The DNA repair enzyme MutY plays an important role in the prevention of DNA mutations resulting from the presence of the oxidatively damaged lesion 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG). MutY is a base excision repair (BER) glycosylase that removes misincorporated adenine residues from OG:A mispairs, as well as G:A and C:A mispairs. We have previously shown that, under conditions of low MutY concentrations relative to an OG:A or G:A substrate, the time course of the adenine glycosylase reaction exhibits biphasic kinetic behavior due to slow release of the DNA product by MutY. The dissociation of MutY from its product may require the recruitment of other proteins from the BER pathway, such as an apurinic-apyrimidinic (AP) endonuclease, as turnover-enhancing cofactors. The effect of the AP endonucleases endonuclease IV (Endo IV), exonuclease III (Exo III), and Ape1 on the reaction kinetics of MutY with G:A- and OG:A-containing substrates was investigated. The effect of the glycosylases UDG and MutM and the DNA polymerase pol I was also characterized. Endo IV and Exo III, unlike Ape1, UDG, and pol I, greatly enhance the rate of product release with a G:A substrate, whereas the rate constant for the adenine removal step remains unchanged. Furthermore, the turnover rate with a truncated form of MutY, Stop 225, which lacks 125 amino acids of the C terminus, is unaffected by the presence of Endo IV or Exo III. These results constitute the first evidence of an interaction between the MutY-product DNA complex and Endo IV or Exo III. Furthermore, they suggest a role for the C-terminal domain of MutY in mediating this interaction.

The base excision repair (BER) pathway is the primary cellular mechanism charged with the task of removing DNA bases modified via hydrolysis, oxidation, and alkylation. BER relies on the action of damage-specific DNA glycosylases that recognize various types of modified or inappropriate bases within the context of normal Watson-Crick DNA. The repair process is initiated by hydrolytic removal of the target base by the relevant DNA glycosylase and proceeds by incision at the abasic site, generation of a gap, reparative DNA synthesis, and ligation of the nicked DNA. The BER pathway has been reconstituted in vitro with cell-free extracts or purified protein components, and these experiments have established the minimal requirements for restoration of the damaged DNA. For example, repair of uracil in DNA was achieved by use of five Escherichia coli proteins: uracil-DNA glycosylase (UDG), endonuclease IV (an AP endonuclease), RecJ protein, DNA polymerase I, and DNA ligase. Both "short patch" and "long patch" BER pathways have been observed, which differ in the number of nucleotides and the various protein components involved. The type of BER pathway utilized depends on the organism and the type of DNA damage; however, minimally the BER pathway requires a damage-specific glycosylase, an AP endonuclease, DNA polymerase, and DNA ligase.

The DNA glycosylases of BER may be classified into two distinct classes: 1) monofunctional glycosylases, which catalyze the removal of the damaged base to generate an apurinic or apyrimidinic (AP) site and 2) bifunctional glycosylase/AP-lyases, which catalyze base displacement and an associated β-elimination reaction to provide strand scission. The AP site products of monofunctional glycosylases are unstable and degrade autocatalytically to generate DNA strand breaks. The presence of AP sites in DNA impedes DNA synthesis with many DNA polymerases and may also be potentially mutagenic due to lack of base coding information. The potent cytotoxicity of these sites behooves the repair machinery to insert the appropriate base without leaving the AP site exposed at any time. In keeping with this imperative, many DNA glycosylases, including the human thymine DNA glycosylase (hTDG), human uracil DNA glycosylase (hUDG), human OG glycosylase (hOGG1), human MED1 protein, E. coli double-stranded uracil DNA glycosylase (DUG), and the adenine glycosylase MutY have been reported to bind very tightly to their respective AP site products. Presumably, these glycosylases remain bound to their AP site product until cellular components, which will complete the repair process, are recruited.

The enzymes in charge of recognizing AP sites and catalyzing cleavage of the DNA backbone are AP endonucleases. The ability of the human AP endonuclease Ape1 (also called HAP1, APEX, REF1) to enhance release of the DNA product in vitro from hUDG (19), hTDG (17), hOGG1 (20, 21), and murine MutY homolog (26) has been demonstrated recently. This suggests a direct interaction between the AP endonuclease and the BER glycosylase/AP site DNA product complex. In eukaryotes,
specific interactions between enzymes downstream of the glycosylase in the BER pathway have also been uncovered. For example, Apel1 has been found to make protein-protein contacts with polymerase β (27), which, in turn, interacts directly with DNA ligase I (28). Some interactions occur indirectly using a third protein as a mediator, as is the case for polymerase β and ligase III, which are connected via the scaffolding protein XRCC1 (9). Structural studies of BER enzymes and their substrate and product complexes have also suggested coordination between enzymes of the BER pathway (3, 29).

Two AP endonucleases, exonuclease III (Exo III) and endonuclease IV (Endo IV), are well characterized in E. coli (2, 30). Both enzymes nick the DNA strand on the 5'-side of the AP site resulting in 5'-deoxyribose phosphate and 3’-deoxyribose-hydroxyl end products. Despite their similar substrate specificity, the primary sequences of these two enzymes are different. Exo III is the major AP endonuclease in E. coli (31, 32) and is homologous to the human AP endonuclease Apel1 (33–35). Exo III also possesses a potent 3’-5’ exonuclease activity. Endo IV belongs to a second group of AP endonucleases that includes a variety of homologs thus far only identified in bacteria and simple eukaryotic organisms such as APN-1 in yeast (36, 37) and CeAPN-1 in the nematode Caenorhabditis elegans (38). The in vitro reconstitution studies reported for the bacterial BER pathway utilized Endo IV as the AP endonuclease, suggesting that this protein may be responsible for the step following base removal by DNA glycosylases in vivo (7, 10). Indeed, APN-1 yeast strains are strong mutants resulting in a 60-fold increase in the rate of AT → GC transversions and a hypersensitivity to AP site-generating species (39).

The prevention of mutations associated with formation of OG within duplex DNA in E. coli relies on two BER glycosylases: MutY and MutM (also called Fpg) (40). MutM is an OG glycosylase specific for removal of OG opposite C. The MutY enzyme prevents mutations caused by OG by removing misincorporated adenine residues, thereby short-circuiting G:C to T:A transversion mutations (41). Homologs to both MutY (42) and MutM (43–48) have been identified in human cells (2). Indeed, the importance of the “GO” repair system in humans has recently been highlighted by the finding of a family with colorectal tumors that have inherited variants of human MutY (hMYH) (49).

MutY also exhibits adenine glycosylase activity toward G:A and C:A mismatches (50–52). The adenine glycosylase MutY is composed of two domains that are susceptible to separation upon partial proteolytic digestion (53). The N-terminal domain (Met-1 to Lys-225) retains catalytic activity (54) and exhibits a sequence (55) and structure (56) that place MutY within the family of DNA glycosylases (57). The C-terminal domain has sequence (58) and structural homology to the dOG/TPase MutT (59), indicating a role in recognition of OG. Kinetic experiments with substrates and binding assays with substrate analogs are consistent with a role of the C-terminal domain in OG recognition and nucleotide flipping (58, 60). However, it is possible that this domain has additional functions. In studies of the kinetic properties of MutY reported previously by our laboratory (24, 60), the product release step of the glycosylase reaction with G:A and OG:A substrates was shown to be rate-limiting, suggesting a high affinity of MutY for the AP site product. Though MutY binds tightly to the AP site product produced from both OG:A and G:A substrates, this interaction is particularly strong with the OG:AP site, such that the half-life (t1/2) of the complex is approximately 3 h (24). The strong interaction between MutY and OG:AP was also shown by the ability to observe a distinct MutY “footprint” in methidium-propyl EDTA-Fe(II) hydroxyl radical footprinting experiments on the MutY-product complex (25). The tight binding of MutY to the AP site may be important in vivo to avoid the adverse effects of the abasic site lesion and the premature removal of OG by MutM.

It is conceivable that the in vivo turnover rate of MutY may be higher than that observed in vitro. The dissociation of MutY from its product may require the recruitment of other proteins from the BER pathway, such as an AP endonuclease, as turn-over-enhancing cofactors. A study of the effect of the AP endonucleases Endo IV, Exo III, and Apel1 on the kinetics of the reaction of MutY with G:A and OG:A substrates is described herein. The effect of the glycosylases UDG and MutM and the DNA polymerase pol I was also characterized. It was found that Endo IV and Exo III, unlike Apel1, UDG, and polymerase I, greatly enhances the rate of product release with a G:A substrate, whereas the rate constant for the adenine removal step remained unchanged. However, neither Endo IV nor Exo III enhanced turnover with an OG:A substrate. Furthermore, the turnover rate for a truncated form of MutY, Stop 225, which lacks 125 amino acids on the C terminus, is not affected by the presence of Endo IV or Exo III. These results constitute the first evidence of an interaction between MutY and E. coli AP endonucleases and suggest a role for the C-terminal domain in mediating these interactions.

Experimental Procedures

General Methods, Materials, and Instrumentation—The plasmids pKKY2eco, containing mutOY, and pKKApary2, containing Fpg, were kindly provided by M. Michaels and J. H. Miller (UCLA). The plasmid containing endonuclease IV (pET24 Eco Nfo) was provided by Dr. Richard Cunningham (SUNY, Albany, NY). All common DNA manipulations were performed using the standard protocols (61). All β-cyanoethyl phosphoramidites were purchased from ABI, except the 7,8-dihy- dro-8-oxo-2-deoxyguanosine, and the tetrahydrofuran (displacer) phosphoramidites, which were purchased from Glen Research. All other reagents used were purchased from Fisher, Sigma Chemical Co., or USB. 5'-End labeling was performed with T4 polynucleotide kinase (New England BioLabs) using [γ-32P]ATP (Amersham Biosciences, Inc.). Labeled oligonucleotides were purified using ProbeQuant G-50 Micro columns (Amersham Biosciences, Inc.). UV-visible spectroscopy was performed on a Hewlett-Packard 8452A diode array spectrophotometer. Storage phosphor autoradiography was performed using a Molecular Dynamics STORM 840 PhosphorImager. All data fitting was performed using GraFit v. 4. All electrophoresis was performed using 1 x 0.5% Tris-borate-EDTA (TBE) buffer (pH 8.3), where 1 x = 90 mM Tris, 90 mM boric acid, 1 mM EDTA. Chromatography for MutY, endonuclease IV, and MutM purification was conducted with a BioLogic (Pharmacia) column (BioRad) at 4 °C.

Enzymes—The E. coli strain JM101 mutY::mini-tn10 harboring the plKKS225 plasmid or pKKY2eco plasmid were used in the production of Stop 225 and WT MutY, respectively, as described previously by our laboratory (60). Endonuclease IV was purified using pET 24-Eco Nfo plasmid in BL21 DE3/pLysS cells. The purification of endonuclease IV was modified from Haas et al. (62) by replacing dialysis steps with an Amersham Biosciences, Inc. HiPrep 26/10 desalting column. E. coli MutM was purified as described previously (63). Purified Apel1 was a generous gift of Dr. David Wilson and Dr. Jan Erzberger (Lawrence Livermore National Laboratory). E. coli uracil-DNA glycosylase (UDG) and pol I were purchased from Invitrogen. Exonuclease III was purchased from New England BioLabs. It should be noted that, for all of the enzymes except MutY and MutM (Fpg), the concentration is listed as the Bradford concentration. MutY concentrations are reported as the active site concentrations, and MutM concentrations are based on the absorbance at 280 nm (ε280 = 3.9 x 104 liters mol −1 cm −1).

Oligonucleotides—DNA oligonucleotides were synthesized by standard phosphoramidite chemistry on an Applied Biosystems model 392 DNA/RNA synthesizer as per the manufacturer’s protocol. Oligonucleo-
dides for enzyme assays and binding experiments were purchased from Invex-charge chromatography (Waters, Protein-Pak DEAE 8H column).

The following DNA oligonucleotides were used: d(5'-CGCATC-GGACCCACCAAXGCTCCCGTTACAG-3') where 30-G, X = 2'-deoxyguanosine (G) and 30-OG, X = 7,8-dihydro-8-oxo-2'-deoxyguanosine (OG); d(3'-GCTAGTACCTCCGGTGYTCGAGGGCCAATGC-5') where 30-A, Y = 2'-deoxyadenosine (A) and 30-THF, Y = tetrahydrofuran
nucleotide (THF); d(5’-CGATCGTGAGCCAGCTGCTCCGGTACAG-
TAGG-3’): 34-G; d(3’-GGATCTGATAGCTCGGTAGAAGCCCAT-
GTC-5’): 34-A; d(5’-CGAAGATTCGCCTGCTGATCGCGAG-
GCTAGTTGCTCAGATGCTACGTAAGATAATCGG-3’): 70-A; d(3’-
GCTACAGTGAGCCAGCTGCTCCGGTACAG-TAGG-5’): 70-GG.

Adenine Glycosylase Assays with MutY—The adenine glycosylase activity of MutY was monitored using a 30-bp oligonucleotide duplex substrate with a centrally located G:A or O:A pair, as described previously by our laboratory (24, 60). The A-containing strand was 3’-P-labeled on the 5’-end, and the rate of adenine removal was monitored by treating the reaction mixture with NaOH to promote strand cleavage at the abasic site. The substrate DNA (30-nucleotide strand) and the product-derived oligonucleotide (14-nucleotide) were separated in an 8% urea-denaturing polyacrylamide gel (15%) and then quantified via storage phosphor autoradiography.

The A-containing strand of the DNA duplexes used was 5’-P-end-labeled using T4 polynucleotide kinase, and the end-labeled A-containing strand was added to a final concentration of 2 nM. The duplex formation was achieved by heating to 90°C in an annealing buffer (20 mM Tris-HCl, pH 7.6, 10 mM EDTA, and 150 mM NaCl) and then slow-cooling to room temperature over 3–4 h.

In all of the kinetic experiments, substrate DNA (20 nM duplex) was equilibrated at 37°C in reaction buffer (20 mM Tris-HCl, pH 7.6, 10 mM EDTA, 30 mM NaCl, 0.1 mg/ml bovine serum albumin). For multiple-turnover experiments, the enzyme concentrations were adjusted to afford a 10–20% product formation for the burst phase of the reaction. Single-turnover experiments were performed in a manner analogous to the multiple-turnover experiments, with a MutY protein concentration of 60 nM. Enzyme concentrations for MutY are the active enzyme concentration determined using an active-site titration method with an O:A substrate (24).

Adenine Glycosylase Assays in the Presence of BER Enzymes—In experiments requiring Endo IV or Apel, these enzymes were added at concentrations ranging from 5 to 500 nM. In experiments using Exo III, the Exo III concentration ranged from 38 to 760 nM. The adenine glycosylase activity was performed as above with NaOH providing cleavage at the abasic site (not the AP endonuclease). For experiments using Apel, the buffer did not contain EDTA and contained 2 mM MgCl$_2$. Experiments were performed in a similar manner with MutM, UDG, and poly I.

Gel Retardation Assays—Gel retardation experiments (64) were performed in a manner similar to that described previously for MutY with substrate analog duplexes (25, 65). However, in these experiments, the DNA duplex was prepared in a manner similar to that for the kinetic experiments, i.e. 2–5% of the duplex was 3’-P-end-labeled and mixed with cold duplex at the appropriate concentration. In addition, buffer conditions were slightly different than previous experiments. Reactions containing 15 nM DNA duplex, 20 mM Tris-HCl, pH 7.5, 25.5 mM NaCl, 10 mM EDTA, 10% glycerol were incubated in the presence of freshly diluted Endo IV, Exo III, Apel-1, or MutM in the presence or absence of 20 nM MutY. After incubation at 37°C for 15 min, the samples were electrophoresed on a 6% non-denaturing polyacrylamide gel (17%). The autoradiogram was quantitated using ImageQuaNT (Molecular Dynamics).

RESULTS

Endo IV Stimulates Release of MutY from the DNA Product with a G:A Substrate—Our laboratory has shown previously that, under multiple-turnover conditions ([MutY] < [DNA]), the reaction of MutY exhibits biphasic kinetics with both O:A and G:A-containing substrates, consistent with rate-limiting product release (24). The rate constant for the exponent of the step involving chemistry ($k_b$), whereas the amplitude of the burst phase is proportional to the rate constant of the steps involving chemistry ($k_c$), whereas the amplitude of the burst phase is proportional to the concentration of active MutY. The linear steady-state portion of the plots is dominated by the rate of product release ($k_p$). We have previously proposed a kinetic scheme to analyze the kinetic properties of MutY (Scheme 1) and have described our analysis using multiple- and single-turnover kinetics to determine the relevant rate constants (24).

The effect of 5, 20, 50, and 100 nM Endo IV on the kinetics of the reaction of 3 nM MutY with 20 nM DNA is shown in Fig. 1A. The presence of increasing concentrations of Endo IV causes a remarkable increase in the steady-state rate of the glycosylase reaction but has a minimal effect on the rate or amplitude of the burst. At sufficiently high Endo IV concentrations (~50 nM), product release is no longer rate-limiting, and the observed plot is no longer biphasic. This result in quantitative conversion to product after approximately 25 min at 100 nM Endo IV, whereas in the absence of Endo IV only 25% product is formed after the same period of time. These data suggest that Endo IV is able to displace MutY from the G:AP site in DNA, thus facilitating the turnover of MutY.

The effect of Endo IV on the chemical events prior to product release was investigated under single-turnover conditions (60 nM MutY with 20 nM substrate DNA). Under these conditions, the observed rate constant remained unchanged (Fig. 1B). The reactions in the presence of 0 or 480 nM Endo IV lead to quantitative conversion of substrate to product with observed rate constant values of (1.0 ± 0.2) and (1.1 ± 0.2) min$^{-1}$, respectively. The combined results indicate that the presence of Endo IV does not affect the intrinsic chemical process associated with the adenine removal and that the effect is limited to the steps governing the steady-state rate (i.e. product release).

Exo III Stimulates the Product Release of MutY with a G:A-containing DNA Substrate—To prevent exonucleolytic degradation of the DNA substrate by Exo III, a DNA duplex containing 3’ overhangs on both the G and the A strand was used, which has previously been shown to inhibit the exonuclease activity of Exo III (66). Unfortunately, at the high Exo III concentrations used in these experiments (77–380 nM), the presence of the 3’ overhanging ends did not effectively inhibit the exonuclease activity; however, reactions in which 10 mM EDTA was present in the buffer completely inhibited the exonuclease activity while only partially inhibiting the AP endonuclease activity. Thus, all Exo III experiments were performed in buffer containing 10 mM EDTA so that the analysis of the experiments would not be hampered by the exonuclease degradation of the substrate.

Experiments in which increasing amounts of Exo III (0, 77, 160, and 380 nM) were added to reactions of 4 nM MutY with 20 nM G:A-containing substrate showed a stimulation of the product release ($k_p$) of MutY, until at high Exo III concentrations (380 nM), the product formation plots no longer retain biphasic character (Fig. 2A). These results are qualitatively similar to those observed with Endo IV; however, a higher concentration of Exo III is required under these conditions to produce the same stimulation of the product release of MutY with G:A-containing DNA by Endo IV.

The effect of Exo III on the chemical events prior to product release was investigated under single-turnover conditions (30 nM MutY with 20 nM substrate DNA). The reactions in the presence of 0 or 380 nM ExoIII lead to quantitative conversion of substrate to product with observed rate constant values of 1.4 ± 0.1 and 2.1 ± 0.1 min$^{-1}$, respectively (Fig. 2B). Under these conditions, the observed rate constant is minimally changed; indeed, there appears to be a slight increase in the observed rate constant in the presence of Exo III. The combined results indicate that the presence of Exo III minimally perturbs the intrinsic chemical process associated with the adenine removal and that the effect is limited to the steps governing the steady-state rate (i.e. product release).
Effect of Ape1 on the Reaction of MutY with a G:A Substrate—The effect of the ExoIII homolog human AP endonuclease Ape1 on the reaction of 3 nM active MutY with 20 nM G:A-containing substrate DNA was also investigated. In these reactions, EDTA was absent from the buffer to prevent removal of the catalytic metal ions from Ape1. As shown in Fig. 3, increasing concentrations of Ape1, in the range of 5–100 nM had no measurable effect on the kinetics of the glycosylase reaction. Ape1 experiments performed in the presence and absence of Mg\(^{2+}\)/EGTA gave similar results (data not shown). Because the AP site is the substrate for all of the AP endonucleases, but an effect is seen only with Endo IV and Exo III, there must be specific interactions between these E. coli endonucleases and either the DNA and/or enzyme in the MutY/product DNA complex. In short, the recognition of the AP site as a substrate is not sufficient to displace MutY from the product-bound complex.

Endo IV Does Not Significantly Increase the Product Release of Stop 225 with G:A- and OG:A-containing DNA—We (60) and others (58, 67) have previously shown that removal of the C-terminal domain to form a truncated enzyme (henceforth referred to as Stop 225) affects the intrinsic rate for adenine removal. However, Stop 225 retains biphasic kinetic behavior under conditions of substrate excess, indicating that the rate of product release remains rate-limiting in a manner similar to that observed with the WT enzyme (60). Indeed, the measured \(k_r\) values for Stop 225 and WT MutY with a G:A substrate are nearly identical (60). Thus, the effect of Endo IV on the rate of

---

**Fig. 1.** Effect of endonuclease IV on the reaction of MutY with a G:A substrate. A, a representative plot of product formation at 37 °C as a function of time, under multiple-turnover conditions, with MutY in the presence of increasing amounts of Endo IV is shown. Conditions: substrate DNA, 20 nM; active MutY, 3 nM; Endo IV, 0 nM (closed circles), 5 nM (open circles), 20 nM (open squares), 50 nM (open triangles), and 100 nM (open diamonds). The steady-state rates \(k_{ss}\) for this particular experiment: 0.04 ± 0.01, 0.06 ± 0.01, and 0.08 ± 0.02 min\(^{-1}\) with 0, 5, and 20 nM Endo IV, respectively. B, representative plot of product formation as a function of time, under single-turnover conditions, with MutY on a G:A-containing DNA duplex in the presence and absence of Endo IV at 37 °C. Conditions: substrate DNA (20 nM) and 60 nM MutY (active site concentration) were reacted in the absence (closed circles) or presence (open circles) of 480 nM Endo IV.

**Fig. 2.** Effect of exonuclease III on the glycosylase activity of MutY with a G:A substrate. A, a representative plot of product formation as a function of time at 37 °C, under multiple-turnover conditions, with MutY in the presence of increasing amounts of Exo III is shown. Conditions: DNA (34G:34A), 20 nM; active MutY, 4 nM; Exo III, 0 nM (closed circles), 77 nM (open circles), 160 nM (open squares), and 380 nM (open diamonds). B, a representative plot of product formation as a function of time at 37 °C, under single-turnover conditions, with MutY (30 nM), DNA (30G:30A, 20 nM), and Exo III, 0 nM (closed circles) and 380 nM (open circles).
the product release step with the truncated form of MutY was studied under similar conditions. In these experiments, 20 nM G:A-containing oligonucleotide was incubated with 4 nM active Stop 225 in the presence of 0, 5, 20, 50, and 100 nM Endo IV (Fig. 4). The calculated rate constant for the steady-state phase in the absence of Endo IV was (0.015 ± 0.005) min⁻¹ and remained essentially unchanged by the presence of Endo IV at all concentrations. This result indicates that the C-terminal domain of MutY, which has been deleted in Stop 225, is at least partially responsible for the enhancement of the steady-state rate by Endo IV on the native enzyme reaction. Similar results were also obtained with an OG:A-containing substrate. The effect of Endo IV on the reaction of MutY with an OG:A-containing duplex was also analyzed under multiple-turnover conditions. No change was observed on the steady-state rate of the reaction of 20 nM OG:A-containing duplex DNA and 3 nM MutY even at the highest concentration (480 nM) of Endo IV assayed (Fig. 7). This suggests that Endo IV is not able to enhance the release of the OG:AP product from MutY.

Because an OG:AP duplex has not yet been characterized as
a substrate for Endo IV, the ability of this enzyme to recognize and cleave a DNA strand containing an AP site opposite OG was tested. The AP site analog tetrahydrofuran (THF) was used due to its increased stability when compared with an abasic site. THF has been previously used in biochemical studies of Endo IV, and it was shown to constitute a good substrate for the enzyme (68). In this study, THF was incorporated into a duplex DNA opposite G and OG, and the ability of Endo IV to cleave the THF-containing DNA strand was determined. Analysis of the reaction of Endo IV with G:THF- and OG:THF-containing oligonucleotides (data not shown) demonstrated that OG:THF is comparable to G:THF as a substrate for Endo IV. This result is consistent with a previous report by Erzberger et al. (68), where the recognition elements for efficient AP endonuclease binding and subsequent activity were analyzed. The authors concluded that the base opposite the AP site is not an important recognition element for Endo IV. Thus, the inability of Endo IV to enhance the OG:AP release from MutY must be a consequence of unique properties of the MutY-OG:AP product complex. This further highlights the differences in recognition and catalysis by MutY of OG:A relative to G:A base pairs.

Displacement of MutY from the OG:AP site product may require formation of a multienzyme complex of BER enzymes. Thus, we also investigated the effect of various combinations of Endo IV, pol I, and MutM on the product release rate \( k_3 \) in the presence of MutY (data not shown). Essentially, all combinations of these enzymes did not enhance the measured \( k_3 \), indicating that the presence of these enzymes under these conditions was unable to enhance release of MutY from the OG:AP product. In addition, we considered the possibility that the 30-bp duplex may be too short to mediate interactions between these various BER enzymes and MutY, and therefore, these experiments were also performed using a 70-bp duplex. Similar results were observed with the 70-bp duplex (data not shown).

Exo III Does Not Increase the Release of MutY from the Product with OG:A-containing DNA Substrate—Similar to Endo IV, Exo III does not increase the product release rate of MutY from OG:A-containing substrate (Fig. 8). Under conditions of 4 nM MutY with 20 nM OG:A-containing DNA, the presence of a relatively high Exo III concentration (380 nM) resulted in no measurable increase in the product release rate \( k_3 \). However, unlike Endo IV, under single-turnover conditions with the buffer conditions used in previous experiments, Exo III processes OG:THF-containing DNA more slowly than G:THF-containing DNA (Fig. 9). This slow processing of the OG:THF-containing DNA may contribute to lack of stimulation of MutY by Exo III with OG:A-containing substrate. However, at high concentrations of Exo III, it is unlikely that the lack of stimulation is solely due to the slow processing of the OG:AP-containing DNA.

We also investigated the effect of various combinations of Exo III, pol I, and MutM on the product release rate \( k_3 \) in the presence of MutY using the 70-bp OG:A-containing duplex substrates (data not shown). Essentially, all combinations of these enzymes did not enhance the measured \( k_3 \), indicating that the presence of these enzymes under these conditions was unable to enhance release of MutY from the OG:AP product.

Displacement of MutY from the DNA Product by Endo IV—The enhancement of the rate of product release \( k_3 \) of MutY by Endo IV suggested that Endo IV may interact with MutY to displace MutY from the DNA, or, alternatively, both Endo IV and MutY may remain bound to the DNA. Non-denaturing PAGE gel-retardation experiments were performed to examine the interaction of Endo IV and \(^{32}\)P-end-labeled DNA duplexes containing OG:A, OG:THF, G:A, or G:THF base pairs, in the
The PAGE analysis indicated that Endo IV was able to displace MutY when a duplex containing a G:A or G:THF base pair was used (Fig. 10). Endo IV (Fig. 10, A and B, lanes 2–4) or MutY (Fig. 10, A and B, lane 5) with the duplex alone exhibit a band with a distinct retarded mobility relative to the free duplex. The mobility differences in the retarded bands are consistent with the larger molecular mass of MutY (~39 kDa) relative to Endo IV (~32 kDa). In addition, in the case of MutY, the retarded band is likely due to binding to the GAP site product that would have formed during the incubation period prior to loading on the gel. As Endo IV was added with increasing concentrations to the MutY-DNA complex, a band with altered migration increased in intensity (Fig. 10, A and B, lanes 6–8). This band comigrates with the lanes for Endo IV with the DNA duplex, indicating that increasing concentrations of Endo IV displace MutY from the DNA duplex. Analogous experiments with Ape1 instead of Endo IV in the presence and absence of Mg\(^{2+}\) with the G:A and G:THF duplex (data not shown) did not result in displacement of MutY from the DNA product or product analog by Ape1. Similar experiments were performed with OG:A- or OG:THF-containing duplex (data not shown); however, no displacement of MutY from the DNA duplex in the presence of Endo IV was observed. This result is consistent with the lack of an effect by Endo IV on the glycosylase activity of MutY with OG:A substrates.

A Super-shifted Band Is Observed in Gel Retardation Assays of MutY, Exo III, and G:A or OG:A-containing DNA—The enhancement of the rate of product release (k\(_{3}\)) of MutY by Exo III may also be due to the ability of Exo III to displace MutY from the DNA duplex. Non-denaturing PAGE gel retardation experiments were performed to examine the interaction of Exo III and 32P-end-labeled DNA duplexes containing OG:A, OG:THF, G:A, or G:THF base pairs, in the presence and absence of MutY.

Gel retardation experiments were performed with increasing concentrations of Exo III (0, 38, 77, 380 nM) with 15 nM OG:A-, G:A-, G:THF-, or OG:THF-containing DNA and 20 nM of active MutY. As shown in Fig. 11A, with the G:A substrate DNA, increasing concentrations of Exo III in the absence of MutY (Fig. 11, lanes 1–4) did not produce a retarded band consistent with an Exo III-DNA complex; this suggests that Exo III has a relatively low affinity for DNA under the assay conditions. However, increasing concentrations of Exo III in the presence of MutY resulted in a super-shifted band with significantly retarded mobility relative to MutY and DNA in the absence of Exo III (Fig. 11, lanes 5–8). Exo III has a molecular mass of 28 kDa whereas MutY has a molecular mass of 39 kDa; therefore, Exo III bound to DNA is not likely to produce a retarded band with slower migration than MutY. Moreover, no retarded bands were observed with Exo III and DNA alone. Thus, it is likely that the slow migrating band that is observed is due to the DNA-MutY-Exo III complex. This indicates that Exo III may interact directly with the MutY product-DNA complex. This interaction appears to be strong enough to be observed in the gel retardation experiments; however, this interaction must also mediate the displacement of MutY from the G:AP product to enhance turnover of MutY.

Similar results were seen with the DNA containing the THF product analog (Fig. 12A). Exo III appeared to not bind to DNA under these conditions in the absence of MutY (Fig. 12A, lanes 1–4) even though G:THF-containing DNA is a substrate for Exo III. However, a similar super-shifted band was observed as the Exo III concentration was increased in the presence of MutY (Fig. 12A, lanes 5–8). Interestingly, a super-shifted band with similar mobility to that observed with the G:THF- and G:A-containing duplex was also seen when an OG:THF-containing DNA duplex was used (Fig. 12B, lanes 5–8) or an OG:A-containing duplex (Fig. 11B). This suggests that the DNA-MutY-Exo III complex forms with both OG:AP- and G:AP-containing product duplexes even though ExoIII only stimulates the release of MutY from G:AP-product DNA.

MutM Displaces MutY from G:A-containing DNA but Not OG:A-containing DNA in Gel Retardation Assays—Low concentrations of MutM have also been shown to stimulate the turnover of MutY; however, as the concentration of MutM was increased, inhibition of the adenine glycosylase activity of MutY was observed. This suggests that the two enzymes may compete for the DNA duplex. Gel retardation assays were performed with increasing concentrations of MutM (0 to 200 nM) with 15 nM G:A-, G:THF-, OG:A-, and OG:THF-containing DNA and 20 nM of active MutY. In Fig. 13 the resulting autoradiogram from the experiment with the G:A-containing DNA is shown. Similar results were obtained with the G:THF duplex (data not shown). Increasing concentrations of MutM in the absence of MutY (Fig. 13, lanes 1–4) resulted in a significant amount of retarded band due to the MutM-DNA complex; thus, MutM has a high affinity for the DNA duplex under these assay conditions. When the concentration of MutM increases in the pres-
ence of MutY, the amount of DNA bound to MutY decreases as the amount of DNA bound to MutM increases (Fig. 13, lanes 5–8). Analysis of samples used in the gel retardation assay by denaturing PAGE (12%) indicates that all of the G:A-containing duplex had been converted to product during the incubation time prior to loading on the gel in the gel retardation experiment. Thus, these experiments illustrate that MutM is able to displace MutY from the G:AP site DNA product. However, displacement of MutY by MutM was not observed when an OG:A- or OG:THF-containing DNA duplex was used.
MutY, and 50 nM MutM; substrate. Conditions: DNA, 15 nM; active MutY, 20 nM. The presence of increasing amounts of MutM results in displacement of MutY by MutM from G:A-containing DNA duplex (30G:30A). The gel resolution of DNA bound to MutY and free G:A-containing DNA duplex (30G:30A).

**DISCUSSION**

During the past several years, considerable advances have been made in the understanding of the structural and mechanistic details of BER DNA glycosylases. Many studies on the substrate specificity and kinetics have been performed, aimed at characterizing the behavior of these important enzymes. In particular, pre-steady-state kinetics of the reactions of E. coli MutY (24, 60), hTDG (17, 18), and the human mismatch-specific glycosylase MED1 (22) have been thoroughly investigated. In addition, analyses of the reactions of hUDG (19), E. coli DUG (23), hOGG1 (20, 21), and mMYH (26) reveal a similar type of kinetic behavior, and therefore a common theme for this class of enzymes is emerging. In all of these cases, the overall rate for the catalytic cycle is controlled by a step following base removal as evidenced by characteristic burst kinetics for product formation. This rate-limiting step is dominated by the rate of the release of the enzyme from the DNA product. Our laboratory has used this kinetic behavior to our advantage to determine the magnitude of rate constants for both steps of mutagenesis.

The release of the product by these enzymes in vivo may require the aid of cellular factors to protect the cell from the deleterious effect of the AP site that remains after base removal. For example, Ape1 has been shown to enhance the steady-state reaction of hTDG, hUDG, hOGG1, and mMYH (17, 19–21, 26). In addition, previous work with E. coli DUG (also called MUG) indicated that the presence of the E. coli AP endonuclease Endo IV enhances product release (23). In the case of the reaction of MutY with a G:A substrate, a dramatic change in the steady-state rate of the reaction was observed upon addition of Endo IV. The rate is significantly enhanced to a degree that is dependent on the Endo IV concentration. At the higher Endo IV concentrations tested, the product release step is no longer rate-limiting and the product formation curve is no longer biphasic. Similar results were also obtained using the E. coli AP endonuclease Exo III (67); however, the stimulation of the turnover of MutY with Exo III required a significantly higher concentration of protein. This may be due to the reduced activity of Exo III or may suggest a weaker interaction of Exo III with the MutY-DNA complex. Surprisingly, the human homolog to Exo III, Ape1, did not stimulate turnover of MutY. This indicates that recognition of the AP site alone is not responsible for the enhanced turnover and suggests that there may be specific recognition of the E. coli MutY-product complex by the E. coli AP endonucleases.

Many enzymes of the BER pathway have significant affinity for undamaged DNA and have been proposed to recognize their substrates through a DNA-scanning process. To rule out nonspecific binding as a cause for the Endo IV and Exo III effect, three other enzymes belonging to the BER pathway have been assayed for potential effects on the glycosylase activity of MutY. For two of them, UDG and DNA polymerase I, no effect on the reaction of MutY with a G:A substrate was detected. At low concentrations of MutM, on the other hand, enhancement of the turnover of MutY in a fashion similar to Endo IV and Exo III was observed. However, in contrast to the effect observed with the E. coli AP endonucleases, the glycosylase reaction of MutY is inhibited at high concentrations of MutM. Indeed, even the highest concentrations of Endo IV or Exo III tested did not diminish the glycosylase activity of MutY. This suggests that MutM and MutY compete for the DNA duplex and that this may be part of the mechanism by which MutM stimulates the turnover of MutY. Because Endo IV and Exo III do not seem to inhibit the glycosylase reaction at any concentration, this suggests that competition between Endo IV or Exo III and MutY for non-target DNA does not contribute to the effect observed.

Endo IV, Exo III, and MutM are all able to enhance the turnover of MutY with a G:A substrate; however, there are hints from the effects on the glycosylase activity of MutY and the gel retardation experiments that the exact mechanism of the enhanced turnover may be different for the three enzymes. The fact that the presence of MutM inhibits the glycosylase activity of MutY whereas Exo III and Endo IV do not suggests that MutM may be able to more effectively compete with MutY for the DNA duplex. Indeed, MutM binds to the G:A-containing duplex in the absence of MutY. A distinct feature of Exo III, is that, unlike Endo IV and MutM, it does not bind to the DNA duplex under the conditions used; however, Exo III does bind to the MutY-DNA complex to give a super-shifted band in the gel retardation assay. In contrast, in the gel retardation assay, both Endo IV and MutM result in displacement of MutY from the DNA duplex and formation of an Endo IV-DNA or MutM-DNA complex. This is highly suggestive that Exo III may be recognizing a specific structure of MutY bound to the product DNA. Experiments are in progress to further explore both the interaction of Endo IV and Exo III with MutY, and this may provide additional insight into the features required for the enhanced turnover.

Specific recognition of the MutY-AP site DNA product by the E. coli AP endonucleases may be responsible for the enhanced turnover of MutY. In the three-dimensional structure of Endo IV bound to an AP site-containing oligonucleotide, a major distortion of the DNA structure was observed (72). Enzyme loops intercalate amino acid side chains at the AP site, compress the phosphate backbone, and bend the DNA by ~90°. This DNA distortion upon Endo IV binding could be one of the factors reducing the affinity of MutY for the DNA and promoting its dissociation from the duplex. Indeed, in a thorough analysis of the structural characteristics of the enzymes in the human BER pathway, Tainer et al. (29) showed that the extent of DNA bending increases as the different enzymes in the pathway bind to the DNA. For example, Ape1 has an extensive DNA binding surface and causes considerable distortion of the bound DNA (73). The degree of kinking of the DNA by Ape1 is considerably more than that observed by the preceding glycosylase (such as hUDG). This has led Tainer and coworkers (73) to suggest that these features of Ape1 recognition of AP site substrate DNA allow for displacement of the glycosylase bound to its AP site product. A similar mechanism would allow po-
lymerase β to displace Ape1 bound to its nicked AP site product, and therefore, this generic mechanism would ensure direct and sequential transfer of the DNA duplex to the next enzyme action of various proteins in the BER pathway as well. However, at present, there is less evidence than in the mammalian counterparts for a coordinated repair effort.

Other elements may also be important for the effect of Endo IV and Exo III on the adenine glycosylase activity of MutY. The lack of enhancement in the turnover rate of Stop 225 by Endo IV or Exo III indicates that the C-terminal domain of MutY plays an important role in mediating the E. coli AP endonucleases effect on the product release. It is possible that Endo IV/Exo III binds to the MutY-G-AP-DNA complex by recognizing features of MutY, such as the C-terminal domain, and the Watson-Crick portion of the DNA duplex to promote MutY dissociation. Subsequent formation of the specific complex between Endo IV or Exo III and its substrate, the AP site, leads to strand cleavage catalysis. These results suggest that there may be a direct interaction of the E. coli AP endonucleases and MutY, possibly involving the C-terminal domain of MutY. A direct interaction between the human homolog of MutY, hMutY, and Ape1 in vivo has been demonstrated using immunoprecipitation and Western blotting (75). It is also possible that the C-terminal domain of MutY may be playing a more indirect role in mediating the effect with Endo IV or Exo III. The presence of the C-terminal domain may be necessary to provide the proper degree of DNA bending and/or distortion that is needed for recognition of the MutY product complex by Endo IV and Exo III. Indeed, recognition of the unusual structure of the AP site duplex in the presence of MutY by an E. coli AP endonuclease may be more important than recognition of MutY.

Surprisingly, an enhancement by Endo IV or Exo III of the glycosylase reaction of MutY with an OG:AP substrate was not detected. The reported differences in the MutY reaction with G:A compared with OG:A substrates suggest distinct properties of the recognition and catalysis by MutY with these two base pairs (24). Indeed, the faster turnover of MutY with the G:A substrate suggests that the affinity of the enzyme for the G:AP site product is lower than for the OG:AP product (24). This has also been reflected by smaller dissociation constants for OG:THF compared with G:THF (60). Waters et al. (17) also observed that Ape1 was able to enhance the turnover of hTDG more efficiently with a C:U mismatch than a G:T mismatch. This observation also corresponded to the weaker affinity of hTDG for the C:AP product compared with the G:AP product such that steady-state turnover was much faster with a C:U substrate. The different characteristics of MutY complexed with its two products (G:AP and OG:AP) could also affect the interaction with Endo IV and Exo III. Interestingly, in the gel retardation experiments, a “super-shifted” band was observed in the presence of Exo III, MutY, and OG:AP or G:THF duplex. This indicates that Exo III recognizes the complex between MutY and the OG:THF duplex, even though this interaction does not lead to enhancement of turnover. Thus, stimulation of turnover of MutY with an OG:AP substrate may require the participation of other proteins, in addition to Exo III or Endo IV, that are not necessary with the G:AP substrate. In contrast, in experiments with the murine homolog of MutY with an OG:AP substrate, enhancement of turnover with Ape1 was observed (26). Thus, this further underscores the unusual behavior of E. coli MutY with its OG:AP product.

The endonuclease action of Endo IV or Exo III on OG:AP may be harmful, because it would result in the formation of a strand break opposite the damaged OG base producing an extremely unstable site in the DNA. This type of site in DNA is a substrate for the OG glycosylase MutM, and therefore, action of MutM may result in double-strand breaks that may further promote loss of genetic information or cell death. As suggested earlier, the observed enhancement of the turnover of MutY by MutM may be the result of competition of the two enzymes for the non-target DNA. Alternatively, it is possible that MutY and MutM interact in a multienzyme complex to modulate each other’s activity to some extent. A multienzyme complex has been isolated from bovine testis that is capable of repair of uracil in DNA (76), and this suggests that BER enzymes may exist in a preformed “reparasome” that can carry out repair in a concerted, rather than stepwise, manner. The formation of such a complex may be necessary for the activity of MutY with OG:A substrates due to the unique properties of the OG:AP product, which differs from the products of other BER glycosylases in that both strands are “damaged.” Indeed, the high affinity of MutY for the OG:AP site may serve as a mechanism for blocking DNA replication or transcription until the “OG” and “AP” sites have been fully repaired. Further investigation of the unusual properties of MutY with its OG:AP site product will be required and will likely provide unique insight into the coordination of repair events in the cell.

REFERENCES
1. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, ASM Press, Washington, D. C.
2. David, S. S., and Williams, S. D. (1998) Chem. Rev. 1221–1261
3. Scharer, O. D., and Jiricny, J. (2001) Bioessays 23, 270–281
4. Matsumoto, Y., and Bogenhagen, D. F. (1989) Mol. Cell. Biol. 9, 3750–3757
5. Wang, Z., Wu, X., and Friedberg, E. C. (1993) Mol. Cell. Biol. 13, 1051–1058
6. Diano, G., Price, A., and Lindahl, T. (1992) Mol. Cell. Biol. 12, 1605–1612
7. Diano, G., and Lindahl, T. (1994) Curr. Biol. 4, 1069–1076
8. Wiebauer, K., and Jiricny, J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5842–5845
9. Kubota, Y., Nash, R. A., Klungland, A., Schar, P., Barnes, D., and Lindahl, T. (1999) EMBO J. 18, 6662–6670
10. Harrison, L., Hatahet, Z., and Wallace, S. S. (1999) J. Mol. Biol. 290, 667–684
11. Lane, D. P., Abbondandolo, A., and Dogliotti, E. (1999) J. Biol. Chem. 274, 2276–2285
12. Frosina, G., Fortini, P., Rossi, O., Carrozzino, F., Rasapaglio, G., Cox, L. S., and Peroni, G. (1996) J. Biol. Chem. 271, 9573–9578
13. Klungland, A., and Lindahl, T. (1997) EMBO J. 16, 3341–3348
14. Podson, M. L., Michaels, M. L., and Lloyd, S. R. (1994) J. Biol. Chem. 269, 32079–32712
15. Lindahl, T. (1990) Mutat. Res. 227, 305–311
16. Barbazil, G., and Hickson, I. D. (1995) Bioessays 17, 713–719
17. Waters, T. R., Gallinari, P., Jiricny, J., and Swann, P. F. (1999) J. Biol. Chem. 274, 67–74
18. Waters, T. R., and Swann, P. F. (1998) J. Biol. Chem. 273, 20007–20014
19. Parikh, S. S., Mol, C. D., Slupphaug, G., Bharati, S., Krokak, H. E., and Tainer, J. A. (1998) EMBO J. 17, 5214–5226
20. Vidal, A. E., Hickson, I. D., Boiteux, S., and Radicella, J. P. (2001) Nucleic Acids Res. 29, 1285–1292
21. Hill, V. J., Hazra, T. K., Izuomi, T., and Mitra, S. (2001) Nucleic Acids Res. 29, 430–438
22. Petronzelli, F., Riccio, A., Markham, G. D., Seelohrer, S. H., Steecker, J., Genuardi, M., Yeung, A. T., Matsumoto, Y., and Bellacosa, A. (2000) J. Biol. Chem. 275, 32422–32429
23. Sung, J.-S., and Mosbaugh, D. W. (2000) Biochemistry 39, 10224–10225
24. Parikh, S. S., Lelys, A. E., and David, S. S. (1998) Biochemistry 37, 14756–14764
25. Parse, S. L., Williams, S. D., Kuhn, H., Michaels, M. L., and David, S. S. (1999) J. Am. Chem. Soc. 121, 10694–10692
26. Yang, H., Clendenin, W. M., Wong, D., Demple, B., Slupska, M. M., Chiang, J.-H., and Miller, J. H. (2001) Nucleic Acids Res. 29, 743–752
27. Bennett, R. O., Wilson, D. M., Wong, D., and Demple, B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7158–7163
28. Caldecott, K. W., Aoufouchi, S., Johnson, P., and Shall, S. (1996) Nucleic Acids Res. 24, 4387–4394
29. Parikh, S. S., Mol, C. D., Hosfield, D. J., and Tainer, J. A. (1999) Curr. Opin. Struct. Biol. 9, 37–47
30. Demple, B., Bailey, E., Bennett, R. A. O., Masuda, Y., Wong, D., and Xu, Y.-J. (1999) in Advances in DNA Damage and Repair (Dizdaroglu, M., and Karakaya, A. E., eds) pp. 59–66, Karakaya Kluwer Academic/Plenum, New York
31. Doetsch, P. W., and Cunningham, R. P. (1998) Mutation Res. 438, 173–201
32. Mal, C. D., Kuo, C.-F., Thayer, M. M., Cunningham, R. P., and Tainer, J. A. (1995) Nature 374, 381–386
33. Demple, B., Herman, T., and Chen, D. S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11450–11454
Escherichia coli Apurinic-Apyrimidinic Endonucleases Enhance the Turnover of the Adenine Glycosylase MutY with G:A Substrates
Mary Ann Pope, Silvia L. Porello and Sheila S. David

J. Biol. Chem. 2002, 277:22605-22615.
doi: 10.1074/jbc.M203037200 originally published online April 17, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203037200

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 72 references, 32 of which can be accessed free at http://www.jbc.org/content/277/25/22605.full.html#ref-list-1