Substrate Specificity of *Escherichia coli* MutY Protein†

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ABSTRACT: The MutY protein of *Escherichia coli* removes mismatched deoxyadenine residues from DNA. In this study, duplex oligodeoxynucleotides containing modified bases are used as model substrates for this enzyme. In contrast to a recent report [Lu, A.-L., et al. (1995) *J. Biol. Chem. 270*, 23582], dA:8-oxo-dG appears to be the preferred natural substrate for MutY, as evidenced by the specificity constants (k_{cat}/K_m) for dA:8-oxo-dG and dA:dG of 39 600 × 10^{-6} and 383 × 10^{-6} (min^{-1} nM^{-1}), respectively. k_{cat} for the duplex containing dA:dG was highest at lower pH; the rate of cleavage for the duplex containing dA:8-oxo-dG was unaffected over a pH range of 5.5−8.0. The presence of an 8-oxo function in dG increased significantly the rate of removal of dA from all substrates tested. Replacement of dA by rA reduced the specificity constant of dA:8-oxo-dG to 294 × 10^{-6} (min^{-1} nM^{-1}), whereas replacement of dA by 2′-*O*-methyladenosine virtually abolished enzymatic activity. Modifications of the dG moiety generally were better tolerated than those of dA; however, introduction of a methyl ether at the 6 position of dG produced a noncleavable substrate and replacement of dG by 2′-*O*-methylguanosine generated a substrate with a low specificity constant. Rates of cleavage of duplexes containing dA:dC and dA:tetrahydrofuran were three orders of magnitude lower than the reference substrate. Duplexes containing a carbocyclic analog of dA were not cleaved. A model is proposed to explain the recognition of DNA substrates by MutY and the catalytic properties of this enzyme.

Reactive oxygen species produced by endogenous or exogenous sources attack cellular DNA, forming a variety of modified bases and sugars, including 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG)[1] (Halliwell & Gutteridge, 1989). During DNA synthesis, dAMP may be incorporated opposite dG (Echols & Goodman, 1991) or 8-oxo-dG (Shibutani *et al.*, 1991), leading to G:C→T:A transversion mutations during the second round of replication in bacteria (Radicella *et al.*, 1988; Ngheim *et al.*, 1988; Michaels *et al.*, 1992 Wood *et al.*, 1990; Moriya *et al.*, 1991; Cheng *et al.*, 1992) and mammalian cells (Moriya *et al.*, 1993). Additionally, 8-oxo-dGTP in the nucleotide triphosphate pool can be incorporated, pairing with dA in the DNA template and generating A:T→C:G transversions (Minnic *et al.*, 1994).

MutM, MutT, and MutY proteins defend *Escherichia coli* against the mutagenic effects of oxidative damage [reviewed by Michaels and Miller (1992) and Tchou and Grollman (1993)]. MutM (*fpg*) protein removes 8-oxo-dG and formamidopyrimidines efficiently from duplex DNA except when the former is paired with dA (Tchou *et al.*, 1991, 1993). Several MutM-like activities have been found in human cells (Bessho *et al.*, 1993) and in yeast (deOliveira *et al.*, 1994). MutT protein is an 8-oxo-dGTPase that eliminates 8-oxo-dGTP from the dNTP pool in *E. coli* (Maki & Sekiguchi, 1992). The human analog of MutT protein has been cloned and sequenced (Sakumi *et al.*, 1993).

MutY protein corrects errors arising from misincorporation of dAMP opposite dG (Ngheim *et al.*, 1988; Au *et al.*, 1989) or 8-oxo-dG (Michaels *et al.*, 1992) during DNA replication in *E. coli*. Initially, MutY was reported to act strictly as a DNA glycosylase, the purified enzyme being free of significant AP endonuclease activity (Au *et al.*, 1988). Later, the protein was reported to contain AP-lyase activity (Tsai-Wu *et al.*, 1992). A protein homologous to the MutY protein of *E. coli* has been identified in nuclear extracts of calf thymus and human HeLa cells (McGoldrick *et al.*, 1995).

Mechanistic studies on five selected DNA glycosylases led to the suggestion that two general nucleophilic mechanisms are operating (Dodson *et al.*, 1994; Sun *et al.*, 1995; Tchou & Grollman, 1995). One mechanism concerns DNA glycosylases that exhibit similar rates for both glycosylase and AP-lyase activities. It was proposed that an amino group in these enzymes could serve as a nucleophile, generating an imino enzyme−DNA intermediate (Kow & Wallace, 1987). The second mechanism involves DNA glycosylases lacking concomitant AP-lyase activity. These enzymes could utilize a nucleophile from the medium, such as an activated water molecule, to effect base displacement.

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[1] Abbreviations: AP, apurinic/apyrimidinic; F, D-1,4-anhydroribitol; 2′-O-meA, 2′-*O*-methyladenosine; rA, adenosine; 8-oxo-dA, 8-oxodeoxyadenosine; dI, 2′-deoxyinosine; dTu, 7-deaza-2′-deoxyadenosine; 8-oxo-dG, 8-oxo-2′-deoxyguanosine; 8-oxo-dI, 8-oxo-2′-deoxyinosine; 6-meo-8-oxo-dG, 6-*O*-methyl-8-oxo-2′-deoxyguanosine; 8-meoxo-dG, 8-methoxy-2′-deoxyguanosine; 2′-O-meG, 2′-*O*-methylguanosine; dAris, deoxyaristeromycin; 8-oxo-dNeb, 8-oxo-2′-deoxynebularine; dGpme, 2′-(2-amino-2-hydroxymethyl-1,3-dihydroxypropane hydrochloride)-deoxyguanosine-3′-methylphosphonate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride buffer; ITPG, isopropyl 1-thio-β-*O*-galactopyranoside; PAGE, polyacrylamide gel electrophoresis.

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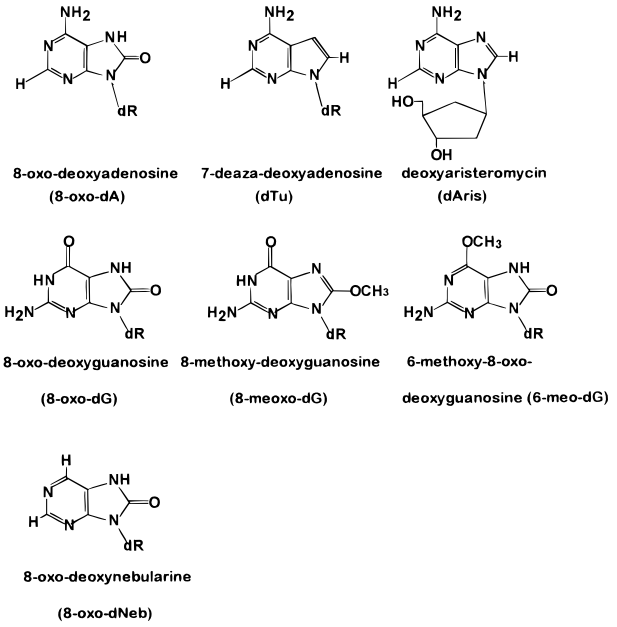

FIGURE 1: Structures of deoxynucleoside analogs.

In this paper, MutY is shown to bind tightly to duplex oligodeoxynucleotides containing dA:8-oxo-dG or structurally related mispairs. MutY cleaves the glycosidic bond of dA in dA:8-oxo-dG at a rate higher than in dA:dG or other mismatches tested. AP-lyase activity was not detected in our preparations of MutY. The structure−function studies reported here lead us to conclude that MutY recognizes and binds a unique configuration of hydrogen-bond donors and acceptors exposed in the major groove of duplexes containing dA(*anti*):8-oxo-dG(*syn*) or dA(*anti*):dG(*syn*). We propose a model for this reaction in which binding of substrate to MutY leads to eversion of dA from the interior of the DNA helix, facilitating nucleophilic attack at C1′ of dA, thereby initiating cleavage of the glycosidic bond.

EXPERIMENTAL PROCEDURES

Materials. Reagents of the highest grade commercially available were obtained from Fisher, Boehringer Mannheim, Sigma, Difco Laboratories, Bio-Rad, and Gibco-BRL.

Oligonucleotides. Unmodified and modified oligonucleotides were prepared by solid-state synthesis on a DuPont Coder 300 automated DNA synthesizer. Phosphoramidites of rA, 2′-O-meA, 2′-O-meG, dI, dTu, and dG-pme were purchased from Glen Research. The syntheses of oligonucleotides containing 8-oxo-dG, 8-oxo-dA (Bodepudi *et al.*, 1992), tetrahydrofuran (Takeshita *et al.*, 1987), 8-methoxy-dG (Kuchino *et al.*, 1987), 6-methoxy-8-oxo-dG (Chamakura *et al.*, 1996), dAris (G. Dorman and F. Johnson, unpublished data), and 8-oxo-dNeb (V. Bodepudi and F. Johnson, unpublished data) were carried out as described. Structures of the modified nucleosides used in these experiments are shown in Figure 1 and sequences of oligonucleotides are listed in Table 1. Oligonucleotides were purified by HPLC and PAGE (7 M urea, 20% acrylamide), as described previously (Tchou *et al.*, 1994), phosphorylated at the 5′-end with T4 polynucleotide kinase and [γ-^{32}P]ATP, desalted with Nensorb 20 (Du Pont), using the manufacturer's procedure, and dried *in vacuo*.

Table 1: Sequences of Oligonucleotides Used in Gel Mobility Shift and DNA Cleavage Assays[a]

5'-CTCTCCCTTC**A**CTCCTTTCCTCT,
5'-CTCTCCCTTC**G**CTCCTTTCCTCT,
5'-CTCTCCCTTC**F**CTCCTTTCCTCT,
5'-CTCTCCCTTC-**2'-O-meA**CTCCTTTCCTCT,
5'-CTCTCCCTTC-**rA**CTCCTTTCCTCT,
5'-CTCTCCCTTC-**8-oxoA**CTCCTTTCCTCT,
5'-CTCTCCCTTC-**6-meo-dG**CTCCTTTCCTCT
5'-CTCTCCCTTC-**8-meoxo-dG**CTCCTTTCCTCT,
5'-CTCTCCCTTC-**dAris**CTCCTTTCCTCT,
5'-CTCTCCCTTC**dI**CTCCTTTCCTCT,
5'-CTCTCCCTTC-**dTu**ACTCCTTTCCTCT,
5'-CTCTCCCTTC-**8-oxo-dNeb**CTCCTTTCCTCT
5'-CTCTCCCTTC-**8-oxo-dG**CTCCTTTCCTCT
5'-AGAGGAAAGGAG**G**GAAGGGAGAG,
5'-AGAGGAAAGGAG-**8-oxo-dG**GAAGGGAGAG,
5'-AGAGGAAAGGAG-**8-oxo-dI**GAAGGGAGAG,
5'-AGAGGAAAGGAG**A**GAAGGGAGAG,
5'-AGAGGAAAGGAG**C**GAAGGGAGAG,
5'-AGAGGAAAGGAG**T**GAAGGGAGAG
5'-AGAGGAAAGGAG**F**GAAGGGAGAG,
5'-AGAGGAAAGGAG**dI**GAAGGGAGAG,
5'-AGAGGAAAGGAG-**8-oxo-dA**GAAGGGAGAG,
5'-AGAGGAAAGGAG-**2'-O-meG**GAAGGGAGAG
5'-AGAGGAAAGGAG-**dGpme**GAAGGGAGAG

[a] Where F, furan; 2′-O-meA, 2′-*O*-methyladenosine; rA, adenosine; 8-oxo-dA, 8-oxodeoxyadenosine; dI, deoxyinosine; dTu, 7-deazadeoxyadenosine; 8-oxo-dG, 8-oxodeoxyguanosine; 8-oxo-dI, 8-oxodeoxyinosine; 6-meo-dG, 6-*O*-methyl-8-oxodeoxyguanosine; 8-meoxo-dG, 8-methoxydeoxyguanosine; 2′-O-meG, 2′-*O*-methylguanosine; dAris, deoxyaristeromycin; 8-oxo-dNeb, 8-oxodeoxynebularine; and dGpme, deoxyguanosine-3′-methylphosphonate.

Purification of MutY Protein. *E. coli* JM109 bearing the MutY gene overexpression plasmid pKKY was grown at 37 °C in 3 L flasks containing 1 L of yeast-tryptone media, pH 7.5, and 50 μg/mL ampicillin. The culture was grown to an A_{600} of 0.8−1.0; IPTG (0.5 mM, final concentration) was then added. Cells were incubated at 37 °C for 1 h, at which time ferrous chloride was added to a final concentration of 50 μM. Cells were grown for 3 h at 37 °C and harvested. The cell paste was stored at −80 °C. Generally, a yield of 5−5.5 g of cells/1 L of medium was obtained. Purification of MutY protein was carried out using a modification of the procedure of Au *et al.* (1989). All fractionation procedures were performed at 0−4 °C. Frozen cell paste (20−68 g) was thawed at 4 °C and resuspended in 60−210 mL of 50 mM Tris-HCl, pH 7.5/1 mM dithiothreitol/1 mM EDTA/0.5 mM phenylmethanesulfonyl fluoride (buffer A) containing 0.1 M KCl. Cells were then disrupted by sonication. After centrifugation for 30 min at 9000 rpm, the lysate was treated with an aqueous solution of 25% (w/v) streptomycin sulfate, added slowly with stirring to a final concentration of 5%. Stirring was continued for 1 h; the solution was centrifuged for 30 min, and the supernatant solution was treated with ammonium sulfate, added slowly as a solid with stirring to a final concentration of 45%. The solution was stirred for 1 h and centrifuged. The resulting pellet was dissolved in a minimum volume of buffer A, filtered through a GF/C filter (Whatman, U.K.), diluted with buffer A to a final A_{280} of 6−8 OD, and loaded at 1 mL/min onto a 40 cm

3 L (XK 50/70 Pharmacia) phosphocellulose column equilibrated with buffer A. The column was washed with three column volumes of buffer A followed by three volumes of buffer A containing 0.15 M KCl and then developed with 10 volumes of buffer A, using a linear (0.15−0.5 M) gradient of KCI. A large peak was eluted with 0.45 M KCl; fractions were collected and combined (leading and trailing edges were discarded), concentrated using Centriprep 10 (Amicon), diluted 10-fold with 5 mM potassium phosphate containing 1 mM dithiothreitol (pH 7.5), and then loaded onto a 20 cm, 150 mL (XK 16/20 Pharmacia) hydroxylapatite column. The column was washed with two column volumes of 5 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol and then developed with 10 volumes of a linear (0.005−0.2 M) gradient of potassium phosphate, pH 7.5 containing 1 mM dithiothreitol. Material eluted by 0.1 M potassium phosphate was collected, concentrated by Centriprep, and then diluted 3-fold with buffer A. The solution was loaded in small portions (1.5−2 mg of protein) onto a HR5/5 MonoS column (Pharmacia). The latter was washed with 5 mL of buffer A and developed with 10 column volumes of buffer A containing KCl (0−0.5 M) in a linear gradient. Fractions corresponding to the peak containing MutY activity were collected and divided into two portions. One portion was stored and frozen at −80 °C. These samples were used for binding assays and kinetic studies. The second portion was concentrated with Centriprep and glycerol added to a final concentration of 50% (v/v). This material was stored at −20 °C. Protein concentration was determined by means of a Bio-Rad protein assay solution, using the standard procedure and bovine serum albumin as a standard. The specific activity of the enzyme was determined at 25 °C as described below, using duplex oligodeoxynucleotides containing a single dA:dG mismatch. Specific activities were in the range of 95−165 × 10^6 units per mg of protein.

Binding Assay. Duplicate reaction mixtures, containing 80 fmol of oligomer, 62.5 mM Tris-HCl (pH 7.5), 0.125 M KCl, and 6.25 mM potassium EDTA in a final volume of 8 μL, were incubated for 15 min at 4 °C with varying amounts of MutY protein. As described previously (Tchou *et al.*, 1994), samples of reaction mixtures (5 μL) were loaded onto a 7% nondenaturating polyacrylamide (acrylamide/bisacrylamide = 29:1) gel (19 × 15 cm), which was 50 mM in Tris-borate and 0.05 mM in EDTA. Samples were run at 80 V at 4 °C until the marker dyes (loaded separately in one well) penetrated the gel, at which point the voltage was increased to 180 V. Gel electrophoresis was stopped after the xylene cyanole marker migrated for 4−5 cm. Following autoradiography, bands corresponding to bound and free oligodeoxynucleotide were excised from the gel. Radioactivity was determined by liquid scintillation counting, using Liquiscint (National Diagnostics). The fraction of DNA bound was plotted against the concentration of free enzyme. The apparent dissociation constant (K_d) is defined as the amount of enzyme required to bind 50% of the DNA present. The K_d and standard errors of deviation for these constants were determined using the Enzfitter program (Leatherbarrow, 1987).

Kinetic Studies. The standard reaction mixture contained 62.5 mM Tris-HCl, pH 7.5; 0.125 M KCl; 6.25 mM in EDTA (potassium salt), and varying amounts of an oligonucleotide duplex labeled with ^{32}P at the 5′ terminus in a total volume of 8 μL. The range of DNA concentrations used was 0.1−200 nM. For kinetic analysis, duplicate reaction mixtures were preincubated for 30 min at 25 °C. MutY protein was diluted in 10 mM Tris-HCl buffer, pH 7.5, containing 0.5 mg/mL bovine serum albumin and 50% glycerol (v/v). Aliquots (2 μL) of this freshly prepared solution were added to each reaction mixture and incubated at 25 °C for varying times (2 min to 17 h), depending on the rate of substrate cleavage. 2-(*N*-Morpholino)ethanesulfonic acid replaced Tris in experiments in which pH dependence was determined. Reactions were stopped by adding 10 μL of 20% aqueous piperidine (v/v), followed by heating at 95 °C for 30 min to cleave abasic sites. Reaction mixtures were evaporated *in vacuo*, and the residue was dissolved in 10 μL of 9 M urea containing 0.01% bromphenol blue and 0.01% xylene cyanole. An aliquot (4 μL) was subjected to 7 M urea−20% PAGE. Following autoradiography, bands corresponding to cleavage products and unreacted substrates were excised from the gel, and their radioactivity was determined by liquid scintillation counting. The initial reaction rate was plotted versus substrate concentration. Values for K_m, V_{max}, and standard errors were derived from a computer-fitted curve, using Enzfitter.

RESULTS

Cleavage by MutY protein of duplexes containing a single mispair is shown in Figure 2. The apparent binding affinity (K_d) of this enzyme for various substrates is summarized in Table 2. Binding of MutY to a duplex containing dA:8-oxo-dG (K_d = 5.5 nM) is approximately 4-fold higher than to an unmodified duplex, 5-fold higher than to a duplex containing dA:dG, and 8-fold higher than to duplexes containing 2′-O-meA:dG or F:dG. The presence of an 8-oxo function in the base opposite dA invariably increases apparent K_d of the enzyme for modified DNA (compare K_d values of dA:dG, rA:dG, 2′-O-meA:dG, F:dG, dI:dG, dTu:dG, and 8-oxo-dA:dG to their 8-oxo-dG analogs). K_d values for MutY did not correlate with k_{cat} or K_m/k_{cat} for all substrates; for example, MutY binds tightly (K_d = 5.8−7.2 nM) to noncleavable duplexes containing dA:6-methoxy-8-oxo-dG or dTu:8-oxo-dG.

The deoxyribose moiety of dA appears to play a relatively minor role in binding MutY to the naturally occurring mispairs, dA:8-oxo-dG and dA:dG. When dA in either pair is replaced by rA or by a carbocyclic analog of dA, deoxyaristeromycin, binding was not impaired; a significant increase in apparent K_d was noted only for 2′-*O*-meA:8-oxo-dG (K_d = 27). The presence of a 2′-*O*-methyl group in the sugar ring opposite the group being cleaved (dA:2′-O-meG) did not reduce apparent K_d for this mismatch.

The effect of structural modifications of DNA on the catalytic properties of MutY protein may be deduced from the results of kinetic studies summarized in Table 2. The specificity constant (k_{cat}/K_m) for duplex DNA containing dA:8-oxo-dG (39 600) serves as a point of reference. Duplex substrates containing other mismatches are classified as moderate (294−1333), weak (35−81), minimal (cleavage after extended incubation), or poor (no cleavage after extended incubation in the presence of excess enzyme). The enhancing effect of the 8-oxo substituent in the dG moiety can be appreciated by noting the k_{cat} values for the several pairs of substrates in which cleavage is detected in at least

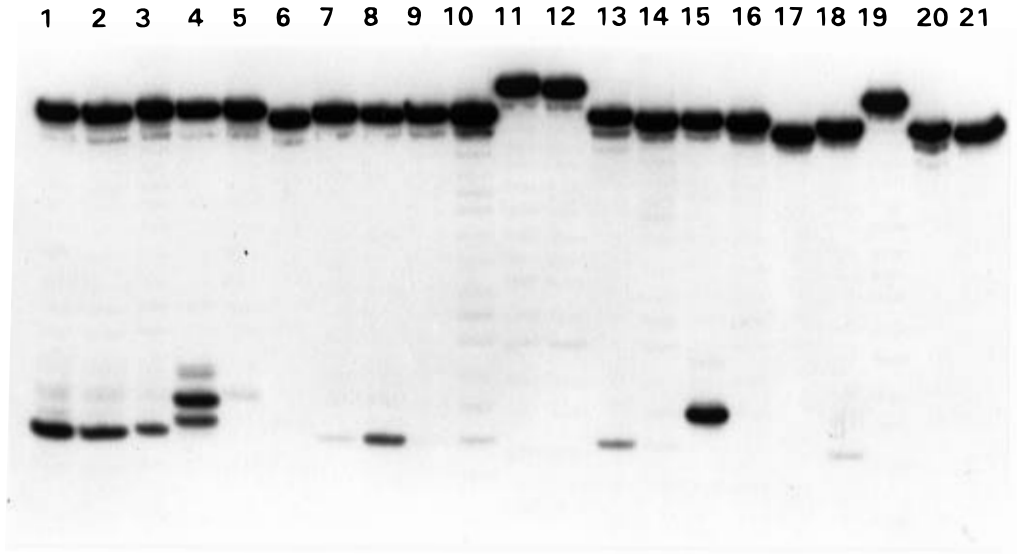

FIGURE 2: Cleavage of mismatch-containing oligonucleotides by MutY protein. DNA substrates (53.6 nM) were assayed for glycosylase activity at 25 °C with 20 nM (lanes 1−3 and 8−13) or 60 nM (lane 4−7) MutY. Incubations were for 20 min (lane 1), 1.5 h (lanes 2 and 3), and 7 h (lanes 4−13). Reaction mixtures were treated as described under Experimental Procedures. MutY was omitted from reactions in lanes 14−21. Oligonucleotides containing the following mismatches were used as substrates: dA:8-oxo-dG (lanes 1 and 14), dA:dG (lane 2), dA:2′-O-meG (lane 3), rA:8-oxo-dG (lanes 4 and 15), 2′-O-meA:8-oxo-dG (lanes 5 and 16), F:8-oxo-dG (lanes 6 and 17), dTu:8-oxo-dG (lanes 7 and 18), dI:8-oxo-dG (lanes 8 and 21), dI:dG (lane 9), dA:dI (lane 10), dA:dI* (lanes 11 and 19), 8-oxo-dA:dI* (lane 12), 8-oxo-dA:8-oxo-dG (lanes 13 and 20). The nucleosides shown first were located in pyrimidine-rich chains (see Table 1); these oligonucleotides were labeled at the 5′-end. In lanes 11, 12, and 19, the purine-rich chains are labeled ("*" indicates labeled chain). The extent of cleavage of dTu:8-oxo-dG and dI:dG mismatches in the presence or absence of MutY, following piperidine treatment, were equal (compare lanes 7 and 18, and 9 and 21, respectively).

Table 2: Kinetic and Binding Parameters for MutY Protein

DNA duplex	K_d (nM)	K_m (nM)	V_{max} (nM min^{-1} × 10^2)	k_{cat} (min^{-1} × 10^3)	k_{cat}/K_m (min^{-1} nM^{-1} × 10^6)
dA:T	21 ± 3.8			noncleavable	
dA:dG	26 ± 5.3	12 ± 1.7	4.6 ± 0.2	4.6 ± 0.2	383
dA:8-oxo-dG	5.5 ± 0.7	2.4 ± 0.5	95 ± 3.5	95 ± 3.5	39600
rA:dG	25 ± 3			<0.1[a]	
rA:8-oxo-dG	10 ± 2.1	16 ± 2.6	1 ± 0.04	4.7 ± 0.02	294
2′-O-meA:dG	48 ± 6.7			noncleavable	
2′-O-meA:8-oxo-dG	27 ± 3.6			<0.1[a]	
dA:2′-O-meG	18 ± 2.5	12 ± 2.6	4.9 ± 0.4	0.98 ± 0.05	81
dA:dI	17 ± 3.1			<0.1[a]	
dA:8-oxo-dI	8.0 ± 1.2	3.3 ± 0.5	3.2 ± 1.0	32.6 ± 1.0	9890
F:dG	42 ± 9.5			noncleavable	
F:8-oxo-dG	11 ± 1.4			noncleavable	
dI:dG	46 ± 10			<0.1[a]	
dI:8-oxo-dG	8.2 ± 1.0	17 ± 2.7	3 ± 0.2	0.59 ± 0.04	35
dTu:dG	28 ± 3.5			noncleavable	
dTu:8-oxo-dG	7.2 ± 1.1			noncleavable	
8-oxo-dA:dG	29 ± 7.2			noncleavable	
8-oxo-dA:8-oxo-dG	11 ± 1.8			<0.5[a]	
dA:8-oxo-dA	14 ± 2.4	11 ± 3.3	20 ± 0.9	10 ± 0.5	909
dA:8-oxo-dNeb	25 ± 4.7	9.1 ± 1.7	15 ± 0.6	7.5 ± 0.3	824
8-oxo-dNeb:8-oxo-dG	12 ± 3.0	12 ± 2.7	3 ± 0.2	0.61 ± 0.04	51
dA:8-methoxy-dG	20 ± 4.1	2.4 ± 0.5	6.4 ± 0.2	3.2 ± 0.1	1333
dA:6-methoxy-8-oxo-dG	5.8 ± 0.8			noncleavable	
dAris:8-oxo-dG	13 ± 1.9			noncleavable	
dA:dGpme		11 ± 4.7	5.6 ± 0.6	0.28 ± 0.03	

[a] Values calculated from initial rates of cleavage.

one of the two duplexes (compare dA:dG, rA:dG, 2′-O-meA:dG, dI:dG, and 8-oxo-dA:dG with the corresponding pair in which 8-oxo-dG replaces dG).

The adenosine moiety of dA:8-oxo-dG was selectively modified at N7, N6, H8, and C2′ and the guanosine residue of this mispair at C8, O6, N2, and C2′ (Table 3). In addition, the 3′-methylphosphonate of dG was prepared. Replacement of adenine N7 by carbon abolished enzymatic activity when the base opposite 7-deaza-2′-deoxyadenosine was 8-oxo-dG or dG. Replacement of the 6-amino group by a keto function (dI:dG or dI:8-oxo-dG) or by oxidation at C8 (8-oxo-dA:8-oxo-dG or 8-oxo-dA:dG) reduced the specificity constant of MutY by three orders of magnitude compared to the reference duplex. Weak enzymatic activity ($k_{cat}/K_m = 51$) was retained in the pair where the 6-amino group of dA was replaced by H and an 8-oxo group was introduced (8-oxo-dNeb:8-oxo-dG).

Modifications of the deoxyribose moiety of dA also were explored. Replacement of dA by rA reduced the specificity constant of 8-oxo-dG:dA from 39 600 to 294. Introduc-

Table 3: Structure−Function Relationships[a]

modification	series dA:oxo-dG	series dA:dG
none	39 600 (6)	383 (26)
adenine		
N7→C	0 (7)	0 (28)
N6→O	35 (8)	tr
N6→H1; H8→O	51 (12)	
H8→O	tr (11)	0 (29)
2H′→OH	294 (10)	tr
2H′ →Ome	tr (27)	0 (48)
8-oxoguanine		
O8→H	383 (26)	
O8→OMe	1333 (20)	
O6→H; N2→H	824 (25)	0
N2→H	9280 (8)	tr (17)
O6→OMe	0 (5.6)	
O6→NH2; N2→H	909 (11)	0
2H′→2′OMe	81 (14)	

[a] Values for k_{cat}/K_m are given in min^{-1} nM^{-1} × 10^6; K_d is shown in parentheses.

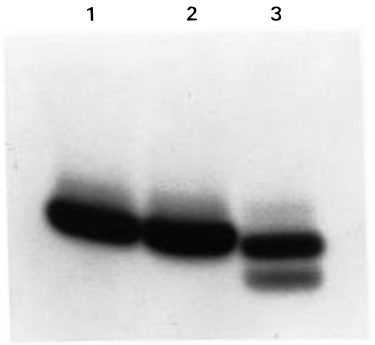

FIGURE 3: Cleavage of mismatch-containing oligonucleotides by MutY protein. DNA substrates (30 nM) were assayed for glycosylase activity at 25 °C with 30 nM MutY. Oligonucleotides containing the following mismatches were used: dAris:8-oxo-dG (lanes 1 and 2); dA:8-oxo-dG (lane 3). MutY was omitted from the reaction in lane 2. Incubations were for 3 min for dA:8-oxo-dG and 11 h for dAris:8-oxo-dG mismatches. Pyrimidine-rich oligonucleotides containing a single dA or dAris (see Table 1) were labeled. Reactions were stopped by adding an equal volume of 10 M urea containing dyes and immediately loaded on a 75 cm 20% polyacrylamide gel. The lower band in lane 3 corresponds to an oligonucleotide containing an abasic site.

tion of a 2′-hydroxyl function reduced the specificity constant of dG:dA from 383 to negligible levels. A 2′-*O*-methyl substitution in adenosine similarly reduced enzymatic activity.

Modifications of 8-oxoguanine appear to be better tolerated than those of the adenine moiety (Table 3). Replacement of O8 by O-me or by H reduced the specificity constant 30- and 103-fold, respectively. The contribution of the C2-amino function was established by comparing structural analogs of dG and 8-oxo-dG. Duplexes containing dA:dI possessed minimal substrate activity, but only a 4-fold decrease in the specificity constant was observed for dA:8-oxo-dI, compared to dA:8-oxo-dG. Replacement of O6 by NH_2 or by H in 8-oxo-dG, combined with substitution of the 2-amino group by H, generated substrates with specificity constants of 909 and 824, respectively. Conversion of 8-oxoguanine to the 8-*O*-methyl ether reduced the specificity constant to 1333; introduction of a methyl ether at the 6 position produced a noncleavable substrate. Methylation of the 2′-O position of dG produced a substrate with a specificity constant of 81 and conversion of the 3′-phosphate to a methylphosphonate group reduced k_{cat} 30-fold.

Initial rates of cleavage of duplexes containing dA:dC and dA:F mispairs were estimated to be 0.00175 and 0.002 nM/min, respectively, 3 orders of magnitude lower than the corresponding value for dA:8-oxo-dG. The carbocyclic analog of dA, deoxyaristeromycin, was completely resistant to cleavage by equimolar concentrations of MutY (Figure 3, lane 1). The labeled strand of duplexes containing *dI:dA, *dI:8-oxo-dA, *dG:8-oxo-dG, *dA:dA, *8-oxo-dG:8-oxo-dG, *T:8-oxo-dG (the "*" indicates the ^{32}P-labeled chain) were not cleaved under the conditions employed; namely, 6−12 h of incubation at 25 °C with a 5−10-fold molar excess of enzyme (data not shown).

The effect of pH on the initial rate of cleavage of the naturally occurring duplexes used in this study is shown in Figure 4. The range of pH investigated, 5.5−7.5, was limited by the activity of the enzyme. The duplex containing dG:dA was cleaved significantly more rapidly at lower pH; the rate of cleavage for a duplex containing dA:8-oxo-dG was unaffected by pH over the range tested.

DISCUSSION

The results of this investigation provide insight into the structural requirements for the recognition of mismatched base pairs by the MutY protein of *E. coli* and, in turn, suggest a molecular mechanism by which dA is removed from DNA. As originally conceived (Ngheim *et al.*, 1988; Radicella *et al.*, 1988), the function of MutY involved correction of dG:dA mispairs arising during DNA replication in *E. coli*. Later, MutY was shown to act on DNA containing 8-oxo-dG:dA (Michaels *et al.*, 1992), suggestive of a role for this enzyme in cellular defenses against oxidative DNA damage. The process of repair involves binding of MutY to duplex DNA and preferential recognition of dG:dA and 8-oxo-dG:dA mispairs, followed by cleavage of the glycosidic bond to generate an abasic site. The strand containing the abasic site then is restored by the several enzymes participating in DNA repair (Demple & Harrison, 1994).

Binding of MutY to DNA. Initial binding of MutY protein to DNA appears to be non-sequence-specific, mediated most likely through electrostatic interactions with the phosphodiester backbone (Dowd & Lloyd, 1989; Lu *et al.*, 1995). The enzyme shows relatively high apparent affinity (K_d = 21 nM) for unmodified duplex DNA. The K_d for binding of MutY to duplexes containing 8-oxo-dG:dA is 4-fold lower than binding to unmodified DNA and 5-fold lower than to dG:dA, reflecting a tighter complex for the oxidized substrate. These results parallel the enhanced thermodynamic stability of 8-oxo-dG:dA relative to the dG:dA duplex (Plum *et al.*, 1995).

Lu *et al.* (1995) performed a similar binding study of MutY in the presence of poly(dI:dC). By providing an excess of competing nonspecific sites, the relative contribution of specific binding is magnified at the expense of overestimating the concentration of free protein. This study also combines singly- and multiply-bound complexes in their analysis. Using this model, binding of MutY to 8-oxo-dG:dA was calculated to be approximately 80- and 5200-fold stronger than binding to dG:dA and unmodified DNA duplexes, respectively.

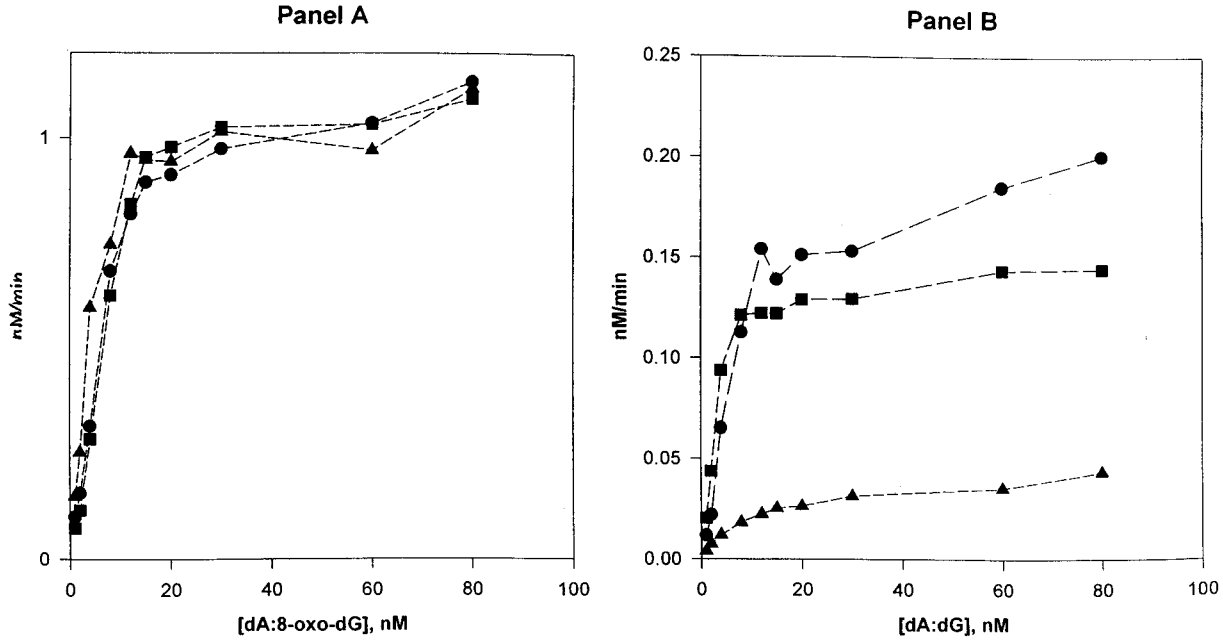

FIGURE 4: pH dependence of the catalytic activity of MutY protein. Experiments were performed as described under Experimental Procedures except that the time of incubation for the higher concentrations of mispairs at pH 5.55 and 6.0 was reduced to 20 min. Panel A: dA:8-oxo-dG. Panel B: dA:dG. ●, ■, and ▲ represent data for pH 5.55, 6.0, and 7.5, respectively.

The relatively low K_d observed for binding of MutY to duplexes containing 8-oxo-dG:dA may be relevant to the repair of oxidative DNA damage in *E. coli*. MutY creates an abasic site on the strand opposite 8-oxoguanine, generating a substrate for *fpg* protein. Removal of 8-oxoguanine from this intermediate by *fpg* protein, together with its AP-lyase activity, could lead to double strand cleavage with lethal consequences for the cell. The K_d for MutY binding to duplexes containing a synthetic abasic site, determined under similar experimental conditions (Tchou *et al.*, 1994), provides support for the proposal (Michaels *et al.*, 1992) that MutY protein remains bound to its substrate, preventing *fpg* protein from acting until the abasic site created by MutY is repaired by the concerted actions of an AP endonuclease, DNA polymerase and DNA ligase (Demple & Harrison, 1994). Repair synthesis involves preferential incorporation of dCMP opposite the lesion (Shibutani *et al.*, 1991), creating a lesion that is relatively resistant to cleavage by MutY but which can be readily incised by *fpg* protein and subsequently repaired (Tchou *et al.*, 1991).

Recognition of Mispairs in DNA. Modification of potential hydrogen bond donor and acceptor sites in DNA strongly affects the enzymatic activity of MutY. All base modifications tested reduce k_{cat}/K_m; in some cases, activity is inhibited with little effect on overall binding of MutY to DNA (Table 3); however, ability to recognize the mispair and/or performance of catalytic functions appear to be impaired. As with other DNA-binding proteins (Seeman *et al.*, 1976; Steitz, 1990), recognition of substrates by MutY involves, but may not be limited to, interactions with hydrogen bond donors and acceptors exposed at the edges of base pairs in the grooves of DNA.

Using 2D NMR techniques combined with restrained molecular dynamics, we have determined the structure of oligodeoxynucleotides containing 8-oxo-dG:dA, the preferred substrate for MutY, establishing that 8-oxoguanine and dA form a Hoogstein pair with the modified base assuming a *syn* conformation (Kouchakdjian *et al.*, 1991). This solution structure has been confirmed by X-ray crystallographic analysis (McAuley-Hecht *et al.*, 1994).

In duplex DNA, conformation of G:A mispairs is sequence- and pH-dependent (Privé *et al.*, 1987; Hunter *et al.*, 1986; Leonard *et al.*, 1990; Brown *et al.*, 1990). NMR studies (Gao & Patel, 1988; Carbonnaux *et al.*, 1991) have shown that the G:A mismatch has an *anti:anti* conformation at neutral pH, adopting the dA(*anti*):dG(*syn*) conformation at lower pH (pK_a of transition is 5.9−6.0). The specificity constant for duplexes containing dA:dG increases when the pH is lowered; in contrast, the action of MutY on dA:8-oxo-dG is not pH-dependent (Figure 4). These results suggest that a dA(*anti*):dG(*syn*) alignment is required for optimal catalytic activity of the enzyme.

Introduction of bulky substituents at the 8-position tends to convert purine nucleosides from *anti* to *syn* conformation (Usegi & Ikehara, 1978; Kanaya *et al.*, 1984; Howard *et al.*, 1985). In the present analysis, we will assume that 8-oxo-2′-dG, 8-oxo-deoxynebularine, 8-methoxy-dG, and 6-methoxy-8-oxo-dG are in the *syn* conformation when paired with dA (*anti*), in which case N2, N1, and O6 of guanine and its analogs, and N6, N7, and H8 of dA and structurally related purines are exposed in the major groove of DNA, and H8 of guanine or O8 of 8-oxoguanine, along with N3 of dA, are found in the minor groove (Figure 5). The mispairs examined in this study represent modifications of purine bases designed to selectively alter their ability to act as hydrogen bond acceptor or donors. In some cases, these modifications also create steric constraints.

In DNA containing a dG(*syn*):dA(*anti*) or 8-oxo-dG (*syn*):dA(*anti*) pair, N2 and N1 of guanine and N6 of adenine appear in the major groove as potential hydrogen-bond donors; O6 of guanine and N7 of adenine are hydrogen-bond acceptors (Figure 5a). N3 of adenine and O8 of oxo-dG are hydrogen-bond acceptors and appear in the minor groove (Figure 5b). For substrates in which N7, N6, or H8 of adenine are replaced by C, O, and O, respectively, specificity constants for MutY are reduced by three orders

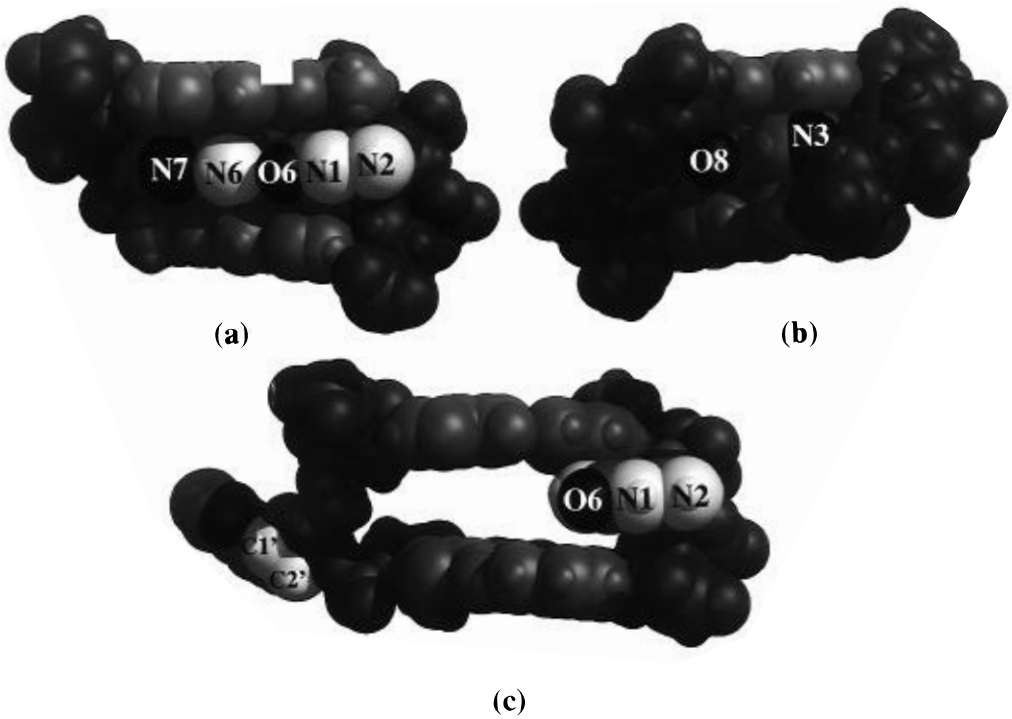

FIGURE 5: Models a and b represent the central three base pairs of an 11-mer DNA duplex showing views from the major and minor groove, respectively. The structure of the dA(*anti*):8-oxo-dG(*syn*) base pair was determined by 2D NMR analysis (Kouchakdjian *et al.*, 1991) coupled with restrained molecular dynamics. Hydrogen-bond donors and acceptors are colored light and dark, respectively. In model c, dA has been everted 180°, leaving a gap in the duplex while 8-oxo-dG remains in the *syn* conformation. This model was constructed by analogy to the crystal structure of the Hha DNA methyltransferase−DNA complex in which dC in a dG:dC pair is everted (Klimasauskas *et al.*, 1994).

of magnitude. The contribution of N1 of adenine was not directly explored although, in its protonated form, this position plays an important role in stabilizing a mispaired dG in the *syn* conformation. In each pair of substrates, in which the dG moiety contains an 8-oxo group, the specificity constant of the 8-oxo derivative is higher by approximately two orders of magnitude. This result could indicate binding of MutY to O8 of the modified base but more likely represents the preference of dG in dG:dA mispairs to remain in the *anti* conformation at pH 7.5 (Carbonnaux *et al.*, 1991; Brown *et al.*, 1990). This partially stacked structure, stabilized by bifurcated hydrogen bonds, binds much less effectively to MutY (Lu *et al.*, 1995; this paper).

8-oxo-dI differs from 8-oxo-dG in lacking a 2-amino group. Absence of this group is associated with a small increase in apparent K_d and only a 4-fold decrease in the specificity constant, suggesting that the presence of N2 of guanine is not of critical importance to enzymatic activity. The effect of removing N2 (dI:dA) is much more marked in the dG:dA series (Table 3). The specificity constant of MutY for dA:8-oxo-dG is reduced by at least several orders of magnitude when O8 and O6 are converted to *O*-methyl ethers. Duplexes in which O6 is removed and which also lack the 2-amino group lose biological activity, suggesting that O6 and O8 of guanine play a role in recognition of substrates by MutY.

In B-DNA containing a dG:dA or 8-oxo-dG:dA mismatch, the sugar residues of adenine are largely shielded from protein interactions; thus, C1′, the primary site of nucleophilic attack by MutY, is not accessible to the enzyme in either groove. The inhibitory effects of replacing deoxyribose with ribose (compare dA:dG with rA:dG and dA:8-oxo-dG with rA:oxo-dG) or of modifying the 2′ position of adenine may relate to structural interference with the catalytic functions of this enzyme. Binding remains strong, a conclusion supported by the low K_d (10 nM) observed for rA:8-oxo-dG. In contrast, the decrease of specificity constant observed after methylating the 2′ position of 8-oxoguanine or by converting the 3′-phosphate to a methylphosphonate more likely is related to steric factors governing the position of enzyme and substrate or electrostatic interactions.

We conclude from this analysis that the edges of dG(*syn*):dA(*anti*) and 8-oxo-dG(*syn*):dA(*anti*) mispairs present a unique configuration of hydrogen-bond donors and acceptors which are recognized by complementary groups in MutY protein. Specific binding is conferred in the major groove by *simultaneous* interaction of MutY with donors and/or acceptors on each base in the mispair. 8-oxo-dG:dA is associated with a 100-fold greater specificity constant compared with dG:dA. This preference is consistent with the fact that, at pH 7.5, dG and dA assume the *anti* conformation with protonation of dA being required to form a Hoogstein pair with dG in the *syn* conformation. There is no indication that either of the hydrogen bond acceptors exposed in the minor groove [O8 of 8-oxo-dG(*syn*) and N3 of dA(*anti*)], which have identical counterparts in the dA(*anti*):dT(*anti*) Watson−Crick pair, are involved in binding to MutY.

Model for the Catalytic Action of MutY. We envisage several discrete steps in the MutY-catalyzed removal of adenine from DNA. Initially, MutY is seen as binding nonspecifically to DNA, an interaction mediated primarily by electrostatic interactions between the protein and phosphodiester backbone (von Hippel & Berg, 1989; Dowd & Lloyd, 1989). In addition, hydrophobic contacts are made between DNA and the target recognition domain of the

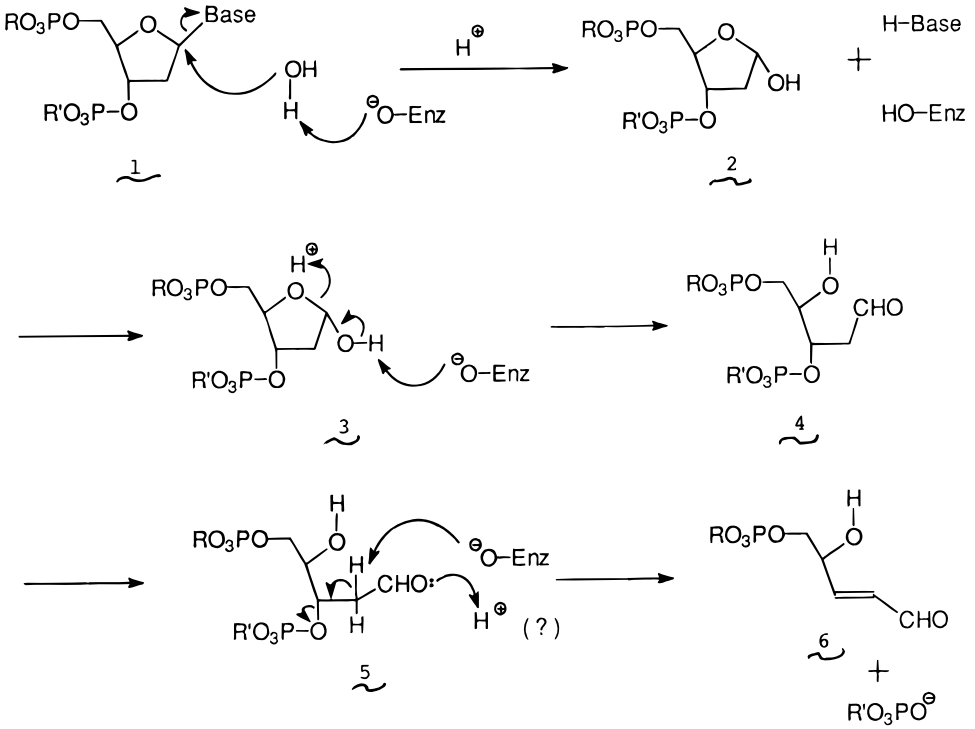

FIGURE 6: Mechanistic scheme showing nucleophilic attack on C1′ by water. See text for details.

protein. The second step involves translation of DNA relative to the protein; a scanning process known as facilitated linear diffusion (Lohman, 1986; von Hippel & Berg, 1989; Dowd & Lloyd, 1990). Through this movement, the protein rapidly reaches the mispair. Scanning of short oligodeoxynucleotides takes only a short time; in macromolecular DNA, the rate of diffusion could be rate limiting. The third step of the process involves recognition of the mispair by MutY. As in other DNA−protein interactions, recognition involves complementary hydrogen bond donor/acceptor pairs in the major groove (Seeman *et al.*, 1976) where the dA:8-oxo-dG mispair displays distinctive features (Figure 5). Additional interactions may stabilize the MutY−DNA complex; for example, methylation and ethylation experiments suggest that phosphate groups on either side of the mispair on both strands of MutY are involved in complex formation (Lu *et al.*, 1995). The importance of nucleotide sequence context in recognition of mispairs by MutY also is suggested by the report of Lu *et al.* (1995) but has not been examined systematically.

The model proposed for subsequent steps (Figure 5c) is based, in part, on an analogy with crystal structures of uracil−DNA glycosylase (Mol *et al.*, 1995), HhaI DNA cytosine-5-methyltransferase (Klimasauskas *et al.*, 1994), and several other enzymes (Roberts *et al.*, 1995). In these structures, the base involved in the enzymatically catalyzed modification is displaced from the interior of the helix. Displacement (eversion) of the base, often referred to as "flipping out", facilitates binding to a pocket in the enzyme and appears to be common in enzymes involved with base modification and DNA repair (Roberts, 1995). Base eversion involves rotations around a number of bonds in the DNA backbone, an increase in the interphosphate distances surrounding the mispair, and conformational changes in the protein (Cheng & Blumenthal, 1996). The protein fills the gap in the helix left by the everted base and provides hydrogen bonding to the orphan base on the opposite strand. In B-DNA, C1′ in the deoxyribose moiety is essentially inaccessible to proteins binding in the major groove. In the everted position (Figure 5c), C1′ can be attacked linearly by a nucleophilic group of the protein or, alternatively, by a water molecule. We propose below a mechanism for this essential step in reactions catalyzed by MutY.

The k_{cat} for MutY increases with decreasing pH when the DNA substrate contains a dG:dA mispair. This effect is not observed for duplexes containing 8-oxo-dG:dA. dG:dA mispairs are stabilized by protonation of dA, with dG assuming the *syn* conformation. Protonation tends to prevent eversion of dA; however, this base competes also for the active site pocket in MutY. This process should be facilitated by the ability of protonated adenine to serve as an electron sink.

Mechanistic Considerations. DNA glycosylases that exhibit similar rates for glycosylase and AP lyase activity are believed to involve a covalent imine (probably protonated) as an intermediate in a reaction that leads ultimately to strand scission (Kow & Wallace, 1987; Dodson *et al.*, 1993). In the absence of an appropriately placed nucleophilic amino group, a specifically positioned water molecule could be deprotonated to generate an hydroxyl anion which then nucleophilically displaces the heterocyclic base (Figure 6, 1→2), generating an abasic site (3). The lyase function of such an enzyme operates on the ring-opened form (4) of the abasic site. The half-life of abasic sites in DNA is measured in hours; a lyase function minimally would require deprotonation of the 1′-hydroxyl group, as shown in 3. A second deprotonation at the 2′ position (5) would induce β-elimination of the 3′-phosphate residue to yield 6. Whether this mechanism also requires protonation of the aldehyde group is debatable. It also is not clear if the same deprotonating groups are involved in both steps. Normally, water deprotonations of this type can be accomplished by a carboxylate anion, a histidine/serine combination or a histidine residue alone, and a combination of such systems cannot be ruled out. At present, little definitive information is available

concerning the mechanistic actions of MutY although there is suggestive evidence, based on homology to the crystal structure of endonuclease III (Kou *et al.*, 1992), that Asp-138 is involved in the glycosylase function of this enzyme (Dodson *et al.*, 1994).

Comparison with Results of Others. After these studies were completed, a paper appeared by Lu *et al.* (1995), describing similar investigations of MutY protein with a more limited series of substrates. Our results differ from that report in several significant ways, including the important question of the preferred natural substrate for this enzyme. Biological evidence suggests that the primary function of MutY in *E. coli* is the removal of dA from oxidatively damaged mispairs (Michaels *et al.*, 1992). We find that specificity constants for MutY for substrates containing dA:8-oxo-dG and dA:dG differ by two orders of magnitude, reflecting an increase in k_{cat} and decrease in K_m for duplexes containing 8-oxo-dG. In contrast, Lu *et al.* (1995) reported that duplexes containing dG:dA were cleaved twice as rapidly as the comparable duplex with 8-oxo-dG; specificity constants were not determined.

MutY also binds more tightly to duplexes containing 8-oxo-dG:dA than to duplexes containing the dG:dA mispair; the apparent K_d was 80-fold lower in the study reported by Lu *et al.* (1995) and 4-fold lower in our investigations. Differences in absolute and relative values for K_d may reflect addition of excess dI:dC in the studies of Lu *et al.* As noted above, nonspecific binding is not treated in their analysis; furthermore, more than one protein appears to bind to a single duplex (Lu *et al.*, 1995). In our experiments, oxidation at C-8 of guanosine results in an increase in K_d in all substrates in which effects of this modification were examined.

Our study differs in other ways from the report of Lu *et al.* (1995); for example, we find for several substrates that N6 of adenine is necessary for biological activity. Also, we show that N2 of 8-oxo-dG is not required for tight binding or cleavage of substrates by MutY.

Some of the differences between these two studies may relate to the assay conditions employed. AP lyase activity is reported to be an intrinsic component of the enzyme (Tsai-Wu *et al.*, 1992). However, we did not detect significant AP lyase activity in highly purified preparations of MutY. Similar results were reported by Au *et al.* (1988, 1989), who first described the properties of MutY. Recently, Lloyd and his colleagues (Latham & Lloyd, 1995) compared the catalytic properties of T4 endonuclease V, *E. coli* endonuclease III, and MutY protein, basing this analysis on sequence homology and the three-dimensional crystal structure of these enzymes. MutY and endonuclease V have identical sequences between Ala-113 and Ala-122, except at position 120, which in endonuclease III is lysine and in MutY is serine. Lysine-120 is proposed as the residue involved both in Schiff base formation and AP lyase activity. In MutY, when serine was replaced by lysine, AP lyase activity and glycosylase activity became approximately equal (Sun *et al.*, 1995).

A direct test for the presence of an AP lyase function involves trapping the Schiff base intermediate formed between enzyme and substrate by reduction with sodium borohydride or cyanoborohydride (Dodson *et al.*, 1993). Sun *et al.* (1995) report that a DNA−MutY complex could not be obtained under conditions used to form a covalent complex between endonuclease V and duplexes containing a single thymine dimer. This suggests that a mechanism such as that shown in Figure 6, which involves H_2O as a nucleophile, is involved in cleavage of the phosphodiester backbone by MutY.

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