Cloning, Overexpression, and Biochemical Characterization of the Catalytic Domain of MutY†

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ABSTRACT: Proteolysis of MutY with trypsin indicated that this DNA mismatch repair enzyme could exist as two modules and that the N-terminal domain (Met1−Lys225), designated as p26, could serve as the catalytic domain [Manuel et al. (1996) *J. Biol. Chem. 271*, 16218−16226]. In this study, the p26 domain has been cloned, overproduced, and purified to homogeneity. Synthetic DNA duplexes containing mismatches, generated with regular bases and nucleotide analogs containing altered functional groups, have been used to characterize the substrate specificity and mismatch repair efficiency of p26. In general, p26 recognized and cleaved most of the substrates which were catalyzed by the intact protein. However, p26 displayed enhanced specificity for DNA containing an inosine·guanine mismatch, and the specificity constant (K_{cat}/K_m) was 2-fold higher. The truncated MutY was able to cleave DNA containing an abasic site with equal efficiency. Dissociation constants (K_d) were obtained for p26 on noncleavable DNA substrates containing a tetrahydrofuran (abasic site analog) or a reduced abasic site. p26 bound these substrates with high specificity, and the K_d values were 3-fold higher when compared to the intact MutY. p26 contains both DNA glycosylase and AP lyase activities, and we provide evidence for a reaction mechanism that proceeds through an imino intermediate. Thus, we have shown for the first time that deletion of 125 amino acids at the C-terminus of MutY generates a stable catalytic domain which retains the functional identity of the intact protein.

Mismatch repair enzymes are vital to preserve the integrity of the genetic material. MutY of *Escherichia coli* is a mismatch repair enzyme with a molecular mass of 39.1 kDa and has significant sequence homology with *E. coli* endonuclease III (*1*) and the pdg UV endonuclease of *Micrococcus luteus* (*2*). Although these enzymes have extensive sequence homology, they have different substrate specificity. The human homolog of MutY (hMYH) has also been cloned and sequenced, and it shows significant homology to *E. coli* MutY (*3*). Another protein homologous to MutY has been identified in *Salmonella typhimurium* with 91% amino acid sequence identity (*4*).

The biological role of MutY is the removal of adenine when paired with 8-oxo-7,8-dihydro-2′-deoxyguanine (8-oxodG)[1] (*5*−*7*) and avoidance of C·G to A·T transversions by removing the adenine mispaired with guanine (*8*). MutY also removes the adenine when mispaired with guanine or cytosine (*5*). The stability of these mismatches may justify the requirement of special DNA repair enzymes to remove the mismatched base and safeguard the genetic information. In vitro studies have shown that MutY also recognizes and removes other nucleotide analogs with modified functional groups (*9, 10*).

In *E. coli*, the excision of adenine from an A·G or A·C mismatch appears to be straightforward. However, the repair of an 8-oxodG lesion in the DNA is relatively complex. During replication, dCMP or dAMP can be selectively incorporated opposite 8-oxodG depending on the DNA polymerase involved (*11*). When the daughter strand contains a C opposite 8-oxodG, FPG (MutM) removes 8-oxodG and allows the base excision repair machinery to fill the gap with a G and thus restore the original sequence (*12*). However, when A is incorporated opposite 8-oxodG, MutY is responsible for the removal of adenine. The complete repair of the lesion is not accomplished until dCMP pairs with 8-oxodG and creates a substrate for FPG (*5*). To supplement these two repair enzymes, MutT functions as an 8-oxodGTPase and degrades this mutagenic substrate from the nucleotide pool (*13*).

X-ray crystallography and NMR spectroscopy have been used to study the structural anomalies of the mismatched bases recognized and catalyzed by MutY. NMR spectroscopic studies show that the A^{anti}·8-$oxodG^{syn}$ conformation is stable in the interior of the DNA helix (*14*) and the syn conformation of 8-oxodG facilitates the correct geometry to form hydrogen bonds with adenine (*15*). The structure of an A^{anti}·G^{syn} mispair is shown to be similar to A^{anti}·8-$oxodG^{syn}$, although the former may be more efficiently corrected by the 3′−5′ exonuclease activity of DNA polymerase, due to its relatively low thermodynamic stability (*16*). However, other structural studies with DNA containing an A·G mismatch have yielded contradictory results with respect to the syn and anti conformations of the mismatched bases (*17*−*19*).

We are beginning to understand the structural and functional aspects of MutY. Ethylation interference studies show

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[1] Abbreviations: AP, apurinic/apyrimidinic; APE, AP endonuclease; BSA, bovine serum albumin; dhU, dihydrouracil; dRpase, deoxyribophosphodiesterase; FPG, 2,6-dihydroxy-5-*N*-formamidopyrimidine; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanine; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; ssDNA, single-stranded DNA; THF, tetrahydrofuran; RAP, reduced AP site.

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that MutY interacts electrostatically with at least 5 phosphates in the DNA backbone and covers 12 base pairs around the mismatch (*9*). Also methylation interference experiments have shown that MutY interacts with the mispaired A and G in addition to the two bases on either side of the mismatch, indicating that the nature of the flanking sequences of the mismatch may have a role in the efficiency of the enzyme (*9*). The domain structure of MutY has been deduced by proteolytic studies (*20, 21*). A N-terminal domain (p26) and a C-terminal domain (p13) were isolated, and p26 was similar in size to endonuclease III with high sequence homology. Molecular modeling studies suggested that they could share a common three-dimensional structure (*20*).

Here we report the cloning and overproduction of the catalytic domain of MutY (Met1 to Lys225). Using DNA containing mismatches created by regular bases and purine analogs, we have characterized the substrate specificity and catalytic activity of p26 and compared it to intact MutY. A controversy which has been lingering for some time now is the functional identity of MutY itself. It has not been categorically classified as a glycosylase or as a bifunctional glycosylase/AP lyase. We provide experimental evidence with the truncated version of MutY which leads us to believe that MutY can possess both the glycosylase and AP lyase activities, and that the AP lyase reaction mechanism could go through an imino intermediate similar to other DNA glycosylases/AP lyases.

EXPERIMENTAL PROCEDURES

Cloning of the p26 Domain. The region of the *mutY* gene corresponding to the p26 domain (Met1 to Lys225) was PCR (polymerase chain reaction) amplified out of the plasmid pKKYEco (gift grom Drs. J. H. Miller and M. L. Michaels), containing the entire *mutY* structural gene, using primers (P1 and P2) which placed *Eco*RI restriction sites at both ends of the coding sequence (Figure 1). Primers were designed using sequence information from the intact *mutY* gene (*1*). PCR reactions were performed in a GeneAmp PCR system 9600 (Perkin Elmer) in a 100 μL reaction volume with 50 pmol of each primer and 5 units of Taq DNA polymerase (Stratagene) in a buffer containing 10 μL of 10 $\times$ TAQ buffer (Stratagene), 1.5 mM $MgCl_2$, 1 μg of bovine serum albumin (BSA), and 0.2 mM of each dNTP. The thermocycler settings were 94 °C for 30 s, 68 °C for 45 s, and 72 °C for 3 min, for 30 cycles. PCR products were separated by agarose gel electrophoresis and purified. This portion of the *mutY* gene harboring *Eco*RI restriction sites at both ends was inserted in a pCR II vector, digested with *Eco*RI. DNA sequence analyses (Applied Biosystems 373A DNA Sequencer) were performed to verify the sequence of the cloned region of the *mutY* gene. The pCR II vector containing the coding region of the p26 domain and pKKYEco containing the entire *mutY* gene were digested with *Mlu*I and *Pst*I. As shown in Figure 1, the C-terminal portion of the p26 domain released from pCR II was ligated to pKKYEco containing the N-terminal portion of the p26 domain. The resulting vector, designated as pKKYEco-p26, contains the entire coding region of the p26 domain between *Eco*RI sites. This expression vector is derived from pKK223-3 (Pharmacia Biotech).

Overexpression and Purification of the p26 Domain of MutY. *E. coli* strain CC104 (*mutY*$^-$) harboring the expression

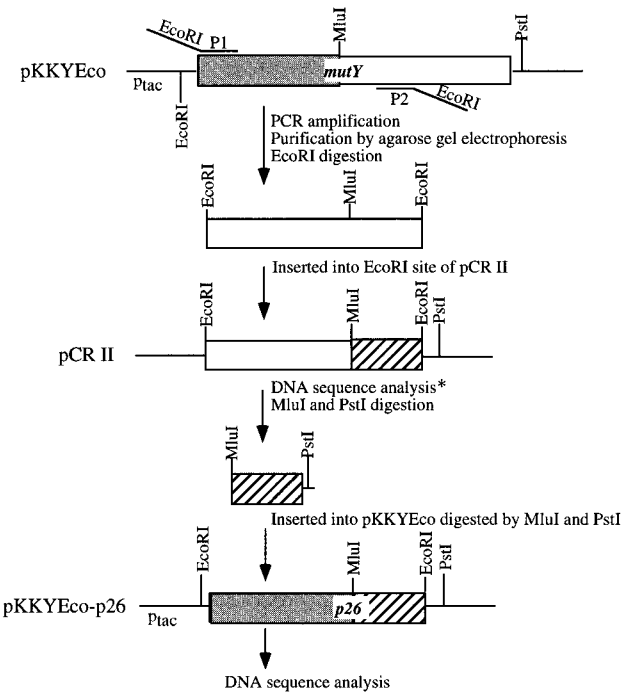

FIGURE 1: Cloning of the p26 domain of MutY. As described under Experimental Procedures, the p26 domain of MutY was cloned into an expression vector, designated as pKKYEco-p26 (derived from pKK223-3). The coding sequence of the p26 domain is downstream of the *tac* promoter. Asterisk: A mutation was detected at the 5′-end of the cloned region of the p26 domain. Therefore, we identified the restriction sites *Mlu*I and *Pst*I in the vectors pCR II and pKKYEco which allowed us to exchange the portion of the *mutY* gene between these two restriction sites in the plasmids pCR II and pKKYEco.

vector pKKYEco-p26, containing the portion of the *mutY* structural gene corresponding to the p26 domain (Met1 to Lys225) downstream of the *tac* promoter, was grown in LB medium containing 100 μg/mL ampicillin at 34 °C in a New Brunswick Scientific BioFlo III fermenter, by induction with 1 mM isopropyl β-D-thiogalactoside when A_{600} reached 0.8. The p26 domain was purified as described earlier for the intact MutY protein (*20*). However, pure protein was obtained in two chromatographic steps: Q-Sepharose and SP-Sepharose (Pharmacia) columns in tandem followed by a single-stranded DNA−cellulose (USB) column. Purification was monitored by a combination of Coomassie Brilliant Blue staining of p26 in SDS−polyacrylamide gels and by adenine glycosylase activity on a DNA substrate with a site-specific A·G mismatch (*20*).

Purification of MutY Protein. MutY was overexpressed from the plasmid pKKYEco containing the *mutY* structural gene downstream of the *tac* promoter in *E. coli* strain GX1200. The procedure for the preparation of MutY has been described elsewhere (*20*).

Amino Acid Sequencing. The intact MutY and p26 were subjected to SDS−polyacrylamide gel electrophoresis, and then electroblotted onto a poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad), as described previously (*22*). The protein band was excised from the membrane and the amino-terminal sequence determined in a Applied Biosystems 120A Analyzer 477A protein sequencer.

Gel Mobility Shift Assay To Survey Substrate Specificity. This assay was used to demonstrate the binding of MutY and the p26 domain to different DNA substrates. The sequence of the DNA substrate used is as follows:

Sequence I
5′-*TACGAATTGCTTA**X**TTCGTGCAGGCATGGT-3′
3′-ATGCTTAACGAAT**Y**AAGCACGTCCGTACCT-5′
* = [γ-^{32}P]ATP

where **X** = adenine, nebularine (a purine riboside), 2-aminopurine, or inosine (hypoxanthine) and **Y** = guanine, 8-oxoguanine, or cytosine. The structures of adenine, guanine, and the purine analogs used in this study are provided in Figure 2A. Nebularine, 2-aminopurine, inosine, and 8-oxoguanine phosphoramidite monomers were purchased from Glen Research (Sterling, VA) for synthesis of the oligonucleotides shown in sequence I. The synthetic oligonucleotides were purified by electrophoresis on a 15% polyacrylamide gel. The strands containing adenine or its analogs were 5′-end-labeled with [γ-^{32}P]ATP and annealed to their complementary strand. The molar ratio between the labeled vs unlabeled strand was 1:2. The binding reaction mixture contained 20 mM Tris-HCl (pH 7.5), 80 mM NaCl, 1 mM DTT, 1 mM EDTA, and 2.9% glycerol. The DNA substrate (2.5 nM) was incubated with 15 nM MutY or p26 for 30 min at 37 °C. Protein−DNA complexes were separated in 8% nondenaturing polyacrylamide gels and subjected to autoradiography and PhosphorImager analysis (Molecular Dynamics).

Mismatch Cleavage Assay. The heteroduplex DNA substrates that are described above were used in the following experiment. The DNA substrate (2.5 nM) was reacted with 25 nM intact MutY or p26 in a reaction buffer containing 25 mM sodium phosphate (pH 6.8), 1 mM EDTA, 50 mM NaCl, and 100 μg/mL bovine serum albumin (BSA). The 20 μL reaction mixture was incubated at 37 °C for 30 min. The reactions were terminated in formamide loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue) and heated at 90 °C for 4 min before separating the reaction products in 15% polyacrylamide gels containing 7 M urea. Oligonucleotide sizing markers were obtained from Pharmacia Biotech. The nicked products were visualized by autoradiography and quantified using the PhosphorImager.

Kinetic Studies. The standard reaction mixture contained 25 mM sodium phosphate (pH 6.8), 50 mM NaCl, 1 mM EDTA, and 100 μg/mL BSA. The oligonucleotide containing adenine or inosine was 5′-end-labeled with [γ-^{32}P]ATP and annealed to a complementary strand (sequence I) to create a DNA substrate with an A·G or I·G mismatch. The molar ratio between the labeled vs unlabeled strand was 1:2. The range of substrate concentrations were from 1 nM to 50 nM for DNA containing A·G mismatch and from 0.1 nM to 10 nM for DNA containing I·G mismatch. An aliquot (5 μL) was withdrawn, and this was considered as the zero time point. Intact MutY and p26 were diluted in the reaction buffer. Aliquots of the intact MutY or p26 were added, and the reactions were initiated at 37 °C. Aliquots (5 μL) were withdrawn after 0.5, 1, 2, and 3 min. At each time point, the reactions were terminated with 5 μL of formamide loading buffer. The reaction products were separated by electrophoresis on 15% polyacrylamide gels containing 7 M urea. The cleaved products and the remaining substrate were quantified by PhosphorImager analysis. The initial reaction rate was plotted against the substrate concentration. The curves were fitted with the Michaelis−Menten equation using KaleidaGraph software, and the Michaelis constant (K_m) and V_{max} values were determined. The turnover number (K_{cat}) values were derived from the V_{max} values and enzyme concentration.

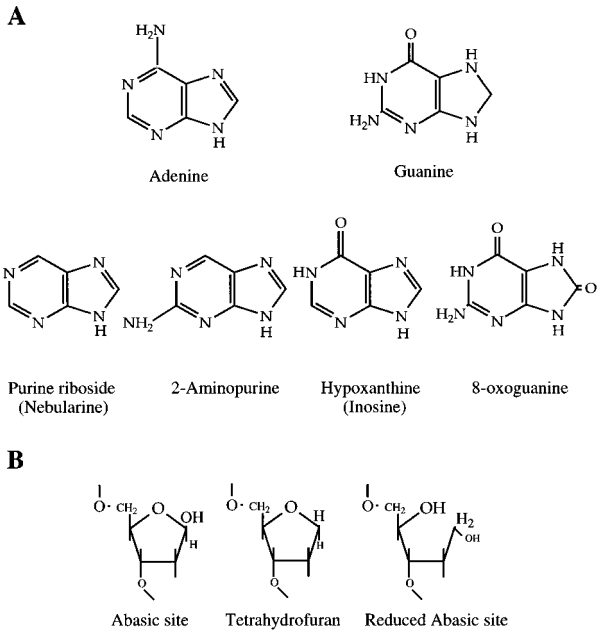

FIGURE 2: (A) Structures of adenine, guanine, and purine analogs used in this study. (B) Structure of an abasic site compared with tetrahydrofuran (an abasic site analog) and a reduced abasic site.

Enzyme−DNA Covalent Complex Formation with $NaBH_4$. Duplex DNA (10 nM), containing a single 8-oxodG or guanine opposite adenine or nebularine (sequence I), was reacted with a 100-fold molar excess of MutY or p26 in the buffer used for the mismatch repair assay. The reaction was incubated at 37 °C in the presence of 100 mM $NaBH_4$, and allowed to proceed for 30 min before it was stopped with loading buffer [50 mM Tris-HCl (pH 6.8), 10 mM DTT, 2% SDS, 10% glycerol, 0.1% bromphenol blue]. The reaction products were heated at 90 °C for 5 min and separated by electrophoresis in a 15% SDS−polyacrylamide gel. The covalently trapped enzyme−substrate complex was visualized by autoradiography.

Activity on Abasic Sites. A 49-base oligonucleotide with the sequence 5′-AGCTACCATGCCTGCACGAA**U**TAAG-CAATTCGTAATCATGGTCATAGCT-3′ containing a single uracil was 5′-end-labeled with [γ-^{32}P]ATP and annealed to its complementary strand in a 1:2 molar ratio. The uracil base was removed with uracil DNA glycosylase, and a DNA substrate with a single abasic site was created as previously described (*22*). To 5 nM of this substrate were added different amounts (5−50 nM) of MutY or p26 under the reaction conditions used for the mismatch repair assay. The reactions were terminated in the formamide loading buffer and the products analyzed in a 15% polyacrylamide gel containing 7 M urea. The nicked product was visualized by autoradiography, and the percentage cleavage at abasic sites was obtained by PhosphorImager analysis.

Activity Assay on a DNA Substrate Containing Tetrahydrofuran (THF). To demonstrate that DNA containing THF (an AP site analog) is not cleavable by MutY and the truncated p26 domain, 3.5 nM of the substrate shown below:

Sequence II
5′-*GGATAGTGTCCA**F**GTTACTCGAAG-3′
3′-CCTATCACAGGTGCAATGAGCTTC-5′
F = tetrahydofuran; * = [γ-^{32}P]ATP

was incubated with different concentrations of MutY or p26 domain (2.5−125 nM). The reaction components and conditions were similar to those of the mismatch repair assay.

Specific Binding to DNA Containing an AP Site Analog or a Reduced AP Site (RAP). To determine the specificity of MutY and p26, DNA (1 nM) containing a THF (sequence II) or DNA (1 nM) containing a RAP as shown below:

Sequence III 5′ - *GTGAACCTGAGC**(RAP)**TAGCTCAGTAAC - 3′
3′ - CACTTGGACTCG C ATCGAGTCATTG - 5′

RAP = reduced abasic site; ∗ = [γ-^{32}P]ATP

was incubated with 50 nM MutY or 100 nM p26 domain in a buffer containing 25 mM HEPES (pH 7.8), 0.5 mM EDTA, 0.5 mM DTT, 5% glycerol, 100 mM NaCl, and 100 μg/mL BSA, at 25 °C for 30 min. Reactions were performed in duplicate, and one set of reactions contained a 50 molar excess of nontarget DNA (sequence II with cytosine in place of THF). The reactions were terminated with equal amounts of 50% glycerol containing 0.01% bromphenol blue and loaded on an 8% nondenaturing polyacrylamide gel. The DNA−protein complex was visualized by autoradiography, and the fraction of the substrate bound was determined by PhosphorImager analysis.

Determination of the Equilibrium Dissociation Constant (K_d) of p26 and MutY. To determine the binding affinity of the intact MutY and the truncated p26 domain, DNA duplex (1 nM) containing a THF (sequence II) or a reduced AP site (sequence III) was incubated with different amounts of MutY (3−150 nM) or p26 domain (10−200 nM). The reaction conditions and the separation of the DNA−protein complexes were similar to those in the assay described above to determine the specific binding of MutY and p26 domain to the same substrates. Through PhosphorImager analysis, the fraction of the bound substrate was determined and plotted against enzyme concentration. The curve was fitted with the Michaelis−Menton equation using KaleidaGraph software.

Nature of 3′ Ends Resulting from Abasic Site Nicking. Heteroduplex DNA shown in sequence I, containing a single A·G mismatch, was used in this experiment. Reactions were performed in triplicate, containing 10 nM DNA, 50 nM MutY or p26 domain, and the buffer used in the mismatch repair assay. After incubation at 37 °C for 30 min, one reaction was terminated with the formamide loading buffer, another was treated with piperidine and incubated at 90 °C for 15 min before adding the formamide buffer, and the third was again incubated at 37 °C for 15 min with 2 mM $MgCl_2$ and 50 nM human AP endonuclease (gift from Drs. P. Strauss and S. H. Wilson). The reaction products were separated in a 20% polyacrylamide gel containing 7 M urea. The nicked products were visualized by autoradiography.

Absence of Activity on DNA Containing the dhU·G Lesion. An oligonucleotide (37-mer) containing a site-specific 5,6-dihydrouracil (gift from Drs. P. Doetsch and L. Augeri, Emory University) with the sequence 5′-CTTGGACTG-GATGTCGGCAC**(dhU)**AGCGGATACAGGAGC-3′ was 5′-end-labeled with [γ-^{32}P]ATP before being annealed with twice the amount of its complementary strand containing a guanine opposite dhU. 25 nM of MutY, p26, or endonuclease III was incubated with the above DNA substrate (2.5 nM). The reaction conditions and analyses of the products were similar to those in the mismatch repair assay.

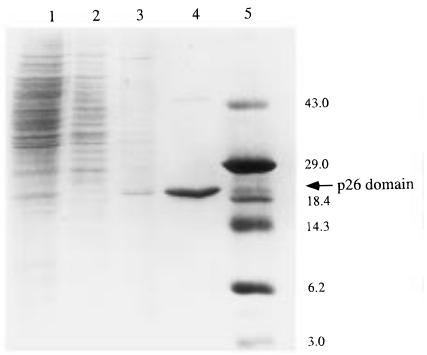

FIGURE 3: Purification of the overexpressed p26 domain. Photograph of Coomassie Brilliant Blue stained gel after 15% SDS−PAGE. Lane 1, crude cell lyste. Lane 2, flow-through from Q-Sepharose and SP-Sepharose columns in tandem. Lane 3, pooled fractions containing adenine glycosylase and AP lyase activity from a dual chromatography step using Q-Sepharose and SP-Sepharose columns. Lane 4, pooled fractions containing pure p26 eluted from single-stranded DNA−cellulose column. Lane 5, prestained molecular weight markers.

Absence of Activity on DNA with an 8-oxodG·C Lesion. Another oligonucleotide (21-mer) with a single 8-oxodG with the sequence 5′-CAGCCAATCA**(8-oxodG)**TGCACCATCC-3′ was 5′-end-labeled with [γ-^{32}P]ATP before being annealed to a complementary strand containing a cytosine opposite 8-oxodG. This DNA substrate (2.5 nM) was reacted with 25 nM MutY, p26, or FPG. The reaction conditions and analyses of the products were similar to those in the mismatch repair assay.

RESULTS

Cloning of the p26 Domain. Using the methods described under Experimental Procedures, we generated the recombinant plasmid pKKYEco-p26 containing the coding region of the p26 domain (Met1−Lys225) of MutY. The entire coding region was verified by DNA sequence analysis. The structure of the expression vector pKKYEco-p26 is shown in Figure 1. The cloning vector pCR II was used to facilitate the insertion of the PCR-amplified region of the *mutY* gene. This truncated version of MutY is identical to the N-terminal domain isolated by proteolysis of MutY with trypsin (*20*). In addition, it is homologous to *E. coli* endonuclease III in having 60% similarity and 20% identity. The p26 domain also contains the four cysteine residues (192, 199, 202, and 208) which are likely to coordinate the $[4Fe\text{-}4S]^{2+}$ cluster.

Protein Expression and Purification. To characterize the structural and functional properties of p26, it was expressed in *E. coli* strain CC104, which contains a disrupted chromosomal *mutY* gene (*8*). The purification steps for p26 are presented in Figure 3. The purified p26 was found to be free from contaminants in silver-stained SDS−polyacrylamide gels (data not shown). The first step using the Q-Sepharose and SP-Sepharose columns in tandem eliminated the bulk of the soluble *E. coli* proteins (lane 3). The next step using ssDNA−cellulose matrix yielded the p26 domain with an apparent molecular mass of 26 kDa (lane 4). The purified protein did not have any nonspecific DNA nicking activity. Fractions containing the p26 were yellow in color, as observed for the intact MutY. This is presumably due to the presence of the iron−sulfur cluster. Like the intact MutY, p26 did not remain in solution at high concentrations. However the protein was extremely stable and active when

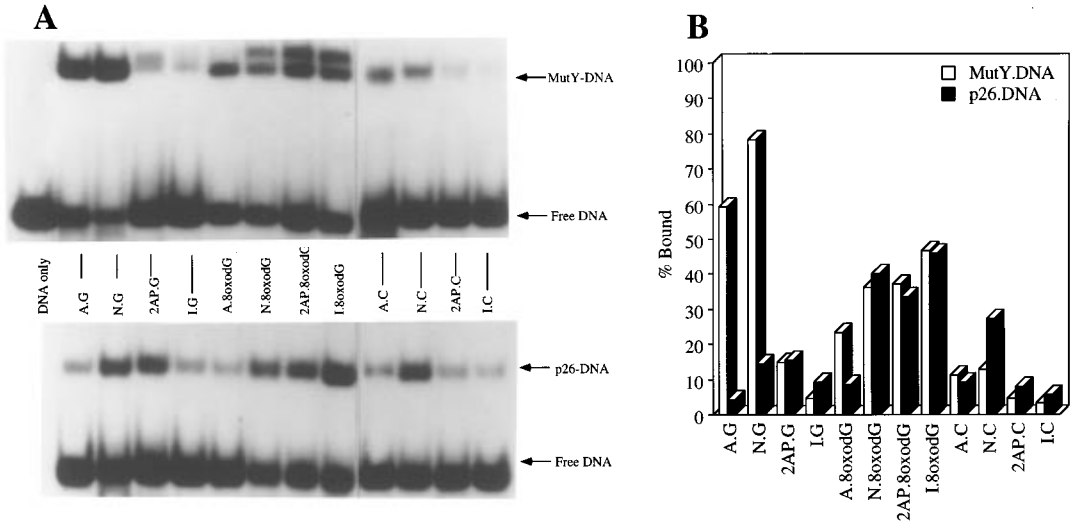

FIGURE 4: Gel mobility shift assay to determine the substrate specificity of MutY and p26 to DNA containing mismatches. (A) Autoradiograph showing the binding of MutY and p26 to synthetic DNA (sequence I) containing a mismatch created with normal bases and nucleotide analogs (Figure 2A). The oligonucleotides containing the mismatched adenine, nebularine, 2-aminopurine, or inosine were 5′-end-labeled with [γ-^{32}P]ATP. The DNA substrate (2.5 nM) was incubated with 15 nM MutY or p26 for 30 min at 25 °C. The reaction products were separated in 8% native polyacrylamide gels. The autoradiograph shows the MutY−DNA and p26−DNA complexes formed. In the reaction containing DNA only, no complexes were found. (B) Histogram depicting the relative amount of MutY−DNA and p26−DNA complexes formed. The percents protein−DNA complexes were obtained by PhosphorImager analysis. All the gel-shifted products were taken into account for quantitation.

stored at a concentration of 0.5 mg/mL at 4 °C. The N-terminal sequence of the overproduced p26 was MQASQF-SAQVLD, perfectly matching the N-terminal sequence of intact MutY. This confirmed that p26 was expressed in the correct reading frame. The N-terminal methionine is not modified posttranslationally. MutY was produced from the expression plasmid pKKYEco containing the *mutY* structural gene downstream of the *tac* promoter and purified as described elsewhere (*20*).

Binding Affinity for Various Substrates. The relative efficiencies of mismatch recognition and binding by p26 versus intact MutY on DNA substrates containing mismatches were analyzed. Synthetic oligonucleotides containing adenine or purine analogs (Figure 2A) were annealed with oligonucleotides containing guanine, 8-oxoguanine, or cytosine to generate mismatches in duplex DNA (sequence I). The above combinations generated DNA substrates with 12 different mismatches, some of them with modified functional groups within the mismatch. The binding affinity of p26 to the above substrates was compared with that of the intact protein. A representative autoradiograph of gel retardation assays and the percentage of protein−DNA complexes formed are shown in Figure 4. The binding affinity of the truncated protein was significantly different from the intact protein when adenine or its analog nebularine, which lacks the C-6 amino group, was paired with guanine. The affinity of p26 for DNA containing an A·G mismatch was 12-fold lower than that of the intact protein (Figure 4). When the C-6 amino group was absent, the difference was only 5-fold. Intact MutY also had a higher affinity for DNA containing an A·8-oxodG mismatch.

The p26 domain had a 2-fold higher affinity for DNA containing an inosine where the C-2 amino group of guanine had been deleted and paired with guanine. With DNA containing nebularine opposite cytosine, p26 had a marginal increase in binding affinity. p26 and MutY had similar affinities for all of the remaining mismatch containing DNA substrates, including those containing 2-aminopurine where the C-6 amino group of adenine is displaced to the C-2 position. DNA containing adenine, nebularine, or inosine paired with 8-oxodG had a similar binding affinity with MutY and p26, although with MutY an additional band with lower mobility was formed (Figure 4A). Although this survey with a single enzyme and substrate concentration in a fixed time point does not provide the kinetics of protein−DNA interaction, it is a good indicator for the relative affinity of p26 and MutY to DNA containing specific mismatches. The affinity of p26 and MutY to nontarget DNA will be discussed later.

Mismatch Cleavage Activity. To assess the mismatch cleavage efficiency of p26 on DNA substrates containing mismatches generated by regular bases and nucleotide analogs with altered functional groups, the substrates used in the binding assays described above were reacted with p26 or MutY, and the products were separated in polyacrylamide gels under denaturing conditions. An autoradiograph showing the cleaved products and a histogram depicting the relative amounts of nicking are shown in Figure 5. In comparing these data with the binding affinities shown in Figure 4, it is evident that there is no direct correlation between the relative binding affinity and repair efficiency of p26 and intact MutY with DNA substrates containing mismatches used in this study. The truncated protein had amounts of nicking activity similar to that of the intact MutY, with a DNA substrate containing a mismatch with nebularine opposite guanine. On substrates containing adenine or 2-aminopurine opposite guanine, the increase in activity by intact protein was 3- and 4-fold, respectively. p26 was not able to cleave DNA with mismatch containing adenine or its analogs opposite cytosine. However, when the guanine analog inosine (lacking the C-2 amino group) was paired with guanine, 8-oxoguanine, or cytosine, p26 was able to incise inosine much more efficiently than the intact MutY. The increase in p26 activity on DNA containing I·G and I·8-oxodG mismatches is 8- and 6-fold, respectively. Nicking activity was completely absent when intact protein was

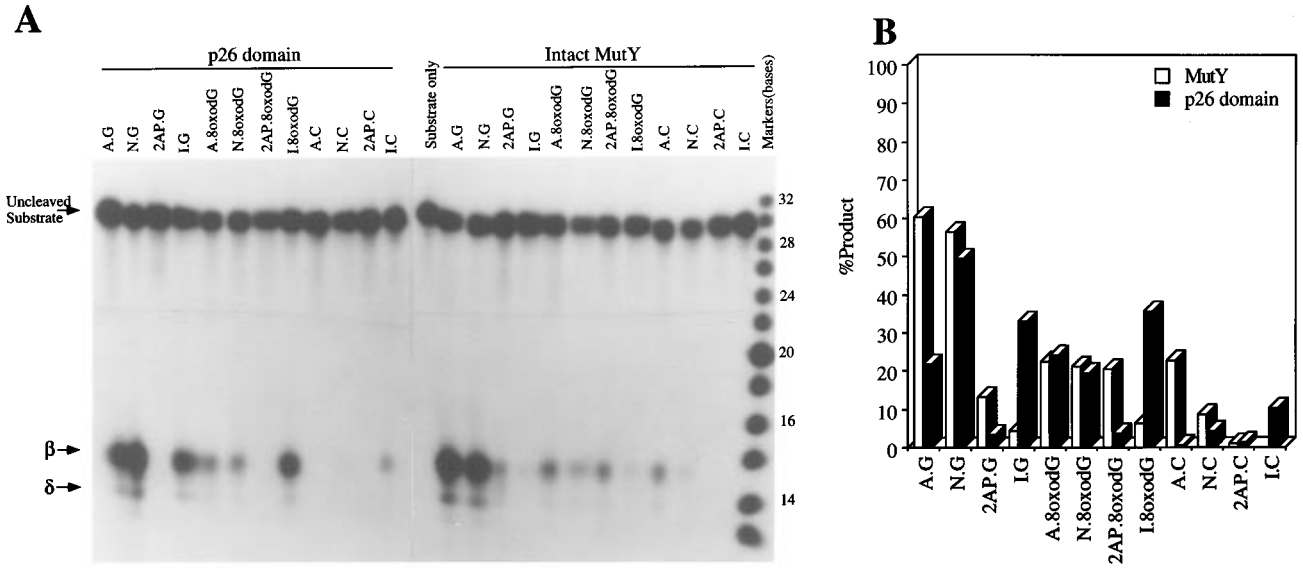

FIGURE 5: Relative mismatch repair activity of MutY compared with p26 on DNA substrates containing a mismatch. (A) Autoradiograph showing the β- and δ-elimination products after incubation of MutY (25 nM) or p26 (25 nM) with synthetic DNA (2.5 nM) containing a single mismatch as shown in sequence I. The strand containing the mismatched adenine, nebularine, 2-aminopurine, or inosine was 5′-end-labeled with [γ-^{32}P]ATP before annealing to their complementary strands. After incubating the reaction at 37 °C for 30 min, the products were separated in a 15% denaturing polyacrylamide gel and autoradiographed. Oligonucleotide sizing markers are shown in the margin. The lane which was loaded with the DNA substrate only did not show any nicked products. (B) Graphical representation of the relative amounts of nicked products generated by MutY or p26. The percent of nicked products (β- and δ-elimination) was quantified by PhosphorImager analysis.

Table 1: Steady-State Kinetic Parameters for MutY and p26 on DNA Substrate Containing a Single A·G or I·G Mismatch (Sequence I)[a]

DNA mismatch	enzyme	K_m (nM)	V_{max} (pM min^{-1})	K_{cat} (min^{-1})	K_{cat}/K_m (min^{-1} μM^{-1})
A·G	MutY	1.43	557	5.57×10^{-2}	38.95
	p26 domain	4.66	82	0.82×10^{-2}	1.76
I·G	MutY	1.50	62	0.62×10^{-2}	4.13
	p26 domain	1.85	163	1.63×10^{-2}	8.81

[a] Assays were performed as outlined under Experimental Procedures. Velocities were fitted to the Michaelis−Menten equation using KaleidaGraph.

incubated with DNA containing an I·C mismatch, while p26 had 10% activity. In all of the nicking assays, glycosylase activity was followed by AP lyase activity, as there was no increase in nicked products with the addition of piperidine following the treatment of DNA substrates with the enzymes (data not shown). The nicked product was predominantly from a β-elimination reaction, although some δ-elimination product was distinguishable. The δ-elimination product did not increase by increasing the time of incubation or with a higher enzyme concentration (data not shown).

Kinetics of Adenine and Inosine Excision. Since we observed a significant increase in inosine glycosylase activity by p26 when inosine is paired with guanine, 8-oxoguanine, or cytosine, we obtained kinetic parameters for p26 and MutY on adenine and inosine excision with DNA containing an A·G or I·G mismatch (Table 1). The Michaelis constant (K_m) is quite comparable for p26 and MutY when adenine or inosine is paired with guanine, except that p26 has a K_m which is 3-fold higher on a A·G substrate, indicating reduced affinity for this mismatch. The turnover number (K_{cat}) for p26 on an I·G mismatch is 3-fold higher than that of the intact MutY, although it is 3-fold lower than that of the K_{cat} for the intact MutY on an A·G mismatch. We also found that deletion of the C-terminal domain diminished the turnover number 6-fold for an A·G mismatch. The specificity constant (K_{cat}/K_m) for p26 on an A·G mismatch is about 20-fold less than that of MutY. The specificity constant for an I·G mismatch was 4-fold higher for p26 compared to its value for an A·G mismatch, and this validates the enhanced nicking observed in DNA containing I·G mismatches in the mismatch repair assay (Figure 5).

Formation of a Covalent Enzyme−Substrate Complex by Reduction with $NaBH_4$. If the AP lyase catalytic mechanism of MutY proceeds via an imino intermediate, as shown with T4 endonuclease V (*23*), *E. coli* endonuclease III (*24*), and *E. coli* FPG (*25*), the protein−DNA complex can be covalently trapped by reduction with $NaBH_4$. DNAs containing either an adenine or a nebularine opposite a 8-oxodG or guanine (sequence I) were incubated with p26 or MutY in the presence of 100 mM $NaBH_4$. When the reaction products were separated in an SDS−polyacrylamide gel, the covalently linked protein−DNA complex had a slower mobility and was distinctly separated from free DNA (Figure 6). p26 and the intact protein were able to covalently cross-link with DNA containing A·G, N·G, and N·8-oxodG mismatches. In addition, intact MutY was able to form a covalent complex with DNA containing a A·8-oxodG mismatch. A covalent complex between p26 and A·8-oxodG was below detection levels. The p26·DNA complex had a faster mobility than the MutY·DNA complex, and these complexes should display different mobilities since they are predicted to have molecular masses of 42 and 55 kDa, respectively. The trapped complexes in the SDS−polyacrylamide gel were visualized by autoradiography since the substrate was labeled with [γ-^{32}P]ATP.

Activity on Abasic Sites. Since we observed the cleavage of the phosphodiester bond along with the incision of the *N*-glycosyl bond, a DNA duplex with a single abasic site was created, as described earlier (*22*), and treated with different concentrations of intact MutY or p26. The amount

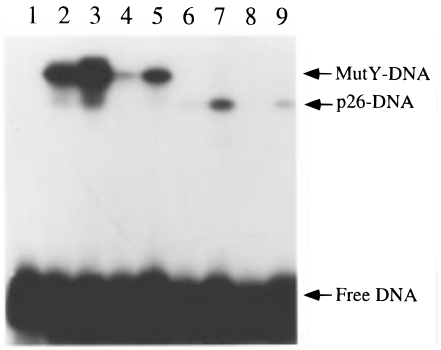

FIGURE 6: SDS−PAGE analysis of covalent protein−DNA complex formation in the presence of sodium borohydride. Mismatched DNAs (10 nM) containing A·G (lanes 2 and 6), N·G (lanes 3 and 7), A·8-oxodG (lanes 4 and 8), or N·8oxodG (lanes 5 and 9) were incubated with a 100-fold molar excess of MutY (lanes 2−5) or p26 (lanes 6−9) in the presence of 100 mM $NaBH_4$. Lane 1 was loaded with DNA substrate containing a A·G mismatch treated with 100 mM $NaBH_4$. The reaction components and conditions are provided under Experimental Procedures. The covalently trapped protein−DNA complex was separated by 15% SDS−PAGE, and the gel was autoradiographed.

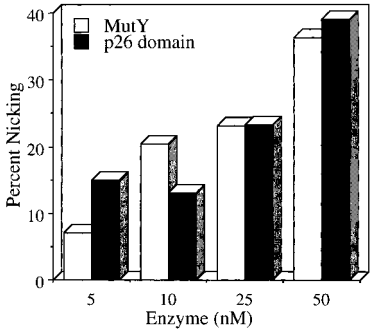

FIGURE 7: Activity of MutY and p26 on a DNA substrate containing a single abasic site. The DNA duplex containing a single abasic site at a defined position was prepared as described in Manuel et al. (1995). MutY or p26 at different concentrations (5, 10, 25, and 50 nM) was added to the substrate (5 nM) and incubated at 37 °C for 30 min. Nicked products were separated in a 15% denaturing polyacrylamide gel and quantified by PhosphorImager analysis.

of nicked products was more with increasing concentrations of MutY or p26. Up to 40% of the DNA substrate was nicked when the protein concentration was 50 nM (Figure 7). The increase in the percentage of nicking by the truncated version was comparable to that of the intact protein. This clearly demonstrates that phosphodiester bond nicking activity was not compromised by the deletion of 125 amino acids which constitute the C-terminal domain of MutY.

Specificity for DNA Containing an AP Site Analog or a Reduced AP Site. To demonstrate the specificity of p26 and MutY on DNA containing the AP site analog (THF) or the reduced AP site (sequence II and III, respectively), the protein was allowed to react with the DNA substrates and analyzed in nondenaturing polyacrylamide gels. The ability of the p26 domain to form a complex with the modified AP site containing DNA was investigated, and the efficiency of p26 to form this DNA−protein complex was compared to that of the intact protein (Figure 8). We also show that MutY and p26 failed to cleave duplex DNA containing a THF residue (see below). A single band with lower mobility establishes that a stable DNA−protein complex is formed. The addition of competitor DNA (duplex DNA with sequence II containing cytosine instead of THF) in 50 molar

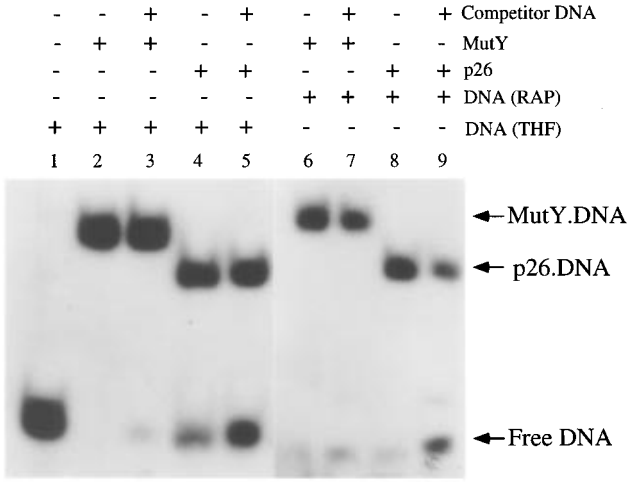

FIGURE 8: Gel mobility shift assay to demonstrate specific binding of MutY and p26 to DNA containing an AP site analog (THF) or a reduced AP site. DNA (1 nM) containing a single tetrahydrofuran (sequence II) was reacted with 50 nM MutY (lanes 2 and 3) or 100 nM p26 (lanes 4 and 5). DNA containing the reduced AP site (sequence III) was reacted with MutY (lanes 6 and 7) or p26 (lanes 8 and 9) at the same concentrations. To determine substrate specificity, nontarget DNA (sequence II with THF replaced by cytosine) was added as a competitor in 50 molar excess to lanes 3, 5, 7, and 9. Lane 1 did not contain any protein. BSA was added as a nonspecific protein competitor at a final concentration of 100 μg/mL in all the reactions. All reactions were carried out at 25 °C for 30 min. The protein−DNA complexes were separated in 8% native gels and autoradiographed.

excess reduced the binding of MutY to THF and RAP containing substrate by 5% and 10%, respectively (lanes 3 and 7), compared to the binding reactions without competitor DNA (lanes 2 and 6). The binding affinity of the p26 domain to the same substrates was reduced by 9% and 38%, respectively (lanes 5 and 9), compared to lanes 4 and 8 without the competitor. These results illustrate that the specificity of p26 for DNA containing modified AP sites is not altered significantly, except when p26 interacts with DNA containing a reduced AP site, where the affinity was reduced by 38% upon adding nontarget DNA in 50 molar excess. The concentration of p26 used in this experiment to bind the same amount of substrate (1 nM) was twice (100 nM) that of MutY (50 nM). p26 was used in excess, since we found that the K_d values for p26 on the same DNA substrates were higher than those for the intact protein as described below.

Dissociation Constants (K_d) of p26 and MutY. The dissociation constants of MutY and p26 on DNA substrates containing THF (sequence II) or a RAP (sequence III) were determined using gel-mobility shift assays. The amount of MutY required to bind 50% of the DNA substrate containing THF or RAP was 12.8 nM and 8.7 nM, respectively. The concentration of the catalytic domain of MutY (p26) required to bind 50% of the same DNA substrates was 37.5 nM and 22.5 nM, respectively, which indicates approximately 3-fold lower affinity than that of the intact MutY (Figure 9). The binding of MutY and p26 to nontarget DNA (sequence II containing cytosine instead of THF) was found to be in the 2 μM range (data not shown). This value was obtained by extrapolation, since the maximum binding we could achieve with 100 nM MutY or p26 was only about 2%.

Evidence for the β-Elimination Mechanism. Following cleavage of abasic sites, AP endonucleases and AP lyases

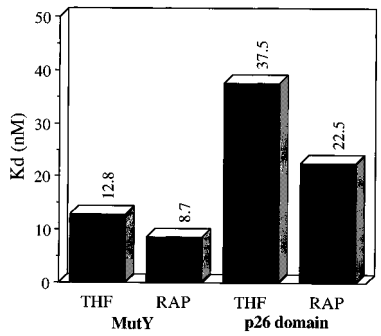

FIGURE 9: Apparent dissociation constants (K_d) for MutY and p26 on DNA containing an AP site analog (THF) or a reduced AP site. MutY (3−150 nM) and p26 (10−200 nM) were incubated with DNA containing an AP site analog and with DNA containing a reduced AP site. The DNA substrates are shown in sequences II and III in the text. The concentration of the substrate was 1 nM in all the reactions. The buffer conditions are provided under Experimental Procedures. Following incubation at 25 °C for 30 min, the protein−DNA complexes were separated by electrophoresis in 8% native polyacrylamide gels. The bands corresponding to the bound and free DNA were quantified by PhosphorImager analysis. The values of percent DNA bound to MutY or p26 were plotted versus enzyme concentration, and the K_d values are the enzyme concentration required to bind 50% of the DNA substrate.

can generate different 3′-termini in the nicked substrate. AP lyases generate a 3′-α,β-unsaturated aldehyde via a β-elimination reaction, and AP endonucleases leave behind a 3′-hydroxyl. AP endonucleases can also process the 3′-α,β-unsaturated aldehyde ends to yield a 3′-hydroxyl. The presence of the unsaturated aldehyde is expected to retard the mobility of the incised DNA fragment relative to the DNA fragment containing the 3′-hydroxyl. Labeling the 5′-end of the mismatched adenine-containing strand enabled us to visualize the difference in mobility of these oligonucleotide species in a 20% denaturing gel (Figure 10) following treatment of the substrate with p26 (lanes 5 and 7) or the intact protein (lanes 2 and 4). When the β-elimination reaction of an AP lyase is accompanied by δ-elimination, the deoxyribose moiety of the aldehydic abasic site is removed, resulting in a 3′-phosphate end. This DNA species will have faster mobility compared to the DNA fragment having a 3′-OH generated by an AP endonuclease. In the absence of a concerted β,δ-elimination reaction by an AP lyase, the δ-elimination reaction can be mimicked with the addition of piperidine (lanes 3 and 6). The results obtained from this experiment provide evidence favoring a β-elimination reaction mechanism for p26 and the intact protein.

p26 and Intact MutY Do Not Cleave DNA Containing a THF. In Figures 8 and 9, we demonstrated that both p26 and intact MutY bind to DNA containing THF residues. However, unlike the hydrolytic AP endonucleases, neither p26 nor the intact protein is able to incise an DNA duplex containing a single THF residue (Figure 11A). When the same DNA substrate (sequence II) was incubated with *E. coli* exonuclease III, it was completely cleaved at the THF position (data not shown).

p26 Does Not Acquire the Catalytic Activities of E. coli Endonuclease III and FPG. Deletion of the C-terminal domain (p13) of MutY does not render the enzyme active on DNA substrates specific for the structurally related *E. coli* endonuclease III and the functionally related FPG. Saturated pyrimidines like 5,6-dihydrouracil are known to be released by *E. coli* endonuclease III (*24*). Since p26 and

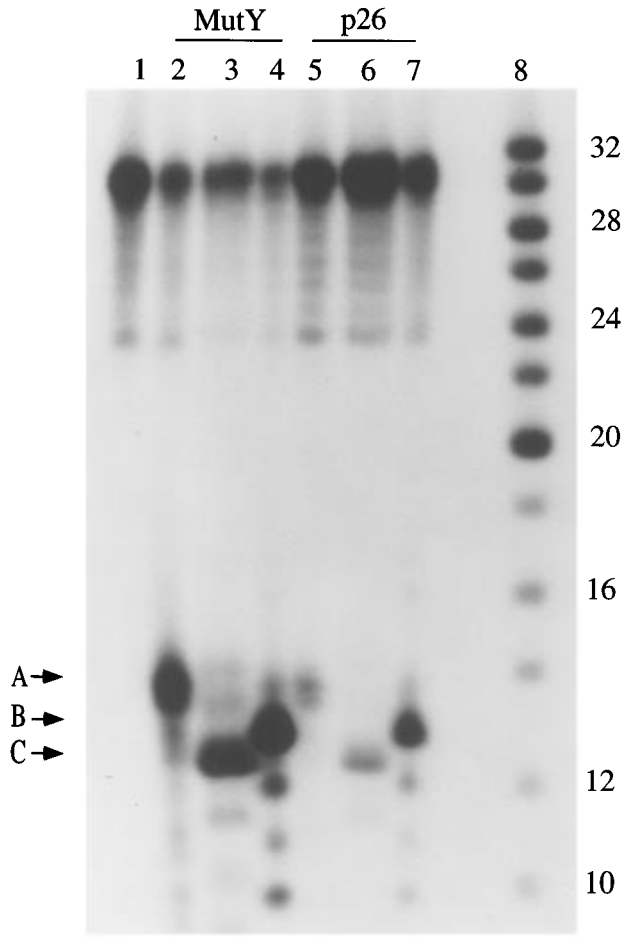

FIGURE 10: Autoradiograph showing the mobility of DNA fragments after treating DNA containing an A·G mismatch (sequence I) with p26 or MutY followed by incubation with piperidine or AP endonuclease. All reactions were performed with 10 nM DNA substrate. Lane 1, DNA substrate only. Lane 2, DNA treated with 50 nM MutY for 30 min at 37 °C. Lane 3, similar to lane 2 with additional incubation with piperidine for 15 min at 85 °C. Lane 4, similar to lane 2 with additional incubation with 50 nM AP endonuclease for 15 min at 37 °C. Lanes 5−7, similar to lanes 2−4 with 50 nM p26 substituted for MutY. Lane 8, oligonucleotide sizing markers. The reaction products were visualized by autoradiography after separating them through a 20% denaturing polyacrylamide gel. The reaction products are indicated in the margin with the following 3′-termini: A, 3′-α,β-unsaturated aldehyde terminus; B, 3′-hydroxyl terminus; C, 3′-phosphate terminus.

endonuclease III are highly conserved in their primary structure (*1, 20*) and tertiary structure based on molecular modeling (*20*), and are almost equal in size, one might expect them to have similar activities on DNA substrates with similar lesions. Using a 37-base pair oligonucleotide duplex containing a site-specific 5,6-dihydrouracil, we show that p26 does not have glycosylase or AP lyase activity on this substrate which is specific for endonuclease III (Figure 11C, lanes 4 and 5). MutY also was not able to cleave this substrate (lanes 2 and 3). The lack of glycosylase activity is evident from the absence of any nicked products after treatment with piperidine following the nicking reaction with MutY and p26 (Figure 11C, lanes 3 and 5, respectively). Endonuclease III was treated with the same substrate and had robust glycosylase and AP lyase activity (Figure 11C, lanes 6 and 7).

FPG removes 8-oxodG lesions from DNA when they are paired with cytosine. When this lesion remains in DNA

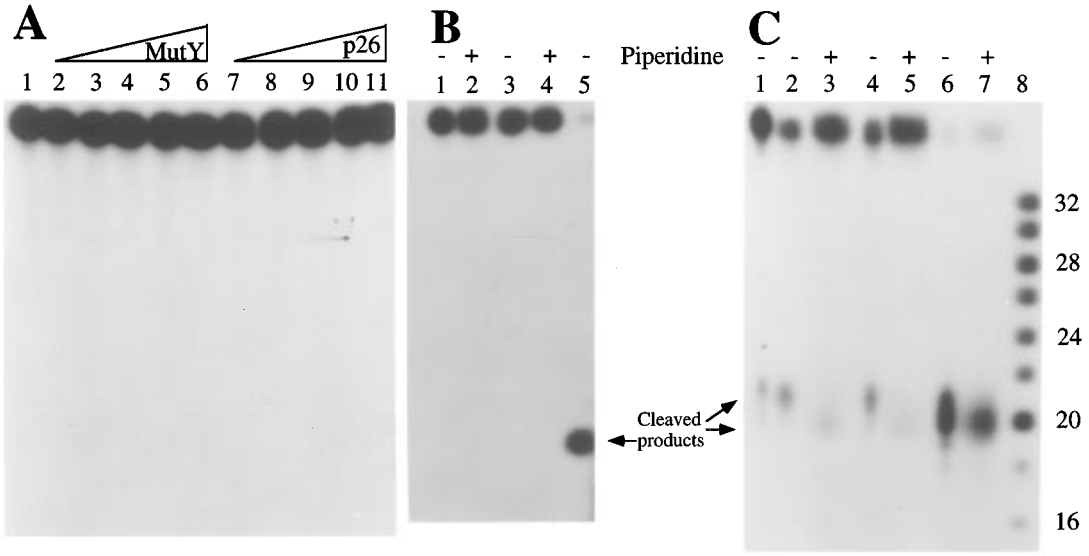

FIGURE 11: DNA substrates resistant to cleavage by MutY and p26. The photographs of autoradiograms shown in panels A, B, and C were obtained after separating the reaction products (described below) in 15% denaturing polyacrylamide gels. (A) Panel A shows the absence of any nicked product after treating DNA substrate (3.5 nM) containing a single tetrahydrofuran (sequence II) with increasing concentrations of MutY (lanes 2−6) or p26 (lanes 7−11). The range of MutY and p26 concentration was from 2.5 to 125 nM. In lane 1, the substrate was not treated with the protein. All reactions were incubated at 37 °C for 30 min in the buffer conditions described under Experimental Procedures. (B) An oligonucleotide (21-mer) containing a single 8-oxodG was 5′-end-labeled with [γ-^{32}P]ATP and annealed to its complementary strand containing a cytosine opposite 8-oxodG. The DNA substrate (2.5 nM) was treated with 25 nM MutY (lanes 1 and 2) for 30 min at 37 °C. In lane 2, the reaction was further incubated with piperidine for 15 min at 85 °C. Lanes 3 and 4, similar to lanes 1 and 2 except that 25 nM p26 was substituted for MutY. Lane 5, the DNA substrate described above was incubated with 25 nM FPG for 30 min at 37 °C. (C) An oligonucleotide (37-mer) containing a 5,6-dihydrouracil (dhU) at position 21 was 5′-end-labeled with [γ-^{32}P]ATP and annealed to its complementary strand containing a guanine opposite dhU. This DNA substrate (2.5 nM) was treated with 25 nM MutY (lanes 2 and 3), 25 nM p26 (lanes 4 and 5), or 25 nM *E. coli* endonuclease III (lanes 6 and 7). All reactions were for 30 min at 37 °C. Lanes 3, 5, and 7 were further incubated with piperidine for 15 min at 85 °C. Lane 8, oligonucleotide sizing markers. Lane 1, control lane containing DNA substrate only.

without being repaired, adenine can be incorporated opposite 8-oxodG in the next round of replication (*11*). MutY prevents further damage to DNA by removing the adenine mispaired with 8-oxodG. In this study, we show that the catalytic domain of MutY (p26) maintains its identity in the repair of 8-oxodG lesions. It does not remove 8-oxodG when it is mispaired with cytosine (Figure 11B, lanes 3 and 4) like the intact MutY (lanes 1 and 2). FPG can readily remove this 8-oxodG lesion and cleave the phosphodiester bond (lane 5).

DISCUSSION

Domain mapping studies using limited proteolysis have shown that MutY is comprised of two stable domains: an N-terminal domain (Met1−Lys225) and a C-terminal domain (Gln226−Val350). These domains were designated as p26 and p13, respectively (*20*). The p26 fragment, generated by proteolysis, was catalytically competent to incise adenine in DNA containing A·G and A·8-oxodG mismatches, and molecular modeling studies indicated that this region of MutY could have a tertiary structure with a similar fold to endonuclease III (*20*). In this study, the region of the *mutY* gene corresponding to the p26 domain was amplified by PCR, cloned, and overexpressed. The purified p26 domain was stable and was characterized for substrate recognition, binding, and mismatch repair activity. Since the p26 was overexpressed in an *E. coli* strain (CC104 *mutY*$^-$) where the chromosomal *mutY* gene is disrupted, all the functional activity observed is intrinsic to the p26 domain.

Mismatch Recognition and Cleavage by p26 and MutY. MutY does not have a wide range of substrate specificity like endonuclease III (*26*, *27*) and FPG (*28*). It is known to function primarily as an adenine glycosylase (*5*−*7*). However, MutY is known to recognize and cleave nucleotide analogs, and the significance of modified functional groups in substrate binding and catalysis has been investigated (*9*, *10*). These altered functional groups in the mismatched bases can potentially change the electronic environment in the mismatch, by affecting the hydrogen bond donor/acceptor properties. They could also induce steric variations in the mismatch which can reveal the potential contacts or interactions with the enzyme. Thus, using nucleotide analogs in the mismatched region is a valuable approach to study the geometric and electronic interfaces between the enzyme and the DNA substrate.

In this study, we have investigated the binding affinity and mismatch repair efficiency of the p26 domain lacking 125 amino acids at the C-terminus of MutY. Synthetic oligonucleotide substrates (sequence I) containing mismatches generated with adenine or other nucleotide analogs paired with guanine, 8-oxoguanine, or cytosine have been used in an attempt to probe the role of specific functional groups in mismatch recognition, binding, and catalysis. Two analogs of adenine and two analogs of guanine have been used (Figure 2A). In the adenine analog, nebularine (a purine riboside), the C-6 amino group has been eliminated. In 2-aminopurine, the C-6 amino group of adenine has been displaced to C-2. 8-Oxoguanine has an additional keto function at C-8, and in the guanine analog inosine (hypoxanthine), the C-2 amino group has been deleted. All the studies were conducted in parallel with the intact protein to

delineate the functional properties of the truncated p26 domain.

We show that p26 can recognize and bind to DNA containing a range of mismatches generated with adenine, guanine, or their nucleotide analogs used in this study (Figure 4). The mismatch containing 8-oxodG opposite adenine or other nucleotide analogs was recognized by p26 and the intact protein with equal affinity. This indicates that the 8-oxo function in deoxyguanine could be one of the determinants of specificity for this mismatch repair enzyme. This is significant because of its role in repairing oxidative lesions in DNA within biological systems. Previous kinetic studies have also demonstrated the significance of the C-8 keto group in substrate recognition and preferential binding of the intact MutY (*10*) and FPG (*28*). This also implies that the C-6 amino group of adenine is not critical in the recognition and binding by p26 to a A·8-oxodG mismatch. An equal or increased binding affinity was observed with p26 on DNA substrates containing a mismatch with inosine opposite guanine, 8-oxoguanine, or cytosine. Deletion of the C-terminal domain of MutY results in reduced affinity for DNA containing an A·G mismatch.

There were no absolute relationships between the binding affinity of p26 and the mismatch repair efficiency (Figures 4 and 5). The only correlation was that p26 recognized DNA containing mismatches opposite inosine and cleaved these substrates more efficiently than the intact protein. The catalytic properties were not compromised in the truncated MutY when nebularine was paired with guanine, 8-oxoguanine, or cytosine. These findings are in agreement with earlier studies with the intact protein (*9*). When adenine or nebularine was paired with 8-oxodG, p26 was able to cleave these DNA substrates as efficiently as the intact protein. When 2-aminopurine was paired with 8-oxodG, the nicking rate was reduced 5-fold. The low binding affinity to substrates containing a cytosine in the mismatch was also reflected in their nicking activity. However, when inosine was paired with cytosine, there was a significant amount of catalytic activity by p26. p26 did not cleave DNA with an A·C mismatch, although it did bind to this substrate with reduced affinity. The kinetic behavior of p26 on DNA containing an I·G or A·G mismatch (Table 1) provides additional evidence to believe that p26 is the catalytic domain of MutY. The K_m values were comparable for MutY and p26 on DNA containing an A·G or I·G mismatch. Although p26 was less efficient in removing an adenine opposite guanine, the specificity constant (K_{cat}/K_m) of p26 was 2-fold higher on DNA containing an I·G mismatch. There was a 5-fold increase in the specificity constant of p26 on DNA containing a inosine opposite guanine, compared with adenine opposite guanine. This is biologically relevant because inosine is known to be incorporated into DNA from the dNTP pool. A deoxyinosine 3′-endonuclease has been identified in *E. coli*, although this enzyme seems to have a broad specificity (*29*). It is clear from this study that p26 is the catalytic domain of MutY with subtle changes in substrate specificity.

We also show that p26 has not acquired the activity of endonuclease III (Figure 11C), despite their resemblance in size, primary sequence, and possibly in the tertiary structure. Finely crafted damage-specific features in the tertiary structure are likely to be responsible for distinguishing different lesions in DNA. We also show that p26 retains its functional identity in the combined defense mechanism against oxidative lesions. p26 was not able to remove 8-oxodG when paired with cytosine, which is a natural substrate for FPG (Figure 11B).

p26 and MutY on Abasic Sites. An abasic site in DNA consists of a 2-deoxyribose residue attached to the adjacent nucleotide through 3′- and 5′-phosphodiester bonds. Repair of an abasic site in DNA is a multistep process involving many enzymes. However, the cleavage of the phosphodiester bond at an AP site can be accomplished by a single enzyme which has AP lyase activity (*22*). Since the mismatch repair assays in this study suggested that the p26 and the native MutY have an associated phosphodiester bond scission activity, we proceeded to determine whether the pure enzyme was competent to incise DNA at AP sites. A synthetic DNA substrate with a site-specific AP site was cleaved by p26 and the intact protein. The amount of nicked products increased with increasing enzyme concentrations, and the catalytic activity was comparable between p26 and the intact protein (Figure 7). This adds further credence to the claim that p26 is the catalytic domain of MutY.

To obtain the kinetic properties of the protein−DNA interaction, we needed a substrate which was not cleavable and was specific for the enzyme. Determination of equilibrium dissociation constants with cleavable substrates may not be accurate, as the equilibrium changes constantly during the course of the reaction. If cleavable substrates are used, the nicked products should be accounted for while determining the K_d, and the binding constants should be interpreted in relation to the specificity constants (K_{cat}/K_m) of the enzyme with those substrates. This problem can be overcome by obtaining dissociation constants with catalytically compromised mutant enzymes or with modified substrates which cannot be metabolized. In this study, we have avoided using DNA substrates which can be incised by p26 or the intact protein. Since AP lyase activity was consistently observed in our mismatch repair assays with p26 and MutY, in addition to having activity on abasic sites, we chose synthetic DNA substrates containing an AP site analog (THF) or a reduced AP site (Figure 2B). The tetrahydrofuran moiety is isosteric with 2′-deoxyribofuranose and serves as a stable structural analog to the natural AP site (*30*). The reduced deoxyribose moiety is incapable of ring closure and yields a noncleavable substrate. DNA's containing these modified AP sites can serve as ideal substrates to determine K_d values, as they are not metabolized in the course of the reaction. We show that p26 and MutY were unable to cleave a DNA substrate containing a THF residue (Figure 11A). Cleavage on the reduced AP site was not expected, as the aldehyde of the deoxyribose is reduced to an alcohol. Binding constants have been obtained previously for FPG on DNA containing a reduced AP site (*31*), and it is also known that DNA containing a reduced AP site is not cleaved by FPG (*32*).

Gel-retardation assays indicate that a strong complex is formed with DNA containing an AP site analog or a reduced AP site. The dissociation constants of p26 and MutY on the above substrates range between 8.7 nM and 37.5 nM (Figure 9). To evaluate the specificity of p26 on DNA substrates containing the modified AP sites (THF and RAP), competitor DNA was added in 50 molar excess, and this reduced the affinity of p26 on THF containing substrate and RAP containing substrate by 9% and 38%, respectively (Figure 8). With the intact MutY, the corresponding

reduction in specificity was 5% and 10% (Figure 8).

We also evaluated the affinity of p26 and MutY to nontarget DNA. The K_d was estimated to be about 2 μM, and this is comparable to the K_d for another DNA repair enzyme, T4 endonuclease V, on nontarget DNA (*33*). In a recent study (*10*), it was reported that the binding affinity of MutY to nontarget DNA was greater than that to DNA containing an A·G mismatch and only 4-fold less than that to DNA containing an A·8-oxodG mismatch. This finding is difficult to reconcile with the function of MutY in the recognition of these mismatches in vivo. By the addition of competitor DNA and competitor enzyme, we have demonstrated that MutY and p26 can specifically bind to target DNA with much greater affinity.

p26 and MutY Are Glycosylase/AP Lyases. A few reports classify MutY as a simple glycosylase (*5*, *10*, *34*), while others have shown it to be a glycosylase with 3′ AP "endonuclease" or lyase activity (*9*, *20*, *21*, *35*−*37*). It appears that the catalytic properties of MutY may depend on the procedures used during purification. This is evident from an earlier report from our laboratory (*24*) where we were able to confirm the results of Drs. J. H. Miller and M. L. Michaels when we used the enzyme prepared in their laboratory. However, several preparations of the intact MutY and the p26 domain from our laboratory consistently show glycosylase activity accompanied by AP lyase activity.

This discrepancy has to be resolved in order to decipher the catalytic mechanism of this mismatch repair enzyme. AP sites in duplex DNA are substrates for AP endonucleases and AP lyases. However, the products of cleavage by these two groups of enzymes differ. AP endonucleases hydrolytically incise the phosphodiester backbone of DNA at the 5′ side of the AP site by P−O bond cleavage, leaving a 3′-OH and a 5′-deoxyribose phosphate (*38*, *39*). The 5′-deoxyribose 5-phosphate has to be removed by deoxyribophosphodiesterase (dRpase) to generate a single-nucleotide gap (*40*), which is required for repair synthesis. On the contrary, AP lyases cleave the phosphodiester bond 3′ to the abasic site via a β-elimination reaction, leaving a 3′-α,β-unsaturated aldehyde and a 5′-phosphate by cleaving the C−O bond (*41*−*43*). The 3′-α,β-unsaturated aldehyde is removed by an AP endonuclease to generate a 3′-hydroxyl, which will generate a suitable substrate for 3′−5′ exonuclease activity of DNA polymerase. AP lyases leave a different sugar−phosphate product (*trans*-4-hydroxy-2-pentanal 5-phosphate) at the 3′ incised AP site, and it has been shown in *E. coli* that dRpase can release this sugar−phosphate (*44*).

In this study, we provide several lines of evidence to show that the p26 catalytic domain and the intact MutY have glycosylase and AP lyase activities and that the AP site nicking reaction proceeds via β-elimination: (1) The addition of piperidine after reacting the DNA substrate with p26 does not increase the amount of cleaved products. This was observed in all the cleavable DNA substrates containing mismatches with regular bases or nucleotide analogs (data not shown). This was shown earlier with intact MutY on DNA containing an A·G or A·8-oxodG mismatch (*20*). (2) Intact MutY and p26 have strong affinity (K_d range = 9−37 nM) for DNA containing AP sites which are noncleavable (Figure 9). In addition, they were able to cleave DNA containing an unmodified AP site (Figure 7). AP site nicking activity and affinity toward DNA containing AP site analogs are generally observed with DNA glycosylases/AP lyases. (3) Nicked products were not observed when p26 or MutY were incubated with DNA containing an AP site analog, THF (Figure 11A). In contrast, *E. coli* exonuclease III and endonuclease IV, the primary AP endonucleases in *E. coli*, are known to cleave DNA containing THF (*45*). (4) All mismatch repair activity assays were performed in the presence of 1 mM EDTA, and it has been shown that EDTA inactivates exonuclease III (*46*). (5) Through silver-stained gels, we have established that there is no contamination of exonuclease III or endonuclease IV to catalyze an AP endonuclease activity. In addition, by Western blot analysis we find that our preparation of the p26 domain does not react with anti-exonuclease III antibody (data not shown). (6) As shown in Figure 6, a covalent enzyme−DNA imino intermediate was formed when p26 or intact MutY was treated with DNA containing different mismatches. A β-elimination reaction is generally associated with the formation of this covalent imino intermediate (*47*). (7) When DNA containing an AP site is cleaved by an AP lyase, we expect the DNA fragment to have an α,β-unsaturated aldehyde at the 3′ terminus, and when this reaction product is further processed by an AP endonuclease, we expect a DNA fragment with a 3′-hydroxyl. In this study, we were able to resolve the difference in mobility between these cleaved products containing different 3′ termini (Figure 10), demonstrating that p26 and the intact protein proceed, via the β-elimination reaction mechanism, in the repair of an A·G mismatch in DNA.

It has been reported that abasic sites in DNA can selectively decompose by heat to yield 3′-termini containing an α,β-unsaturated aldose and 5′-phosphate termini by β-elimination (*48*). However, in earlier studies (*9*, *36*), nicked products were detected in a native gel during gel-shift analysis, where the samples were not heated, suggesting that this may not necessarily be the case. Additionally, when MutY was originally isolated, it was shown that A·G to C·G repair can be reconstituted with MutY, AP endonuclease, DNA polymerase I, and DNA ligase I (*34*). With our current understanding of the repair of substrates nicked by 5′-AP endonucleases, we conclude that a dRpase or lyase is required to remove 5′-deoxyribose 5-phosphate to create a suitable substrate for DNA polymerase I. Therefore, A·G to C·G repair would not have been efficient or possible without providing a dRpase or AP lyase if the AP site left behind by MutY was cleaved by a 5′-AP endonuclease. Thus, we consider it is likely that MutY cleaved the phosphodiester bond through a β-elimination mechanism and the 5′-AP endonuclease created a 3′-OH terminus to enable repair synthesis. The above experimental evidence, combined with other observations made here, suggests that the mechanism of AP site nicking by p26 and MutY is via a β-elimination reaction.

Reaction Mechanism. A catalytic mechanism integrating all DNA glycosylases and DNA glycosylase/AP lyases has been proposed (*47*). These two groups of enzymes differ in their reaction mechanism by the type of nucleophile used to attack C-1′ of the damaged/mismatched base. When the phosphodiester bond incision is at the same rate as the glycosylase step, it is postulated that a primary amine acts as a nucleophile, resulting in an imino enzyme−DNA intermediate. This type of mechanism can be diagnosed by reducing the intermediate with $NaBH_4$ and isolating the covalent complex in a SDS−polyacrylamide gel (*23*−*25*).

The class of enzymes which lack the lyase activity may use a nucleophile from the medium such as an activated water molecule. In this mechanism, there are no enzyme−DNA covalent intermediates formed, and therefore an enzyme−DNA complex cannot be isolated under denaturing conditions. In fact, trapping the covalent protein−DNA complex with $NaBH_4$ has been used as a diagnostic tool to distinguish bifunctional glycosylase/lyases from monofunctional glycosylases (*24*, *49*).

With regard to the reaction mechanism of MutY, we bring additional data to the earlier report (*10*) which postulates that MutY is a monofunctional glycosylase having a reaction mechanism similar to that of AlkA, using a nucleophile from the medium (*50*). This proposal was based solely on the inability to isolate the imino enzyme−DNA intermediate in the presence of $NaBH_4$. Although our data do not address directly the glycosylase reaction, at least relative to the AP lyase step, we have been able to isolate this covalent complex (Figure 6) by reacting the p26 domain or the intact protein with DNA containing mismatches. Another study has also been successful in isolating the covalent intermediate between MutY and DNA containing A·8-oxodG or A·G mismatch (*36*). The ability to trap the p26 domain with different mismatches, as a covalent complex, adds additional evidence to establish p26 as the catalytic domain of MutY.

In classical glycosylase/AP lyases, the amine nucleophile which attacks the glycosidic bond that connects the damaged base to the sugar moiety has been identified. The amine nucleophile of FPG is the N-terminial proline (*25*), and in T4 endonuclease V, it is the N-terminal threonine (*51*, *52*). In endonuclease III, it is postulated that Lys120 is the primary amine involved in this mechanism (*53*). When the primary sequence of MutY or p26 is aligned with endonuclease III, we find that Lys120 of endonuclease III is not conserved in MutY (*1*). In fact, this allowed us to speculate that MutY is a simple glycosylase. Structural analysis of p26 or the intact protein by X-ray crystallography and site-directed mutagenesis studies will reveal the primary amine that acts as a nucleophile in the AP lyase reaction mechanism.

It is becoming more evident that base flipping is the common mechanism by which mismatched or damaged bases are removed from DNA (reviewed in *54*, *55*). The enzyme flips the base extrahelical to facilitate the cleavage of the glycosyl bond. This mechanism has been illustrated with uracil−DNA glycosylase (*56*−*58*), DNA cytosine-5-methyltransferase (*59*), and methyltransferase *Hae*III (*60*). In T4 endonuclease V, the base opposite the target site is flipped out (*61*). The X-ray crystal structure of *E. coli* endonuclease III exhibits the structural requirements to accommodate a flipped out base within the substrate binding pocket (*53*, *62*). However, this mechanism will only be confirmed for endonuclease III by solving a co-crystal structure. Also, in endonuclease III, the two residues (Lys120 and Asp138), presumed to be involved in catalysis, embrace the interdomain cleft that could accommodate an extrahelical base (*53*). The molecular model of p26 (20) shows a tertiary fold similar to endonuclease III, indicating that p26 may possess this interdomain pocket required to bind DNA and flip the mismatched adenine extrahelical. Based on the molecular model, we also find that Asp138, which is conserved between endonuclease III and MutY, is in the vicinity of this interdomain cleft. The different rates of catalytic activity seen by p26 and the intact protein on different substrates containing the nucleotide analogs and regular bases may depend on the relative ease in flipping out the target base and the spatial constraints required within the binding pocket of the enzyme to accommodate the flipped out base. MutY may very well use the flip out mechanism to discriminate between mismatches and remove the mismatched adenine in biological systems. To demonstrate this novel mechanism, we have to engineer catalytically deficient enzymes with enhanced binding and obtain enzyme−substrate co-crystals.

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