Single-Turnover and Pre-Steady-State Kinetics of the Reaction of the Adenine Glycosylase MutY with Mismatch-Containing DNA Substrates†

Silvia L. Porello, Aquiles E. Leyes, and Sheila S. David*

Department of Chemistry, University of Utah, Salt Lake City, Utah 84112

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ABSTRACT: The DNA repair enzyme MutY plays an important role in the prevention of DNA mutations resulting from the presence of the oxidatively damaged lesion 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG) in DNA by the removal of misincorporated adenine residues in OG:A mispairs. MutY also exhibits adenine glycosylase activity toward adenine in G:A and C:A mismatches, although the importance of this activity in vivo has not been established. We have investigated the kinetic properties of MutY's glycosylase activity with OG:A and G:A containing DNA duplexes. Our results indicate that MutY's processing of these two substrates is distinctly different. By using single-turnover experiments, the *intrinsic* rate for adenine removal by MutY from an OG:A substrate was found to be at least 6-fold faster than that from the corresponding G:A substrate. However, under conditions where [MutY] ≪ [DNA], OG:A substrates are not quantitatively converted to product due to the inefficient turnover resulting from slow product release. In contrast, with a G:A substrate MutY's dissociation from the corresponding product is more facile, such that complete conversion of the substrate to product can be achieved under similar conditions. The kinetic results illustrate that the glycosylase reaction catalyzed by MutY has significant differences depending on the characteristics of the substrate. The lingering of MutY with the product of its reaction with OG:A mispairs may be biologically significant to prevent premature removal of OG. Thus, this approach is providing insight into factors that may be influencing the repair of damaged and mismatched DNA in vivo by base-excision repair glycosylases.

Precise DNA replication is mandatory for survival of all organisms. In general, DNA replication occurs with high fidelity; however, an occasional mismatch may persist in DNA. Correction of these mismatches by mismatch repair systems is critical for preventing mutagenesis and carcinogenesis (*1*). The presence of DNA damage can exacerbate DNA replication errors and significantly increase the frequency of DNA mutations. A particularly detrimental lesion resulting from oxidative damage is 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG)[1] (*2*). The presence of OG in DNA can result in misincorporation of 2′-deoxyadenosine by DNA polymerase to form OG:A base pairs (*3*). Subsequent replication events secure G-to-T transversion mutations. Fortunately, repair systems for OG appear to be present across all phylogeny (*4*−*6*). The repair system for OG in *Escherichia coli*, the "GO" system, requires the action of three proteins, MutM, MutY, and MutT (*4*). MutM (or FPG protein) is an OG glycosylase which removes OG from OG:C base pairs, while MutY is an adenine glycosylase which removes misincorporated adenine from OG:A base pairs. MutT hydrolyzes d(OGTP) to remove it from the dNTP pool and therefore prevent its misincorporation opposite A.

MutY is representative of a class of base-excision repair (BER) glycosylases that remove a wide variety of inappropriate bases (*7*). However, MutY is unique among this enzyme class in that it catalyzes the removal of an undamaged adenine that is mispaired with a damaged base (OG) (*8*). Though the mechanism employed by MutY has not been fully elucidated, significant details have been suggested on the basis of biochemical and structural data available for a number of glycosylases (*7*). In all BER glycosylases studied to date, the removal of the base has been proposed to be assisted by nucleophilic attack at C1′ by either a protein-derived amino group or an activated water molecule (*9*). In the case of adenine removal, the assistance of an acidic protein residue in protonating the adenine at N-7 is also likely involved to promote departure of the adenine base (*10, 11*). This type of mechanism is supported by several studies of MutY with substrate and transition-state analogues (*12*−*14*).

Genetic evidence based on mutation frequencies in *E. coli* suggests a critical role of MutY in the removal of adenine from OG:A base-pairs. MutY has also been shown to remove adenine in vitro and in vivo from G:A and C:A mismatches (*8, 15, 16*). NMR and X-ray crystallographic studies on oligonucleotides containing G:A mismatches have indicated that this particular mismatch can form a variety of stable structures within the DNA double helix. The observed structures of G:A mismatches, G(*anti*):A(*anti*), G(*anti*):

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* To whom correspondence and reprint requests should be addressed. Tel: 801-585-9718. Fax: 801-581-8433. E-mail: david@chemistry.utah.edu.

[1] Abbreviations: PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; AP, apurinic/apyrimidinic; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; OG, 8-oxo-7,8-dihydro-2′-deoxyguanosine or 8-hydroxy-2′-deoxyguanosine; HPLC, high-performance liquid chromatography; TBE, tris-borate-EDTA buffer; F, 2′-deoxyformycin A.

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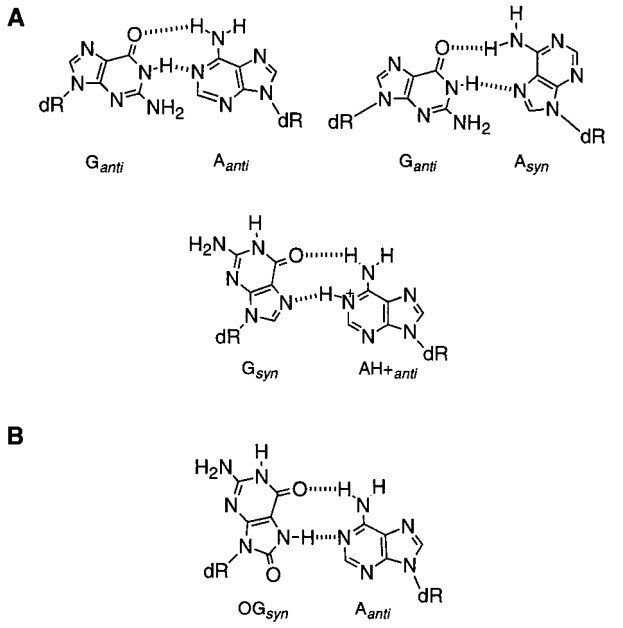

FIGURE 1: (A) Possible conformations for isolated G:A mismatches within B-form DNA observed by NMR spectroscopy and X-ray crystallography (*17*−*21*). (B) Single conformation for an OG:A mismatch in B-form DNA observed in both NMR and X-ray structural studies (*22, 23, 34*).

A(*syn*), and G(*syn*):AH$^+$(*anti*) (Figure 1A), differ in the conformation of the N-glycosidic bond (*17*−*21*). In contrast, OG:A mismatches have been observed only in the OG(*syn*):A(*anti*) conformation due to properties of OG (Figure 1B), such as the presence of the oxo group at the 8-position which favors the syn conformation for OG (*22*). Furthermore, the presence of the keto tautomer of 8-oxoguanine places a H-bond donor at N-7 which results in a stabilization of the OG(syn):A(anti) base pair conformation without protonation. These important distinctions between OG:A and G:A base pairs suggest that MutY's recognition and repair chemistry of these two substrates may be significantly different. However, the fact that *both* are substrates for MutY indicates that there are common features between them that may be important recognition and repair determinants. Thus, a detailed understanding of MutY's enzymatic properties with these two substrates will provide insight into its recognition and repair properties.

Surprisingly, on the basis of studies from many laboratories working with MutY, the relative activity of MutY with G:A mismatch substrates with respect to OG:A substrates is unclear. This issue was first addressed by Michaels et al. in qualitative assays which indicated similar rates for adenine removal from OG:A and G:A base pairs (*8, 23*). In contrast, in similar qualitative experiments from Lu et al. (*24*) and Manuel et al. (*25*), a G:A substrate duplex appears more efficiently processed than the corresponding OG:A duplex. The differences in these results could stem from using a qualitative assay that may not properly reflect the enzymatic properties of MutY. However, quantitative measurements of k_{cat} and K_M for MutY's interaction with G:A- and OG:A-containing substrates are also contradictory as to the preferred substrate. Lu et al. (*26*) reported k_{cat}/K_M values of10 and 3.3 min^{-1} μM^{-1} for the reaction of MutY with the corresponding G:A- and OG:A-containing duplexes, respectively. In contrast, Bulychev et al. (*13*) reported k_{cat}/K_M values of 0.383 and 39.6 min^{-1} μM^{-1} for MutY's activity with G:A- and OG:A-containing duplexes, respectively, indicating OG:A base pairs are the superior substrate. Furthermore, substrate preference is often reflected by the greater affinity of an enzyme for its favored substrate. Although significant scatter exists in reported K_d measurements, OG:A duplexes are generally reported to bind more tightly to MutY. Importantly, studies using noncleavable substrate analogues have shown that MutY has a higher affinity for OG-containing duplexes compared to the corresponding G-containing duplexes (*12*−*14, 27*). For example, we recently reported that, in experiments using duplexes containing the noncleavable 2′-deoxyadenosine analogue, 2′-deoxyformycin A (F), MutY binds approximately 30-fold more tightly to the OG:F duplex than to the corresponding G:F duplex (*12*). The greater affinity for OG-containing duplexes is suggestive of OG:A as the preferred MutY substrate.

Considerable evidence has also been provided indicating that MutY has an unusually high affinity for the product of its glycosylase action on an OG:A substrate, presumably an OG:(AP site). This has been illustrated by the observation of a defined MutY "footprint" in MPE-Fe(II) hydroxyl radical footprinting experiments of MutY bound to its product formed from an OG:A duplex (*12*). Moreover, Miller and Michaels reported that, in the presence of MutY, MutM is unable to remove OG from the OG:(AP site) produced by MutY (*8*) suggesting that MutY remains bound to its product such that access by MutM is precluded. This lingering of MutY with its product may be biologically significant in order to prevent double-strand breaks by the subsequent action of MutM. Importantly, this high affinity for the product also suggests that the differences observed in the reported substrate specificities from the various laboratories may be a consequence of not only the intrinsic rates of processing of the different substrates but also the relative efficiency with which MutY undergoes multiple turnover events with different substrates. Under conditions of multiple enzyme turnover, k_{cat} measurements may be influenced by the dissociation rate of the enzyme from the product, as well as the intrinsic rate for the chemical step.

To clarify these issues regarding substrate preference and to provide insight into the mechanisms of DNA damage recognition and repair, we have initiated a thorough analysis of MutY's enzymatic properties. We have found that MutY's processing of OG:A relative to G:A substrates is distinctly different. In particular, single-turnover experiments where [MutY] $\gg$ [DNA] indicate that the rate of removal of A from OG:A mispairs by MutY is significantly faster than from G:A mispairs. However, MutY's ability to *turn over* with an OG:A substrate is significantly impeded relative to the reaction with a G:A-containing substrate. In the case of OG:A substrates, MutY appears to remain bound to its product, thus precluding additional enzymatic turnover. These results indicate the importance of a thorough kinetic analysis when evaluating the substrate specificity of MutY. It is possible that other base-excision repair glycosylases exhibit similar properties toward their substrates, and therefore, these results may be revealing important features of the chemistry of BER glycosylases that are critical to their in vivo function.

MATERIALS AND METHODS

General Methods. All chemicals were purchased from Fisher Scientific. Standard 2-cyanoethylphosphoramidites were purchased from Applied Biosystems, Inc. (ABI). 5′-(γ-^{32}P)-ATP was obtained from Amersham, and T4-polynucleotide kinase was purchased from New England Biolabs. Reagents for Bradford assays were obtained from BioRad. Distilled, deionized water (Milli-Q) was used for all aqueous solutions. Storage phosphor autoradiography was performed using a Molecular Dynamics STORM 840 Phosphorimager. Quantitation of storage phosphor autoradiograms was performed using the software ImageQuant (Molecular Dynamics). Electrophoresis experiments were performed with TBE buffer (90 mM Tris, 90 mM boric acid, 1 mM EDTA).

MutY Purification. MutY was purified as described previously (*12*) and stored in liquid nitrogen. The protein concentration was determined by the method of Bradford (*28*) and aliquoted in 15 μL fractions. Each fraction was used in only one experiment due to the instability of MutY toward repeated freeze−thaw cycles.

Oligonucleotides. 7,8-Dihydro-8-oxo-2′-deoxyguanosine (OG) and 2′-deoxyformycin A (F) phosphoramidite monomers were synthesized as reported previously (*12, 29*). Oligonucleotides were synthesized on an ABI 392B automated DNA synthesizer using the manufacturer's protocols and then purified by HPLC as described previously (12). All oligonucleotide duplexes used in this work have the following sequence: d(5′-CGATCATGGAGCCAC**X**AGCTCCCGTTACAG-3′)·d(3′-GCTAGTACCTCGGTG**Y**TCGAGGGCAATGTC-5′), where X = G or OG and Y = A, C, or F.

Substrate DNA Preparation. The A-containing 30-mer (10 pmol) was ^{32}P-5′-end-labeled with T4-polynucleotide kinase using the manufacturer's protocol. Unincorporated ^{32}P-ATP was removed from the 5′-^{32}P-end-labeled oligonucleotide using a Nensorb column (Dupont-NEN). The labeled oligonucleotide was mixed with unlabeled A-containing oligonucleotide to make a solution where 1%−2% of the DNA was ^{32}P-labeled. A solution containing 100 nM A-containing strand and 100 nM complementary strand (either G- or OG-containing 30-mer) was heated to 95 °C and then slowly cooled to room temperature over approximately a 4 h time period to promote duplex formation. The buffer used for the annealing procedure contained 20 mM Tris-HCl, pH 7.6, 10 mM EDTA, and 150 mM NaCl.

Glycosylase Activity Assays. Substrate DNA, 20 nM, was equilibrated at 37 °C in 20 mM Tris-HCl, 10 mM EDTA, 0.1 mg/mL BSA, pH 7.5. Reactions were started by the addition of MutY to final concentrations ranging from 1.3 to 100 nM as indicated for each experiment. Aliquots (10 μL) of the reaction mixture were taken at various times (10 s to 60 min), quenched with 2 μL of 1 M NaOH, and heated at 90 °C for 5 min. Subsequently, 10 μL of denaturing loading dye (80% formamide, 0.025% xylene cylanol, 0.025% bromophenol blue in TBE buffer) was added to the samples which were then heat-denaturated and electrophoresed in a 15% denaturing polyacrylamide gel in TBE buffer for several hours at 1600 V. The gel was exposed for 12−15 h to a Molecular Dynamics phosphor imager screen. The resulting storage phosphor autoradiogram was quantitated to determine the percent conversion of substrate to product. Control reactions with no MutY and with excess MutY (200 nM) were performed to determine 0% and 100% reaction. The excess MutY control serves as a sensitive indicator of the efficiency of the duplex annealing procedure. Raw data from autoradiography analysis were corrected for the background signal in the control without enzyme.

Active Site Titration. These experiments were performed using the same buffer and conditions described above for glycosylase assays. The DNA concentration used was 20 nM, and the MutY concentration was adjusted to provide less than 15% conversion to product to preserve pseudo-first-order conditions. These MutY concentrations typically were 1.3, 2.6, and 3.9 nM as determined by the Bradford assay. Time points collected ranged from 15 s to 60 min. Fitting of the data to the appropriate rate equations provided an accurate value for the amplitude of the burst that relates directly to the active enzyme concentration. The active site concentration of MutY is most easily obtained using an OG:A duplex as the substrate. In this case, the amount of active enzyme can be estimated by extrapolating the linear portion to determine the *y* intercept. The active enzyme concentration is calculated as a percentage of the total protein concentration based on the Bradford method.

Single Turnover Kinetics. These experiments are performed using the same buffer and conditions described for adenine glycosylase assays above. A notable difference is that the MutY concentration used was 100 nM.

MutY Stability Assay. MutY (3.9 nM) was incubated in the presence of 20 nM nonspecific 30-mer (G:C base pair instead of OG:A or G:A) at 37 °C in 20 mM Tris-HCl, 10 mM EDTA, 0.1 mg/mL BSA, pH 7.5, to simulate the reaction conditions used in the glycosylase assay. Aliquots of that solution were assayed for activity with a G:A-containing duplex after 0, 15, 30, and 60 min of incubation. Each aliquot reaction was monitored for 60 min in order to obtain a complete time trace. An identical set of experiments was performed in the presence of a duplex containing a noncleavable 2′-deoxyadenosine analogue, 2′-deoxyformycin (F), opposite G (G:F base pair) instead of the G:C-containing duplex. Parallel experiments were also performed which did not include the nonspecific DNA or the substrate analogue DNA.

RESULTS

General Features of Adenine Glycosylase Experiments with MutY. Kinetics of adenine removal opposite G and OG by MutY were carried out under multiple-turnover ($[DNA]_o \gg [MutY]_o$) as well as under single-turnover ($[DNA]_o \ll [MutY]_o$) conditions. In all cases, kinetic analysis of the adenine glycosylase reaction was based on complete time traces rather than on initial rates. The adenine glycosylase activity of MutY was monitored using 30 base pair DNA duplex substrates of the following sequence: d(5′-CGATCATGGAGCCAC**X**AGCTCCCGTTACAG-3′)·d(3′-GCTAGTACCTCGGTG**A**TCGAGGGCAATGTC-5′) where **X** = OG or G and **A** indicates the adenine in the mismatch. The A-containing strand was ^{32}P-end-labeled prior to annealing of the duplexes. The duplex substrate was then incubated with MutY, and aliquots were removed at times ranging from 10 s to 60 min. The reactions were quenched by the addition of base which also produces strand scission at the abasic

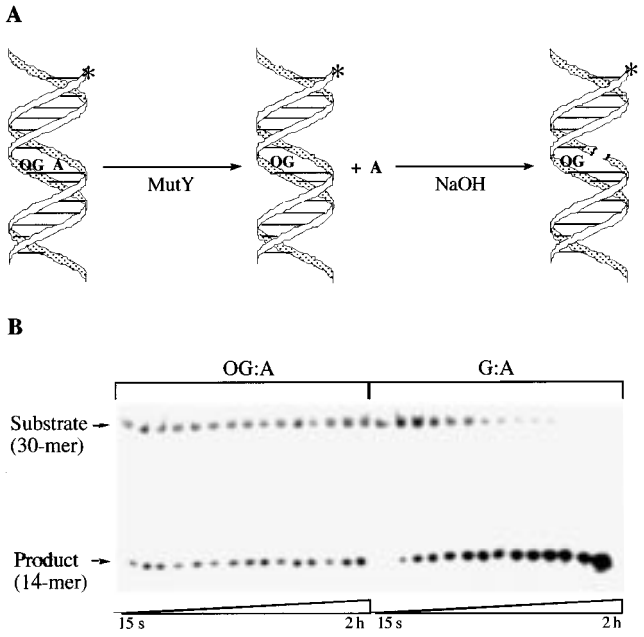

FIGURE 2: (A) Schematic representation of the glycosylase activity assay for MutY. The A strand of a DNA substrate containing either an OG:A or a G:A mismatch is labeled in its 5′-end with ^{32}P (as indicated with *). The duplex DNA is then incubated at 37 °C in the presence of MutY, and an abasic site is obtained as the result of adenine removal. Subsequently, the product is subjected to base treatment to cause strand scission resulting in a smaller labeled DNA fragment. The DNA is then electrophoresed under denaturing conditions, and the substrate (large fragment) and product (smaller fragment) are quantified by phosphorimagery. (B) Storage phosphor autoradiogram of a denaturing polyacrylamide gel experiment illustrating the differential processing of OG:A substrates relative to G:A substrates by MutY. The A-containing strand is 5′-end-labeled with ^{32}P prior to annealing to the complementary strand to form the duplex. In this particular experiment, a 30-mer duplex (20 nM) containing either an OG:A or a G:A mismatch was incubated with 10 nM MutY for 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 8, 10, 15, 30, 60, 90, and 120 min. After base treatment, the individual samples were electrophoresed on a 15% denaturing polyacrylamide gel. The small DNA fragment, 14 bases long, represents the base-treated product of the glycosylase reaction while the larger fragment, 30 bases long, corresponds to the unreacted labeled A-containing strand of the duplex substrate.

site generating a labeled 14-nucleotide strand from the product. A schematic representation of the adenine glycosylase assay is shown in Figure 2A. The labeled 14-nucleotide strand (*product*) is then separated from any remaining labeled 30-nucleotide A-containing strand of the duplex (*substrate)* by denaturing polyacrylamide gel electrophoresis. The relative amounts of product and substrate are visualized and quantitated by phosphorimagery. It is important to note that this assay does not distinguish free and enzyme-bound product. Control reactions with no MutY and with excess MutY (200 nM) were performed to determine 0% and 100% reaction. MutY is highly specific for adenine removal from the target base pair within *duplex* DNA, and therefore, the excess MutY control serves as a sensitive indicator of the efficiency of the duplex annealing procedure. Raw data from autoradiography analysis were corrected for the background signal in the control without enzyme.

The stability of the enzyme under the reaction conditions was assessed to ensure that loss of enzyme activity as a function of time was not contributing to the observed

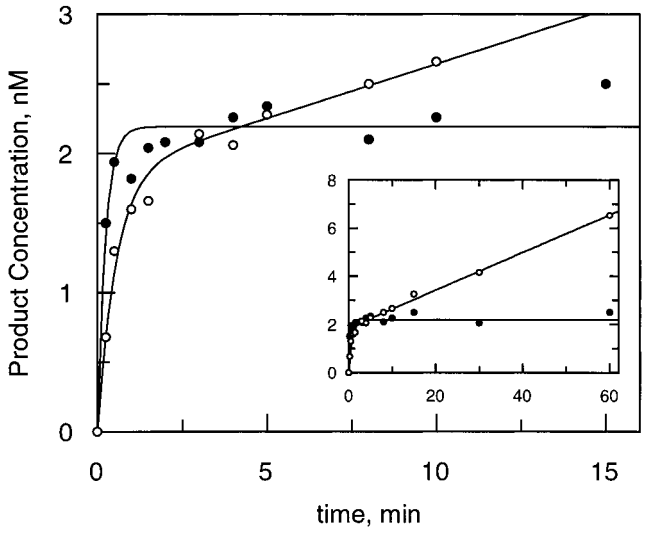

FIGURE 3: Extent of adenine glycosylase activity of MutY with an OG:A- (closed circles) and a G:A- (open circles) containing duplex as a function of time. Substrate DNA, 20 nM, was incubated with 3 nM MutY at 37 °C in Tris buffer (20 mM Tris-HCl, 10 mM EDTA, 0.1 mg/mL BSA, pH 7.5) in a 150 μL reaction volume. Aliquots (10 μL) were taken from the reaction mixture at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 8, 10, 15, 30, and 60 min, quenched, and analyzed. The concentration of product was plotted as a function of time.

progression of the adenine glycosylase activity during the 60 min period. In these experiments, a solution of MutY was prepared and incubated at 37 °C in the presence of nonspecific DNA for various time periods (0, 15, 30, and 60 min) and subsequently the adenine glycosylase activity with a G:A-containing substrate was determined. No loss of activity toward a G:A-containing duplex was detected during the course of 60 min (Supplemental Information). Similar results were also obtained when incubating MutY with a DNA duplex containing a noncleavable 2′-deoxyadenosine analogue, 2′-deoxyformycin A (F), in a G:F base pair instead of nonspecific DNA. Interestingly, in the absence of nonspecific DNA or the substrate analogue DNA, almost complete loss of enzymatic activity was observed within 40 min. This indicates that the association of MutY with DNA is important for providing stability under the reaction conditions.

OG:A Versus G:A Mispairs as Substrates for MutY: Multiple-Turnover Experiments. Figure 2B shows the results of a representative kinetic experiment with the OG:A and G:A mispair substrates under multiple-turnover conditions ([MutY] < [DNA]). The difference in the reactivity of MutY toward either substrate is so remarkable that it can be ascertained by inspection of the autoradiogram. With the OG:A duplex, the amount of product formed is relatively constant from the initial time point of 15 s to the ending time point of 120 min. In contrast, the appearance of product from MutY's reaction with a G:A duplex continues until all of the substrate has been consumed.

To further compare the observed relative reactivities of G:A- and OG:A-containing duplexes, pre-steady-state kinetic experiments of the reaction of 3 nM MutY with G:A and OG:A duplexes (20 nM) were performed (Figure 3). Under these multiple-turnover conditions, the reactions for both G:A and OG:A were characterized by biphasic kinetics, displaying an exponential burst of product followed by a steady-state

Scheme 1

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

phase. The rate of the burst was considerably faster for the OG:A substrate relative to the G:A substrate, indicating a higher intrinsic reactivity for the former. However, after the first turnover, the amount of product from the OG:A-containing duplex reached a plateau at approximately 10%, while the reaction with the G:A substrate attained a steady state. These results indicate that multiple catalytic turnovers are possible with a G:A substrate, while with an OG:A substrate MutY remains essentially "trapped" in a product-bound state hence unable to process any additional substrate beyond the first turnover. As a consequence, observed relative reactivities (i.e., substrate preference) evaluated from single-time point experiments will be highly dependent on the time at which the reaction is assayed.

The simplest mechanism consistent with the kinetic data presented in this and the following sections is shown in Scheme 1. This scheme depicts the three basic steps necessary for the enzymatic cycle: substrate binding (k_1, k_{-1}), chemical reaction (k_2), and product release (k_3). The burst kinetics observed with the G:A as well as the OG:A duplexes indicates that $k_3 < k_2$ in both cases, although the k_2/k_3 ratio is much larger for the latter. Experiments aimed at evaluating the k_2 and k_3 parameters are described below.

Single-Turnover Kinetics with OG:A Versus G:A Mispair Substrates. Prompted by the initial observations above, single-turnover experiments were conducted to isolate the chemical step of the reaction (k_2), and thus ascertain the *intrinsic* reactivities of the OG:A- versus G:A-containing substrates. Reactions were run at $[\text{OG:A}]_o = 19$ nM or $[\text{G:A}]_o = 18.4$ nM, and $[\text{MutY}]_o = 100$ nM.

Adenine removal from a G:A mismatch by MutY followed first-order kinetics, the reaction leading to a quantitative amount of product (Figure 4). The raw data were analyzed using eq 1, where A_o represents the amplitude of the

$$[\text{P}]_t = A_o\{1 - \exp(-k_{obs}\, t)\} \quad (1)$$

exponential phase and k_{obs} is the observed rate constant associated with that process.

The observed rate constant under the present pseudo-first-order conditions ($[\text{MutY}]_o \gg [\text{DNA}]_o$) is given by eq 2,

$$k_{obs} = \frac{[\text{E}]_o}{K_d + [\text{E}]_o} k_2 \quad (2)$$

assuming enzyme−substrate binding is a rapid equilibrium ($k_{-1} \gg k_2$). However, since the enzyme concentration utilized in the experiment is well above the K_d values for both substrates (*12*), eq 2 simplifies to $k_{obs} = k_2$. Accordingly, an analysis of the data for the G:A duplex using eq 1 afforded $k_2 = (1.6 \pm 0.2)$ min^{-1}.

The reaction of MutY with an OG:A-containing duplex under these conditions is too fast for our manual assay (Figure 4) and hence an accurate value for k_{obs} (k_2) cannot be obtained by curve fitting the data. Nevertheless, since the first time point assayed (15 s) corresponds to about 90% product (i.e., approximately four half-lives), an upper limit for $t_{1/2} \leq 4$ s and thus a lower limit for $k_2 \geq 10$ min^{-1} could

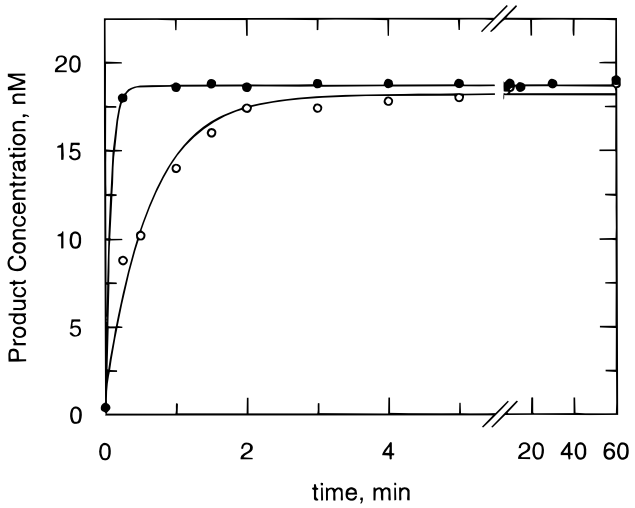

FIGURE 4: Single-turnover kinetics of MutY with DNA duplex containing either an OG:A (closed circles) or a G:A (open circles) mismatch. In this experiment, 20 nM substrate DNA and 100 nM MutY were incubated at 37 °C in 20 mM Tris-HCl, 10 mM EDTA, 0.1 mg/mL BSA, pH 7.5. Aliquots (10 μL) were taken from the reaction mixture at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 8, 10, 15, 30, 60, 90, and 120 min, quenched with 2 μL of 1 M NaOH, and heated at 90 °C for 5 min. The concentration of product is plotted as a function of time for both substrates.

be estimated. The combined results allowed us to set a lower limit on the intrinsic relative reactivities of the OG:A and G:A substrates, $k_2^{OG:A}/k_2^{G:A} \geq 6$.

Kinetics of Adenine Excision by MutY from an OG:A Mismatch. Active Site Titration Experiments. We wished to examine the kinetics of the reaction of MutY with the OG:A-containing duplex in more detail, and attempt to dissect the individual rate constants shown in Scheme 1. The extremely slow turnover rate of MutY with this substrate provided the opportunity for us to easily determine the concentration of active enzyme in a MutY sample by the active site titration method (*30*). Reactions were carried out at 20 nM DNA and 1.3, 2.6, and 3.9 nM nominal MutY concentration and assayed as a function of time as described in the Experimental Section. Plots of product concentration versus time (Figure 5) were analyzed using eq 3 to determine

$$[\text{P}]_t = A_o\{1 - \exp(-k_{obs}t)\} + k_{ss}t \quad (3)$$

the kinetic parameters A_o (amplitude of the burst), k_{obs} (rate constant for the burst), and k_{ss} (slope of the linear phase).

In terms of the microscopic rate constants in Scheme 1, A_o, k_{obs}, and k_{ss} are defined by eqs 4−6. Equation 7, in turn, defines the effective rate constant k' for the process MutY + $(\text{DNA})_S \rightarrow \text{MutY}\cdot(\text{DNA})_P$.

$$A_o = \{k'/(k' + k_3)\}^2[\text{MutY}]_o \quad (4)$$

$$k_{obs} = k' + k_3 \quad (5)$$

$$k_{ss} = k'k_3/(k' + k_3)[\text{MutY}]_o \quad (6)$$

where,

$$k' = \frac{k_2[S]_o}{\left(\frac{k_{-1} + k_2}{k_1}\right) + [S]_o} \quad (7)$$

Table 1 summarizes the results of fitting the experimental data to eq 2. Figure 4 clearly shows that $k' \gg k_3$, reducing eqs 4−6 to $A_o = [MutY]_o$, $k_{obs} = k'$, and $k_{ss} = k_3[MutY]_o$, respectively. Once again, these reactions were too fast for our manual assay, so that even the earliest points in the time traces were at the end of the exponential phase. For this reason, k_{obs} values obtained from the computer fit are affected by large errors and are not reported. On the other hand, the curve fit affords values for the amplitude of the burst that correlate well with the amount of active enzyme, that is, an increase in the nominal enzyme concentration results in a proportional increase in the size of the burst. From these experiments, it was determined that this particular enzyme preparation was 26% active (average of all three determinations). Various preparations assayed by this method in our laboratory have afforded values between 25% and 65%. On average, MutY preparations were approximately 60% active. These values are in agreement with active enzyme concentrations determined by monitoring MutY binding to a duplex containing a noncleavable OG:F base-pair (data not shown). It should be noted that the percent active enzyme concentration is determined using the Bradford assay which may overestimate the total protein concentration.

The rate constant for product release was determined from the slopes of the linear portions of the plots, affording an average value for $k_3 = (0.004 \pm 0.002)$ min^{-1}.[2] This extremely low k_3 value reveals that determination of k_{cat} and K_m for an OG:A-containing duplex by standard steady-state kinetics (Michaelis−Menten) will require very long reaction times since the enzyme does not turn over to any significant extent within a reasonable time. Problems associated with conducting enzyme kinetics over extended periods of time will make this an undesirable practice.

Kinetics of Adenine Excision by MutY from a G:A Mismatch. Kinetics of adenine glycosylase activity by MutY on a G:A mismatch-containing substrate were conducted under pseudo-first-order conditions, with $[G:A]_o = 20$ nM and 1.3, 2.6, and 3.9 nM enzyme concentrations as determined by the Bradford assay. In all cases the reactions were characterized by an initial burst of product followed by a linear phase (Figure 6). The corresponding A_o, k_{obs}, and k_{ss} parameters, as defined by eqs 4−6, were determined by fitting the experimental data to eq 3 (Table 1). As observed in the case of OG:A, the rate of the burst is significantly faster than the steady-state initial rate (i.e., $k' \gg k_3$) so that the simplifications of eqs 4−6 mentioned above apply here as well.

As expected, the rate of the burst is independent of the enzyme concentration within error. The scatter in the values is due to the error involved in the assay; however, they agree well with the values obtained from the single-turnover experiments. The average percent value for active enzyme concentration of 20% compares with that determined using the OG:A duplex. Although there is a large difference between the k' and k_3 values in both cases, the fact that k'(G:A) < k'(OG:A) and k_3(G:A) > k_3(OG:A) makes the $\{k'/(k' + k_3)\}^2$ term in eq 4 slightly lower for the G:A reaction, thus resulting in a somewhat lower value for MutY concentration.

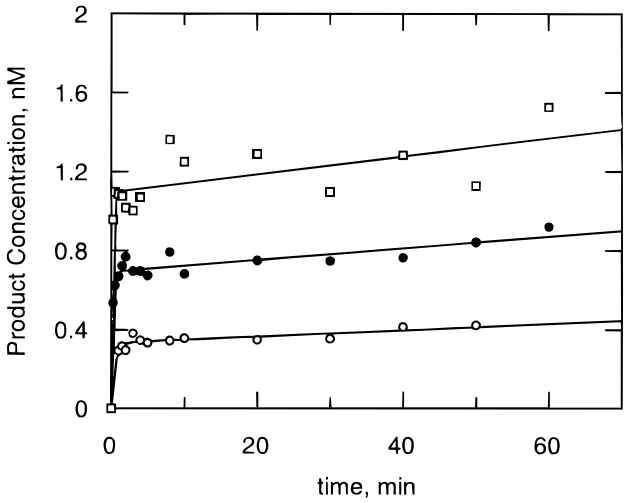

FIGURE 5: Kinetics of the reaction of MutY with OG:A-containing duplex (active site titration experiment). Substrate DNA, 20 nM, containing an OG:A mismatch was incubated with 1.3 (○), 2.6 (●), and 3.9 (□) nM MutY at 37 °C in Tris buffer (20 mM Tris-HCl, 10 mM EDTA, 0.1 mg/mL BSA, pH 7.5). Aliquots of 10 μL were taken from the reaction mixture at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 8, 10, 15, 30, and 60 min, quenched with 2 μL of 1 M NaOH, and heated at 90 °C for 5 min. The concentration of product was plotted versus time. The concentration of active sites was calculated as indicated in the text.

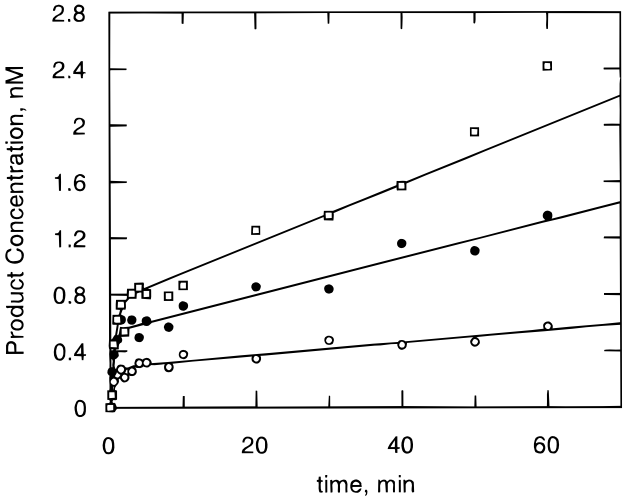

FIGURE 6: Kinetics of the reaction of MutY with G:A-containing duplex. Substrate DNA, 20 nM, containing a G:A mismatch was incubated with 1.3 (○), 2.6 (●), and 3.9 (□) nM MutY at 37 °C in Tris buffer (20 mM Tris-HCl, 10 mM EDTA, 0.1 mg/mL BSA, pH 7.5). Aliquots (10 μL) were taken from the reaction mixture at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 8, 10, 15, 30, and 60 min, quenched with 2 μL of 1 M NaOH, and heated at 90 °C for 5 min. The concentration of product was plotted as a function of time.

Values of k_3 as determined from the slopes of the linear portions of the plots reveal that release of product from the G:A reaction is approximately 8-fold faster than from the OG:A substrate. An average value for k_3 including several determinations carried out under similar conditions (data not shown) affords $k_3 = (0.03 \pm 0.01)$ min^{-1}.[3]

[2] The error reported (50%) is an overestimation based on the largest error for an individual k_3 value (37%).

Table 1: Pre-Steady-State Kinetics of the Reaction of MutY with OG:A and G:A Containing DNA Duplexes[a]

substrate	$[MutY]_0$, nM[b]	k_{obs} ($\approx k'$), min^{-1}	A_0 ($\approx[MutY]_0$), nM	active enzyme[c], %	k_{ss} ($\approx k_3[MutY]_0$), nM min^{-1}	k_3, min^{-1}
OG:A	1.3	NR[d]	0.33 ± 0.01	25	0.0016 ± 0.0004	0.005 ± 0.002
	2.6	NR[d]	0.69 ± 0.02	26	0.0030 ± 0.0006	0.004 ± 0.001
	3.9	NR[d]	1.10 ± 0.05	27	0.0045 ± 0.0017	0.004 ± 0.002
G:A	1.3	1.3 ± 0.4	0.28 ± 0.02	21	0.005 ± 0.001	0.018 ± 0.005[e]
	2.6	2.5 ± 0.6	0.53 ± 0.03	20	0.013 ± 0.001	0.025 ± 0.004[e]
	3.9	1.5 ± 0.4	0.74 ± 0.04	18	0.020 ± 0.002	0.027 ± 0.004[e]

[a] Data Correspond to Figures 5 and 6. [b] Concentrations determined by the Bradford assay. [c] Calculated as $(A_0/[MutY]_0) \times 100$. Average active site concentration = 26%. [d] NR: Rate constants from the burst phase are not reported due to large errors. [e] The average $k_3 = (0.03 \pm 0.01)$ min^{-1} reported in the text includes these and other data (not shown).

DISCUSSION

Base-excision repair enzymes attend to a wide variety of damaged and mismatched bases (*7*). MutY belongs to a superfamily within this class that has a similar overall fold with specific conserved DNA binding and catalytic motifs. However, within this superfamily, the substrate specificity varies considerably. For example, the BER glycosylase, AlkA, is specific for alkylated bases and has been shown to be active on at least 12 different substrates. In contrast, MutY is quite specific for removal of adenine in the inappropriate base-pairs OG:A, G:A, and C:A within duplex DNA. Among these substrates, the relative activity of MutY in vitro has not been clearly established, even though this has been the subject of considerable investigation. An important aspect of understanding the in vivo processing of these substrates involves delineating the intrinsic chemistry of MutY with all substrates. This knowledge will shed light on the factors influencing the mutagenic potential of specific types of DNA damage and DNA mismatches. In addition, delineating the molecular details associated with the processing of different substrates by MutY will provide important insight into the modes of damage recognition and mechanism of the base-excision process for this important class of DNA repair enzymes.

In this paper, we have investigated the properties of MutY's glycosylase activity toward an OG:A- and G:A-containing substrate in detail. We have shown that MutY's processing of OG:A-containing substrates relative to G:A-containing substrates is surprisingly and distinctly different. Under conditions of multiple turnover with both substrates, an initial "burst" of product is produced that corresponds to the amount of active MutY and, subsequently, a slower rate for product formation is observed. However, the rate of product formation after the burst is extremely slow for the OG:A substrate relative to the G:A substrate. This indicates that the dissociation rates for MutY from the products produced from these two substrates are significantly different. With OG:A substrates, this dissociation step is remarkably slow and the consequence of this is that when an OG:A substrate duplex is incubated with MutY under conditions where [MutY] < [DNA], incomplete conversion to product is observed. In the case of a G:A substrate, the product dissociation is considerably more facile (~8-fold) such that more efficient conversion of all the substrate to product under similar conditions ([MutY] < [DNA]) is observed. However, MutY's intrinsic rate for adenine removal from an OG:A substrate is at least 6-fold faster than from the corresponding G:A substrate. Therefore, under conditions of single turnover ([MutY] ≫ [DNA]), duplex substrates containing OG:A are completely converted to product and this occurs more rapidly than in the corresponding reaction with a G:A substrate. These results illustrate that slight differences in substrates can have a profound effect on their enzymatic processing.

The kinetic results presented herein provide insight into the discrepancy surrounding MutY's relative activity with OG:A- and G:A-containing substrates that is present in the literature. In Figure 3, after approximately 5 min, the extent of product formation from an OG:A substrate has begun to level off, while the extent of product formation for a G:A substrate is increasing. This results in a crossover point between the respective curves for product formation for the two substrates. Thus, monitoring the reaction at short time points indicates that OG:A substrates are more reactive, while monitoring at longer time points, more product is observed with a G:A substrate. In many qualitative assays with MutY, a single time point (typically, 30 min) has been used and the results shown here indicate that such an analysis can be misleading (*24*, *25*).

It is also important to consider the *concentration* of MutY relative to substrate. MutY's reaction with an OG:A substrate is essentially stoichiometric with an amount of product produced that equals the concentration of active enzyme. The presence of excess enzyme is usually required to convert all of an OG:A substrate into product. In contrast, a G:A substrate can be converted completely to product even under conditions of [E] < [S]. Thus, the relative concentrations of enzyme and substrate used can strongly influence the observed reactivity of MutY with different substrates. At long reactions times, a G:A substrate will appear to be more efficiently processed when [E] < [S] while the two substrates may appear to be similarly processed when [E] > [S]. Furthermore, the observed amount of product will also be influenced by the concentration of "active enzyme" rather than total protein concentration in the sample and therefore a determination of the active enzyme concentration should be performed when comparing the specificity of MutY with different substrates. Knowledge of the active enzyme concentration will also aid in comparing activities of MutY from different preparations and laboratories.

The magnitude of the differences observed between the two substrates may vary with the conditions of the glycosylase reaction such as the nature and concentration of salt used, the temperature, and the presence of stabilizers such as BSA, DTT, and glycerol. In the studies from various laboratories, these conditions vary considerably and may contribute in part to the differences that have been observed.

[3] The error reported is the standard deviation of the average.

Another factor may be the sequence environment surrounding the mismatch. An evaluation of the importance of the buffer components and sequence environment on MutY's glycosylase activity is in progress in this laboratory. A third possible contributing factor may depend on the use of base treatment in the analysis of glycosylase activity. The inconsistent use of base treatment is due to the lack of agreement regarding whether MutY has an associated β-lyase activity to provide strand scission. If the base removal step and the strand scission reaction are *not* directly correlated for *all of MutY's substrates*, reliance on MutY to provide strand scission may underestimate the extent of glycosylase action. In our experiments, MutY behaved as a simple glycosylase (*31*) and therefore base treatment was consistently employed.

These results underscore the need to obtain the entire time profile under defined conditions when evaluating the activity of MutY and related DNA repair enzymes with different substrates. The results that we have obtained with an OG:A substrate indicate that standard Michaelis−Menten kinetics parameters, k_{cat} and K_m, cannot be determined under these conditions. Literature reports of determinations of K_m and k_{cat} for OG:A are likely derived from measuring initial velocities of the pre-steady-state portion of the reaction and therefore are not true "steady-state" parameters. To perform steady-state kinetics with an OG:A substrate, conditions would have to be identified which promote more facile enzymatic turnover. Unlike OG:A substrates, steady-state kinetics of the reaction with a G:A substrate *can* be performed. However, since the reaction of MutY with a G:A substrate also provides a "burst" of product, several points in the *linear* portion rather than the burst region of the product curves would need to be obtained. Importantly, under these conditions, the determined k_{cat} parameter will be dominated by the product dissociation rate and therefore may not be as informative as to MutY's mechanism as measuring the rate constants for the burst under pre-steady-state conditions.

The extremely inefficient turnover of MutY with an OG:A duplex makes it an ideal substrate for performing active site titration experiments. An accurate concentration of active sites can be determined directly from the amplitude of the burst phase if the ratio k'/k_3 is greater than 20. Under these conditions, eq 1 simplifies to $A_o = [E]_o$ and the absolute value of the burst equals the active enzyme concentration. Indeed, the active site concentration can be estimated directly from inspection of the kinetic plots. Use of this active site titration method should aid in the comparison of results obtained from different preparations of MutY. In addition, if mutated forms of MutY also undergo similar "burst" kinetics with OG:A substrates, this method may also be applicable and may therefore be useful in comparison of these forms to native MutY.

Relative DNA binding experiments and reported K_d values for OG:A- and G:A-containing substrates, in general, indicate that MutY binds with higher affinity to OG:A-containing duplexes; however, there is considerable scatter in the reported K_d values (*13, 24*). This may be due to the extent of conversion of the substrate to product during the gel retardation DNA binding experiment. Since the reported K_d values were performed under different conditions, it is possible that the extent of conversion to products differs in the various measurements. In previous experiments, we measured the affinity of MutY to OG:A and G:A duplexes, while in parallel the glycosylase activity was monitored (*12*). With the G:A duplex, conditions were identified to measure the affinity to the *product* which gave a K_d of 30 nM. In contrast with the OG:A substrate, the measured K_d was subnanomolar in value and represented binding to a mixture of substrate and product. The differences in the extent of product formed in the K_d measurements of G:A and OG:A substrates are consistent with the observed differences in enzyme turnover with these substrates illustrated in this work.

The slow dissociation of MutY from the OG:(AP site) also provides further evidence for the importance of OG in the substrate recognition process of MutY. The slower dissociation rate of MutY from an OG:(AP site) compared to the corresponding G:(AP site) is suggestive of specific contacts between MutY and OG that persist after the adenine has been removed. The specific contacts between MutY and OG may serve as an anchoring point that significantly impedes dissociation of MutY from the DNA. These contacts may be formed in an OG-specific cleft or pocket such that G may also be accommodated within this "OG"-specific site thus facilitating recognition of G:A base-pairs; however, G may not be as tightly held such that product dissociation is more facile. The more efficient recognition and removal of adenine from OG:A relative to G:A base-pairs may also suggest that the stable conformational properties resulting from the presence of OG [e.g., OG(syn):A(anti)] provide for more facile recognition by MutY.

The determinants responsible for more efficient removal of adenine from OG:A substrates by MutY remain to be elucidated. However, it is possible to speculate as to potential contributing factors. One possibility is that the hydrogen-bonding pattern of OG:A is important since OG possesses an extra "proton" at N7 that may be transferred to adenine N-3 to make adenine a better leaving group. Alternatively, the single-turnover rate constant (k_2) that we have measured may include an additional conformational change involving DNA distortion to provide access of MutY to the C1′−N glycosidic bond of 2′-deoxyadenosine, as illustrated below:

$$\text{MutY}\cdot(\text{DNA})_S \rightarrow [\text{MutY}\cdot(\text{DNA})_S]^* \rightarrow \text{MutY}\cdot(\text{DNA})_P$$

This seems plausible since many DNA repair glycosylases have been shown to use a "base" or "nucleotide-flipping" mechanism for substrate recognition (*7*). Indeed, this mechanism has been suggested on the basis of the structural characterization of human Uracil-DNA glycoyslase (hUDG) bound to a DNA duplex substrate (*32*). The structural analysis reveals that specific nucleotide flipping of the target nucleotide/base is achieved by both an enzyme "push" from base and phosphate contacts with specific amino acid side chains and an enzyme "pull" by specific base contacts provided within a base-specific pocket. The structural properties of OG:A base-pairs may facilitate such a nucleotide-flipping process by MutY. Importantly, OG:A base-pairs adopt the stable OG(syn):A(anti) conformation and are less destabilizing to the DNA duplex than G:A base-pairs (*22, 23, 34*). On the basis of this information, the "flipping" process may be expected to be more facile with G:A base-pairs. However, if MutY makes specific contacts with OG

in the OG:A base-pair that are not similarly possible with G in a G:A base-pair, the "push−pull" process may be more efficient with an OG:A base-pair. This idea may imply that MutY extrudes the OG out of the DNA helix rather than the A. However, the presence of OG-specific contacts may also promote flipping out of the A from the helix. Expulsion of either base from the duplex would be expected to facilitate the catalysis of hydrolysis of the target 2′-deoxyadenosine C1′−N glycosidic bond. These details may be elaborated by further kinetic analysis as well as structural studies of MutY bound to DNA.

It will also be of considerable interest to determine whether other DNA repair glycosylases exhibit similar kinetics with different substrates or whether the observed kinetic behavior is a distinguishing feature of MutY. MutY is quite unique among DNA repair glycosylases in being active toward an undamaged base in a damaged and mismatched base-pair (OG:A) to produce an OG:(AP site). Previously, it has been shown that MutM is active toward the removal of OG opposite an AP site (*8*). The ability of MutM to act on an OG:(AP site) suggests that successive action of MutY and MutM could result in complete loss of one base-pair and a DNA double-strand break. Such an event would be extremely deleterious to the cell. Thus, MutY's tight binding to an OG:(AP site) may be important to prevent the OG glycosylase activity of MutM at OG opposite AP sites in vivo. It is also possible that in vivo turnover of MutY may be more facile. Dissociation of MutY from its product may require the recruitment of an AP endonuclease and/or DNA polymerase. In this way, the OG:(AP site) would be "protected" from MutM until it has been partially restored to the appropriate OG:C substrate for MutM. In eukaryotes, interactions between various enzymes in the BER pathway have been uncovered. For example, the human AP endonuclease has been found to make protein−protein contacts with polymerase β (*35*). Thus, it is possible that recognition and repair of damaged DNA mediated by glycosylases in vivo in *E. coli* may also involve the coordinated action of various proteins in the BER pathway.

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NOTE ADDED IN PROOF

Since the submission of this manuscript, a report on the kinetics of human thymine-DNA glycosylase (hTDG) has appeared (Waters, T. R., Swann, P. F. (1998) *J. Biol. Chem. 273*, 20007−20014). Similar kinetic behavior (i.e., slow turnover) as we have observed with MutY was observed with hTDG.

SUPPORTING INFORMATION AVAILABLE

A figure showing the determination of MutY's stability under the reaction conditions (2 pages). Ordering information is given on any current masthead page.

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