An Escherichia coli MutY Mutant without the Six-helix Barrel Domain Is a Dimer in Solution and Assembles Cooperatively into Multisubunit Complexes with DNA*

Chih-Yung Lee‡, Haibo Bai§§, Rebecca Houle §§, Gerald M. Wilson §§§, and A-Lien Lu §§§

From the ‡Department of Biochemistry and Molecular Biology, the §Combined Ph.D. Program in Biochemistry, and the ¶Center for Fluorescence Spectroscopy, Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland 21201

Escherichia coli MutY is an adenine and weak guanine DNA glycosylase involved in reducing the mutagenic effects of 7,8-dihydro-8-oxoguanine (GO). MutY contains three structural domains: an iron-sulfur module, a six-helix barrel module with the helix-hairpin-helix motif, and a C-terminal domain. Here, we demonstrate that the mutant MutY(Δ26–134), which lacks the six-helix barrel domain, cannot complement the mutator phenotype of a mutY mutant in vivo. However, the mutant can still bind DNA and has weak catalytic activity at high enzyme concentrations. The mutant is a dimer in solution and assembled into two and multiple (up to five) complexes with 20- and 44-bp DNA fragments, respectively, in a concentration-dependent manner. Higher order complexes with DNA substrates containing A/GO mismatches were formed at lower protein concentrations than with the A/G mismatch and homoduplex DNA. Measurement of equilibrium binding using fluorescence anisotropy showed that the mutant protein retains some specificity for A/GO-containing DNA substrates and that the binding event is highly cooperative. This is consistent with the MutY structure determined, which indicates that GO specificity is contributed by both the six-helix barrel and C-terminal domains. The non-specific binding of MutY(Δ26–134) to DNA suggests a model in which the specific binding of mismatched DNA by MutY involves sequential interactions, in which one MutY molecule scans the DNA and enhances binding of another MutY molecule to the A/GO mismatch.

DNA bases are subjected to oxidative damage from cellular metabolism as well as exogenous stimuli such as ionizing radiation and degenerative conditions, including aging and cancer. 7,8-Dihydro-8-oxoguanine (GO) is one of the most stable products of oxidative DNA damage and has the most deleterious effects because it can mispair with adenine during DNA replication (1, 3). Several repair pathways are involved in the repair of DNA lesions caused by oxidative stress (4). In Escherichia coli, MutM, MutS, MutT, and MutY are involved in defending against the mutagenic effects of GO lesions. The MutT protein eliminates 8-oxo-dGTP from the nucleotide pool with its parapophosphohydrolase activity (5), whereas the MutM glycosylase (Fpg protein) removes both mutagenic GO adducts and ring-opened purine lesions (6). When C/GO mismatches are not repaired by MutM, adenines are frequently incorporated opposite GO bases during DNA replication (7, 8) and can subsequently cause G to A transversions (8, 9). MutS and MutY are involved in increasing replication fidelity by removing the adenine misincorporated opposite GO or G (2, 10–12). The MutS-dependent mismatch repair removes mismatched A on the daughter DNA strands (13). MutY is an adenine and weak guanine DNA glycosylase active on A/G, A/AC, or G/GO mismatches (11, 14–17). AGO mismatches are particularly important biological substrates of MutY glycosylase.

Proteolysis of MutY demonstrated that its catalytic activity resides within the N-terminal domain (18, 19). The catalytic domain of the MutY(D138N) mutant protein consists of iron-sulfur and six-helix barrel modules (20) and shares structure similarity with AlkA (3-methyladenine DNA glycosylase II), endonuclease III, and OGG1 (7,8-dihydro-8-oxoguanine glycosylase-1) (20–22). The x-ray crystal structure of the MutY catalytic domain shows that the bound adenine is buried in the active-site pocket, suggesting that the mismatched adenine must flip out of the DNA helix for glycosylase action (20). The C-terminal domain of MutY has structural similarity to MutT (23, 24) and plays an important role in the recognition of GO lesions (15, 18, 23). The structure of MutY from Bacillus stea-thermophilus in complex with A/G-containing DNA was recently determined (25). Similar to endonuclease III, AlkA, and OGG1 (21, 22, 26), MutY distorts the bound DNA and flips substrate bases out of the helix. All three domains of MutY wrap around the DNA substrate. The mismatched GO remains intrahelical and is sandwiched by the six-helix barrel and the C-terminal domains.

One central issue concerning MutY function is how MutY searches for the mismatches within a vast excess of normal DNA in the genome. Although the MutY-DNA crystal structure offers some insight into the preferential recognition of GO and the binding event preceding catalysis (25), the search mecha-

*This work was supported by NIGMS Grant GM05132 (to A-L. L.) and NCI Grant CA102428 (to G. M. W.) from the National Institutes of Health and by National Center for Research Resources Grant P41 RR 08119 from the National Institutes of Health to the Center for Fluorescence Spectroscopy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 108 N. Greene St., Baltimore, MD 21201. Tel.: 410-706–4356; Fax: 410-706-1787; E-mail: aluchang@umaryland.edu.

¶The abbreviations used are: GO, 7,8-dihydro-8-oxoguanine; FI, fluorescence; FRET, fluorescence resonance energy transfer; A/GO44, for example, 44-mer A/GO-containing DNA; Δ-DNA, MutY(Δ26–134) mutant protein-DNA complex; Y-DNA, intact MutY-DNA complex; PD, P,D, and P,D, monomer-DNA, dimer-DNA, and tetramer-DNA complexes, respectively.

This paper is available online at http://www.jbc.org

52653
nism remains unclear. To further investigate the substrate recognition of MutY on A/GO-containing DNA, we have constructed the mutant MutYΔ26–134, without the six-helix barrel domain. The six-helix barrel module with the helix-hairpin-helix motif has been shown to directly contact the backbone of the GO strand and has substantial interaction with the C-terminal domain (25). This MutY deletion mutant has no in vivo activity. In solution, the mutant protein is a dimer, whereas intact MutY is a monomer as measured by both gel filtration and sedimentation velocity. To our surprise, the mutant binds to DNA and has very weak catalytic activity. Upon gel mobility shift assay, the mutant-DNA complex migrated slower than Y-DNA. The mutant protein could form two and multiple complexes with 20- and 44-bp DNA fragments, respectively, but higher order complexes with DNA substrates containing A/GO were formed at lower protein concentrations than with those containing A/G or homoduplex DNA. Measurement of equilibrium binding between the mutant protein and fluorescent DNA substrates by fluorescence anisotropy showed that the binding event is highly cooperative and that the specificity for A/GO mismatches is partially retained in the mutant. These data support a model in which MutY scans the DNA cooperatively as a dimer or a multimeric complex to locate base-base mismatches.

**EXPERIMENTAL PROCEDURES**

**Bacteria**—E. coli strain GM7724 is a MutY knockout strain derived from AB1157 (ara14 argE3 lux-gpt-proA62 galK2 hisG4 kgdK5 leuB6 lacY1 mtl-1 rac) (27). The mtl-1 gene was replaced with a chloramphenicol resistance gene (Cam) by Jen-Yen Wang in the laboratory of Dr. Michael Volkert and was moved into AB1157 by Dr. Martin G. Marinus. Strain CC101 containing a lacZ mutation at residue 461 of β-galactosidase and its derivative CC101mutM-mini kan mutY-mini-Tn10 were derived from Dr. Jeffery H. Miller. DE3 lyogenic strains were constructed according to the procedures described by Ingraveson.

**Construction of the MutYΔ26–134 Mutant**—The mutant mutY gene was constructed by the PCR splicing overlap extension method (27). The first PCR with pMYW-1 (28), which contains the mutY gene, as template and primers ChangYGSTF (5′-GGCGCGCTTGGTGACCCAGCGACGCGCTGCGACGCGGCGACGCAGGGAGTTTCTTCCCGCCCGG) and Chang349 (5′-GGCCGAGGCGGAGGGCCGATCCTGGATGACCAACTCGAGTGTA) generated PCR fragment 1, which contains the first 25 amino acid residues of MutY with an extension of 14 nucleotides covering residues 135–138. The second PCR with pMYW-1 as template and primers Chang377 (5′-TCGAGAATCGTGGGCGACGAGGCGACGACGCCGGCGACGCCAGGGAGTTTCTTCCCGCCCGG) and Chang576 (5′-TCGAGAATCGTGGGCGACGAGGCGACGACGCCGGCGACGCCAGGGAGTTTCTTCCCGCCCGG) generated PCR fragment 2, which contains amino acid residues 135–350 of MutY with an extension of 14 nucleotides covering residues 22–25. PCR fragments 1 and 2 contain a 28-bp overlapping region and were used as templates for the third PCR with primers ChangYGSTF and Chang349. The final PCR product was digested with BamHI and XhoI and ligated into the BamHI/XhoI-digested fragment and transferred into pET21a (Novagen) to generate plasmid pETΔ26–134. The sequence of pETΔ26–134 was confirmed by DNA sequencing. The mutY gene in pETΔ26–134 was isolated as a BamHI/Xhol-digested fragment and cloned into pET21a (Novagen) to generate plasmid pETΔ26–134.

**Protein Expression and Purification**—E. coli strain GM7724DE3 harboring expression plasmid pETΔ26–134 was grown in LB broth containing 100 μg/ml ampicillin at 37 °C. The host GM7724DE3 cell was the chromosomal mutY gene replaced with a transposon and does not contain any MutY activity. Protein expression was induced at A<sub>600</sub> = 0.6 by adding isopropyl β-D-thiogalactoside to a final concentration of 0.4 mM to the culture at 20 °C. The cells were harvested 16 h after induction. The mutant protein that was purified using a strategy similar to that employed for the wild-type MutY enzyme (29). Cells (11 g of cell paste) were resuspended in 45 ml of buffer A (20 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, 0.5 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 100 mM KCl and disrupted with a bead beater. In brief, the MutYΔ26–134) protein was purified by 45% ammonium sulfate precipitation and phosphocellulose, hydroxyapatite, and heparin chromatography. Fraction IV (the pool from the hydroxylapatite column) was loaded onto a 1-mL High-Trap heparin column (Amersham Biosciences) equilibrated with buffer A containing 50 mM KCl and 10% glycerol. Upon washing with 5 ml of the equilibration buffer, the column was eluted with a 30-ml linear gradient of KCl (0.05-0.7 M) in buffer A. Two MutY peaks were observed on the heparin column: one eluted at 0.23 M KCl, and the other eluted at 0.4 M KCl. Fractions containing the majority of the MutY protein that eluted at 0.4 M KCl were pooled (Fraction VIB), divided into small aliquots, and stored at −80 °C. Protein concentration was determined by the method of Bradford (30).

**Oligonucleotide Substrates**—The DNA substrates used in this study were as follows: 19-mer, 5′-CCCGAGGAATTTXCGCTTGTCTG-3′ (top) and 3′-GCTCTTAAAYCGGAGAACG-5′ (bottom strand); and 40-mer, 5′-ATTGGGCCTTCTCGGAAATTTGCGCTTGGACGGCTG-3′ and 3′-GGCGAGAACTTAAAYCGGAGAACGCGCTTCG-5′ (bottom strand) (where X = A or C and Y = G or O). The top strands were labeled at 5′-end with [γ<sup>32</sup>P]ATP and polyacrylamide and kinase then annealed with the bottom strands. The single-stranded overhangs were filled in with the Klenow fragment of DNA polymerase I and unlabeled deoxynucleotide triphosphates as described by Lu et al. (10). Radioactively labeled DNA substrates were used for gel mobility shift, trapping, and glycosylase assays. For fluorescence anisotropy assays, a fluorescein (Fl) tag (6-carboxyfluorescein) was linked to the 5′-end of the top strand of the 19-mer oligonucleotide. Applicable DNA substrates are indicated by the prefix “Fl.” For fluorescence resonance energy transfer (FRET) assays, the top strand of the 19-mer oligonucleotide containing the mismatched adenine was linked with Cy3 at either the 5′- or 3′-end. DNA oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

**MutY Gel Mobility Shift Assays**—The MutY binding assay with labeled DNA was performed as described (33) with some modifications. The MutY binding reaction mixture contained 20 mM Tris-HCl (pH 7.6), 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 2.9% glycerol, 20 ng of poly(dC·dC), and 1.8 fmol of labeled DNA (90 pmol) in a total volume of 20 μl. After incubation at 37 °C for 30 min, the mixture was supplemented with 2 μl of 50% glycerol and analyzed on a 6% polyacrylamide gel in 50 mM Tris borate (pH 8.3) and 1 mM EDTA. The fluorescence anisotropy assay was performed by incubating the reactions with 0.2 μM MutY and the appropriate labeled DNA substrates. Fluorescence anisotropy was measured with a Cary Eclipse Spectrophotometer (Varian). The fluorescence anisotropy (r) was calculated according to the equation:

\[
\text{r} = \frac{I_h - I_v}{I_h + 2I_v}
\]

where I<sub>h</sub> and I<sub>v</sub> are the horizontal and vertical fluorescence intensities, respectively.

**MutY Trapping and Glycosylase Assays**—Covalent complexes of MutY with DNA substrates were formed by the trapping assay in a 10-μl reaction containing 20 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, 1 mM EDTA, 2.9% glycerol, and 0.1 mM NaBH<sub>4</sub>. A stock solution of NaBH<sub>4</sub> was diluted with water, and 0.1 μl of this solution was added to each reaction (100 μM final concentration). After incubation at 37 °C for 30 min, 2.5 μl of 5-fold concentrated dye buffer containing 25% glycerol, 5% SDS, 155 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, and 0.5 mg/ml bromophenol blue was added to the samples, which were heated at 90 °C for 3 min and separated on a 12% polyacrylamide gel in the presence of SDS according to Laemmli (32).

The glycosylase assay was carried out similarly to the trapping assay, except that 50 μg/ml bovine serum albumin was added, and NaBH<sub>4</sub> was omitted. After incubation at 37 °C for 30 min, the reaction mixtures were supplemented with 1 μl of 1 M NaOH and heated at 90 °C for 30 min. Five μl of formamide dye (90% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) was added to the sample, which was heated at 90 °C for 2 min; and 5 μl of the mixture was loaded onto a 14% polyacrylamide gel containing 7% urea.

**Fluorescence Anisotropy Assays**—Fluorescence anisotropy was measured using the Beacon 2000 variable temperature fluorescence polarization system (Panvera, Madison, WI) equipped with fluorescein excitation (490 nm) and emission (535 nm) filters. Unless otherwise noted, binding reactions were performed as described for gel mobility shift assay. The concentration of MutY protein that was added, the DNA concentration was 2 μM, and the reaction volume was 100 μl. Each sample was read as a blank prior to addition of Fl-DNA. After adding Fl-DNA, samples were incubated for 1 min before measurement of anisotropy (r<sub>0</sub>) and total fluorescence emission (F<sub>0</sub>). Preliminary on-rate analyses indicated that anisotropic equilibrium was reached within 15 s under these conditions (data not shown). Each data point represents the mean of 10 measurements for each binding reaction.
MutY binding to DNA substrates was considered in terms of a binding model in which a single fluorescent DNA substrate (Fl-DNA) may be sequentially targeted by multiple protein molecules (P) to yield a saturated protein-DNA complex (Equation 1).

\[
\text{Fl-DNA} + P \Rightarrow \text{Fl-DNA-P}
\]

\[
\text{Fl-DNA-P} + P \Rightarrow \text{Fl-DNA-P}_2
\]

(Eq. 1)

\[
\text{Fl-DNA-P}_1 + P = \text{Fl-DNA-P}_2
\]

Association of the MutY protein with Fl-DNA substrates was detected by an increase in the anisotropy of the Fl-DNA emission because of restricted segmental motion and retarded rotational correlation time in the protein-DNA complexes relative to the free DNA substrate (33–35). However, association of MutY proteins with Fl-DNA substrates also induced a concomitant decrease in the fluorescence quantum yield (Q), thus diminishing the contributions of protein-bound Fl-DNA to Am relative to unbound Fl-DNA. To correct for this effect, fractional contributions of bound and unbound Fl-DNAs to relative fluorescence quantum yield change concomitantly as a function of [P], suggesting that fractional concentrations of intermediate peptide-Fl-DNA complex; [P]

\[
A_	ext{DNA} = A_	ext{DNA} + (A_	ext{DNA}/A_	ext{complex})Q_	ext{DNA}/Q_	ext{complex}
\]

where \(A_	ext{DNA}\) and \(A_	ext{complex}\) are the measured anisotropy values of the free Fl-DNA and the saturated protein-Fl-DNA complex (Fl-DNA-P), respectively, solved as the averaged \(A_m\) of at least three independent samples lacking protein (\(A_{DNA}\)) or in the presence of saturating protein concentrations (\(A_{complex}\); \([P] > 700 \text{ nM}\)). Averaged \(F_{P50}\) values for comparable samples resolved the quantum yield ratio (\(Q_{DNA}/Q_{complex}\)). Although this model does not explicitly assess contributions of intermediate peptide-Fl-DNA complexes to \(A_m\), its utility for approximation of multistep MutY/Fl-DNA equilibria in this study is supported by (i) the cooperative nature of binding events with respect to [P], which ensures that fractional concentrations of intermediate peptide-Fl-DNA complexes remain low; and (ii) observations that fluorescence anisotropy and quantum yield change concomitantly as a function of [P], suggesting that individual protein binding events may simultaneously influence both emission parameters.

Although it was not possible to assess binding constants describing individual steps of oligomeric protein assembly on Fl-DNA substrates by this model, indices of binding affinity and cooperativity were estimated by Equation 4.

\[
A_m = A_	ext{DNA} + (A_	ext{DNA}/A_	ext{complex})Q_	ext{DNA}/Q_	ext{complex}
\]

(Eq. 2)

where \(A_m\) and \(A_	ext{complex}\) are the corrected anisotropy values of the unbound and protein-saturated Fl-DNA substrates, respectively; \([P]_{50}\) is the protein concentration yielding half-maximal DNA saturation; and \(h\) is the Hill coefficient. Nonlinear regression analyses of \(A_m\) data sets taken across at least 20 protein concentrations were performed using PRISM Version 3.0 (GraphPad, San Diego, CA).

**FRET Analyses—**Protein-DNA binding reactions were assembled as described for fluorescence anisotropy analyses (above), except that the A/GO-containing Fl-DNA substrate (5 nm) was mixed with an equal amount of unlabeled or 5'- or 3'-Cy3-labeled A/GO-containing DNA and then incubated with 500 nm MutY (26–134) or MutY protein at 37 °C for 5 min in a 60-μl reaction mixture. For controls, unlabeled A/GO-containing DNA substrate (5 nm) was mixed with an equal amount of 5'- or 3'-Cy3-labeled A/GO-containing DNA in similar reactions. Potential co-localization of DNA substrates containing fluorescent donor (Fl) and acceptor (Cy3) moieties was assessed by measurement of FRET efficiency (\(E_{\text{FRET}}\)) derived from the decrease in emission of the A/GO-containing Fl-DNA substrate (\(A_m = 490 \text{ nm}\) and \(A_m = 518 \text{ nm}\)) in the presence of Cy3-labeled DNA (\(F_{\text{DNA}}\)) relative to samples containing the donor alone (\(F_{\text{D}}\)) using Equation 5 (37, 38).

\[
E_{\text{FRET}} = \frac{F_{\text{D}} - F_{\text{D}}}{F_{\text{D}}}
\]

(Eq. 5)

If protein-DNA complexes include more than one DNA molecule, an equimolar mixture of A/GO-containing Fl-DNA and Cy3-conjugated DNA substrates would result in donor-acceptor co-localization in at least 50% of all cases. Accordingly, the maximal scalar distance \(r_0\) between Fl and Cy3 moieties in such complexes could be resolved by Equation 6,

\[
E_{\text{FRET}} = 0.5 \left( R_0^2/R_0^2 + r^2 \right)
\]

(Eq. 6)

where \(R_0\) is the distance yielding \(E_{\text{FRET}}\) of 50%. For the Fl donor-Cy3 acceptor pair linked to DNA, \(R_0\) has been calculated as 55.7 Å (39). All fluorescence readings were taken using a Cary Eclipse fluorescence spectrophotometer (Varian Instruments, Walnut Creek, CA) with the Peltier multicell holder and temperature controller accessories.

**Gel Filtration Analysis—**A Superose 12 10/300 gel filtration column (24 ml; Amersham Biosciences) was equilibrated with buffer A containing 200 mM KCl and 10% glycerol. MutY (26–134) (0.2 ml of Fraction VI, 1 mg/ml) was loaded onto the column, and the column was developed with the same buffer at flow rate of 0.25 ml/min. Size markers (carbonic anhydrase, ovalbumin, bovine serum albumin, and β-amy-

**Glyceraldehyde 3-phosphate Dehydrogenase**—Intact MutY (4 μl of 7 mg/ml) or MutY (26–134) (25 μl of 1 mg/ml) was mixed with size markers in buffer A containing 50 mM KCl in a final volume of 0.1 ml and layered on top of a 50-ml 15–35% (v/v) glycerol gradient. The gradients were spun for 22 h at 45,000 rpm in a Sorvall AH650 rotor at 4 °C. Fractions of one drop were collected from the bottom of the tube, and aliquots were analyzed by SDS-PAGE. To determine the sedimentation coefficient of MutY-DNA complexes, 1.5 μl 19-mer Fl-DNA was incubated with 1.5 or 6 μM MutY or MutY (26–134) protein at 37 °C for 1 min as described for fluorescence anisotropy assays, except that no poly(dI-dC) was added. The protein-DNA complexes were loaded onto the glycerol gradient and processed as free protein. The elution positions of the marker proteins (β-amyrase, transferrin, bovine serum albumin, and carbonic anhydrase) were determined by SDS-PAGE followed by Coomassie Blue staining, and the MutY protein were determined by Western blot analyses.

**Other Methods—**Measurement of mutation frequency and iron assay were performed as described by Lu and Wright (40).

**RESULTS**

**In Vivo Complementation Activity of the MutY(26–134) Mutant—**E. coli cells with a single mutation in the mutY or mutM gene are moderate mutators; however, the MutY/MutM double mutant is a strong mutator because it fails to remove GO lesions and to correct replication errors (41, 42). In the absence of functional MutY and MutM, a high level of mutations in the rifampin-binding site of RNA polymerase renders the cell resistant to rifampin. As shown in Table 1 (second line), cells with mutations in both the mutY and mutM genes have a very high mutation frequency (11). To study the in vivo complementation activity of the MutY (26–134) mutant, the pET26–134 plasmid was transformed into CC104mutYmutM/DE3 cells. This cell strain was used to test the in vivo activity of the

**Table 1**

<table>
<thead>
<tr>
<th>Strain**</th>
<th>Mutation frequency** Increase</th>
<th>RifR colonies/107 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC104/pET21a (vector)</td>
<td>1.2 ± 0.6</td>
<td>1</td>
</tr>
<tr>
<td>CC104mutYmutM</td>
<td>569 ± 122</td>
<td>474</td>
</tr>
<tr>
<td>CC104mutYmutMc/pET21a (vector)</td>
<td>330 ± 125</td>
<td>275</td>
</tr>
<tr>
<td>CC104mutYmutM/pET26–134</td>
<td>350 ± 70</td>
<td>299</td>
</tr>
<tr>
<td>CC104mutYmutM/pET-MYW1</td>
<td>2 ± 0.9</td>
<td>1.7</td>
</tr>
</tbody>
</table>

**CC104 contains the wild-type mutY gene, and CC104mutYmutM is a double mutant of mutY and mutM. All strains contain ΔDE3 lysogen, but the lysogen in CC104 derivatives is not properly established.

**The in vivo activity of MutY was measured by the frequency of rifampicin-resistant (RifR) colonies on average in at least three separate experiments. Standard deviations are reported.

**Values are the fold increase compared with the wild-type strain containing the vector pET21a.

**The mutation frequency for the wild-type mutY gene cloned into pET11a is from Li and Lu (43).
MutY(Δ26–134) mutant because we have shown that it offers a very sensitive assay due to the low protein expression level in this strain (43). Expression of the MutY(Δ26–134) protein in CC104mutYmutM cells did not reduce the mutation rate (Table I, fourth line) compared with the vector itself (Table I, third line). In contrast, expression of wild-type MutY reduced the mutation rate of CC104mutYmutM cells to the normal level (Table I, fifth line). Thus, the MutY(Δ26–134) protein is functionally defective in vivo.

Expression and Purification of Mutant MutY Proteins—The mutY gene in pET21a can produce a fusion protein with 14 residues (MASMTGGQQMGRGS) of the T7 tag from the pET21a vector. The expression and solubility of the MutY(Δ26–134) protein were suboptimum. Thus, the yield of the purified protein was moderate: 2.4 mg of MutY(Δ26–134) protein was purified from 11 g of cell paste, whereas 60 mg of wild-type MutY protein could be purified from the same amount of cell paste. The reason for their difference in elution profile is not clear. Fractions 51–55, containing the majority of the MutY protein, were pooled as Fraction VIB. Both the major and minor bands migrating at 26.6 kDa in Fraction VIB were sequenced and shown to contain the N-terminal processed T7 tag (ASMTGGQQMGRGS) from pET21a. Thus, both bands are presumably the mutant MutY protein, but the reason for their mobility difference is not known. The purified MutY(Δ26–134) protein has a brown color and contains 4.06 iron atoms/protein molecule as measured by chemical iron analysis. Therefore, the MutY(Δ26–134) protein contains an intact [4Fe-4S] cluster.

DNA Binding and Catalytic Activities of the MutY(Δ26–134) Mutant Protein—Using gel retardation assays, we assayed the fractions from the heparin column for binding to the 44-mer A/GO-containing DNA (A/GO44) substrate. As shown in Fig. 1B, the DNA binding activity correlated with the MutY(Δ26–134) protein amount in each fraction (Fig. 1A). Some weak binding activity was observed in fractions 37–39, and strong binding activity
was observed in fractions 51–55. There were multiple protein-DNA complexes in the major peak (fractions 51–55). Surprisingly, the smallest complex, -DNA1 (a complex between the MutY(26–134) mutant protein and DNA), had a slower mobility compared with the intact MutY-DNA complex, Y-DNA (Fig. 1B, lane 2), although the deletion mutant protein is 27 kDa, and the intact MutY protein is 39 kDa.

The MutY protein has been shown to form a covalent Schiff base intermediate with DNA through Lys 142 (28, 44–46) that can be trapped as a stable protein-DNA complex in the presence of sodium borohydride. The fractions from the heparin column were assayed for the trapping activity with A/GO44 on 12% SDS-polyacrylamide gels. A weak trapping activity was observed in fractions 49–55, but not in fractions 37–39 (Fig. 1C).

In this assay, the covalent protein-DNA complex of -DNA had a faster mobility compared with Y-DNA (Fig. 1C, lane 2). The mobility of -DNA on the denaturing gel (Fig. 1C) was significantly different from the mobility of -DNA1 on the native gel (Fig. 1B) relative to that of Y-DNA complexes.

The pooled Fraction VIB also had very weak glycosylase activity on A/GO20 (Fig. 1D). The glycosylase activity of the MutY(26–134) protein could be detected only at concentrations >0.9 μM, whereas intact MutY had strong glycosylase activity at 0.11 nM. The relative rate of the glycosylase activity of the MutY(26–134) protein was reduced by ~5000-fold compared with that of intact MutY.

Binding of the MutY(26–134) Mutant Protein to DNA Detected by Gel Mobility Shift Assays—To determine the apparent dissociation constant ($K_d$) values for the MutY(26–134) protein with different DNA substrates, we performed gel mobility shift assays with eight enzyme concentrations ranging from 0.028 to 1.8 μM as shown in Fig. 2. For the 20-mer DNA substrates, the MutY(26–134) protein bound to A/G-, A/GO-, and C/G-containing DNAs at high protein concentrations (Fig. 2, A–C). At enzyme concentrations <0.6 μM, only one complex (-DNA1) was observed. The mobility of -DNA1 was slower than that of Y-DNA (Fig. 2, A and B, lane 2). At enzyme concentrations >0.9 μM, another complex (-DNA2) was formed with all three DNA substrates (Fig. 2, A and B, lanes 3 and 4; and C, lanes 2 and 3). The levels of -DNA1 diminished as the abundance of -DNA2 increased, suggesting a precursor-product relationship (Fig. 2B). At 1.8 μM MutY(26–134), -DNA2 was the major complex with the A/GO-containing DNA (Fig. 2B, lane 3). Plotting the total amounts of bound DNA versus the enzyme concentrations from three independent experiments yielded the binding curves shown in Fig. 3A. Attempts to determine the $K_d$ values by Enzfitter program (47) were unsuccessful because the curves are sigmoidal. Thus, the enzyme concentrations that gave 50% maximal binding were taken as $K_d$ values (Table II). The $K_d$ value for MutY(26–134) with A/GO20 was slightly lower than those with A/G20 and C/G20 (Table II).
The MutY(H900426–134) protein also bound to 44-mer DNA substrates containing A/G, A/GO, and C/G at high protein concentrations to form multiple complexes (Fig. 2, D–F). At enzyme concentrations >0.056 μM, Δ-DNA2 appeared with all three DNA substrates (Fig. 2, D and E, lane 8; and F, lane 8). Thus, the formation of Δ-DNA2 with 44-mer DNA occurred at 15-fold lower enzyme concentrations than with 20-mer DNA. Moreover, up to five complexes could be observed with the 44-mer DNA substrates (for example, see Fig. 2D, lanes 4–6) in a concentration-dependent manner. Similar to the 20-mer, higher order complexes with A/GO44 were formed at lower protein concentrations than with A/G- and C/G-containing DNAs, e.g. the formation of MutY(H9004)-DNA3 peaks at 0.6, 0.45, and 0.9 μM enzyme for A/G44, A/GO44, and C/G44, respectively. Plotting the total amounts of bound DNA versus the enzyme concentrations from three independent experiments yielded the binding curves shown in Fig. 3B. The curves are also sigmoidal. The enzyme concentration that gave 50% maximal binding (apparent Kd) with A/GO44 was slightly lower than those with A/G44 and C/G44 (Table II).

The dissociation constants for all DNA substrates determined by gel mobility shift assays are high (Table II). These values are similar to those for intact MutY with C/G-containing DNAs, e.g. the formation of Δ-DNA3 peaks at 0.6, 0.45, and 0.9 μM enzyme for A/G44, A/GO44, and C/G44, respectively. Plotting the total amounts of bound DNA versus the enzyme concentrations from three independent experiments yielded the binding curves shown in Fig. 3B. The curves are also sigmoidal. The enzyme concentration that gave 50% maximal binding (apparent Kd) with A/GO44 was slightly lower than those with A/G44 and C/G44 (Table II). The specific activity for A/GO was compromised (compare 0.066 nm for intact MutY and 470 nm for the mutant with A/GO20) but not completely abrogated because the Kd values for MutY(H26–134) with A/GO were slightly lower than those with A/G and C/G (Table II). The binding curves for the MutY(H26–134) protein with all six DNA substrates have a sigmoidal character (Fig. 3). This suggests an allosteric en-

**TABLE II**

<table>
<thead>
<tr>
<th>DNA</th>
<th>Kd (nM)</th>
<th>h for MutY(H26–134)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type MutY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/G20</td>
<td>5.3 ± 0.5</td>
<td>9.8 ± 1.5</td>
</tr>
<tr>
<td>A/GO20</td>
<td>0.066 ± 0.052</td>
<td>9.4 ± 4.8</td>
</tr>
<tr>
<td>C/G20</td>
<td>370 ± 80</td>
<td>4.2 ± 0.7</td>
</tr>
<tr>
<td>A/G44</td>
<td>1.8 ± 0.3</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>A/GO44</td>
<td>0.141 ± 0.008</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>C/G44</td>
<td>315 ± 49</td>
<td>1.7 ± 0.3</td>
</tr>
</tbody>
</table>

* Kd values were determined from three experiments using eight protein concentrations.

* The cooperative constants were determined from Fig. 3 using the following equation, Y = Y₀ + H (X^h/K + X^h)) where X is the enzyme concentration, Y is percent bound, Y₀ is the basal line, H is maximal binding, K is the dissociation constant, and h is the cooperative constant.

* Dissociation constants for wild-type MutY are from Lu et al. (10).

* Dissociation constants for wild-type MutY are from Lu and Fawcett (48). Standard errors are reported.

The MutY(H26–134) protein also bound to 44-mer DNA substrates containing A/G, A/GO, and C/G at high protein concentrations to form multiple complexes (Fig. 2, D–F). At enzyme concentrations >0.056 μM, Δ-DNA2 appeared with all three DNA substrates (Fig. 2, D and E, lane 8; and F, lane 8). Thus, the formation of Δ-DNA2 with 44-mer DNA occurred at 15-fold lower enzyme concentrations than with 20-mer DNA. Moreover, up to five complexes could be observed with the 44-mer DNA substrates (for example, see Fig. 2D, lanes 4–6) in a concentration-dependent manner. Similar to the 20-mer, higher order complexes with A/GO44 were formed at lower protein concentrations than with A/G- and C/G-containing DNAs, e.g. the formation of Δ-DNA3 peaks at 0.6, 0.45, and 0.9 μM enzyme for A/G44, A/GO44, and C/G44, respectively. Plotting the total amounts of bound DNA versus the enzyme concentrations from three independent experiments yielded the binding curves shown in Fig. 3B. The curves are also sigmoidal. The enzyme concentration that gave 50% maximal binding (apparent Kd) with A/GO44 was slightly lower than those with A/G44 and C/G44 (Table II). The dissociation constants for all DNA substrates determined by gel mobility shift assays are high (>240 nM) (Table II). These values are similar to those for intact MutY with C/G-containing DNA (also listed in Table II) (10, 48). The specificity for A/GO was compromised (compare 0.066 nm for intact MutY and 470 nm for the mutant with A/GO20 with 0.141 nm for intact MutY and 245 nm for the mutant with A/GO44) but not completely abrogated because the Kd values for MutY(H26–134) with A/GO were slightly lower than those with A/G and C/G (Table II). The binding curves for the MutY(H26–134) protein with all six DNA substrates have a sigmoidal character (Fig. 3). This suggests an allosteric en-
hancement of enzyme binding to DNA. Using the Hill cooperative binding equation, the cooperative constants (h) for 20-mer DNA substrates were calculated to be 4 for CG20 and 9.5 for both A/G20 and A/GO20; the cooperative constants for 44-mer DNA substrates were calculated to be 1.7 for CG44 and 2.5 for both A/G44 and A/GO44 (Table II, fourth column).

Equilibrium Binding of the MutY(Δ26–134) Mutant and Intact MutY Proteins with DNA Detected by Fluorescence Anisotropy—From the gel mobility shift assay, the cooperative constants (h) for 20-mer DNA substrates were calculated to be 9.5 for both A/G20 and A/GO20 (Table II, fourth column). The data suggest a minimal number of binding sites of 10. However, the maximal number of complexes observed by the gel mobility shift assay was two (Fig. 2, A–C). One possibility for the high cooperative constants is a gel effect whereby the amounts of complexes detectable at lower protein concentrations are reduced due to rapid complex dissociation in the sample wells or during electrophoresis. Therefore, we performed equilibrium binding experiments in solution by fluorescence anisotropy to eliminate this potential artifact.

A fluorescence anisotropy-based assay was adapted to assess the binding properties of the MutY(Δ26–134) protein with selected DNA substrates under true equilibrium conditions. In the case of MutY(Δ26–134) protein binding to the Fl-A/GO19 substrate, a significant decrease in total fluorescence emission was observed as the protein concentration increased (Fig. 4A). This may be due to some contact between the protein and fluorescein possibly involving the [4Fe-4S] cluster since ionic iron is a potent collisional quencher of fluorescein emission (49). Accordingly, the anisotropy measured for each binding reaction was corrected to compensate for the decreased quantum yield of protein-bound Fl-DNA substrates relative to free Fl-DNA using Equation 2. Protein-dependent changes in the corrected anisotropy of the Fl-A/GO19 substrate were well resolved using Equation 3 (Fig. 4B), as evidenced by a strong coefficient of determination (R² = 0.9908) and tight distribution of residuals about the regression solution. Evaluation of regression solutions from triplicate experiments indicated that formation of saturated MutY(Δ26–134)-Fl-A/GO19 complexes was significantly cooperative with respect to protein concentration (h = 1.79 ± 0.21). Similar cooperativity was observed for MutY(Δ26–134) binding to Fl-A/G19 and Fl-C/G19 substrates (Table III).

The [P]½ values for MutY(Δ26–134) with all DNA substrates determined by fluorescence anisotropy assay (Table III) are substantially lower (~8-fold) than those determined by gel mobility assay (Table II). The cooperative constants determined by fluorescence anisotropy assays were in the range of 1.7–1.9 (Table III). The data suggest a minimal number of binding sites of two and that the binding event is highly cooperative. This is consistent with the number of complexes observed by the gel mobility shift assay (Fig. 2, A–C). Thus, the high cooperative constants obtained from the gel mobility assay (Table II) are likely due to poor complex retention during electrophoresis. The MutY(Δ26–134) mutant protein retained some specificity for A/GO, as the [P]½ value with A/GO (53.9 ± 4.9 nM) was significantly lower than that with C/G (76.7 ± 3.4 nM) (Table III).

The binding of intact MutY to Fl-C/G19 was also measured by the fluorescence anisotropy assay. The binding was significantly cooperative with respect to protein concentration (h = 2.10 ± 0.08). As indicated in Table III, the binding affinities of intact MutY and MutY(Δ26–134) for homoduplex DNA were similar.

The MutY(Δ26–134) Protein Is a Dimer in Solution—The unusual mobility of Δ-DNA1 on native gels suggests that the

![Figure 4](https://example.com/figure4.png)

**TABLE III**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>[P]½ (nM)</th>
<th>h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/G20</td>
<td>61.1 ± 7.7</td>
<td>1.93 ± 0.21</td>
</tr>
<tr>
<td>A/GO20</td>
<td>53.9 ± 4.9</td>
<td>1.79 ± 0.21</td>
</tr>
<tr>
<td>C/G20</td>
<td>76.7 ± 9.1</td>
<td>1.68 ± 0.15</td>
</tr>
<tr>
<td>C/G20</td>
<td>71.5 ± 3.4</td>
<td>2.10 ± 0.08</td>
</tr>
</tbody>
</table>

MutY(Δ26–134) protein may not be a monomer, as is intact MutY. Thus, we determined the native molecular weights (Mₙ) of both the wild-type and mutant proteins by the method of Siegel and Monty (50). By this method, Mₙ is calculated from Equation 7,

$$ M_n = \frac{6πη_0 Na_s}{(1 - \nu)} $$

where s is the sedimentation coefficient, a is the Stokes radius, η₀ is the viscosity of the solvent, N is Avogadro’s number, ν is the partial specific volume of the protein, and ρ is the solution density. Gel filtration through a Superose 12 column yielded Stokes radii of 34 and 28.4 Å for the MutY(Δ26–134) protein.
and intact MutY, respectively, relative to size markers (data not shown). By sedimentation velocity through 15–35% glycerol gradients (Fig. 5, A and B, ■), the sedimentation coefficients of Δ26–134 and intact MutY proteins were estimated to be 3.8 S and 3.5 S, respectively. The partial specific volumes of the intact MutY and MutY(Δ26–134) proteins were calculated as 0.7303 and 0.7272 using the AASTAT5 application of Biology Workbench Version 3.2 (San Diego Supercomputer Center, www.workbench.sdscc.edu). Based on these parameters, Equation 7 returned hydrodynamic $M_r$ values for MutY(Δ26–134) and intact MutY of 53,700 and 41,800, respectively. These values are in very close agreement with the calculated $M_r$ values for a MutY(Δ26–134) dimer ($M_r = 54,654$) and a MutY monomer ($M_r = 39,381$). Thus, the MutY(Δ26–134) protein is a dimer in solution and binds to DNA as a dimer or higher order form (Fig. 1B).

**Determination of the Compositions of Protein-DNA Complexes**—Because the free MutY(Δ26–134) mutant protein is dimeric and Δ-DNA complexes migrated slower than Y-DNA in gel mobility shift assays, we postulated that Δ-DNA1 may be a dimer-DNA complex (P$_2$D). To verify this assignment, we determined the sedimentation coefficients of MutY-DNA complexes on 15–35% glycerol gradients. At a 1:1 ratio of protein monomer to A/GO19, the MutY(Δ26–134) mutant protein sedimented at a position of 68 kDa or 4.5 S (Fig. 5A, ●). This is in agreement with the theoretical value of a dimeric protein with one DNA duplex (67 kDa). At a 4:1 ratio of protein monomer to DNA, the MutY(Δ26–134) mutant protein sedimented over a wide range, with a major peak at $\sim 110$ kDa or 5.8 S and some at 71 kDa or 4.6 S. The size of 110 kDa is reasonably close to the predicted mass (121 kDa) of the tetramer-DNA complex (P$_4$D). The chance for the Δ-DNA1 and Δ-DNA2 complexes to be P$_2$D$_2$ and P$_4$D$_2$, respectively, is highly improbable because we did not observe any significant FRET between 5'-Fl-labeled DNA and either 5' or 3'-Cy3-labeled DNA (data not shown). Base on Equation 6, 5% FRET is expected to occur if the donor and acceptor are 80 Å apart. This would correspond approximately to the predicted size of the MutY(Δ26–134) tetramer complexed to DNA. Thus, the Δ-DNA1 and Δ-DNA2 complexes detected by the gel mobility shift assay in Fig. 2B are likely dimer-DNA (P$_2$D) and tetramer-DNA (P$_4$D) complexes.

The Y-DNA complexes sedimented as two peaks at 42 and 62 kDa (Fig. 5B, ●) at 1:1 and 4:1 ratios of protein monomer to C/G19, respectively. The 62-kDa (4.4 S) peak is larger than the predicted size of the monomer-DNA complex (P$_d$), but smaller than that of the monomer-DNA tetramer complex (P$_d$ D$_4$). Similarly to the MutY(Δ26–134) mutant protein, we ruled out the possibility of P$_d$ D$_2$ for intact MutY because we did not observe any FRET between 5'-Fl-labeled DNA and either 5'- or 3'-Cy3-labeled DNA (data not shown). The size of 88 kDa (5.2 S) is close to the predicted mass (91 kDa) of the dimer-DNA complex (P$_d$ D). Thus, the Y-DNA1 and Y-DNA2 complexes detected by the gel mobility shift assay in Fig. 2B are likely monomer-DNA (P$_d$) and dimer-DNA (P$_d$ P$_d$) complexes. These complexes were also applied to a Superose 12 column, but the protein was eluted at the same position as free MutY. Thus, MutY-DNA complexes were likely dissociated during passage through the gel filtration resin.

In addition, the sizes of the protein-DNA complexes were...
plexes were assigned as P2D (67 kDa) and P4D (121 kDa), respectively. The Y-DNA1 and Y-DNA2 complexes were assigned as PD (52 kDa), whereas the Y-DNA2 complexes are dimer-DNA complexes (P2D, 91 kDa).

**DISCUSSION**

The six-helix barrel module of MutY containing the conserved helix-hairpin-helix motif has been shown to directly contact the mismatched adenine, the mismatched GO, and the backbone of the GO strand in addition to substantial interaction with the C-terminal domain (20, 25). The adenine-binding pocket of *E. coli* MutY consists of Glu<sup>37</sup>, Leu<sup>40</sup>, Gln<sup>122</sup>, and Asp<sup>186</sup> (20, 25). The specific recognition of GO involves Gln<sup>42</sup>, Thr<sup>63</sup>, Leu<sup>86</sup>, Tyr<sup>88</sup>, and Ser<sup>209</sup> of *E. coli* MutY based on the MutY-DNA structure from *B. stearothermophilus* (25). Glu<sup>37</sup>, Leu<sup>40</sup>, Gln<sup>122</sup>, Thr<sup>63</sup>, Leu<sup>86</sup>, and Tyr<sup>88</sup> are missing in the MutY<sup>26–134</sup> mutant. Therefore, deletion of the six-helix barrel module of MutY is expected to cause major functional defects. We have shown here that the MutY<sup>26–134</sup> mutant protein containing only the half-adenine-binding pocket still has very weak glycosylase activity, with an 5000-fold reduced rate compared with the intact MutY protein.

We have shown that the MutY<sup>26–134</sup> protein without bound DNA is a dimer, whereas the intact MutY protein is a monomer in solution by both gel filtration and sedimentation velocity analyses. Thus, a dimerization domain is likely exposed when the six-helix barrel module is deleted. Because the free MutY<sup>26–134</sup> mutant protein is dimeric and because Δ-DNA1 migrated slower than Y-DNA in gel mobility shifting assays, Δ-DNA1 is likely a dimer-DNA complex (P2D), and Δ-DNA2 is a likely a tetramer-DNA complex (P2D). This assignment was confirmed by three independent methods. First, the sizes of the complexes determined by sedimentation velocity analysis were in reasonable agreement with the predicted complex sizes. Second, FRET analyses showed that the chance of the formation of protein-DNA complexes containing two DNA molecules is very slim. Third, the Ferguson plot (Fig. 6E) showed that the line for Δ-DNA2 has a steeper slope than that for Δ-DNA1 and that both lines have the same y intercepts.

Intact MutY also formed two complexes with the C/G<sub>20</sub> subsequence (Fig. 6A–D). The binding affinities are low because the *K<sub>D</sub>* values were reported as 370 nM for C/G<sub>20</sub> (10). The Ferguson plot (51) of the migration distance (in log scale) of Y-DNA1 and Y-DNA2 as a function of the polyacrylamide percentage in the gel (Fig. 6E) showed a linear relationship, and both lines have the same y intercepts as those for free DNA and the Δ-DNA complexes. The slopes for Y-DNA1 (Fig. 6E, ○ and Y-DNA2 (●) are steeper and steeper, respectively, than those for Δ-DNA1 (□). These results indicate that the Y-DNA1 complexes are monomer-DNA complexes (PD, 52 kDa), whereas the Y-DNA2 complexes are dimer-DNA complexes (P2D, 91 kDa).

FIG. 6. Gel mobility shift assays using different percentages of polyacrylamide gels. C/G<sub>20</sub> (1.8 fmol) was incubated with 1.8 μM MutY<sup>26–134</sup> mutant protein (Fraction VIB) (lane 1) and 0.9 μM MutY (lane 2) at 37 °C for 30 min. Products were fractionated on 4% (A), 6% (B), 8% (C), and 10% (D) native polyacrylamide gels. Free DNA (F) is indicated. Ferguson analysis was performed by plotting the log of the mobility (log mm) versus the polyacrylamide concentrations of each band (E). All of the linear lines have the same y intercepts. Complexes with higher masses have steeper slopes. Free DNA (Δ), MutY-DNA complexes (Y-DNA1 (○) and Y-DNA2 (●), and MutY<sup>26–134</sup> mutant protein-DNA complexes (Δ-DNA1 (□) and Δ-DNA2 (■)) are shown. The Y-DNA1 and Y-DNA2 complexes were assigned as PD (52 kDa) and P2D (91 kDa), respectively. The Δ-DNA1 and Δ-DNA2 complexes were assigned as PD (67 kDa) and P2D (121 kDa), respectively.

Determined by their sensitivity to gel sieving, gel mobility shift experiments with MutY<sup>26–134</sup> mutant with C/G<sub>20</sub> (Fig. 6, A–D, lane 1) were performed using 4, 6, 8, and 10% polyacrylamide gels. The Ferguson plot (51) showed a linear relationship between the migration distance (in log scale) and the polyacrylamide percentage in the gel for free DNA (Fig. 6E, △, Δ-DNA1 (□), and Δ-DNA2 (■)). The lines have steeper slopes for larger complexes because they are more sensitive to sieving by the gel matrix. As shown in Fig. 6E, all lines have the same y intercepts. This indicates that the complexes have similar charges. These results indicate that the Δ-DNA1 complexes are dimer-DNA complexes (P2D, 67 kDa), whereas the Δ-DNA2 complexes are tetramer-DNA complexes (P2D, 121 kDa). Similar gel mobility shift experiments with the MutY<sup>26–134</sup> mutant with C/G<sub>20</sub> were performed using 4, 6, 8, and 10% polyacrylamide gels (data not shown). The results indicate that Δ-DNA1 is a dimer-DNA complex (P2D, 83 kDa) and Δ-DNA2 is a tetramer-DNA complex (P2D, 137 kDa).

Intact MutY also formed two complexes with the C/G<sub>20</sub> substrate (Fig. 6, A–D, lane 2). These binding affinities are low because the *K<sub>D</sub>* values were reported as 370 nM for C/G<sub>20</sub> (10). The Ferguson plot (51) of the migration distance (in log scale) of Y-DNA1 and Y-DNA2 as a function of the polyacrylamide percentage in the gel (Fig. 6E) showed a linear relationship, and both lines have the same y intercepts as those for free DNA and the Δ-DNA complexes. The slopes for Y-DNA1 (Fig. 6E, ○ and Y-DNA2 (●) are steeper and steeper, respectively, than those for Δ-DNA1 (□). These results indicate that the Y-DNA1 complexes are monomer-DNA complexes (PD, 52 kDa), whereas the Y-DNA2 complexes are dimer-DNA complexes (P2D, 91 kDa).
this method provide a better index of solution binding affinity. We applied this assay, for the first time, to measure DNA binding of MutY. The $[P]_{1/2}$ values for MutY with all DNA substrates were $>50$ nM as determined by fluorescence anisotropy assay (Table III) and $240$ nM as determined by gel mobility shift assay (Table II). The substantial differences in $[P]_{1/2}$ values determined by these methods indicate that weak DNA binding is likely more sensitive to gel sieving. Three lines of evidence show that the specificity of MutY for A/GO was compromised but not completely abrogated by deletion of the six-helix barrel domain. First, higher order complexes with A/GO-containing DNA were formed at lower protein concentrations than with A/G and homoduplex DNA (Fig. 2). Second, the $K_d$ values for MutY with A/GO determined by gel mobility shift assay were slightly lower than those with A/G and C/G (Table II). Third, the $[P]_{1/2}$ value for MutY with A/GO determined by fluorescence anisotropy assay was lower than those with A/G and C/G (Table III). Some specificity for GO may be contributed by the C-terminal domain retained in the MutY mutant. This is consistent with the previous findings that the C-terminal domain of MutY plays an important role in the recognition of GO lesions (15, 18, 23) and that the six-helix barrel module directly contacts the mismatched GO and backbone of the GO strand (25).

DNA glycosylases recognize damaged bases within a vast excess of normal DNA and then cleave the target nucleotides. How a DNA glycosylase searches for base damage remains unclear. Based on the structures of several glycosylases complexed to DNA, a pinch-push-pull mechanism has been proposed for DNA damage detection (53). The glycosylase scans the DNA substrate by compressing the DNA intrastrand phosphate distance and binds to the lesion site in a specific way that causes the DNA to kink. The enzyme then forces the target base to flip out the distorted DNA into a pocket on the enzyme surface. Base flipping makes the glycosyl bond accessible for cleavage. The specificity of the glycosylase for the target base is determined by the base flipping specificity of the push and by the chemical specificity of the pull by the binding pocket. The data we reported here for DNA binding by the MutY protein and C/G binding by intact MutY add information to the MutY searching mechanism for mismatches. For each binding event, the MutY protein is a dimer, whereas intact MutY is a monomer. The binding of both intact MutY and MutY proteins to homoduplexes as assayed by fluorescence anisotropy indicated that MutY binds DNA cooperatively, i.e. binding of the first MutY monomer or MutY dimer can enhance the second protein binding to DNA. We propose a model in which MutY scans the DNA cooperatively as

![Sequential dimer binding model for MutY binding to DNA.](http://www.jbc.org/)

(A) $P + D \rightarrow PD$ (or $PD^*$)
(B) $P + D \rightarrow P + D$ (or $P + D^*$)
(C) $P + D \rightarrow P + D$ (or $P + D^*$)
(D) $P + D \rightarrow P + D$ (or $P + D^*$)
(E) $P + D \rightarrow P + D$ (or $P + D^*$)
(F) $P + D \rightarrow P + D$ (or $P + D^*$)
(G) $P + D \rightarrow P + D$ (or $P + D^*$)
(H) $P + D \rightarrow P + D$ (or $P + D^*$)
(I) $P + D \rightarrow P + D$ (or $P + D^*$)
(J) $P + D \rightarrow P + D$ (or $P + D^*$)
(K) $P + D \rightarrow P + D$ (or $P + D^*$)
(L) $P + D \rightarrow P + D$ (or $P + D^*$)
(M) $P + D \rightarrow P + D$ (or $P + D^*$)
(N) $P + D \rightarrow P + D$ (or $P + D^*$)
(O) $P + D \rightarrow P + D$ (or $P + D^*$)
(P) $P + D \rightarrow P + D$ (or $P + D^*$)
(Q) $P + D \rightarrow P + D$ (or $P + D^*$)
(R) $P + D \rightarrow P + D$ (or $P + D^*$)
(S) $P + D \rightarrow P + D$ (or $P + D^*$)
(T) $P + D \rightarrow P + D$ (or $P + D^*$)
(U) $P + D \rightarrow P + D$ (or $P + D^*$)
(V) $P + D \rightarrow P + D$ (or $P + D^*$)
(W) $P + D \rightarrow P + D$ (or $P + D^*$)
(X) $P + D \rightarrow P + D$ (or $P + D^*$)
(Y) $P + D \rightarrow P + D$ (or $P + D^*$)
(Z) $P + D \rightarrow P + D$ (or $P + D^*$)

DNA glycosylases recognize damaged bases within a vast excess of normal DNA and then cleave the target nucleotides. How a DNA glycosylase searches for base damage remains unclear. Based on the structures of several glycosylases complexed to DNA, a pinch-push-pull mechanism has been proposed for DNA damage detection (53). The glycosylase scans the DNA substrate by compressing the DNA intrastrand phosphate distance and binds to the lesion site in a specific way that causes the DNA to kink. The enzyme then forces the target base to flip out the distorted DNA into a pocket on the enzyme surface. Base flipping makes the glycosyl bond accessible for cleavage. The specificity of the glycosylase for the target base is determined by the base flipping specificity of the push and by the chemical specificity of the pull by the binding pocket. The data we reported here for DNA binding by the MutY protein and C/G binding by intact MutY add information to the MutY searching mechanism for mismatches. For each binding event, the MutY protein is a dimer, whereas intact MutY is a monomer. The binding of both intact MutY and MutY proteins to homoduplexes as assayed by fluorescence anisotropy indicated that MutY binds DNA cooperatively, i.e. binding of the first MutY monomer or MutY dimer can enhance the second protein binding to DNA. We propose a model in which MutY scans the DNA cooperatively as
a dimer or multisubunit complex to locate the mismatches (Fig. 7). The MutY monomer binds randomly to the DNA. A second monomer then binds to the same DNA cooperatively to form a dimer with higher affinity. This MutY dimer then slides along the DNA until one of the MutY monomers binds specifically to the mismatch site. Our model is consistent with the findings of Wong et al. (52) that MutY assembles into a dimer upon binding DNA and that the dimer is the functionally active form. However, our findings indicate that a MutY dimer is present only transiently while MutY searches for a mismatched site.

Acknowledgments—We thank Drs. Michael Volkert, Martin G. Marinus, and Jeffery H. Miller for providing E. coli strains. We thank Dr. Enrico Bucci (Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine) for helpful discussion and Dr. Enrico Bucci (Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine) for helpful discussion and

REFERENCES

An *Escherichia coli* MutY Mutant without the Six-helix Barrel Domain Is a Dimer in Solution and Assembles Cooperatively into Multisubunit Complexes with DNA
Chih-Yung Lee, Haibo Bai, Rebecca Houle, Gerald M. Wilson and A-Lien Lu

doi: 10.1074/jbc.M405271200 originally published online September 28, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405271200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 49 references, 18 of which can be accessed free at http://www.jbc.org/content/279/50/52653.full.html#ref-list-1