.[36] We have previously characterized interactions of MutY with the hydrophobic analogue, 4-methylindole $\beta$-deoxynucleoside (M)[37] opposite OG in duplex DNA.[25] M is a nonpolar variant of 7-deaza-2′-deoxyadenosine (Z), and both were shown to be resistant to processing by MutY. The origin of the resistance of M to the glycosylase action of MutY may be simply related to the absence of nitrogen at the position of the N7 of adenine, as is the case for the Z analogue.

In this work, the importance of hydrogen-bonding interactions with adenine in the glycosylase activity of Fpg and MutY was examined using the hydrophobic 2′-deoxyadenosine isosteres, 4-methylbenzimidazole $\beta$-deoxynucleoside (B)[38] and 9-methyl-1*H*-imidazo[4,5-*b*]pyridine $\beta$-deoxynucleoside (Q)[39] (Figure 2), in DNA duplexes opposite OG and G. We observed an increase in the rate of *OG* removal by Fpg when OG is paired with B or Q relative to A, providing further evidence that the mechanism of Fpg involves base flipping as part of the base removal process. In contrast, the removal of *B* from OG:B substrates by MutY was severely reduced, establishing that the presence of a

(15) Roldan-Arjona, T.; Wei, Y. F.; Carter, K. C.; Klungland, A.; Anselmino, C.; Wang, R. P.; Augustus, M.; Lindahl, T. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 8016−8020.
(16) Leipold, M. D.; Muller, J. G.; Burrows, C. J.; David, S. S. *Biochemistry* **2000**, *39*, 14984−14992.
(17) Leipold, M. D.; Workman, H.; Muller, J. G.; Burrows, C. J.; David, S. S. *Biochemistry* **2003**, *42*, 11373−11381.
(18) Hazra, T. K.; Muller, J. G.; Manuel, R. C.; Burrows, C. J.; Lloyd, R. S.; Mitra, S. *Nucleic Acids Res.* **2001**, *29*, 1967−1974.
(19) Radicella, J. P.; Clark, E. A.; Fox, M. S. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 9674−9678.
(20) Tsai-Wu, J.-J.; Liu, H.-F.; Lu, A.-L. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8779−8783.
(21) Porello, S. L.; Williams, S. D.; Kuhn, H.; Michaels, M. L.; David, S. S. *J. Am. Chem. Soc.* **1996**, *118*, 10684−10692.
(22) Chepanoske, C. L.; Porello, S. L.; Fujiwara, T.; Sugiyama, H.; David, S. S. *Nucleic Acids Res.* **1999**, *27*, 3197−3204.
(23) Deng, L.; Scharer, O. D.; Verdine, G. L. *J. Am. Chem. Soc.* **1997**, *119*, 7865−7866.
(24) Bulychev, N. V.; Varaprasad, C. V.; Dorman, G.; Miller, J. H.; Eisenberg, M.; Grollman, A. P.; Johnson, F. *Biochemistry* **1996**, *35*, 13147−13156.
(25) Chepanoske, C. L.; Langelier, C. R.; Chmiel, N. H.; David, S. S. *Org. Lett.* **2000**, *2*, 1341−1344.
(26) Porello, S. L.; Leyes, A. E.; David, S. S. *Biochemistry* **1998**, *37*, 14756−14764.
(27) Guan, Y.; Manuel, R. C.; Arvai, A. S.; Parikh, S. S.; Mol, C. D.; Miller, J. H.; Lloyd, R. S.; Tainer, J. A. *Nat. Struct. Biol.* **1998**, *5*, 1058−1064.
(28) Sugahara, M.; Mikawa, T.; Kumasaka, T.; Yamamoto, M.; Kato, R.; Fukuyama, K.; Inoue, Y.; Kuramitsu, S. *EMBO J.* **2000**, *19*, 3857−3869.
(29) Serre, L.; Pereira de Jesus, K.; Boiteux, S.; Zelwer, C.; Castaing, B. *EMBO J.* **2002**, *21*, 2854−2865.
(30) Fromme, J. C.; Verdine, G. L. *Nat. Struct. Biol.* **2002**, *9*, 544−552.
(31) Gilboa, R.; Zharkov, D.; Golan, G.; Fernandez, A.; Gerchman, S.; Matz, E.; Kycia, J.; Grollman, A. P.; Shoham, G. *J. Biol. Chem.* **2002**, *277*, 19811−19816.
(32) Chepanoske, C. L.; Golinelli, M.-P.; Williams, S. D.; David, S. S. *Arch. Biochem. Biophys.* **2000**, *380*, 11−19.
(33) Williams, S. D.; David, S. S. *Biochemistry* **2000**, *39*, 10098−10109.
(34) Chmiel, N. H.; Livingston, A. L.; David, S. S. *J. Mol. Biol.* **2003**, *327*, 431−443.
(35) Morales, J. C.; Kool, E. T. *Nat. Struct. Biol.* **1998**, *5*, 950−954.
(36) Kool, E. T.; Morales, J. C.; Guckian, K. M. *Angew. Chem., Int. Ed.* **2000**, *39*, 990−1009.
(37) Moran, S.; Ren, R. X.-F.; Sheils, C. J.; Kool, E. T. *Nucleic Acids Res.* **1996**, *24*, 2044−2052.
(38) Guckian, K. M.; Morales, J. C.; Kool, E. T. *J. Org. Chem.* **1998**, *63*, 9652−9656.
(39) Morales, J. C.; Kool, E. T. *J. Am. Chem. Soc.* **1999**, *121*, 2723−2724.

nitrogen only at the position of N7 of A is insufficient to promote base removal. However, a significant rate for removal of *Q* from OG:Q mismatches was observed, indicating that installation of the second nitrogen in the position of adenine's N3 strongly enhances the ability of MutY to catalyze base removal. Importantly, dissociation constant measurements show that MutY exhibits high affinity for duplexes containing OG:B and OG:Q mispairs. Together, the data suggest that even though the lack of hydrogen-bonding interactions is not deleterious to substrate recognition by MutY, these electrostatic interactions are vital for catalysis of base removal.

## Materials and Methods

**General Methods and Materials.** 4-Methylbenzimidazole $\beta$-deoxynucleoside and 9-methyl-1*H*-imidazo[4,5-*b*]pyridine $\beta$-deoxynucleoside were synthesized as previously described.[38,39] The 2′-deoxy-2′-fluoroadenosine phosphoramidite was kindly provided by Greg Kamilar and Dr. Peter Beal (University of Utah).[40] Standard 2′-deoxynucleotide-$\beta$-cyanoethyl phosphoramidites were purchased from Applied Biosystems Inc. (ABI). The 7,8-dihydro-8-oxo-2′-deoxyguanosine phosphoramidite was purchased from Glen Research. All substrate 2′-deoxyoligonucleotides were synthesized on an Applied Biosystems model 392 automated oligonucleotide synthesizer as per the manufacturer's protocol. The 5′-end-labeling was performed with T4 polynucleotide kinase obtained from New England Biolabs while [$\gamma$-$^{32}$P]ATP was purchased from ICN Radiochemicals. Labeled oligonucleotides were purified using ProbeQuant G-50 microcolumns from Amersham Biosciences. Bovine serum albumin (BSA) and Bradford reagents were obtained from Bio-Rad. All other chemicals were purchased from Fisher Scientific or U.S. Biochemical. Milli-Q distilled, deionized water was used for all reactions. All buffers were filtered through a nylon 0.22-$\mu$m filter prior to use. MutY purification was done on a Bio-Rad Biologic FPLC system. Storage phosphor autoradiography was performed using a Molecular Dynamics Storm 840 Phosphorimager. ImageQuaNT software (v5.2) was used to quantify storage phosphor autoradiograms. Kinetic experiments were performed using a rapid-quench flow instrument (model RQF-3) from Kintek Corporation.

**MutY Purification and Preparation.** Fpg and MutY were purified as described previously with minor modifications.[16,21,41] The enzyme concentration was determined by the method of Bradford using BSA as a standard.[42] The percent active enzyme of the Fpg and MutY protein used was 25 and 61%, respectively, using standard active-site titration methods modified for Fpg and MutY.[16,21,26] All enzyme concentrations listed were corrected for the active enzyme concentration.

**Substrate Preparation for Glycosylase Assays.** The following 2′-deoxyoligonucleotides were synthesized for glycosylase assays: 5′-CGATCATGGAGCCAC**X**AGCTCCCGTTACAG-3′ and 3′-GCTAGTACCTCGGTG**Y**TCGAGGGCAATGTC-5′ where **X** = 2′-deoxyguanosine (G) or 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG) and **Y** = 2′-deoxyadenosine (A), 2′-deoxycytidine (C), 4-methylbenzimidazole $\beta$-deoxynucleoside (B), 9-methyl-1*H*-imidazo[4,5-*b*]pyridine $\beta$-deoxynucleoside (Q), or 2′-deoxy-2′-fluoroadenosine (FA). Glycosylase assays were conducted using these oligonucleotides where the Y-containing strand (MutY) or X-containing strand (Fpg) was $^{32}$P-5′-end-labeled. The end-labeled Y- or X-containing strand was mixed with unlabeled Y- or X-strand, respectively, to provide a stock solution in which 5% of the solution was $^{32}$P-5′-end-labeled. The unlabeled complementary strand was then added in slight excess (20%) to form the duplex substrate. To promote duplex formation the mixture was heated to 90 °C in annealing buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.6, 10 mM EDTA) and allowed to cool to 20 °C over 4−6 h.

**Glycosylase Assays.** Fpg and MutY cleavage assays were performed as previously outlined.[16,17,26] Specific modifications with the Fpg protocol include a total reaction volume of 100 $\mu$L with final duplex and protein concentrations of 20 nM and 200 nM, respectively. It should be noted that Fpg reactions utilizing hand-mixing were not base-treated, and therefore the lyase activity of Fpg is used to provide strand scission. Reactions performed using the rapid-quench flow instrument use NaOH to quench the reaction, which may also cleave abasic sites. With OG substrates, we have observed that base-treatment does not modify the rate of strand scission. Modifications to the MutY protocol included final DNA and enzyme concentrations of 20 and 60 nM, respectively. All kinetic reactions contained final buffer concentrations of 20 mM Tris-HCl, pH 7.6, 0.1 mg/mL BSA, 10 mM EDTA, 15 mM NaCl, and 10% glycerol. Rate constants were derived from the observed rate of reaction under the conditions described above as we have reported for Fpg[16,17] and MutY[26,41] previously. In this analysis, $k_{obs} = k_2$, where $k_2$ describes the overall process of conversion of ES → EP.

**Equilibrium Dissociation Constant ($K_d$) Measurements.** $K_d$ values were determined using a gel retardation assay[43] previously described for MutY with substrate analogue duplexes.[22] Only labeled duplex was used with an estimated upper limit of duplex concentration based on 100% recovery from the end-labeling procedure. Reaction volumes of 40 $\mu$L contained 10 pM duplex DNA, 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1 mg/mL BSA, and MutY concentrations ranging from 13 pM to 220 nM. Samples of the protein−DNA mixture were incubated at 25 °C for 30 min. followed by the addition of 5 $\mu$L of nondenaturing loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in 1× TBE). Each sample was loaded on a 6% nondenaturing polyacrylamide gel (17 cm × 14 cm × 0.3 cm) with 0.5× TBE buffer at 120 V and allowed to run for 2 h at 4 °C. The gels were dried and exposed to a Molecular Dynamics storage phosphor screen for at least 18 h. $K_d$ values were determined by fitting the data (percent bound substrate versus log[MutY]) to the equation for one-site ligand binding using GraFit 4.0.16. Reported $K_d$ values are the average of at least six separate experiments.

**Determination of Thermal Denaturation Temperatures.** UV melting studies were performed as previously described.[25] An 11 bp duplex DNA was synthesized and purified as described above with the following sequence: 5′-GAGCT**OG**GTGGC-3′•3′-CTCGA**Y**CACCG-5′ where Y= A, B, or Q. The duplexes were prepared in 10 mM $KHPO_4$, 1 M NaCl, and 1 mM EDTA, pH 7.0, at 4 and 8 $\mu$M concentrations. The absorbance was monitored at 260 nm from 20 to 80 °C on a Hewlett-Packard 8452A diode array spectrophotometer. The DNA samples were degassed with argon and filtered through a 0.45 $\mu$m filter into a 1 cm path length quartz cell. Obtaining melting temperatures ($T_m$) from the experimental data (absorbance vs temperature) was accomplished from curve fits, assuming the two-state transition model.[44]

**Acid-Catalyzed Depurination Assays.** A modified Maxam−Gilbert G+A sequencing reaction was performed as previously described.[45] Olignucleotides containing B or Q at position 15 used in the glycosylase assays were $^{32}$P-5′-end-labled as described above. A solution (23 $\mu$L) containing 17 nM DNA, 0.11 M piperidine, 15% formic acid, and 0.18 mg/mL calf thymus DNA was incubated at 37 °C for 45 min. The reaction was quenched by addition of 225 $\mu$L of a quenching solution (0.3 M sodium acetate, pH 7, 0.1 mM EDTA, 0.03 mg/mL calf thymus DNA). The DNA strands were then ethanol precipitated followed by the addition of 1.0 M piperidine. The mixture was incubated at 90 °C

(40) Yi-Brunozzi, H.-Y.; Easterwood, L. M., Kamilar, G. M.; Beal, P. A. *Nucleic Acids Res.* **1999**, *27*, 2912−2917.
(41) Chmiel, N. H.; Golinelli, M.-P.; Francis, A. W.; David, S. S. *Nucleic Acids Res.* **2001**, *29*, 553−564.
(42) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248−254.
(43) Carey, J. *Methods Enzymol.* **1991**, *208*, 103−117.
(44) Petersheim, M.; Turner, D. H. *Biochemistry* **1983**, *22*, 256−263.
(45) Sambrook, J.; Russell, D. W. *Molecular Cloning, A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: Plainview NY, 2001; Vol. 1.

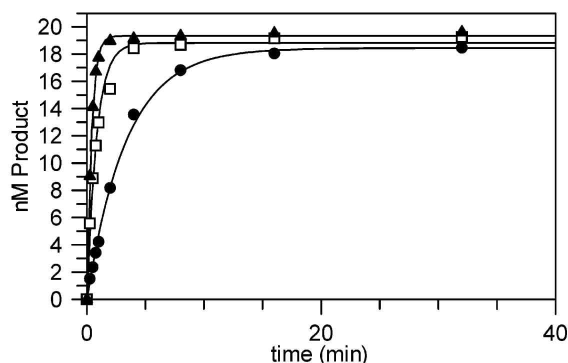


***Figure 3.*** Representative plot of OG removal as a function of time for Fpg performed under conditions of single-turnover with substrates containing OG:A (●), OG:B (□), and OG:Q (▲). Experiments were performed using 220 nM Fpg and 20 nM OG-containing duplex at 37 °C.

for 30 min; denaturing loading dye was added, and the mixture was electrophoresed on a 15% polyacrylamide gel for 2 h at 1600 V. Gels were quantitated using storage phosphor autoradiography.

## Results

**OG Glycosylase Activity of Fpg with OG:C-, OG:A-, OG:B-, and OG:Q-Containing Substrates.** The effects of substituting A in an OG:A mismatch with the nonpolar isosteres B and Q on the OG glycosylase activity of Fpg was evaluated using 30 bp duplexes containing a central OG:X (X = A, B, Q, or C) base pair. Single-turnover kinetics experiments, where [Fpg] > [DNA], were used to evaluate the efficiency of the glycosylase activity of Fpg.[16,17] These reactions followed first-order kinetics, yielding quantitative conversion to products. The data were fitted to the appropriate rate equations to determine the rate constant, $k_2$, which includes all steps involving base excision. A representative plot of the reaction of Fpg with DNA duplexes containing OG:C, OG:A, OG:B, and OG:Q bps is shown in Figure 3. As expected, the removal of *OG* by Fpg from the OG:C-containing duplex was considerably more efficient ($k_2 = 46 \pm 8$ min$^{-1}$) than from the OG:A-containing duplex ($k_2 = 0.30 \pm 0.04$ min$^{-1}$). The 150-fold decrease in $k_2$ for the OG:A-containing substrate relative to the OG:C-containing substrate is consistent with the trends reported previously.[16,46] Importantly, the *OG*-removal activity of Fpg on OG:B- and OG:Q-containing duplexes was robust with the measured $k_2$ values of $1.4 \pm 0.3$ and $2.8 \pm 0.2$ min$^{-1}$, respectively. The significant *increase* in the rate of *OG* removal by substitution of B or Q for A suggests that the lack of hydrogen bonding between OG and B or Q aids in DNA deformation and/or extrusion of OG from the duplex into the enzyme active site.

**Adenine Glycosylase Activity of MutY with OG:A-, OG:B-, and OG:Q-Containing Substrates.** To determine the importance of hydrogen bonding in the catalysis of adenine removal by MutY, the ability of MutY to remove the modified base moieties of B and Q opposite both OG and G in a 30 bp duplex substrate was evaluated. Assays performed under single-turnover conditions revealed a significantly reduced ability of MutY to remove *B* from the OG:B mispair-containing duplex (Table 1). In fact, for adenine removal from the OG:A mispair the rate constant $k_2$ was determined to be $12 \pm 2$ min$^{-1}$ in this duplex substrate compared to an upper limit of <0.002 min$^{-1}$ for the rate of B removal (Table 1). No significant removal of *B* was detected from the G:B-containing duplex. The magnitude of the difference in the rate constants for removal of *B* compared to adenine implies that the presence of an *N*-glycosidic bond, and nitrogen at the seven position is not sufficient for enzymatic base removal by MutY.

***Table 1.*** Rate Constants ($k_2$) Determined under Single-Turnover Conditions for the Reaction of Fpg and MutY with 30 Base Pair Duplexes at 37 °C

| central base pair | rate constants ($k_2$) (min$^{-1}$)[a] | |
|---|---|---|
| | Fpg | MutY |
| OG:A | 0.30 ± 0.02 | 12 ± 2[b] |
| OG:B | 1.4 ± 0.3 | <0.002[c] |
| OG:Q | 2.8 ± 0.2 | 1.2 ± 0.2 |
| OG:C | 46 ± 8 | NR[d] |
| G:A | NR | 1.6 ± 0.2[e] |
| G:B | NR | NC[f] |
| G:Q | NR | <0.03[g] |

[a] The errors reported in the rate constants are the standard deviation of the average. [b] Rate constant determined by Olga Lukianova (David laboratory). This value in this DNA substrate is similar to that reported previously in our laboratory with the same substrate[34] and by other laboratories with different DNA substrates.[57,58] [c] Rate listed is an upper limit estimate calculated using half-lives based on extent of conversion at $t = 30$ min. [d] Not relevant; these bps are not substrates for these enzymes. [e] This value has been previously reported.[26] [f] Not cleaved; Formation of product at the end of the reaction time course was no greater than the no-enzyme control. Estimated rate based on our detection limits is <0.0005 min$^{-1}$. [g] Rate listed is an upper limit estimate calculated using half-lives based on extent of conversion at $t = 30$ min.

However, single-turnover assays with duplexes containing OG:Q reveal a remarkable change in activity relative to duplexes containing OG:B. MutY catalyzes the removal of *Q* opposite OG with a rate constant $k_2$ of $1.2 \pm 0.2$ min$^{-1}$. Thus, the presence of one additional nitrogen in Q at the position of N3 of adenine greatly facilitates the base removal activity of MutY. In the analogous experiment with the G:Q duplex, a detectable amount of cleavage of Q was observed, but with a significantly diminished rate ($k_{obs} < 0.03$ min$^{-1}$) relative to that of the analogous reactions with a G:A substrate ($k_2 = 1.6 \pm 0.4$ min$^{-1}$).

**Equilibrium Dissociation Constant ($K_d$) Determination of MutY with Substrate Analogues.** To determine whether the reduced catalytic activity of MutY toward duplexes containing B or Q analogues opposite OG or G was a result of inefficient recognition of the modified bases, equilibrium dissociation constants ($K_d$) were determined for MutY with these analogue-containing duplexes. We have shown previously that MutY has high affinity for duplexes containing FA or M opposite OG and G;[22,25] thus, duplexes containing these analogues serve as excellent benchmarks for high-affinity MutY binding. Nondenaturing gel retardation assays,[43] performed under conditions where [DNA duplex] < $K_d$, were utilized to quantitate the [MutY−DNA complexes] as a function of [MutY] to determine the relevant dissociation constants, $K_d$ (Table 2). The determined $K_d$ values illustrate the similarity in recognition of the nonpolar isosteres to FA and A by wild type (WT) and E37S MutY. The $K_d$ values determined for duplexes containing OG:FA and OG:B with WT MutY were $0.10 \pm 0.04$ and $0.09 \pm 0.04$ nM, respectively. A $K_d$ measurement of WT MutY with robust substrates, such as OG:A or OG:Q, would be unreliable due to the presence of varying amounts of substrate and product at each MutY concentration in the binding titration. Therefore, a mutant form of MutY, E37S, which has been demonstrated to

(46) Tchou, J.; Bodepudi, V.; Shibutani, S.; Antoshechkin, I.; Miller, J.; Grollman, A. P.; Johnson, F. *J. Biol. Chem.* **1994**, *269*, 15318−15324.

***Table 2.*** Equilibrium Dissociation Constants ($K_d$) for WT and E37S MutY with Substrate Analogs at 25° C

| central base pair | WT MutY $K_d$ (nM)[a] | E37S MutY $K_d$ (nM)[a] |
|---|---|---|
| OG:A | NA[b] | <0.04[c] |
| OG:B | 0.09 ± 0.04 | ND[d] |
| OG:Q | NA | 0.35 ± 0.07 |
| OG:FA | 0.10 ± 0.04[e] | ND |
| OG:M | 0.14 ± 0.05[e] | ND |
| OG:C | 26 ± 13[e] | ND |
| G:A | 21 ± 4 | 27 ± 6 |
| G:B | 27 ± 6 | ND |
| G:Q | NA | 33 ± 6 |
| G:FA | 5.8 ± 0.6[e] | ND |
| G:M | 40 ± 6[e] | ND |
| G:C | 153 ± 64[e] | ND |

[a] Errors reported in dissociation constants are the standard deviation of the average. [b] Not applicable. Accurate $K_d$ measurements cannot be obtained with duplexes that are substrates for the enzyme. [c] Upper limit estimation; DNA concentration (0.02 nM) needed for detection is too close to enzyme concentration for an accurate $K_d$ determination. [d] Not determined. The E37S mutated MutY was only used with duplexes that are substrates for WT MutY. [e] Values previously reported.[21,22,25]

be catalytically inactive,[27] was used for substrates containing OG:Q and OG:A base pairs. A $K_d$ value of 350 ± 7 pM was obtained for substrates containing OG:Q incubated with E37S MutY. For comparison, using E37S with OG:A-containing substrates, the $K_d$ was estimated as <40 pM. This indicates a modest reduction in the binding of E37S MutY with the Q analogue relative to A (∼9-fold) upon consideration of the major structural differences between Q and A.

MutY retains high affinity for substrates containing OG:B and OG:Q despite the lack of potential hydrogen-bonding sites at positions corresponding to N1 and N6 of adenine as shown by the picomolar dissociation constants for the analogue-containing duplexes (Table 2). These values are similar to the $K_d$ value obtained for binding of MutY to duplexes containing OG opposite the hydrophobic analogue M ($K_d = 0.20 \pm 0.05$ nM).[25] Interestingly, the measured affinities are similar to those for analogues that have an unadulterated base, such as FA.[47] Notably, the $K_d$ values for the B and Q duplexes are significantly lower than those obtained with the analogous duplex lacking a mismatch ($K_d = 150 \pm 60$ nM),[21] indicating that the modifications present in B and Q do not have a deleterious effect on MutY recognition. These results imply that MutY does not absolutely require the specific hydrogen-bonding interactions with N1, N3, or the N6 amino group of adenine for recognition of adenine mispaired with OG.

**Thermal Denaturation Studies of DNA Duplexes Containing OG:A and OG:B Mispairs.** Duplexes of 11 bp in length containing a centrally located OG:A, OG:B or OG:Q mispair were prepared to analyze the effects of the Q and B analogues on duplex stability. The melting temperatures ($T_m$) were obtained by monitoring absorbance at 260 nm during thermal denaturation in buffer containing 10 mM potassium phosphate, 1 M NaCl, and 0.1 mM EDTA at pH 7.0. The $T_m$ values for substrates containing OG:A, OG:B, and OG:Q mispairs at 8 $\mu$M were 56.4, 48.9, and 50.2 °C, respectively. The results show that the OG:B and OG:Q base pairs are more destabilizing to the duplex than the OG:A mispair, which is known to hydrogen bond in an $OG_{syn}$:$A_{anti}$ fashion.[3] These results suggest that enzymes utilizing a base flipping mechanism would benefit from this increased duplex destabilization. This is made evident in the case with Fpg acting on the substrate containing OG opposite B or Q wherein the rate of OG cleavage is faster compared to OG opposite A. This also indicates that the steps described by the rate constant $k_2$ include disruption of the DNA duplex, and that base flipping is at least in part rate limiting.

(47) Chepanoske, C. L. Ph.D. Dissertation, University of Utah, Salt Lake City, 2000.

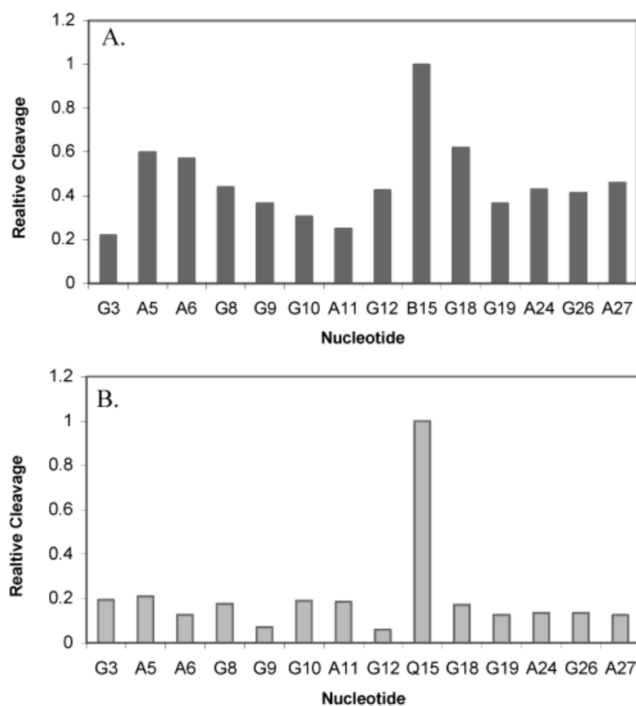


***Figure 4.*** Histograms illustrating extent of acid-catalyzed depurination at purine sites within the B- and Q-containing 30-nucleotide strand (A) 4-methylbenzimidazole or (B) 9-methyl-1*H*-imidazo[4,5-*b*]pyridine at position 15.

**Acid-Catalyzed Depurination of Nonpolar Analogues.** Proposed mechanisms for the adenine glycosylase activity of MutY (Figure 5) implicate protonation of N7 as an important step during the glycosylase reaction. Therefore, an evaluation of the susceptibility of B and Q toward acid-catalyzed depurination would provide insight into constraints for the mechanism of the enzyme-catalyzed adenine removal reaction. Mechanisms that have been proposed for acid-catalyzed depurination invariably invoke protonation at N7 as a major feature of the reaction.[48−50] The same DNA strand used to make the 30 bp duplex substrate for the kinetic reactions was used to test and compare the rate of acid-catalyzed depurination of B and Q to G and A. The $^{32}$P-5′-end-labeled, single-stranded DNA substrate containing B or Q was used in a modified Maxam−Gilbert G + A reaction utilizing piperidine-formate to initiate depurination. Upon analysis, following gel electrophoresis, the relative extents of depurination were gauged by quantitation of the bands at each purine position in the sequence of the oligonucleotide (Figure 4). These results illustrate the extent of depurination of A and G relative to that of B and Q. As shown, B undergoes acid-catalyzed depurination twice as readily as A and G, whereas Q displays a 7-fold increase in depurinated product relative to G or A. This data suggests that these analogues may, in fact,

(48) Zoltewicz, J. A.; Clark, D. F.; Sharpless, T. W.; Grahe, G. *J. Am. Chem. Soc.* **1970**, *92*, 1741−1750.
(49) Garrett, E. R.; Mehta, P. J. *J. Am. Chem. Soc.* **1972**, *94*, 8532−8541.
(50) Mentch, F.; Parkin, D. W.; Schramm, V. L. *Biochemistry* **1987**, *26*, 921−930.

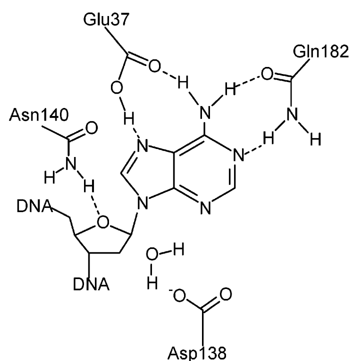


*Figure 5.* Amino acids involved in recognition of 2′-deoxyadenosine by MutY based on X-ray structure with bound adenine.[27]

be superior substrates compared to A, if a purely acid-catalyzed mechanism is used by MutY, since the base moieties appear to be more susceptible to protonation.

## Discussion

Substrate, transition state, and product analogues may provide unique insight into the mechanisms of substrate location, damage recognition, and catalysis of DNA repair enzymes.[9] Previously, we have shown that 2′-deoxy-2′-fluoroadenosine (FA) is resistant to the glycosylase activity of MutY, but retains specific recognition properties akin to those of 2′-deoxyadenosine.[22] Indeed, this and other glycosylase-resistant analogues have been used to evaluate the effects of mutations of MutY on substrate recognition without complications associated with the enzymatic reaction.[32,34,41] The use of synthetic analogues has also allowed for evaluating the importance of hydrogen bonding versus shape in enzyme-catalyzed reactions on DNA. For example, 2,4-difluorotoluene $\beta$-deoxynucleoside (DF) and B have been used as T and A hydrophobic isosteres, respectively, in studies involving DNA polymerases to evaluate the steric and hydrogen-bonding requirements of dNTP insertion.[35,36] Surprisingly, these studies revealed that DNA polymerases do not strictly require correct hydrogen bonding when synthesizing a new base pair. Indeed, the shape of the base and shape complementarity of the base pair appears to be more important in selection of correct base pairs during DNA replication.

In this work, B and Q were employed as 2′-deoxyadenosine analogues to investigate the importance of potential hydrogen-bonding sites on the recognition of A within OG:A mismatches by MutY and Fpg. The results reported herein show that Fpg removes OG from substrates containing OG:B and OG:Q 5-fold and 10-fold faster, respectively, than the corresponding substrates containing OG:A. It has been proposed that BER glycosylases utilize a base-flipping mechanism wherein the base being removed and/or the base opposite are extruded from the helix into a base-specific enzyme pocket. This type of mechanism allows the enzyme greater access for examination of the base and catalysis of its removal. The X-ray structure of Fpg alone and bound to DNA are consistent with use of a base-flipping mechanism.[28−31] The increased rate of *OG* removal by Fpg when OG was paired with the nonpolar analogues suggests that the OG:B/Q base pairs, given the lack of base pair hydrogen bonds, are easier to disrupt to facilitate base flipping. Indeed, as expected, the duplex stability studies indicate that the OG:B- and OG:Q-containing duplexes are less stable than their OG:A counterpart. Likewise, in a related case, a 2-fold increase in the rate of excision of hypoxanthine opposite difluorotoluene $\beta$-deoxynucleoside (DF) compared to T by the human alkyladenine glycosylase (AAG) was observed.[51] The DF analogue is a hydrophobic isostere of T that is unable to hydrogen bond to hypoxanthine, and therefore was similarly proposed to facilitate base flipping by AAG.

Notably, the observed rate constants for the removal of OG by Fpg opposite A, B, and Q are significantly smaller than the rate constants observed for the corresponding reaction with an OG:C-containing duplex. This suggests that Fpg specifically recognizes the shape of the base opposite OG since the rates for processing the OG:B- or OG:Q-containing duplex are more similar to that for the OG:A-containing duplex than for the corresponding OG:C-containing duplex. Thus, Fpg appears to actively discriminate against A, and therefore also the A analogues B and Q. In the X-ray structures of Fpg, recognition of cytosine opposite OG was shown to be mediated by hydrogen bonding of an arginine to cytosine.[30] Interestingly, however, this same arginine was found to be capable of hydrogen bonding to guanine and thymine.[30] Our results call attention to the equal, or perhaps even greater, importance of base *shape* in selection against the incorrect base-pair context for damage removal by DNA glycosylases.

The crystal structure of a truncated form of MutY with a bound adenine uncovers a complex hydrogen bonding-network between MutY and the free adenine base (Figure 5).[27] Specifically, Glu 37 and Gln 182 participate in hydrogen bonds with N7, N1, and the N6 amino group of adenine. The N1 and N6 amino group of adenine are also involved in base pairing with OG in an $OG_{syn}$:$A_{anti}$ mispair. On the basis of the observed interactions with the Watson−Crick face of adenine, MutY has been proposed to utilize a base-flipping mechanism wherein adenine is extruded into an active-site pocket containing Glu 37 and Gln 182.[27] The nonpolar isosteres B and Q prevent the formation of base-pairing hydrogen bonds with OG in a fashion analogous to A. In addition, the hydrogen bonds between the Watson−Crick face of the base portion of the analogue and residues within the adenine-specific pocket of MutY are also eliminated.

This work has shown that removal of *B* by MutY from substrates containing OG:B base pairs was virtually absent ($k_{obs}$ < 0.002 min$^{-1}$) compared to the robust reaction of MutY with its chief substrate, an OG:A-containing duplex ($k_2 = 12 \pm 2$ min$^{-1}$). *B* differs from adenine in that the N1 and N3 are replaced with carbon in addition to replacing the exocyclic amino group with a methyl substituent. These modifications create a Watson−Crick face of the base that is hydrophobic and yet maintain the overall shape of adenine. Therefore, while MutY might benefit from the destabilized duplex arising from the OG:B mispair with respect to base flipping, the loss of hydrogen-bonding contacts or unfavorable electrostatics renders the substrate resistant to catalysis. In contrast, MutY-catalyzed cleavage of *Q* from OG:Q mispair-containing duplexes is dramatically faster ($k_2 = 1.2 \pm 0.2$ min$^{-1}$) than the corresponding reaction with its OG:B-containing cousin. Notably, the rate of processing of the OG:Q substrate by MutY is similar to that observed for the reaction with a G:A substrate (Table 1). The only structural difference between B and Q is the presence of

(51) Vallur, A. C.; Feller, J. A.; Abner, C. W.; Tran, R. K.; Bloom, L. B. *J. Biol. Chem.* **2002**, *277*, 31673−31678.

a nitrogen atom in place of carbon at the position corresponding to that of N3 of adenine. This result demonstrates that specific hydrogen bonding interactions and/or essential electrostatic interactions between the substrate base and the enzyme are critical for catalysis of base removal. The results with the Q analogue indicate the importance nitrogen at the position analogous to N3 of adenine for efficient catalysis of base removal by MutY. The observed 10-fold decrease in the rate of base removal catalyzed by MutY on the OG:Q substrate relative to the OG:A substrate is relatively small in view of the marked differences between the two substrates.

The relevant dissociation constants ($K_d$) measured in gel retardation assays reveal additional insights into the features associated with recognition of OG:A mismatches by MutY. Although MutY exhibits a reduced capacity for removal of the hydrophobic base analogues, the $K_d$ data (Table 2) show that MutY effectively recognizes the B and Q analogues opposite OG and G. Thus, this establishes that the reduced activity of MutY toward these hydrophobic substrates is not a result of deleterious consequences to binding of the modified base. The measured dissociation constants for the OG:Q and OG:B duplexes are essentially identical to those we have measured previously for the analogous duplex containing the FA analogue opposite OG. This is surprising, considering that the FA analogue contains an unadulterated adenine base, while the B and Q analogues have considerably modified base moieties. We have also previously observed high-affinity binding of MutY to an OG:M duplex.[25] The observed high-affinity binding of MutY for the hydrophobic analogues opposite OG may be due to the lack of hydrogen bonding to OG. Indeed, the absence of an energetic cost associated with disrupting the base pair may facilitate a nucleotide-flipping process and therefore compensate for the absence of favorable hydrogen-bonding interactions between the hydrophobic analogues and MutY. These results indicate that the Watson−Crick face of adenine is not of critical importance for initial base recognition by MutY, whereas the unique features of A become important during enzymatic catalysis of the base-removal reaction.

A feature of MutY that distinguishes it from other glycosylases is the recognition of a normal base mispaired with OG. We, and others, have previously shown that MutY preferentially binds to duplexes containing OG versus G when placed opposite the same base.[21−25,41] Indeed, in this work, we observed higher affinity for duplexes containing the B and Q analogues opposite OG (Table 2) and more efficient removal of *Q* opposite OG than G (Table 1). In general, removal of modified purines by MutY is enhanced when paired with OG rather than G (Francis, A. F., David, S. S., unpublished results). Clearly, MutY recognition of its favorite base pair (OG:A) is synergistic with the presence of the correct partner aiding in recognition of the other. One possibility is that MutY *initially* binds relatively tightly to any substrate containing OG, and then utilizes a second screen to ensure that only the appropriate base is removed. Obviously, high-fidelity repair requires that only adenine opposite OG be removed. Thus, it is possible that the active site of MutY may sample a variety of base pairs, but has been optimized toward cleavage of only the base (adenine) containing the proper shape *and* electrostatic interactions.

The X-ray structure of MutY (Figure 5) indicated important contacts with N7, N6, and N1 of adenine.[27] Previous work with purine $\beta$-deoxynucleoside (P) had shown that the N6 amino group is not required for base removal by MutY.[41] Indeed, the observed rate for purine removal by MutY was reduced only by a factor of 2 compared to that for adenine removal. Anticipated mechanisms for MutY based on acid-catalyzed solvolysis and enzymatic hydrolysis of purine nucleosides will require the N9 nitrogen for a proper *N*-glycosidic bond and protonation of the N7 nitrogen to enhance its potential as a leaving group.[49,50,52]

The importance of adenine N7 for the MutY-catalyzed reaction has been previously established in that 7-deaza-A (Z) is completely resistant to the glycosylase action of MutY.[21] However, the N7 group alone is clearly not sufficient, as evidenced by the current finding that analogue B is essentially inactive as a substrate for MutY. The nonpolar base analogues *B* and *Q* retain nitrogen at the positions corresponding to seven and nine of adenine. Thus, we had anticipated that these analogues might be substrates for MutY-catalyzed base removal. However, the absence of potential hydrogen-bond contacts is not the only alteration that occurs by replacement of nitrogen with carbon in the purine ring. Indeed, such replacements alter the electronics of the base. Our acid-catalyzed depurination experiments with single-stranded substrates containing B or Q show that these analogues are actually *more* susceptible to depurination than 2′-deoxyadenosine and 2′-deoxyguanosine. This may well reflect increased basicity of the nonpolar bases. The enhanced acidic depurination suggests that these analogues may be similarly pre-disposed to enzymatic base cleavage. However, glycosylase experiments exhibit the opposite effect; enzymatic base cleavage of B is nonexistent, while cleavage to release *Q* is decreased (relative to that for adenine) by a small amount. The N3 nitrogen alone is responsible for the large difference between Q and B. Future work will be needed to determine the relationship between the increased acid lability arising from the additional nitrogen in Q and its effect in the enzyme active site.

Taken together, the data presented herein suggest that enzymatic removal of adenine by MutY likely occurs by a more complex mechanism than has been previously considered. Indeed, the increased activity of MutY toward the Q analogue relative to that toward the B analogue clearly suggests an importance for the presence of the N3 of adenine. For example, the N3 of adenine might be specifically recognized inside the active-site pocket of MutY in a manner similar to Taq polymerase, wherein protein side chains within the active site confirm the presence of a purine via hydrogen bonding with N3.[53] However, such interactions are not immediately obvious in the published structure of MutY with bound adenine base (Figure 5). The N3 of adenine has also been suggested to be important in the removal of adenine in the ribosome by the toxin ricin.[54,55] Indeed, in crystallographic studies, Arg 180 of ricin A-chain was found to be in close proximity to N3 and therefore was proposed to be involved in hydrogen bonding to N3 of adenine.[55]

The present results suggest that both N7 and N3 of adenine may be protonated and/or involved in hydrogen bonding with

(52) Ehrlich, J. I.; Schramm, V. L. *Biochemistry* **1994**, *33*, 8890−8896.
(53) Eom, S. H.; Wang, J.; Steitz, T. A. *Nature* **1996**, *382*, 278−281.
(54) Chen, X. Y.; Link, T. M.; Schramm, V. L. *Biochemistry* **1998**, *37*, 11605−11613.
(55) Monzingo, A. F.; Robertus, J. D. *J. Mol. Biol.* **1992**, *227*, 1136−1145.

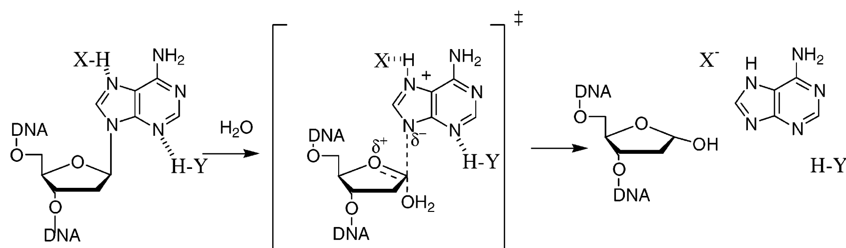


***Figure 6.*** Possible mechanism of MutY utilizing diprotonation/hydrogen-bonding at N7 and N3.[59] The mechanism shown illustrates an oxocarbenium ion-like transition state for *N*-glycosidic bond cleavage based on work with other glycosylases,[60] and related nucleoside or nucleotide hydrolyases.[50,56]

MutY during catalysis (Figure 6). Multiple protonation is consistent with studies of acid-catalyzed solvolysis of adenine nucleosides and nucleotides.[49] Kinetic isotope effect measurements with ricin A-chain indicated a highly dissociative transition state featuring an enzyme stabilized ribooxocarbenium ion species in the transition state.[56] Chen et al. also suggested that diprotonation at N1 and N7 would be a key feature of the reaction in order to stabilize the departing base.[56] Indeed, calculations indicated that the energy required to break the C1′−N9 bond decreases dramatically in the N1H, N7H diprotonated adenosine species.[56] Thus, a similar scenario could be operative in MutY with N7 and N3 protonation. Further studies with additional analogues and kinetic isotope effects with MutY may provide further evidence for such a hypothesis. In particular, analogues addressing the role of N1 may be illuminating with regards to the merits of this potential mechanism (Figure 6).

This work illustrates the usefulness of nonpolar base isosteres in separating the requirements of substrate recognition and catalysis. The results presented herein with Fpg and MutY suggest that non-hydrogen-bonding substrates destabilize duplex DNA and therefore increase the rate of base removal by enzymes utilizing a base-flipping mechanism. However, the loss of hydrogen-bonding interactions that aid in duplex destabilization might also be responsible for loss of enzymatic catalysis in the case of MutY. We have also shown that the hydrophobic nature of B and Q does not interfere with recognition by MutY. However, these analogues have provided insight into the importance of N7 and N3 in the base-removal activity catalyzed by MutY. This work suggests additional analogues that could be synthesized and tested to further illuminate the mechanism of this fascinating enzyme.

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JA0374426

(56) Chen, X.-Y.; Berti, P. J.; Schramm, V. L. *J. Am. Chem. Soc.* **2000**, *122*, 1609−1617.
(57) Noll, D. M.; Gogos, A.; Granek, J. A.; Clarke, N. D. *Biochemistry* **1999**, *38*, 6374−6379.
(58) Bernards, A. S.; Miller, J. K.; Bao, K. K.; Wong, I. *J. Biol. Chem.* **2002**, *277*, 20960−20964.
(59) Our data do not exclude mechanisms in which N3 plays a more indirect role. In addition, it may be that the presence of two nitrogen atoms is required for efficient base removal such that a mechanism with N1/N7 protonation may be operative in A.
(60) Werner, R. M.; Stivers, J. T. *Biochemistry* **2000**, *39*, 14054−14064.