DNA Determinants and Substrate Specificities of Escherichia coli MutY*  

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Multiple mismatch repair pathways with different mismatch specificities and different size repair tracts are utilized by Escherichia coli to reduce replicative errors and to protect its DNA from various types of damage (1). One of the short-patch repair pathways requires mutY gene function and is independent of dam-methylation (2-4). E. coli mutY (or mcA) mutants have higher mutation rates for C-G to A-T transversions (3, 5). The MutY pathway specifically repairs A/G mismatches to C/G base pairs (2-4, 6) and repairs A/C to G/C at a much lower rate (2, 3, 7). MutY can also act on adenines mispaired with 7,8-dihydro-8-oxo-guanine (GO) or 7,8-dihydro-8-oxo-adenine (AO) (8, 9). The GO lesion is one of the most stable products of oxidative damage to DNA known. A role for the MutY pathway in E. coli is to remove misincorporated adenines opposite G or GO following DNA replication (8, 10). Adenines are frequently incorporated opposite GO bases during DNA replication in vitro (11) and in vivo (12). A second round of replication through this mismatch subsequently leads to a C-G to A-T transversion (12-15). E. coli uses MutY, MutM, and MutT to defend against the mutagenic effects of GO lesions (10, 16). The MutT protein eliminates 8-oxo-dGTP from the nucleotide pool by its nucleoside triphosphatase activity (17-19). The MutM protein (FPG protein) provides a second level of defense by removing both ring-opened purine lesions and mutagenic GO adducts (20, 21). MutM removes GO lesions efficiently from C/GO but poorly from A/GO (21). MutY works at a third level by correcting replicative errors that result from misincorporation of A opposite GO (8, 9).

The 39-kDa MutY protein is an iron-sulfur protein, which has homology with E. coli endonuclease III (7, 22, 23). The MutY protein was shown by Tsai-Wu et al. (7) to have both DNA glycosylase and apurinic/apyrimidinic (AP) endonuclease activities, although the AP endonuclease activity could not be detected by Au et al. (24) in their MutY preparation. The DNA glycosylase activity removes the adenine bases from the A/G, A/C, A/GO, and A/AO mismatches (7, 9, 24), and the AP endonuclease activity cleaves the first phosphodiester bond 3’ to the AP site (7, 25).

The activity of MutY on its mismatched DNA substrates is influenced by the neighboring sequence composition (2, 3, 25). Structural studies have demonstrated that A/G mismatches can adopt three possible configurations (A(anti)-G(anti), A(anti)-G(syn), and A(syn)-G(anti)), depending on their neighboring sequences (26-30). It is unclear which configuration of A/G mispair is recognized by MutY. Based on the common features of MutY substrates (A/G, A/GO, A/C, and A/AO), it has been suggested that the N-1 of adenine may be protonated and/or that the guanine is in the syn configuration (7, 9). In the work described here, we have explored the potential purine and phosphate contacts involved in MutY recognition. Alkylation interference experiments have demonstrated that MutY specifically interacts with mismatched A and G as well as neighboring sequences. This may explain the sequence effect on the repair efficiency of MutY. Defined oligonucleotides containing various purines were used in this study to examine the substrate specificity of MutY protein and to establish the role of mismatch functional groups in MutY recognition and catalysis.

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1 The abbreviations used are: GO, 7,8-dihydro-8-oxo-guanine; AP, apurinic/apyrimidinic; AO, 7,8-dihydro-8-oxo-adenine; I, inosine; N, nebularine; 2AP, 2-aminopurine; Z, 7-deaza-adenosine.
Our results add nebularine/G (N/G), inosine/G (I/G), and 2-amino-purine (2AP/G) to the growing list of recognized substrates of MutY, although I/G and 2AP/G are not cleaved well by MutY.

**EXPERIMENTAL PROCEDURES**

**MutY Protein**—The purification of homogenous MutY protein from an overproducer strain of E. coli has been described previously (7). The enzyme used in our studies had a specific activity of about 13 \( \times 10^6 \) pmol/10 min. The standard reaction mixture contained 200 pmol of MutY, 50 pmol of poly(dI-dC) was added to each reaction. Protein-DNA complexes were precipitated with ethanol several times and resuspended in water.

**Duplex oligonucleotides**—Forty- and 44-base-pair duplexes were used in this study. The 40-mer DNA substrate, with four-dithiothreitol, 1 mM EDTA, 2.9% glycerol, and 1.8 fmol labeled DNA in a total volume of 10 \( \mu l \) was incubated with various concentrations of MutY protein, diluted in a buffer containing 20 mM Tris-HCl, pH 7.6, 7 mM KCl, 0.1 M NaCl at 90°C for 30 min to form homoduplexes. The annealed 40-mer or 19-mer duplexes were radiolabeled at the 3'-end with T4 polynucleotide kinase and [\( ^{32} \)P]-dCTP (50 pmol at 3,000 Ci/mmol). The resulting blunt-ended duplex DNA was 44 or 20 base pairs in length, respectively. The reaction mixture was passed through a Quick-Spin column (G-50 for the 40-mer and G-25 for the 20-mer) (Boehringer Mannheim).

**MutY Endonuclease Assay**—The endonuclease activity of MutY, which is the combined action of the glycosylase and AP endonuclease activities, was assayed as described previously (25). The standard reaction mixture contained 200 pmol Tris-HCl (pH 7.6), 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 2.9% glycerol, and 1.8 fmol labeled DNA in a total volume of 10 \( \mu l \). MutY protein, diluted in a buffer containing 20 mM potassium phosphate (pH 7.4), 1.5 mM dithiothreitol, 0.1 mM EDTA, 50 mM KCl, 200 \( \mu l \) bovine serum albumin, and 0.5 mM glycerol, was added to the reaction mixture and incubated at 37°C for 30 min. The reaction products were analyzed on 8 or 14% polyacrylamide DNA sequencing gels.

**MutY Binding Assay**—The binding of MutY to various oligonucleotides was assayed by gel retardation (35, 36). 3'-end-labeled 44- or 20-base-pair oligonucleotides (1.8 fmol) were incubated with various concentrations of MutY as in the endonuclease assay, except that 20 \( ng \) of poly(dI-dC) was added to each reaction. Protein-DNA complexes were analyzed on 4 or 8% polyacrylamide gels in 50 mM Tris borate (pH 8.3) and 1 mM EDTA as described previously.

**Ethylation Interference Assay**—3'-end-labeled 40-mer heteroduplex DNA containing an A/G mismatch was ethylated according to Siebenlist and Gilbert (37) with some modifications. About 2 pmol of DNA in 100 \( \mu l \) of 50 mM sodium cacodylate (pH 7.0) was mixed with an equal volume of freshly prepared ethylnitrosourea and incubated at 45°C for 1 h. The reaction was stopped by adding 20 \( \mu l \) of 3 M sodium acetate, pH 7.0, 5 \( \mu l \) of T2 RNA, and 150 \( \mu l \) of cold ethanol. The ethylated DNA was precipitated with ethanol several times and resuspended in water.

Purified MutY (7.2 pmol) was incubated with the ethylated DNA (0.72 pmol) in a 15-\( \mu l \) reaction containing 20 \( \mu l \) Tris-HCl (pH 7.6), 5 mM dithiothreitol, 0.1 mM EDTA, 0.01% Nonidet P-40, 50 mM methoxamine, and 1 \( \mu l \) of poly(dI-dC) per ml. After incubating at 30°C for 30 min, the reaction mixture was fractionated on a 4% polyacrylamide gel. Protein-bound and free DNA fractions were electroeluted and ethanol precipitated. Strand cleavage at phosphodiester bonds was performed as described (37), and the samples were analyzed on 10% sequencing gels. A control experiment was performed in the same way except that MutY diluent was used in the binding reaction.

**Methylation Interference Assay**—The 40-mer DNA substrate (2 pmol, 5'-end labeled) was partially premethylated by treatment with 0.05% dimethyl sulfate for 10 min at 23°C (37). The reaction (200 \( \mu l \)) was terminated by the addition of 50 \( \mu l \) of 1.5 M sodium acetate, 1 M 2-mercaptoethanol, and 250 \( \mu l \) RNA. DNA was precipitated twice with ethanol and dissolved in water. Binding reactions were performed as in the ethylation interference experiments except that a 5-fold molar excess of MutY protein was added to each reaction. After incubating at 30°C for 30 min, the reaction mixture was fractionated on a 4% polyacrylamide gel. Protein-bound and free DNA fractions were electroeluted, ethanol precipitated, and subjected to the Maxam-Gilbert A → G cleavage reaction (33). The DNA samples were denatured in formamide and electrophoresed through a 10% polyacrylamide sequencing gel. A control experiment was performed in the same way except that MutY diluent was used in the binding reaction.

**RESULTS**

**Ethylation Interference—Ethylnitrosourea ethylates the phosphate backbone and provides a tool to study the contact between proteins and DNA phosphates.** The presence of cleavage activity in MutY AP endonuclease creates one problem in the determination of the ethylation effect on the first phosphodiester bond 3'-to the mispaired A. Therefore, the binding reactions were performed in the presence of Nonidet P-40 and methoxamine. The addition of Nonidet P-40 enhances binding while methoxamine inhibits the AP endonuclease activity. Under this condition, more DNA-protein complexes were formed, and the majority of protein-bound DNA remained intact. Alkaline hydrolysis at ethylated phosphates yields products terminating in a 3'-hydroxyl or an ethylphosphate (appearing as doublet bands at each nucleotide position) (37). The 3'-hydroxyl product of the first phosphodiester bond 3'-to the mispaired A runs at the same position as the MutY cleavage product in sequencing gel. In this case, the band with 3'-ethylphosphate was used to determine the interference effect. Ethylation interference patterns (Fig. 1) on 40-mer DNA with
an A/G mismatch at position 22 (see Table I) suggest that more than five phosphates (with a free/control ratio greater than 3) are involved in interactions between MutY and its DNA sub-
strate. Two of these phosphates are five to seven phosphodi-
ester bonds away from the mismatched G. Interestingly, al-
though MutY has more contacts on the G-strand than on the
A-strand, it is the A-strand that is cleaved by the MutY glyco-
sylase and AP endonuclease.

Methylation Interference—Methylation interference (37) was
utilized to deduce potential purine base contacts involved in
specific complex formation between MutY and A/G-containing
DNA. A 5'-end labeled 40-mer duplex oligonucleotide con-
taining an A/G mismatch at position 22 (see Table I) was used in
this study. The MutY cleavage product at the first phosphodi-
ester bond ran differently from the chemical cleavage products.
The methylation of either the N-7 position of mismatched G or
the N-3 position of mispaired A interfered with MutY binding
(Fig. 2). Besides these mismatched bases, MutY also contacted
purines on both sides of the mismatch. Substantial interfer-
ence was observed when G-23 on the A-strand and A-20 and G-24 on
the G-strand were methylated (Fig. 2). These residues are key
determinants of MutY binding to A/G-containing DNA. Thus,
two nucleotides on both sides of the mismatched base have a
strong influence on MutY reactivity. Methylation of several
other bases farther from the mismatch also showed some in-
terference in MutY binding.

Fig. 3 summarizes the distribution of potential purine and
phosphate contacts of MutY as deduced from the alkylation
interference experiments. MutY covers about 12 base pairs of
the A/G-containing DNA and has more contacts on the
G-strand.

Inosine Substitution Effects—The role of G-23 on the A-
strand and G-24 on the G-strand of 40-mer DNA containing an
A/G mismatch was examined by substitution with inosine, a
guanine base analog lacking a C-2 amino group (Fig. 4). Based
on ethylation interference data, 20-mer oligonucleotides were
designed to investigate base substitution effects (Table I). The
20-mer oligonucleotides containing an A/G mismatch were
bound and cleaved very well by MutY (Fig. 5, lane 1). DNA
containing inosine in place of guanine at position 23 or 24
resulted in a reduced MutY endonuclease activity by 46 and 22%, respectively (Fig. 5, lanes 2 and 6).

Uracil Substitution Effects—Individual thymines within three nucleotides on both sides of the mismatched base were replaced with deoxyuracil. Uracil substitution for T at the first and second nucleotide 5′ to mismatched A had no effect on MutY endonuclease activity (Fig. 5, lanes 3 and 4). When the third nucleotide 3′ to the mismatched G was substituted by uracil, the endonuclease activity was slightly enhanced (Fig. 5, lane 7). Thus, the 5-methyl group of thymine at these positions was not essential for the interaction of MutY protein with its substrates.

Endonuclease Activity of MutY—Four mismatches (A/G, A/C, A/GO, and A/AO) have been reported to be substrates for MutY (7–9, 24). To further delineate the base specificities of MutY and to determine the functional groups in the mispair required for MutY recognition, the A/G mispair was substituted with different purines without changing the surrounding sequence. These purines included neubularine (N), isoines (I), 2-aminopurine (2AP), and 7-deaza-adenine (Z) (Fig. 4). As shown in Fig. 6, MutY nicked different mismatches with different efficiencies. Duplexes containing A/C (Fig. 6, lane 2) and A/GO (Fig. 6, lane 3) were cleaved about one-third and one-half the extent, respectively, of DNA containing an A/G mismatch (Fig. 6, lane 1). The duplex containing N/G (Fig. 6, lane 4) was cleaved as efficiently as the A/G mismatch. Duplexes containing I/G and Z/G (Fig. 6, lanes 5 and 7) and homoduplexes (Fig. 6, lanes 10 and 11) were not cleaved by MutY, and the duplexes containing A/2AP (lane 9), A/I (Fig. 5, lane 5, and Fig. 6, lane 8), and 2AP/G (Fig. 6, lane 6) were very weak substrates for MutY. Further treatment with piperidine after MutY reaction did not increase the amount of cleavage products, suggesting these base substitutions affected the glycosylase activity of MutY (data not shown).

The endonuclease activity of MutY was of the following order: A/G ≫ N/G > A/GO > A/C ≫ A/2AP > A/I = 2AP/G ≫ Z/G = I/G = C/G = A/T = 0.

Binding Affinity of MutY for Different Mismatches—In the gel mobility shift assay, MutY protein formed complexes with 20-mer oligonucleotides containing different mismatches with four slightly different mobility shifts, although the free DNA duplexes had the same mobility (Fig. 7 and data not shown from a similar gel run at longer time). The complex of MutY and A/GO-containing DNA (lane 3) migrated faster than MutY complexed with A/G (lane 1), A/C (lane 2), and N/G (lane 4) containing DNAs. The complex of MutY and 2AP/G-containing DNA (lane 6) had the unique third mobility. The complexes of MutY with I/G (lane 5), Z/G (lane 7), A/I (lane 8), A/2AP (lane 9), C/G (lane 10), and A/T (lane 11) migrated slower. It is interesting to note that weakly cleaved substrates appear to form complexes with MutY with slower mobilities. Higher MutY concentrations were used here to observe weak binding complexes with Z/G, A/I, A/2AP, C/G, and A/T-containing DNAs.

The apparent dissociation constants (Kd) of MutY from the different mismatches were determined. Representative autoradiograms of the binding assay and the corresponding binding curve for MutY to A/G- and A/I-containing 20-mer DNA are shown in Fig. 8 (panels A–D). In A/G binding assays, there was a band that migrated faster than the free DNA and represented about 5 and 15% of input DNA at 3.4 and 53.7 nM MutY, respectively, indicating that some A/G-containing DNA was cleaved by and dissociated from MutY (Fig. 8A). Cleavage products were not used in the Kd calculation because their inclusion would negligibly and improperly increase the apparent values. The cleaved free DNA band in native gel was not observed in the binding of MutY at 53.7 nM to A/G-containing DNA (data not shown). Some slower mobility complexes (either polymers or aggregated forms) were observed in binding assays with low affinity DNA substrates (A/2AP, Z/G, A/I, A/T, and C/G) when the concentration of MutY protein was higher than 0.34 μM (Fig. 8B). The results of these experiments, performed in triplicate, are summarized in Table II.

The apparent Kd values for 20-mer and 44-mer DNA containing an A/G mismatch were 5.3 and 1.8 nM, respectively. MutY bound strongly to the 20-mer duplex with an A/GO mismatch (apparent Kd = 66 pm). This binding is 80-fold greater than that to A/G-containing DNA. The binding affinities of MutY for 20-mer oligonucleotides containing N/G and I/G were comparable to that for A/G. MutY bound weakly to duplex DNA containing A/C and 2AP/G, much weaker to DNA with A/2AP, Z/G, and A/I, and very weakly to duplex DNA containing a matched A/T or C/G base pair. Reproducibly, MutY bound slightly better to A/T than to C/G-containing DNA (Fig. 7, lanes 10 and 11). The binding affinity of MutY was of the following order: A/GO >> A/G = N/G > I/G > 2AP/G > A/C >> A/2AP > Z/G = A/I ≅ A/T ≅ C/G.

**DISCUSSION**

DNA containing A/G, A/C, A/GO, or A/AO mismatch has been shown to be the substrate of MutY protein (7, 9, 24, 25). The MutY protein removes the mispaired adenines from the mismatches by glycosylase activity (7, 24). Tsai-Wu et al. (7) showed that MutY also has AP endonuclease activity. However, the AP endonuclease activity could not be detected by Au et al. (24) in their MutY preparation. The reason for this discrepancy is not clear. The detection of the dissociated nicked product in a native gel (Fig. 8A) argues that the endonuclease activity observed in MutY reaction is not caused by heating at high pH, which may catalyze a β-elimination at the AP site. Therefore, the AP endonuclease (or lyase) activity of MutY appears intrinsic.

DNA phosphate ethylation experiments suggest that MutY interacts with phosphate residues spanning about 12 nucleotide pairs encompassing the A/G mismatch (Fig. 3). MutY binds DNA mainly via five major ionic bonds. Two phosphates with significant effects upon ethylation are located 5 and 7 phosphodiester bonds 3′ to the mismatched G, respectively. This defines a large component of electrostatic binding energy involved in the binding of MutY to phosphates outside of the A/G mismatch. Interestingly, ethylation at the first phosphodiester bond 3′ to the mispaired A that is attacked by the MutY AP endonuclease did not have a substantial interference effect on MutY binding (Fig. 1).

Methylation interference experiments with 40-mer DNA containing an A/G mismatch (Table I) reveal that MutY interacts with purines including the mismatched A and G and two bases on either side of the mismatch (Fig. 3). The N-3 group of A-20 on the G-strand is located in the minor groove while the N-7 groups of G-23 on the A-strand and G-24 on the G-strand are located in the major groove. Substitution of these two guanines by inosines indicates that the 2-amino groups of these guanines, located in the minor groove, are also important in MutY recognition. If substantial helix perturbation is not associated.
with MutY binding, these findings suggest that MutY binds DNA in both the major and minor grooves in the vicinity of the A/G mismatch. The involvement of purines flanking the mismatch in MutY binding may reflect the effect of neighboring sequences on repair and cleavage efficiencies (2, 25, 38). Although flanking sequence effects on MutY reactivity have not been explored systematically, our findings provide a rationale for earlier observations.

The locations of the N-7 group of mismatched G and the N-3 group of mismatched A depend on the structure of the A/G mismatch. Structural analyses have shown that A/G can form three possible conformations: A(anti)-G(anti), A(anti)-G(syn), and A(syn)-G(anti), depending on the neighboring environment (26–30). Because A/O is a substrate for MutY protein and it can form a very stable base pair when A is in the anti and GO is in the syn configuration (39), it is suggested that the A(anti)-G(syn) conformation may be the favored substrate for MutY (9). The protonated N-1 form of adenine is also suggested for the A/C structure (40). Although these DNA conformations with bound MutY have yet to be confirmed, we will assume in the following discussion that the A/G-containing substrate for MutY is in the A(anti)-G(syn) conformation, in which both the N-3 group of mismatched A and the N-7 group of mismatched G are located in the minor groove.

The role of functional groups in mismatch recognition and catalysis by MutY protein were further elucidated by binding and endonuclease assays using defined oligonucleotides containing various purine derivatives. The C-6 amino group of mismatched A is not critical for DNA binding or cleavage since duplex DNA containing N/G is bound and cleaved efficiently by MutY. This is a surprising finding because the C-6 amino group of adenine is involved in hydrogen bonding with guanine in all though flanking sequence effects on MutY reactivity have not been explored systematically, our findings provide a rationale for earlier observations.

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The introduction of a C-2 amino group decreases MutY binding and both binding and endonuclease activities by MutY. The introduction of adenine by carbon as in the Z/G pair results in the loss of glycosidic bond cleavage. The replacement of the N-7 group prevents the use of a protonated N-1 or N-3 as an "electronsink" for catalysis but not binding by MutY. The C-6 keto group may support the presence of a C-6 keto group as in the I/G pair blocks the basis of the stability of the mismatched pair alone. However, the reactivity of MutY protein cannot be rationalized on the basis of the stability of the mismatched pair alone. However, the presence of a C-6 keto group in the I/G pair blocks catalysis but not binding by MutY. The C-6 keto group may prevent the use of a protonated N-1 or N-3 as an "electronsink" in glycosidic bond cleavage. The replacement of the N-7 group of adenine by carbon as in the Z/G pair results in the loss of both binding and endonuclease activities by MutY. The introduction of a C-2 amino group decreases MutY binding and catalysis (compare 2AP/G with N/G). The C-2 amino and the C-6 keto groups of mismatched G, presented in the major groove as in G(syn)-A(anti), are critical for MutY recognition since A/I and A/2AP mismatches are poorly bound by the enzyme.

When the C-8 keto group of GO is present as in GO(syn)-A(anti), the apparent $K_a$ for MutY decreases by 2 orders of magnitude, yet the endonuclease activity is reduced 2-fold as compared to the A/G mispair. The presence of a C-8 keto group in A/GO pair changes the nature of hydrogen bonding between N-7 of GO and N-1 of A. The N-1 of A is protonated in the G(syn)-A(anti) pair (27) but is not protonated in the G(syn)-A(anti) pair (39). These structural differences between A/GO and A/G are located in the minor grove (39). The relative binding and catalytic efficiencies of MutY for A/GO- and A/G-containing DNA were 340 and 8.9 $nm$, respectively. The affinity of MutY to A/GO-containing DNA was determined as the concentration of MutY that results in 50% binding of input DNA. In this case, MutY binding to A/I-20 is not saturated at the highest MutY concentration tested and is not suitable for Enzfitter analysis.

![Image](https://example.com/image)

**Table II**

| DNA duplex | MutY range (nm) | $K_d$ (nm) |
|------------|----------------|------------|
| A/G-44     | 0.052-54       | 1.8 ± 0.3  |
| A/GO-20    | 0.003-0.42     | 0.066 ± 0.013 |
| A/G-20     | 0.42-54        | 5.3 ± 0.5  |
| N/G-20     | 0.42-54        | 5.3 ± 1.3  |
| I/G-20     | 0.42-54        | 6.0 ± 1.6  |
| 2AP/G-20   | 0.84-107       | 12 ± 3     |
| A/C-20     | 0.84-107       | 15 ± 3     |
| A/2AP-20   | 3.4-336        | 94 ± 14    |
| Z/G-20     | 3.4-336        | 120 ± 25   |
| A/I-20     | 6.7-671        | 300 ± 60*  |
| A/T-20     | 6.7-671        | 320 ± 60*  |
| C/G-20     | 6.7-671        | 370 ± 80*  |

*This value represents the concentration of MutY that results in 50% binding of input DNA and is not obtained by Enzfitter (42) because the binding is not saturated at the highest MutY concentration tested.
mainly through three phosphates (between T-15 and C-16, between T-2 and T-21, and between A-26 and A-27). It is interesting to note that these three phosphates are approximately five base pairs apart and form a line nearly parallel to the helical axis. The information obtained in this study provides a basis for understanding the interaction of MutY with DNA and the molecular mechanisms involved in the recognition of damaged or mismatched bases. Identification of I/G-containing DNA as a recognized but not a catalyzed substrate will facilitate the formation of protein-DNA co-crystal.

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