Specific Recognition of Substrate Analogs by the DNA Mismatch Repair Enzyme MutY

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Abstract: The DNA repair enzyme MutY plays an important role in the prevention of DNA mutations caused by the oxidatively damaged lesion 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG) by removal of misincorporated adenine residues in OG:A mismatched base pairs using *N*-glycosylase activity. MutY also has glycosylase activity toward adenine in the mismatched base-pairs G:A and C:A. We have investigated the interaction of MutY with DNA duplexes containing the 2′-deoxyadenosine (A) analogs 2′-deoxytubercidin (7-deaza-2′-deoxyadenosine, Z) and 2′-deoxyformycin A (F). Both F and Z should effectively mimic the *recognition* properties of A but be resistant to the glycosylase activity of MutY, owing to their structural properties. Thus, these derivatives will provide a method for forming a stable MutY−substrate analog complex amenable to structural and biochemical investigation. We find that oligonucleotide duplexes containing OG/G:F and OG/G:Z base-pairs are not *substrates* for MutY as expected. Using a gel retardation method to measure relevant K_d values, we determined that MutY has an increased association with duplexes containing OG/G:F and OG/G:Z base-pairs over their OG/G:C counterparts. Interestingly, MutY has a higher affinity for the F-containing duplexes than the Z counterparts. Additionally, MutY binds to the OG:F and G:F duplexes with a similar, albeit lower, affinity as the substrate OG:A and G:A duplexes. In footprinting experiments using methidiumpropyl-EDTA−Fe(II), a region of the duplex surrounding the OG:F base-pair is observed which is protected by MutY from hydroxyl radical cleavage. These results provide additional evidence for *specific* recognition of the OG:F base-pair within the DNA duplex. Furthermore, these results also illustrate the utility of OG:F duplexes for providing information regarding the MutY-mismatched DNA complex which could not be obtained with the normal OG:A substrate since a footprint *on both strands of the duplex* could only be observed with the OG:F-containing duplex. These substrate analog duplexes will provide avenues for structural analysis of the MutY-mismatched DNA complex and for investigating the properties of the unusual [4Fe−4S] center in MutY.

Introduction

Cellular DNA continually suffers damage from reactive alkylating and oxidative agents, spontaneous hydrolysis, and deamination as well as errors made during DNA replication.[1] Elaborate DNA repair pathways exist in all organisms to protect against the potential deleterious and mutagenic effects of DNA damage and DNA mismatches. Indeed, the importance of DNA repair in the prevention of carcinogenesis has recently been highlighted by the finding of a direct correlation between defective DNA mismatch repair and hereditary colon cancer.[2] Although considerable effort has been focused on DNA repair pathways, the factors influencing the precise *recognition* of DNA damage by DNA repair enzymes are still unclear.

The *Escherichia coli* enzyme MutY plays an important role in the prevention of DNA mutations caused by the oxidatively formed lesion 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG).[3,4] Misincorporation of 2′-deoxyadenosine opposite OG by DNA polymerase[5] often occurs to form stable OG:A base-pairs[6] which results in G to T transversion mutations in subsequent replication events. MutY prevents such mutations by specifically removing the adenine from the OG:A base-pair using *N*-glycosylase activity.[4,7] Additional endonucleases, DNA polymerase, and DNA ligase complete this first step of "repair" by installation of a C opposite to the OG lesion to form an OG:C base-pair.[3] The OG in the OG:C base-pair is subsequently removed by the OG glycosylase, MutM (also called Fpg protein).[8] The recognition properties of MutY are intriguing since MutY must specifically recognize the *damaged* and extremely stable *mismatched* OG:A base-pair in the context of normal Watson−Crick base-pairs and remove the *undamaged* adenine base of the mismatch. MutY is not only functionally important; it also contains a [4Fe−4S] center which is an uncommon metal center

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(4) We are referring to the 2′-deoxynucleotides in the various base-pairs with a single- or two-letter abbreviation. For example, A = 2′-deoxyadenosine and Z = 2′-deoxyturbercidin.

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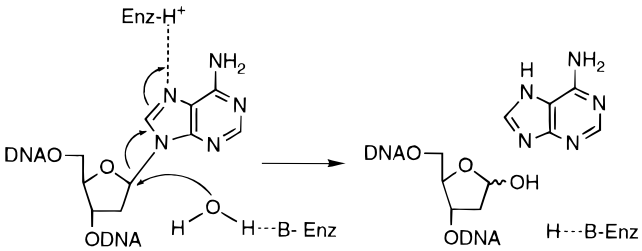

Figure 1. Proposed mechanism for the adenine glycosylase activity of MutY. This figure is adapted from ref 13. In this schematic of the mechanism, the nucleophile is depicted as water (or hydroxide) based on the suggestions and results of Lloyd *et al*. in ref 13. The actual identity of the nucleophile remains to be determined.

in DNA binding proteins and whose role remains to be delineated.[9,10]

MutY has also been shown to be active *in vitro* toward A residues in G:A and C:A mismatch containing duplexes.[10,11] The reaction mechanism employed by MutY has not been investigated in great detail. However, significant details of the mechanism have been suggested by biochemical and structural studies on related enzymes involved in glycosyl transfer reactions.[12] Lloyd *et al.* have proposed a unified mechanism for DNA glycosylases based on the data available for many DNA glycosylases (including MutY).[13] In this mechanism, an acidic amino acid participates by protonating the N7 of adenine to make the base a better leaving group, while an amino acid or solvent-derived nucleophile is involved in attack at C1′ to displace the protonated adenine base yielding an abasic site (Figure 1). The relative importance of the various steps in this proposed mechanism awaits detailed mechanistic evaluation.

As a first step toward delineating the factors influencing the recognition and repair activities of MutY, we have prepared DNA substrate analogs which retain specific recognition and binding properties of the substrate, but are not subject to enzymatic turnover. Stable MutY−substrate analog complexes would be amenable to study by a variety of techniques to provide insight into the structural properties of the MutY−substrate DNA complex. It is difficult to study the MutY−substrate complex due to its short lifetime prior to conversion to products. Schärer and Verdine have recently described an elegant approach for designing substrate analog inhibitors for the base excision repair enzyme alkyl-*N*-purine DNA glycosylase (ANPG) using the concept of transition state destabilization.[14] In their approach, a DNA substrate for ANPG is modified to contain an electron-withdrawing fluorine substituent at C2′ to destabilize the transition state which results in a decreased rate for the glycosidic hydrolysis reaction. The fluoro-substituted substrate analog retains binding affinity to ANPG similar to that of the native substrate, but it is not subject to enzymatic turnover. In a preliminary report on MutY, Varaprasad *et al*. have shown that they can alter the ability of MutY to effectively act on OG:A-containing duplexes by substitution of a methoxy group (OMe) for the oxo group (O6) of OG.[15] The mechanism of this type of inhibition of the glycosylase activity of MutY is unclear; however, these results are intriguing and indicate that efficient recognition *and repair* by MutY are dependent on the entire OG:A base-pair.

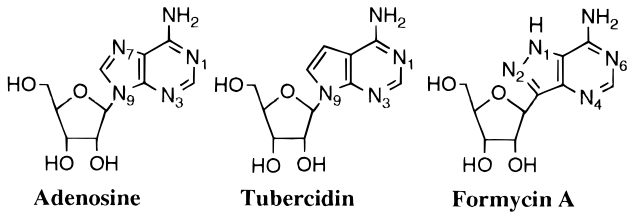

Figure 2. Structures of the ribonucleoside adenosine and its analogs, formycin A and tubercidin. Note that the numbering system for the pyrazole pyrimidine ring system of formycin A is different than that for a purine ring system.

Our approach for preparing suitable substrate analogs for MutY has been to begin with natural products which are structurally similar to adenosine and have been shown to mimic the properties of adenosine and its derivatives *in vitro* and *in vivo*. In particular, the ribonucleosides formycin A and tubercidin (7-deazaadenosine) are structurally similar to adenosine (Figure 2) and exhibit a variety of pharmacological properties including antitumor and antiviral activities which likely stem from their ability to effectively mimic adenosine *in vivo*.[16,17] *In vitro*, formycin, tubercidin, and their derivatives have been shown to effectively substitute for the corresponding adenosine substrates in a variety of enzymatic reactions.[18,19] Indeed, both derivatives are phosphorylated by adenosine kinase and can be incorporated in the RNA and DNA. Furthermore, both formycin A and tubercidin can be converted to the corresponding 2′-deoxyphosphoramidite monomers for site-specific incorporation into DNA using standard phosphoramidite chemistry.

Although formycin and tubercidin can effectively mimic the recognition and hydrogen-bonding capabilities of adenosine (Figures 2 and 3), their structural properties should provide stability toward enzymatic removal of the base. For formycin A, the presence of the ribosyl carbon linkage rather than the normal *N*-glycosidic linkage will provide such stability. Indeed, Schramm *et al*. have found that formycin monophosphate (FMP) is a potent inhibitor for adenosine monophosphate (AMP) nucleosidase and has significantly higher affinity (1200−2600X) than the native substrate for the enzyme.[20] AMP nucleosidase catalyzes the hydrolysis reaction of AMP to yield adenine and ribose-5′-phosphate. This reaction has features similar to the reaction catalyzed by MutY. The ability of FMP to bind to AMP nucleosidase with higher affinity than the substrate is rationalized to stem in part from the similarity of the protonated pyrazole ring of FMP to the protonated adenine

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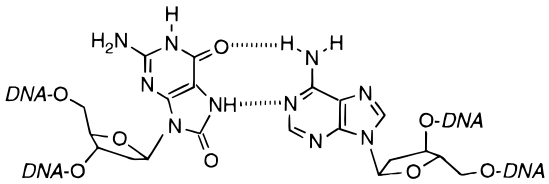
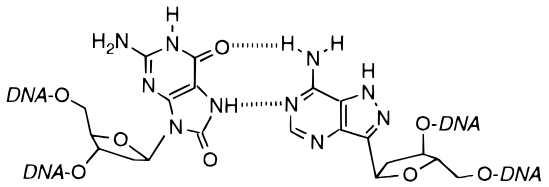
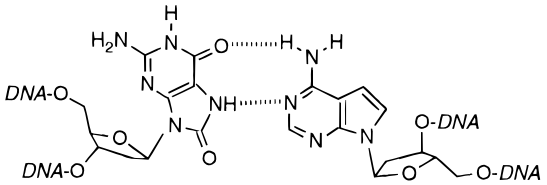


Substrate: OG:A



Substrate Analog: OG:F



Substrate Analog: OG:Z

Figure 3. Substrate and substrate analog DNA duplexes for the adenine glycosylase, MutY. In the substrate OG:A-containing duplex, MutY catalyzes the hydrolysis of the *N*-glycosidic linkage of A to yield an abasic site. In the corresponding OG:F-containing duplex the *C*-glycosidic linkage of F is resistant to hydrolysis by MutY. The hydrogen bonding shown for the OG:A base-pair is based on structural studies.[6]

in the enzyme-stabilized transition state of AMP hydrolysis.[21] For tubercidin, the lack of N7 results in stability toward acid-catalyzed depurination, presumably due to the importance of protonation at this position for activating the adenine toward elimination.[19b] Thus, we reasoned that oligonucleotide *duplexes* incorporating 2′-deoxytubercidin (Z) or 2′-deoxyformycin (F) opposite OG (or G) may retain the required properties as substrates for MutY but would be resistant to MutY's glycosylase activity (Figure 3). Lu *et al*. have previously investigated the interaction of MutY with Z:G-containing duplexes and found that these duplexes are not specifically bound or turned over as substrates by MutY.[22] We have reinvestigated the properties of the interaction of MutY with Z-containing duplexes since the ability of MutY to effectively recognize Z in lieu of A may depend on the sequence context and the identity of the base (OG or G) opposite Z.

We recently described the synthesis of F-containing oligonucleotides and demonstrated that F can effectively mimic A within duplex DNA in terms of hydrogen bonding in a variety of base-pairs.[23] Specifically, herein, we report the characterization of 2′-deoxytubercidin and 2′-deoxyformycin containing oligonucleotide duplexes as substrate mimics for the DNA repair enzyme MutY. Furthermore, we illustrate that 2′-deoxyformycin containing duplexes can be used to provide information regarding the DNA−substrate complex which could not be obtained with the normal substrate.

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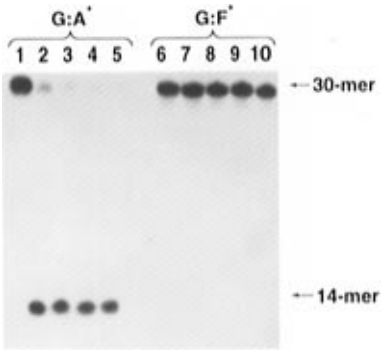

Figure 4. Cleavage assay on G:A- and G:F-containing duplexes with MutY. Excess MutY enzyme (600 nM) and DNA (10 nM) were incubated at 37 °C for various time periods. The asterisk (*) denotes the strand in the duplex which is 5′-^{32}P end labeled. Lanes 2−5: G:A-containing 30-mer duplex at 5, 15, 30, and 60 min. Lanes 7−10: OG:F-containing 30-mer at 5, 15, 30, and 60 min. Lanes 1 and 6: control lanes with no added MutY.

Results

Oligonucleotide Duplexes. Automated solid phase DNA synthesis employing the phosphoramidite method was used to synthesize a series of 30 base-pair oligonucleotide duplexes. The duplexes contained a central **X:Y** base-pair within the sequence d(5′-CGATCATGGAGCCAC**X**AGCTCCCGTTA-CAG3′)·d(3′-GCTAGTACCTCGGTG**Y** TCGAGGGCAATGTC-5′) where **Y** = A, F, Z or C and **X** = OG or G.[24] Experiments were performed with both G and OG opposite **Y** since MutY has activity toward both G:A- and OG:A-containing substrates.[10,11]

Glycosylase Assays. In adenine glycosylase assays with MutY, the strand containing the A (or F or Z) was 5′-end labeled with ^{32}P prior to annealing to the complementary strand. The duplex substrate was then incubated with excess MutY, and the reaction was quenched by the addition of base, which also served to induce strand scission at the abasic site. The amount of cleavage at the mismatched A was monitored by denaturing polyacrylamide gel electrophoresis (Figure 4). Under these assay conditions, a G:A-containing duplex was completely converted to product within 5 min. In the corresponding experiment with a G:F-containing duplex, there is no detectable strand scission at the F even after 1 h (Figure 4). Similarly, experiments with a G:Z-containing duplex also showed no detectable strand scission at the Z after similar time periods (data not shown). MutY also exhibited no activity with OG:F or OG:Z duplexes (data not shown). These results show that the F- and Z-containing duplexes are not substrates for MutY.

***K*$_d$ Measurements of MutY with DNA Duplexes.** To show that the lack of reactivity of MutY toward Z- and F-containing duplexes is not due to inefficient recognition of the substrate analogs Z and F, experiments were performed to monitor binding of MutY to the OG/G:F- and OG/G:Z-containing duplexes and the corresponding OG/G:A and OG/G:C duplexes. These experiments involved using a nondenaturing gel retardation method (Figure 5) to monitor the presence of a specific MutY−DNA complex under the appropriate conditions {[DNA] $\ll K_d$} to determine relative dissociation constants (K_d).[25] These results are listed in Table 1. The ability of the F- and Z-containing duplexes to mimic those of the substrate should be reflected in a comparison of the relative K_d values to those of the corresponding A-containing duplexes (specific binding) and corresponding C-containing duplexes (nonspecific binding).

(24) The OG phosphoramidite was synthesized using a modification of the literature procedure. Koizume, S.; Kamiya, H.; Inoue, H.; Ohtsuka, E. *Nucleosides Nucleotides* **1994**, *13*, 1517−1534.

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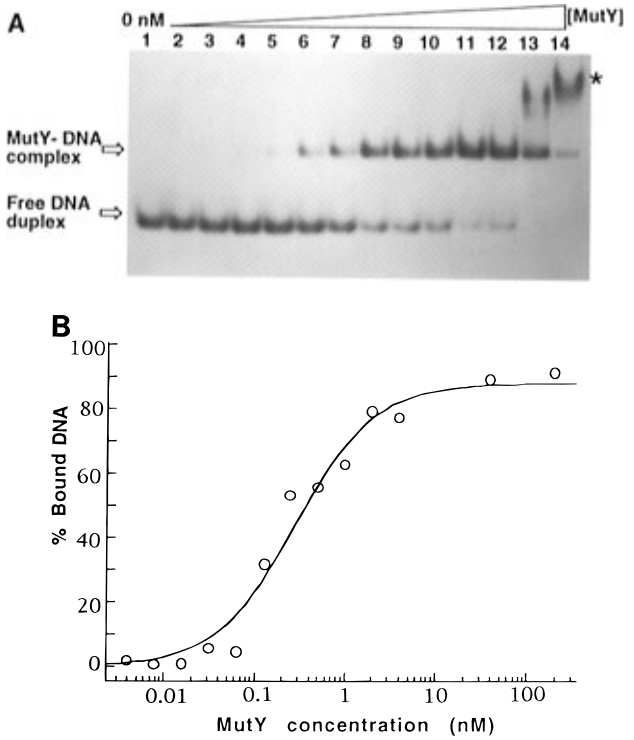

Figure 5. (A) Autoradiogram of the quantitative gel retardation assay used to detect specific binding of MutY to the OG:F-containing duplex. The F-containing strand was 5′-^{32}P end labeled. The DNA duplex concentration was 10 pM, and MutY concentration (from lane 2 to lane 14) was as follows: 0.004, 0.008, 0.016, 0.032, 0.063, 0.125, 0.250, 0.500, 1, 2, 4, 40, and 200 nM. Slower mobility bands indicated by * presumably contain more than one molecule of MutY per DNA duplex. (B) Plot of percent bound DNA as determined from the phosphor imager analysis as a function of MutY concentration. Note that data are plotted on a logarithmic scale. The K_d value obtained for this particular experiment was 0.3 ± 0.1 nM.

Table 1. Dissociation Constants (K_d) of MutY for 30 Base-Pair DNA Duplexes[a]

central X:Y base-pair	K_d (nM)
G:C	255 ± 107
G:Z	58 ± 28
G:F	26 ± 6
OG:C	44 ± 21
OG:Z	5.6 ± 2.8
OG:F	0.8 ± 0.4
G:A (P)[b]	35 ± 7
OG:A (S/P)[c]	0.2 ± 0.1

[a] All measurements were made at room temperature as described in the methods section. [b] Under these conditions, the adenine has been completely removed from the G:A substrate at all of the MutY concentrations used in the K_d titration, and therefore, this K_d represents binding to the product (denoted by the (P)). [c] Under these conditions, the adenine in the OG:A substrate has been removed to varying degrees at the MutY concentrations used in the K_d titrations. At the measured K_d, ~50% of substrate remains uncleaved, and therefore, this measured K_d represents a mixture of the K_d to substrate and product (denoted by (S/P)).

(A) K_d Measurements of MutY with DNA Substrate Analogs (OG:F, OG:Z, G:F, G:Z). The substrate analogs Z and F, when substituted for C in the OG:C or G:C duplexes, provide an increased association of MutY with the DNA duplex as indicated by a smaller K_d value. This is clearly illustrated by a comparison of the K_d values for MutY with the OG:Z duplex (5.6 nM) to the K_d values for MutY with the corresponding OG:C duplex (44 nM), which represents an 8-fold tighter association of MutY. In the comparison of MutY binding to the G:C-containing duplex relative to the G:Z substitution, the Z substitution results in a small increase (approximately 4-fold) in association with MutY. This latter result is similar to that previously reported by Lu *et al*. for the interaction of MutY with G:C- and G:Z-containing 20 base-pair duplexes.[22] They observed under slightly different conditions a decrease in K_d by a factor of 3 from 370 nM to 120 nM by replacement of C with Z. These results indicate that Z mimics some features of A, which allows for an increased association of MutY with these duplexes.

A substantial increase in the association of MutY with F-containing duplexes compared to the C-containing duplexes is noted by inspection of the K_d values in Table 1. The difference is the most dramatic in the comparison of the K_d values for MutY with the OG:C versus the OG:F duplexes (Figure 5) where the K_d diminishes from 44 nM to 0.8 nM, representing a 55-fold tighter association of MutY. The magnitude of the K_d for MutY with the OG:F duplex suggests a specific association with the OG:F base-pair within the duplex which is 300-fold tighter than the association of MutY with nonspecific DNA. The binding of MutY to the OG:F duplex is also 7-fold tighter than to the OG:Z-containing duplex. A similar but not as dramatic trend is observed in the K_d values for MutY with the G:F-containing duplex versus the G:C- and G:Z-containing duplexes. Substitution of F for C or Z in this series of duplexes results in approximately 10-fold and 2-fold decrease in the K_d, respectively. These results show that MutY has a higher affinity for F-containing duplexes versus the Z- or C-containing counterparts, which is consistent with specific recognition of F over C or Z. Notably, the effect of substitution of F or Z for C is much larger in the duplexes where the substrate analog is opposite OG, and this further underscores the importance of specific recognition by MutY of the entire base-pair.

(B) K_d Measurements of MutY with NonSpecific DNA Duplexes (OG:C and G:C). Other interesting trends in the relative dissociation constants for the various duplexes emerge from the data listed in Table 1. Alhough MutY acts upon the adenine in OG:A and G:A duplexes, the K_d values indicate that both members of the base-pair contribute to specific recognition by MutY. In a comparison of duplexes with G versus OG, the K_d values indicate that MutY is specific for recognition of OG, since its presence in the base-pair significantly lowers the K_d. For example, replacement of G in a G:C-containing duplex to form an OG:C-containing duplex lowers the K_d by a factor of 6. MutY also has significant affinity for nonspecific DNA as indicated by the K_d of 255 nM for the duplex containing a central G:C base-pair. The nonspecific binding of MutY to DNA duplexes is often apparent in the K_d determinations of many of the duplexes and is observed as a slower moving fragment in the non-denaturing gel retardation assay (Figure 5A). For the larger K_d values, this likely contributed to the fairly large errors in the K_d determinations.

(C) K_d Measurements of MutY with DNA Substrates (OG:A and G:A). Clearly, MutY binds the F-containing oligonucleotides with higher affinity than their C or Z counterparts, which indicates that the OG:F and G:F duplexes retain some features similar to OG:A and G:A duplexes resulting in a higher affinity to MutY. It is not possible to make direct comparisons of the binding affinity of MutY with the F-containing duplexes relative to the binding of MutY to the A-containing counterparts, since we cannot measure the K_d of MutY with OG:A and G:A substrates without turnover. It is typical in gel retardation experiments to incubate the duplexes with the DNA binding protein for 20−30 min prior to loading on the denaturing gel to establish equilibrium. However, with OG:A- and G:A-containing duplexes, a significant fraction of the duplex is converted to product during this time period and

the amount of product formed will be different at each MutY concentration used in the K_d titration.[26] In order to be consistent in our measurements, we have measured K_d values for the OG:A and G:A duplexes under the same conditions as the nonsubstrate duplexes.[27] However, in order to clarify what the measured K_d represents under these conditions, we have monitored in parallel the glycosylase activity at each point in the K_d titration in order to determine the amount of turnover (Supporting Information).

The percentage of cleavage at the A from the glycosylase assay and percentage of duplex bound to MutY from the gel-shift analysis were plotted as a function of MutY concentration (Supporting Information, Figure 1) for the G:A duplex. This analysis suggests that the measured K_d of 35 nM represents the dissociation constant of MutY to the *product* since, at the approximate midpoint of the binding curve (the approximate K_d), the cleavage at the A indicates that greater than 95% of the substrate has been consumed. These data also indicate that binding to the *substrate* must be tighter than to the product (as expected) since the cleavage does not fall-off until much lower concentrations. These data indicate that MutY has significant affinity for the *product*, since the K_d of 35 nM represents a 7-fold increase in binding affinity over that of nonspecific DNA.

In order to determine the affinity of MutY with an OG:A duplex, a similar analysis was performed (Supporting Information, Figure 2); however, the K_d values determined from the binding analysis were at a concentration of MutY where approximately 50% of the substrate OG:A duplex had been consumed. Interestingly, under these conditions the plot of the percentage of reaction at the "A" versus [MutY] is similar to the plot of percentage MutY bound duplex versus [MutY]. The average measured K_d value of 0.2 nM for MutY with the OG:A duplex obtained under these conditions is therefore representative of a mixture of the K_d of MutY with the OG:A substrate and product. The observation that complete conversion to product is not observed at all concentrations in the titration curve could be a result of a smaller difference in the intrinsic K_d values for MutY with OG:A-containing substrates and the resulting products.

It is difficult to make a *quantitative* comparison between the K_d values for MutY with the substrate analog F and Z duplexes versus the substrate A-containing duplexes. However, the measured K_d values do allow for a *qualitative* comparison. With the G:A substrates, the measured K_d value of 35 nM represents the value of binding of MutY to the product. It is likely that the K_d for MutY with the G:A *substrate* would be significantly lower than 35 nM. The K_d value of 26 nM for MutY with the G:F-containing duplex is slightly lower than that measured with the G:A duplex product. The fact that the binding affinity of MutY for the product is less than that for the G:F duplex suggests that the G:F duplex has more resemblance to the substrate.

With OG:A substrates, the measured K_d value of 0.2 nM represents the affinity of MutY for a mixture of substrate and product based on the corresponding cleavage gels, and therefore, this value represents an upper limit for the K_d of MutY with the substrate. The measured value of 0.2 nM is lower than but in the same range as the K_d of MutY for the OG:F duplex of 0.8 nM. Thus, MutY has an affinity for the OG:F duplex that approaches that of the native OG:A substrate, although not quite as high. This is not unexpected since the substrate analog is not *identical* to the substrate. However, these values indicate that OG:F duplex forms a specific and tight complex with MutY which mimics some of the features of the substrate complex. The inability to *accurately* measure the K_d value to the OG:A and G:A substrates underscores the usefulness of non cleavable substrate analogs for providing insight into the MutY−substrate complex.

Footprinting Experiments. Footprinting techniques can provide useful information on sequence-specific protein−DNA complexes.[28,29] However, these techniques have been used infrequently to investigate DNA repair enzymes involved in base excision repair due to the inherent problem of enzymatic turnover during the footprinting experiment.[30] For example, in dimethyl sulfate footprinting experiments with the pyrimidine dimer glycosylase T4 endonuclease V, only the strand *opposite* the thymine dimer could be analyzed due to the lability of the damaged thymine dimer and the intrinsic cleavage activity of the enzyme.[31] However, since MutY binds to the OG:F-containing duplexes with high affinity and these duplexes are not subject to enzymatic turnover, the OG:F duplexes provide a useful method for further characterization of the interaction of MutY with its substrate.

Footprinting experiments were performed using methidium-propyl-EDTA−Fe(II) [MPE−Fe(II)] to generate hydroxyl radicals as the footprinting reagent.[32] OG:F- and OG:A-containing duplexes in which either the OG-containing strand or A/F-containing strand was 5′-^{32}P-end labeled prior to duplex formation were subject to reaction with MPE−Fe(II)/ascorbate/hydrogen peroxide in the presence and absence of MutY as shown in Figure 6A. In addition, the OG-, F-, and A-containing strands were sequenced using a Maxam−Gilbert G + A reaction[33] to determine the location of the X:Y base-pair in the oligonucleotides (Figure 6, lanes 1, 12, and 18). In the experiments with the OG:A duplex, the OG-containing strand exhibits a region surrounding the OG lesion of approximately eight nucleotides which is protected from hydroxyl radical cleavage in the presence of the MutY enzyme (Figure 6, lanes 4−6). In the corresponding reaction with the A-strand labeled in the OG:A-containing duplex (Figure 6A, lanes 15−17), a "footprint" is not observed and a single cleavage band is observed due the intrinsic activity of MutY which results in strand scission at the A in the OG:A base-pair.[34] It is not surprising that there are no observed hydroxyl radical cleavage bands on the 3′ side of the "A", since these bands would not be

(26) Lu *et al.*[22] have reported K_d values for MutY for a G:A- and OG:A-containing 20-mer duplex of 5.3 nM and 66 pM at 37 °C. It is not clear whether we can compare these numbers to the values we obtained in this work since the data was generated under different conditions and, for the OG:A duplex, the DNA concentration used was larger than the K_d. Additionally, the amount of cleavage at the A by MutY was not reported.

(27) We observe some unusual behavior in determinations of K_d values of OG:A duplexes with long incubation times (1−2 h). This is presently under investigation.

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(29) For examples, see: (a) Ramesh, V.; Nagaraja, V. *J. Mol. Biol.* **1996**, *260*, 22−33. (b) Oakley, M. G.; Mrksich, M.; Dervan, P .B. *Biochemistry* **1992**, *31*, 10969−10975.

(30) (a) Footprinting experiments have been performed on T4-endonuclease V by trapping the intermediate formed *after the glycosylase reaction* by reduction with sodium borohydride. Latham, K. A.; Taylor, J.-S.; Lloyd, R. S. *J. Biol. Chem.* **1995**, *270*, 3765−3771. (b) Footprinting experiments using oligonucleotides containing a reduced apyrimidinic site which are inhibitors of the β-lyase reaction at the AP site generated by the glycosylase reaction with endonuclease III and the FPG protein have also been reported. For endonuclease III see: O'Handley, S.; Scholes, C. P.; Cunningham, R. P. *Biochemistry* **1995**, *34*, 2528−2536. For the FPG protein, see: Castaing, B.; Boiteux, S.; Zelwar, C. *Nucleic Acids. Res.* **1992**, *20*, 389−394.

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(34) We assume that the conditions of the hydroxyl radical footprinting provide the base for cleavage at the abasic site generated by MutY. The band with retarded mobility with respect to the position of the "A" results from incomplete base treatment, since these conditions are not as basic as used in the MutY assay to visualize the glycosylase activity.

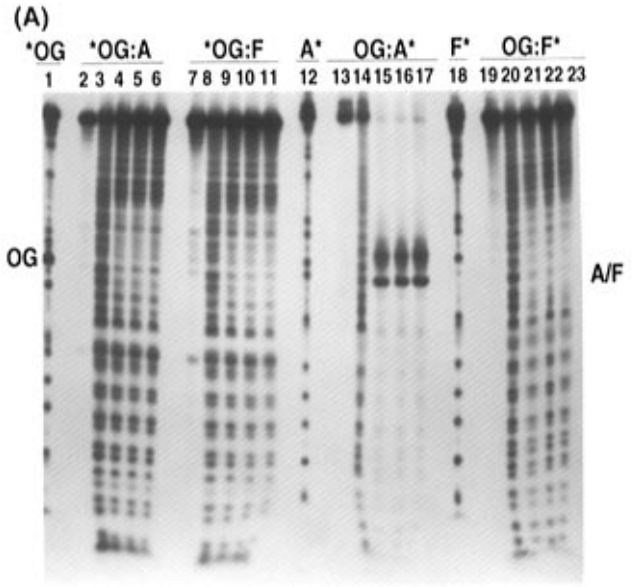

Figure 6. (A) MPE−Fe(II) footprinting of MutY on OG:A- and OG:F-containing 30 base-pair duplexes. The asterisk (*) denotes the strand in the duplex or single stranded oligonucleotide which is 5′-^{32}P end labeled. Lanes 1, 12, and 18: Maxam−Gilbert G+A sequencing reactions. Lanes 2, 7, 13, and 19: control reaction with no MutY and no MPE−Fe(II). Lanes 3, 8, 14, and 20: all reagents except MutY. Lanes 4, 9, 15, and 21: 300 nM MutY. Lanes 5, 10, 16, and 22: 600 nM MutY. Lanes 6, 11, 17, and 23: 900 nM MutY. Other concentrations are as follows: DNA, 20 nM; MPE−Fe(II), 12.5 μM; calf thymus DNA, 500 μM. (B) Sequence of the 30 base-pair oligonucleotide containing a central OG:F base-pair illustrating regions of MutY protection from MPE−Fe(II)-promoted cleavage. The brackets indicate the eight nucleotide region in each strand which is protected by MutY.

visualized owing to the 5′-end location of the ^{32}P. In contrast, one would expect to see a ladder of bands on the 5′-side of the "A" in these footprinting experiments; however, under these conditions only a band at the A is observed with no full-length oligonucleotide remaining.[35] The fact that there is complete cleavage at the "A" during the course of the footprinting experiments indicates that the observed "footprint" on the OG strand in the OG:A duplex is of MutY bound to the *product* of the glycosylase reaction.

In parallel experiments with the OG:F duplex, there is a region of approximately eight nucleotides surrounding the OG lesion on the OG strand, which is protected from hydroxyl radical cleavage in the presence of the MutY enzyme (Figure 6A, lanes 9−11), similar to that observed for the OG strand in the OG:A duplex (Figure 6, lanes 4−6). However, in contrast to the results with the OG:A duplex, an eight nucleotide stretch of protection *surrounding the F* in the OG:F duplex is observed in the presence of MutY when the F strand in the duplex is 5′-^{32}P-end labeled (Figure 6A, lanes 21−23). The pattern of protection of the region surrounding the OG:F in the duplex is illustrated in Figure 6B. Approximately, a 12 base-pair stretch of protection of the duplex is observed with the OG:F base-pair centrally located within the protected region. The region of protection on the two strands is slightly offset by two base-pairs on each side; this pattern is also observed in other examples of MPE−Fe(II) footprinting.[29b,36] The exact size of the observed footprint could be influenced by DNA distortion associated with MutY binding which reduces the ability of MPE−Fe(II) to intercalate at the edge of the MutY binding region.[36] Importantly, the footprinting data provides additional evidence for specific recognition of the OG:F base-pair within the DNA duplex by MutY. Furthermore, these results illustrate the utility of OG:F duplexes for providing information regarding the MutY-mismatched DNA complex, since we were able to obtain footprinting information *on both strands of the duplex* only with the substrate analog OG:F duplexes.

(35) In preliminary data with other footprinting reagents, we observe additional bands on the 5′ side of the "A". Porello, S. L.; David, S. S. Unpublished results.

Discussion

The DNA repair enzyme MutY is unique in being specific for a damaged/mismatched base-pair (OG:A, G:A) with glycosylase activity toward the undamaged 2′-deoxyadenosine. Since a variety of natural products are structurally similar to adenosine and are able to mimic adenosine *in vitro* and *in vivo*, these molecules are good starting points for the development and design of modified DNA oligonucleotide duplexes which retain specific binding properties to MutY but are not subject to enzymatic turnover. The structural properties of the natural product formycin A are particularly interesting since it contains an unusual *C*-glycosidic linkage and may serve as a "prototype" for the development of *C*-nucleoside analogs for investigations of base excision DNA repair enzymes.

In this paper, we have shown that MutY binds with high affinity and specificity to OG:F-containing duplexes using gel retardation assays and footprinting experiments. However, the OG:F-containing duplexes are resistant to the glycosylase activity of MutY and thus eliminate complications associated with turnover when investigating the properties of the MutY−substrate complex. As might be expected, the affinity for the OG:F duplex is not as high as that for the substrate OG:A-containing duplex. This likely results from the structural properties of 2′-deoxyformycin which differ from 2′-deoxyadenosine, namely the *C*-glycosidic linkage and the unusual pyrazole ring. Previously, in measurements of thermodynamic stability of oligonucleotide duplexes containing a single B:F base-pair (where B = G, C, A, T) versus the corresponding B:A base-pair, we observed a minor decrease in thermodynamic stability (1−2 kcal/mol/strand) of the F-containing duplexes.[23] This lower stability may be associated with the conformational properties of the *C*-glycosidic linkage which differ from the normal *N*-glycosidic linkage. In crystallographic studies of the nucleoside formycin and some of its derivatives, the *C*-glycosyl torsion angle was found to be predominantly in the unusual *syn* conformation.[37] A preference of the *syn* conformation of the nucleoside would translate into a less stable double-helical structure which requires the *anti* conformation. Indeed, anomalous behavior of formycin containing polymers and the slow rate of incorporation of FTP into RNA have been suggested to result from the unusual conformational properties of formycin.[38] MutY might be sensitive to any unusual structural features associated with the *C*-glycosidic linkage in the OG:F duplex which is reflected in a lower affinity. Presently, there is no structural information of F within a DNA duplex from NMR spectroscopy or X-ray crystallography. Such structural informa-

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tion would provide considerable insight into the factors which may be influencing the recognition by MutY of OG:F and G:F duplexes.

A factor which may also influence the recognition and repair properties of the G:F and OG:F duplexes by MutY is that 2′-deoxyformycin has an unusual pyrazole ring which places a hydrogen bond donor (N1-H) at the position where 2′-deoxyadenosine has a hydrogen-acceptor (N7). Interestingly, the 7-deaza-2′-deoxyadenosine nucleoside lacks *both* a hydrogen bond donor or acceptor (C7-H) at the position corresponding to N7 in 2′-deoxyadenosine. In the Z-containing duplexes, the lack of N7 in Z should stabilize the base toward depurination since the protonation at N7 is likely important in facilitating the removal of the base. The N7 position may also be an important site for *recognition* of the G:A or OG:A base-pairs within duplex DNA by making specific contacts with the MutY enzyme. The reduced binding of MutY to the OG:Z duplex compared to that with the OG:A duplex may be attributed in part to the importance of recognition at N7. Surprisingly, however, the OG:F- and G:F-containing duplexes which also lack a hydrogen bond acceptor at the position of N7 in A bind to MutY with much higher affinity than their Z-containing counterparts. The origin of this difference is unclear; however, it is possible to speculate on some contributing factors. One possibility is that in the active site pocket of MutY a specific contact can be made with the N2 position in the pyrazole ring of the F in a manner analogous to the contact made with the N7 in A. The position of N2 of F relative to N7 of A may not be ideal, which may contribute to a less-favored interaction with F- versus A-containing duplexes but may be superior to no such interaction as in the Z-containing duplexes. A possible alternative to this may be that in the complex of MutY with the OG:F or G:F duplex the proton on the pyrazole ring of F may shift from N1 to N2. NMR studies have provided evidence for the existence of a minor tautomer of formycin with the proton located at N2 rather than N1.[39] In addition, migration of the proton from N1 to N2 in formycin accompanied by protonation at N6 has been observed in the crystal structure of the salt formed with hydrobromic acid.[40] If transfer of the proton to N2 occurs, this would reveal the N1 position of F for hydrogen-bonding interactions with MutY. Insight into the exact factors influencing the recognition of F oligonucleotide duplexes will require structural investigations of MutY bound to an OG:F- or G:F-containing duplexes. Alternatively, synthetic techniques could be used to prepare new nucleoside analogs which retain or delete features of F to further explore the importance of certain structural features for specific recognition by MutY and resistance to the glycosylase activity of MutY.

Another striking feature of the interaction of MutY with the substrate analogs F and Z relative to their C counterparts is the dependence on the identity of the base opposite (G or OG) the substrate analog in the base-pair. The increased association of MutY with the OG:F versus OG:C duplex is significantly more pronounced than the increased association of MutY with G:F versus the G:C duplex. An interesting feature of this is that the increased affinity of MutY for the OG:F duplex is not simply the addition of the affinity of MutY for OG over G and F over C. If this were the case, we would expect that since G:C versus OG:C results in a 6-fold decrease in K_d and G:C versus G:F results in a 10-fold decrease in K_d, G:C versus OG:F would result in a 60-fold decrease in K_d. However, the actual decrease in K_d with MutY in comparing G:C to OG:F is 300-fold. This comparison indicates that MutY recognition of the individual bases within the damaged/mismatched OG:A occurs in a synergistic manner. Structural investigations of G:A-containing duplexes have shown that this base-pair can exhibit a variety of structures which differ in the conformation at the *N*-glycosidic bond, (e.g., G(*anti*):A(*anti*), G(*syn*):AH$^+$(*anti*), and G(*anti*):A(*syn*)).[41] In contrast, OG:A base-pairs adopt the OG(*syn*):A(*anti*) conformation and are extremely stable with thermodynamic stabilities which rival their Watson−Crick counterparts.[6,42] It might be expected that the increased stability and defined conformation of the OG:F base-pair compared to that of the G:F base-pair could enhance the ability of F to effectively mimic properties of A in the complex formed with MutY. These results further delineate the importance of recognition of the entire damaged/mismatched base-pair by MutY.

Since the OG:F duplex represents an analog of the substrate which retains high affinity to MutY but cannot be enzymatically turned over, the OG:F duplex can be used to trap MutY during the beginning process of catalyzing the adenine glycosylase reaction. These static MutY−substrate analog DNA complexes can then be used in a variety of experiments aimed at determining the structural properties of the complex. In particular, we have illustrated the potential of the OG:F duplex by the use of MPE−Fe(II) footprinting experiments on the stable MutY−OG:F DNA duplex complex. The observation of a region of protection of the duplex surrounding the OG:F base-pair which is protected by MutY from hydroxyl radical cleavage provides additional evidence for *specific* recognition of the OG:F base-pair within the DNA duplex. These results also illustrate the utility of OG:F duplexes for providing information regarding the MutY-mismatched DNA complex which could not be obtained with the normal OG:A substrate, since a footprint *on both strands of the duplex* could only be observed with the OG:F-containing duplex. Additionally, such complexes may be suitable for study by other structural methods, such as X-ray crystallography. Thus, the use of these duplexes will serve as a powerful method for dissecting the course of the glycosylase reaction. In particular, these duplexes will be useful for providing information on the complex formed with MutY prior to the transition state and therefore give insights into the factors influencing damage and mismatch *recognition* within normal duplex DNA.

In addition to the functional importance of MutY in the prevention of oxidatively induced mutations, MutY also is somewhat unusual among DNA repair enzymes in containing a [4Fe−4S] center. Presently, the *role* of the [4Fe−4S] center in these enzymes is unknown; however, the use of the substrate analog OG:F duplexes should allow for additional methods for investigating the properties of the [4Fe−4S] center in MutY which will provide insight into the part this metal center plays in DNA repair.

Conclusions

We have demonstrated that 2′-deoxyformycin in OG:F and G:F base-pairs retains specific *recognition* by the DNA repair enzyme MutY as 2′-deoxyadenosine in OG:A or G:A base-pairs. However, the OG:F- and G:F-containing duplexes are not subsequently turned over as substrates by the MutY enzyme. Footprinting experiments illustrated that information can be obtained using this new material and underscore the usefulness

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of this approach for characterizing features of DNA recognition by DNA repair enzymes. These substrate analog duplexes will provide avenues for structural analysis of the MutY-mismatched DNA complex and for investigating the properties of the unusual [4Fe−4S] center in MutY.

Experimental Section

General Methods. 2′-Deoxyguanosine was purchased from Cruachem. Snake venom phosphodiesterase and alkaline phosphatase were obtained from Boehringer−Mannheim. Standard 2-cyanoethyl phosphoramidites were purchased from Millipore. The 7-deaza-2′-deoxyadenosine phosphoramidite was purchased from Glen Research. 5′-(γ-^{32}P)-ATP was obtained from Amersham. Methidiumpropyl-EDTA and calf thymus DNA were purchased from Sigma. T4-polynucleotide kinase was purchased from USB. All other reagents were obtained from Aldrich or Fisher Scientific. Distilled, deionized water (Milli-Q) was used for all aqueous reactions and dilutions. Mass spectra were recorded at the University of Illinois, Mass Spectrometry Facility. Oligodeoxynucleotides were synthesized on a Cyclone Plus (Milligen/Biosearch) automated DNA synthesizer using the manufacturer's protocols. Autoradiograghy was carried out using Amersham HyperFilm-MP film. All UV−vis absorbance measurements were performed on a HP8452A diode array spectrophotometer. HPLC analysis and purification was performed on a Waters 625LC with diode array detection using a Hamilton PRP-1 or Waters Protein-Pak DEAE 8HR ion exchange column. Quantification of the intensity of bands in gel electrophoresis experiments was performed using a Molecular Dynamics phosphor imager. Electrophoresis experiments were performed with 1X or 0.5X Tris−Borate−EDTA (TBE) buffer (where X = 90 mM Tris, 90 mM boric Acid, 1 mM EDTA).

Synthesis of a Protected 8-Oxo-2′-deoxyguanosine Phosphoramidite. An N^2-dimethylformamidine (dmf) protected 8-oxo-2′-deoxyguanosine phosphoramidite with a 5′-*O*-DMT (dimethoxytrityl) group was used for automated solid phase DNA synthesis. The synthesis of this phosphoramidite is similar to the synthesis of Koizume *et al*. for an N^2-dmf-protected 8-oxo-2′-deoxyguanosine phosphoramidite with a 5′-*O*-MMT (monomethoxytrityl) group.[24] In addition to opting for DMT rather than MMT protection of the 5-hydroxyl group, *N*-bromosuccinimide was used instead of bromine for the bromination of the 8-position of 2′-deoxyguanosine following a literature procedure.[43] This procedure was modified in order to obtain a high yield of the brominated product on a larger scale (10 mmol) than the reported scale (0.1 mmol) by use of a minimal amount of the solvent water.

Synthesis of Oligodeoxynucleotides. The synthesis of the oligodeoxynucleotides was performed on an automated DNA synthesizer using synthesized protected 2′-deoxyformycin A[23] and 8-oxo-2′-deoxyguanosine 2-cyanoethyl phosphoramidite monomers and commercially available conventionally protected 2′-deoxynucleoside 2-cyanoethyl phosphoramidite monomers. The syntheses were carried out at 0.2 and 1.0 μmol scale with retention of the 5′-dimethoxytrityl (DMT) group on the last nucleotide. Coupling efficiencies were in all cases >97%. The stepwise and overall coupling efficiency of the synthesis was determined by collection and spectrophotometric determination (at 495 nm) of the concentration of the dimethoxytrityl cation formed upon deprotection of the 5′-hydroxyl group. This procedure was performed as outlined by the DNA synthesizer manufacturer. All base-labile protecting groups on the oligonucleotides were removed by treatment with concentrated ammonia at room temperature for over 24 h. For the 8-oxo-2′-deoxyguanosine-containing oligonucleotides, concentrated ammonia containing 0.25 M 2-mercaptoethanol was used for the postsynthetic deprotection. The reaction mixture was then evaporated *in vacuo*. The resulting 5′-DMT oligonucleotides were then purified by reverse phase HPLC. Subsequently, the 5′-DMT group was removed using 80% acetic acid. The fully deprotected oligonucleotides were then purified by ion exchange HPLC. Finally, the lyophilized oligonucleotides were desalted on a Waters Sep-Pak C18 Cartridge. The concentration of single-stranded oligonucleotides was determined at 260 nm, using the following molar extinction coefficients for each base: 15 000 (A), 11 700 (G), 8800 (T), 7800 (C), 9900 (OG), and 3950 (F) cm^{-1} M^{-1}. The nucleoside compositions of modified oligodeoxynucleotides (11 nucleotides) were analyzed by reverse phase HPLC after complete digestion with snake venom phosphodiesterase and alkaline phosphatase using the procedure of Eckstein.[44] The deviation between theoretical and experimentally determined nucleoside composition values was in all cases <2%. Additionally, short oligomers of the sequence T-F-T and T-OG-T were analyzed by positive and/or negative FAB-MS. In both cases a strong parent ion at the expected molecular weight of either *m/z* 860 $(M + H)^+$ or *m/z* 912 $(M + Na - 2H)^-$, respectively, was obtained.

Formation of Duplexes for Experiments with MutY. Prior to duplex formation, the single-stranded oligonucleotides were 5′-end labeled with T4 polynucleotide kinase using the manufacturer's protocol. The 5′-^{32}P-end labeled oligonucleotides were then purified using a Nensorb column (Dupont-NEN) to remove unincorporated ^{32}P-ATP. This purification was performed using the manufacturer's protocol. The labeled oligonucleotides were annealed to the complementary nonlabeled strands by heating at 90 °C for 10 min in 150 mM NaCl, 20 mM Tris-HCl, pH 7.6, 10 mM EDTA, and then slowly cooling to room temperature.

MutY Purification. MutY was overexpressed from the plasmid pKKYEco containing the *mutY* gene under the control of an IPTG inducible promoter in *E. coli* JM109 (gift from Drs. M. Micheals and J. H. Miller).[45] The MutY enzyme was purified as previously described by Au *et al*.[11] with the following modifications: the cells were treated with lysozyme (1 mg/g of cells) for 30 min on ice and then disrupted by three freezing and thawing cycles in buffer A without sucrose (50 mM Tris−HCl pH 7.5, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA). An initial purification involved using an SP-Sepharose column as described previously. The fractions collected from the SP-Sepharose column were dialyzed against SFF-1 buffer (50 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, 5% glycerol) with 70 mM NaCl and loaded on a DEAE-Sepharose CL-6B (Pharmacia) column previously equilibrated with the same buffer. MutY is in the effluent which is then directly loaded onto a Heparin-Sepharose affinity column (Pharmacia) previously equilibrated with SFF-1 buffer with 100 mM NaCl. After washing the column with equilibration buffer, the brown MutY protein was eluted using a linear gradient of NaCl (from 100 mM to 500 mM) in SFF-1 buffer. For determination of the MutY-containing fractions, the absorbances at 280 nm and 410 nm were measured and the fractions with $A_{280}/A_{410} \approx 6$ were pooled. The collected enzyme was analyzed by SDS-PAGE followed by silver-staining and appeared to be >95% pure. For storage, the enzyme was dialyzed against 2 L of storage buffer (50 mM sodium phosphate, pH 7.5, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) 100 mM NaCl, and 50% glycerol) and stored at −70 °C. This

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dialysis procedure also served to concentrate the enzyme to an approximate concentration of 8 μM.

Qualitative Adenine Glycosylase Assay. Reactions of 10 μL total volume containing 10 nM 30-mer duplex containing a central X:Y mismatch (with the Y-strand 5′-^{32}P-end labeled) and 300 nM MutY were incubated for 5, 15, 30, and 60 min at 37 °C in 20 mM Tris−HCl, pH 7.5, 10 mM EDTA, and 0.1 μg/μL Bovine serum albumin. The reactions were then quenched by adding 2 μL of 1 M NaOH and heating 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 denatured and electrophoresed on a 15% denaturing polyacrylamide gel in 1X TBE buffer. The gel was then exposed to a Molecular Dynamics phosphor imager screen or autoradiography film for subsequent analysis of the relative activity of MutY with the various duplex substrates.

MPE−Fe(II) Footprinting. Reactions (10 μL) containing 20 nM 5′-^{32}P end labeled 30 base-pair duplex with either a central OG:A or OG:F mismatch and 300, 600, or 900 nM MutY were incubated for 15 min at room temperature in 10 mM Tris-HCl/50 mM NaCl, pH 7.4, 0.1 μg/μL BSA, and 500 μM calf thymus DNA. The MPE−Fe(II) solution was prepared by mixing equal volumes of 1 mM methidiumpropyl-EDTA (MPE) and 1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ followed by 1:4 dilution in water. The MPE solution was stored at −20 °C. The $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and MPE−Fe(II) solution were freshly prepared. A 1.0 μL aliquot of both MPE−Fe(II) and 10 mM sodium ascorbate solutions (stored at −20 °C) were added to each reaction tube. The reaction mixtures were incubated at room temperature for 30 min, and the reactions were quenched by freezing with dry ice. The samples were then lyophilized, resuspended in formamide loading dye, heat denatured, and electrophoresed on a 15% denaturing polyacrylamide gel using 1X TBE buffer. The gel was then exposed to a Molecular Dynamics phosphor imager screen or film. Control reactions were also performed under the same conditions in the absence of MutY.

Gel Retardation Assay. Quantitative gel retardation assays[25] were performed using the duplexes containing a central X:Y base-pair with the Y-containing strand (Y = A, C, F, Z) 5′-^{32}P-end labeled. Reaction volumes of 20−100 μL contained 10 pM duplex (OG:A, OG:F) or 50 pM duplex (all other duplexes), 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1 μg/μL BSA, and various amounts of MutY. To accurately measure K_d values, very low concentrations of duplex DNA (10−50 pM) were used such that [DNA] $\ll K_d$ and without addition of nonspecific DNA. A range of concentrations of MutY were used by diluting stored aliquots of MutY (−70 °C) with dilution buffer (20 mM Tris−HCl, pH 7.5, 10 mM EDTA, 20% glycerol). The samples were incubated together for 20 min at room temperature. Nondenaturing loading dye was added, and the samples were electrophoresed on a 6% nondenaturing polyacrylamide gel (17 cm × 14 cm × 0.3 cm) with 0.5X TBE buffer at 100 V for 2 h at 4 °C. After the gel was dried, it was exposed to a Molecular Dynamics phosphor imager screen for 24−48 h. Additionally, representative dried gels which had previously been exposed to the phosphor imager screen were also exposed to X-ray film for a high-quality image (Figure 5A and Supporting Information, Figures 1A and 2A). The exposed phosphor imager screens were scanned by a Molecular Dynamics phosphor imager, and the intensity of each band was quantitated using ImageQuant Software.

K_d was determined by fitting of the data (percent bound duplex versus [MutY] or log[MutY]) with the equations for one-site ligand binding using the program Ultra Fit (from BIOSOFT). K_d values were determined from data from at least three separate experiments (typically five) using freshly diluted MutY in each case.

Glycosylase Assay on Samples from K_d Measurements. To determine the degree with which the measured K_d values for the G:A and OG:A duplexes reflect binding to substrate or product, denaturing gels were run in parallel with each nondenaturing gel to observe the formation of the abasic site at each MutY concentration. A percentage of the reaction mixture (20 μL from 40 μL for G:A and 30 μL from 100 μL for OG:A) were removed at the end of the incubation, and NaOH was added to give a final concentration of 0.1 M. The removed base-treated aliquots were then heated at 90 °C for 3−5 min. An equal volume of denaturing loading dye was added, and the samples were kept on ice. The samples were then electrophoresed on a gel containing 10% polyacrylamide, 8 M urea (17 cm × 14 cm × 0.1 cm) in 1X TBE buffer at 150 V for 3 h at room temperature. Urea was removed by immersion of the gel in 12% MeOH, 10% glacial acetic acid for 30 min. After the gels were dried, they were exposed to a phosphor imager screen for 48 h and quantitated to determine the percent of cleavage at the abasic site generated by the MutY glycosylase. A representative cleavage experiment on a G:A and OG:A duplex was exposed to autoradiography film for a high-quality image for Supporting Information, Figures 1B and 2B.

Percent Active MutY. In order to estimate the concentration of active enzyme in the MutY samples used, gel retardation experiments were performed under conditions of [DNA] $\gg K_d$ where MutY−DNA binding should proceed via a 1:1 complex. Under these conditions, approximately 60% of the sample appeared to contain active MutY. All concentrations listed in the text represent the concentration of MutY which was determined by using the extinction coefficient of 17 000 M^{-1} cm^{-1} for the absorbance at 410 nM for the [4Fe−4S] cluster. The concentration of protein obtained by using this extinction coefficient is similar to MutY concentration determinations using the method of Bradford (BioRad).[46]

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Supporting Information Available: Autoradiograms of gel retardation assay and adenine glycosylase assays performed in parallel with G:A and OG:A substrates and plots of the K_d determinations and percent substrate consumed as a function of [MutY] from the gel experiments (7 pages). See any current masthead page for ordering and Internet access instructions.

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