Escherichia coli MutY and Fpg Utilize a Processive Mechanism for Target Location†

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Received July 1, 2002; Revised Manuscript Received October 14, 2002

ABSTRACT: MutY and formamidopyrimidine-DNA-glycosylase (Fpg) are base-excision repair (BER) enzymes involved in the 8-oxoguanine repair pathway in *Escherichia coli*. An impressive feature of these enzymes is the ability to locate 8-oxoguanine lesions among a large excess of undamaged DNA. To provide insight into the mechanism of target location, the ability of these enzyme to utilize a one-dimensional processive search (DNA sliding) or distributive (random diffusion-mediated) mechanism was investigated. Each enzyme was incubated with double-stranded concatemeric polynucleotides containing a site-specific target site at 25-nucleotide (nt) intervals. The products of each reaction were analyzed after further treatment and denaturation. A rapid accumulation of predominantly 25-nt fragments would indicate the utilization of a processive mechanism, whereas oligomeric multiples of 25-nt fragments would form if a distributive mechanism were used. Both Fpg and MutY were found to function processively on concatemers containing 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG)·C and G·A mispairs, respectively. An increase in sodium chloride concentration results in the modulation from a processive to distributive mechanism for both enzymes. Interestingly, processive behavior was not observed in the reaction of MutY with concatemers containing OG·A mispairs. A truncated form of MutY (Stop 225) containing only the N-terminal domain was found to behave in a manner consistent with a processive mechanism with both OG·A- and G·A-containing substrates. This suggests that the C-terminal domain of MutY plays an important role in the mechanism by which the enzyme detects OG·A base pairs in DNA.

All organisms are faced with oxidative stress that results in the presence of 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG)[1] within genomic DNA. Indeed, OG is often used as a biomarker indicative of the extent of oxidative damage in tissues (*1*, *2*). OG may accumulate in DNA by two different pathways. First, it may be misincorporated into DNA during replication via polymerase insertion of dOGTP in place of dGTP, forming OG·C or OG·A mispairs (*3*, *4*). Second, oxidative damage at a G·C base pair within a DNA duplex may result in the OG·C lesion. In vitro experiments with DNA polymerases have shown that A is often misinserted opposite OG to form an OG·A base pair (*5*, *6*). The propensity of OG to cause misincorporation of A is consistent with the increased frequency of G·C to T·A transversion mutations caused by the presence of OG in DNA (*6*, *7*). *Escherichia coli* employs two base excision repair (BER) enzymes to prevent G·C to T·A transversion mutations by initiating the repair of OG·C and OG·A mismatches (*8*, *9*). Fpg (MutM) catalyzes the removal of 8-oxoguanine from OG·C mispairs, while MutY catalyzes the removal of the incorrect adenine from OG·A mispairs (*10*). The recruitment of AP endonucleases, phosphodiesterases, a repair polymerase, and DNA ligase effect restoration of the original DNA sequence (*10*, *11*). The importance of the repair of OG to human health has recently been highlighted by the finding of a direct correlation between inherited variants of human MutY (hMYH) and colorectal cancer (*12*).

E. coli MutY is a 39.1 kDa monomeric polypeptide of 350 amino acids which represents a class of BER glycosylases that remove a variety of improper bases (*10*). MutY is classified as a monofunctional glycosylase in that it catalyzes the removal of adenine, forming an AP site, without participating in strand scission (*13*). Previous work has shown that MutY is able to remove adenine from several potentially biologically relevant mispairs including OG·A, G·A, and C·A (*8*, *14*, *15*). Significant differences in the rates for adenine removal have been observed depending on whether the opposite base is OG or G (*16*). In addition, product release is rate-limiting with both OG·A and G·A substrates; however, the observed steady-state rate is considerably smaller with OG·A substrates compared to G·A substrates due to the higher affinity of MutY for the OG·(AP site) product. In contrast, the intrinsic rate for adenine removal is considerably larger for OG·A substrates.

† This work was supported by the National Institutes of Health (CA 67985) and the Department of Chemistry of the University of Utah. S.S.D. is an A. P. Sloan Research Fellow (1998−2002).

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[1] Abbreviations: A, 2′-deoxyadenosine; Abs, absorbance; AP, apurinic−apyrimidinic; bp, base pair; BER, base-excision repair; BSA, bovine serum albumin; C, 2′-deoxycytidine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; endo III, *Escherichia coli* endonuclease III; Fpg, formamidopyrimidine-DNA-glycosylase; Fapy, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; G, 2′-deoxyguanosine; HPLC, high-performance liquid chromatography; MTO, multiple- turnover; Nt, nucleotide; OG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; PAGE, polyacrylamide gel electrophoresis; STO, single-turnover; Stop 225, truncated form of *Escherichia coli* MutY containing residues Met 1−Lys 225; T, thymidine; TBE, tris(hydroxymethyl)aminomethane−borate−ethylenediaminetetraacetic acid buffer; Tris, tris(hydroxymethyl)aminomethane; WT, wild-type.

10.1021/bi026375+ CCC: $25.00
Published on Web 12/31/2002

When subjected to limited proteolytic digestion, MutY may be divided into two distinct domains. The N-terminal domain (1−225), also called Stop 225, exhibits high sequence and structural similarity to *E. coli* endonuclease III (endo III) (*17*, *18*). MutY and endo III, both members of the BER superfamily, share several features in common: a $[4Fe\text{-}4S]^{2+}$ cluster with accompanying iron−sulfur cluster loop (FCL) motif, a conserved helix−hairpin−helix (HhH) motif, and a highly conserved aspartate residue (*10*, *19*). Sequence analysis and NMR studies have shown that the C-terminal domain (226−350) shares sequence and structural homology to MutT, the *E. coli* d(OG)TPase (*20*, *21*). In addition to these homology studies, kinetic experiments have been performed that, when taken together, imply that the C-terminal domain of MutY may contain the required OG binding motif (*20*, *22*).

E. coli Fpg is a 30 kDa monomer of 269 amino acids that is chiefly involved in the catalytic removal of oxidized guanines opposite cytosine in DNA (*10*). However, Fpg is capable of removing a variety of other lesions, including ring-opened purines such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy), guanidinohydantoin, spiroiminodihydantoin, and oxidized pyrimidines such as 5-hydroxyuracil (*10*, *23*). In addition to its glycosylase activity, Fpg also catalyzes β- and δ-elimination of the AP site to result in DNA strand scission (*24*). A number of crystal structures have recently been determined for Fpg bound to the product DNA, and these have provided interesting information on the features involved in product binding, and clues as to the influence of the opposite base (*25*−*27*). However, information relating to the ability of this enzyme to accommodate such a wide variety of substrates is still needed.

One obstacle BER glycosylases must overcome is the challenge of locating a specific damaged base among a sea of similar nondamaged bases. In general, there are two distinct mechanisms these enzymes may use during target location: distributive and processive (*28*). In the distributive mechanism, the enzyme randomly binds to the recognition site, catalyzes its reaction, and then dissociates in search of another target. In the processive, or scanning, mechanism, the enzyme nonspecifically binds to the DNA, translocates along the duplex to the recognition site, catalyzes its reaction, and then continues to scan the DNA without dissociation. This processive mechanism effectively converts a three-dimensional search into a one-dimensional search, thus increasing the efficiency. Several DNA repair enzymes, such as T4 endonuclease V (*29*−*32*), the UvrABC repair complex (*33*), uracil DNA glycosylase (*34*, *35*), and *Micrococcus luteus* UV endonuclease (*36*), have been shown to utilize a processive mechanism. In this study, we demonstrate that *E. coli* MutY and Fpg are able to utilize a processive mechanism in locating their respective damaged sites. In addition, the C-terminal domain of MutY was shown to play a role in target location through conferred substrate specificity.

MATERIALS AND METHODS

General Methods. T4 polynucleotide kinase, T4 DNA ligase, and *Eco*RI was obtained from New England Biolabs, while [γ-^{32}P]ATP was purchased from Amersham Life Sciences. Bovine serum albumin (BSA) and Bradford reagents were obtained from Bio-Rad. Standard (2-cyanoethyl)phosphoramidites were purchased from Applied Biosystems Inc., while 8-oxo-7,8-dihydro-2′-deoxyguanosine phosphoramidite was purchased from Glen Research. All other chemicals were purchased from Fisher Scientific, Sigma, or USB. Milli-Q distilled, deionized water was used for all reactions. All buffers were filtered through a nylon or nitrocellulose 0.22 μm filter prior to use. MutY purification was done on a Bio-Rad Bio-Logic FPLC system. Storage phosphor autoradiography was performed on a Molecular Dynamics Storm 840 phosphorimager. ImageQuaNT software (v4.2a) was used to quantify autoradiograms. UV/Vis spectra were taken on an HP 8452A diode array spectrophotometer.

Oligonucleotides. The oligonucleotides 5′-TTCATGAGCCAC**X**AGCTCCGTCGAA-3′, where X is OG or 2′-deoxyguanosine (G), and the complementary strand 3′-GCTTAAGTACTCGGTG**Y**TCGAGGCA-5′, where Y is 2′-deoxyadenosine (A) or 2′-deoxycytidine (C), were synthesized on an ABI 392B automated synthesizer according to the manufacturer's protocols and purified via ion-exchange HPLC. A 30 nucleotide blunt-ended duplex substrate was also used as a control, which has the following sequence: 5′-CGATCATGGAGCCAC**X**AGCTCCCGTTACAG-3′, where X is OG or G, and 3′-GCTAGTACCTCGGTG**Y**TCGAGGGCAATGTC-5′, where Y is A or C. Synthesis and purification were performed as described above.

Enzymes. Expression and purification of MutY, Stop 225, and Fpg from pKKYEco/JM109, pKKYS225/JM101 *mutY*::mini-Tn10, and pKKFapy2/JM109, respectively, were performed in a manner as previously described (*22*, *23*, *37*). Active-site titration methods for MutY were performed as previously described (*16*). This provided active-site concentrations relative to the total protein concentration determined by Bradford (*38*) for MutY and Stop 225 of 61% and 21%, respectively. The active-site concentration of Fpg was determined to be 25% on the basis of a DNA binding assay (*23*). All enzyme concentrations listed have been corrected for the active enzyme concentration.

5′-End Phosphorylation Reactions. The 25-nt oligonucleotides containing A or OG described above were 5′-end-^{32}P-labeled in 30 μL reaction mixtures containing 6 nmol of 25-mer, 100 μCi of [γ-^{32}P]ATP (6000 Ci/mmol), and 20 units of T4 polynucleotide kinase in buffer K [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.1 mM EDTA]. After these mixtures were incubated at 37 °C for 45 min, 100 μCi of [γ-^{32}P]ATP was added and the mixture was allowed to incubate for an additional 30 min. Excess [γ-^{32}P]ATP was removed by use of a ProbeQuant G-50 microcolumn as per the manufacturer's protocols. Complementary oligonucleotides for ligation experiments were 5′-end-phosphorylated in similar reactions deficient in ^{32}P-labeled ATP.

Glycosylase Assays with MutY and Fpg. The glycosylase activity of MutY and Fpg was monitored by use of either the 25-nt duplex with overhanging ends or the 30-bp oligonucleotide blunt-end duplex substrate, containing a centrally located G·A, OG·A, or OG·C base pair, as described previously by our laboratory (*16*, *22*, *23*). In the MutY reaction, the A-containing strand was ^{32}P-labeled on the 5′-end, and the rate of adenine removal was monitored by removing aliquots at various times and adding NaOH to quench the reaction and promote strand cleavage at the abasic

site. In the Fpg reactions, the OG-containing strand was ^{32}P-labeled on the 5′-end, and the rate of OG removal and associated β- and δ-lyase strand cleavage was monitored by removing aliquots at various times, which were quenched with denaturing loading dye (80% formamide, 0.025% xylene cyanole, 0.025% bromophenol bue in TBE buffer) and then placed on dry ice. The substrate DNA (30-nt strand or 25-nt strand) and the product-derived oligonucleotide (14-, 12-, or 8-nt strand) were separated in an 8 M urea denaturing polyacrylamide gel (15%) and then quantified via storage phosphor autoradiography.

The A- or OG-containing strand of the DNA duplexes used was 5′-^{32}P-end-labeled with T4 polynucleotide kinase, and the end-labeled strand was added to a final concentration of 2−5% to the same unlabeled strand. The complementary strand was then added in slight excess (15%). 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 with MutY, 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, and 0.1 mg/mL BSA). 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 40 nM. Single-turnover experiments with Fpg were performed under similar conditions with 20 nM duplex and 200 nM Fpg. Derivation of rate constants was determined as described previously (*16*, *23*) by use of the minimal kinetic scheme below:

Scheme 1

$$E + (DNA)_S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot(DNA)_S \xrightarrow{k_2} E\cdot(DNA)_P \xrightarrow{k_3} (DNA)_P + E$$

Annealing and Ligation Reactions for Processivity Experiments. For MutY and Stop 225 experiments, ^{32}P-end-labeled A-containing 25-mer was annealed with either unlabeled OG- or G-containing complement 25-mer to form OG·A- and G·A-containing duplexes. For Fpg experiments, ^{32}P-end-labeled OG-containing 25-mer was annealed with either unlabeled C- or A-containing complement 25-mer to form OG·C-and OG·A-containing duplexes. In all cases a slight excess (15%) of unlabeled complementary 25-mer was added. The mixture was heated to 90 °C in a buffer containing 50 mM NaCl, 20 mM Tris-HCl (pH 7.6), and 10 mM EDTA and then allowed to cool to 20 °C over 4−6 h to promote duplex formation.

The annealed products were ligated in a 135 μL reaction mixture containing 3 nmol of annealed duplex, 200 units of T4 DNA ligase, and 20 units of T4 polynucleotide kinase in buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM dithiothreitol, 1 mM ATP, and 25 μg/mL BSA. The ligation mixture was incubated at 16 °C for 2 h, followed by the addition of 200 units of ligase and 50 nmol of ATP and incubation for an additional 16 h. After the ligation, the reaction mixture was fractionated on a 6% polyacrylamide gel. Polynucleotide fragments, 250−450 nucleotides long, were cut from the gel and then eluted by the crush and soak method (*39*). The concentration of substrate after purification was calculated on the basis of known specific radioactivity.

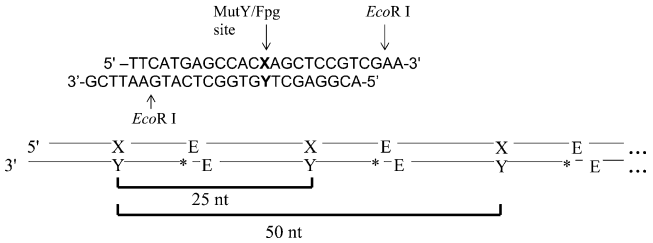

FIGURE 1: Design of substrate concatemers containing nonoverlapping glycosylase and *Eco*RI restriction sites every 25 bp.

Processivity Experiments. The activity of MutY, Stop 225, and Fpg on their respective concatemeric substrates was measured in final reaction mixtures containing 5 μM duplex DNA (average length ∼350 bp), 0.1 mg/mL BSA, and 4 nM enzyme in a buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM EDTA, and 10% glycerol. Various amounts of NaCl were added as indicated in each figure caption. The reaction mixture was incubated at 37 °C, with aliquots removed and quenched at varying time points. MutY and Stop 225 reactions were quenched with 0.1 M NaOH and heated to 90 °C to assist strand cleavage at the product AP sites. Fpg reactions were quenched by adding 90 °C formamide denaturing dye containing 80% formamide, 0.025% xylene cyanol, and 0.025% bromophenol blue in 1× TBE buffer. *Eco*RI reactions were conducted with 4 units enzyme and 5 μM duplex DNA in a buffer containing 50 mM NaCl, 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 0.025% Triton X-100 at 37 °C. Reactions were quenched by heating at 70 °C for 20 min.

All reaction aliquots were combined with equal volumes of denaturing loading dye and then analyzed via electrophoresis through a 15% polyacrylamide gel containing 8 M urea with TBE buffer for 2 h at 1400 V. The extent of strand cleavage was determined by quantitation of the storage phosphor autoradiogram. The intensities of bands are assumed to be proportional to their concentration. In the case of 50-nt and larger fragments, the intensities were corrected for the number of ^{32}P-labeled phosphates.

RESULTS

Construction of Concatemeric Substrates. To determine whether MutY and Fpg utilize a processive or distributive target location mechanism, a concatemeric duplex DNA substrate with specific recognition sites every 25 base pairs was constructed from ^{32}P-end-labeled 25-nt units. Figure 1 illustrates the synthesis and structure of the substrate and possible product fragments. By using a substrate containing recognition sites at regular intervals, the two mechanisms should produce distinct fragment distributions as a function of time. Initial binding of the enzyme could occur at any site along the DNA substrate, resulting in a cleavage event at any one of the evenly dispersed targets. Thus, at early time points, a random distribution of fragment lengths would appear for both mechanisms. If the enzyme dissociates from the duplex after each catalytic event, as would be dictated by a distributive mechanism, subsequent rounds of catalysis will continue to produce a random collection of fragment lengths in multiples of 25 nt. However, if a processive mechanism is operative, the enzyme will translocate one-

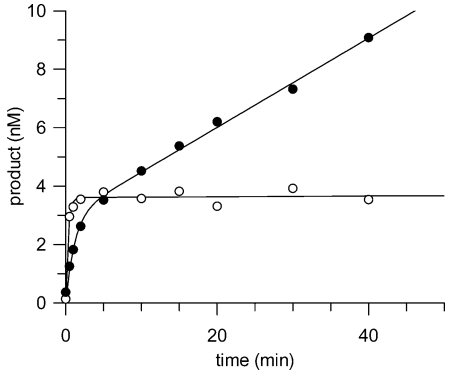

FIGURE 2: Glycosylase activity of MutY on 25-nt monomer substrates containing OG·A (○) or G·A (●) used for ligation to make concatemeric substrates. Shown is a representative plot of product formation as a function of time under multiple-turnover conditions (3 nM MutY and 20 nM 25-mer monomeric substrate). Catalytic rate constants derived from several experiments for MutY, Stop 225, and Fpg are listed in Table 1.

dimensionally along the substrate to the next recognition site resulting in an accumulation of 25-nt fragments without the formation of 50-nt fragments. The interpretation of the data from this type of assay is based upon the assumption that the enzyme would not slide past a recognition site without acting upon it. Additionally, this assay does not provide information regarding the direction of enzymatic sliding.

Monomer Assay. To support the interpretation of data on the enzymatic activity with the concatemeric substrates, it was important to determine the efficiency of base removal by MutY and Fpg from their respective monomeric duplex substrates. Therefore, reactions of each substrate were performed on the labeled, annealed, unligated 25-nt duplex substrates described above. Reactions were performed under single- and multiple-turnover conditions over a 32-min time course to elucidate the rate of catalysis and substrate turnover. A representative plot of product formation as a function of time with MutY and the 25-nt OG·A- or G·A-containing substrate under multiple-turnover conditions ([MutY] < [DNA]) is shown in Figure 2. Under MTO conditions, biphasic kinetic behavior was observed with MutY. This is analogous to previous work in our laboratory and is likely due to slow release of MutY from the DNA product (*16*). Appropriate fitting of the data (*16*), along with data from single-turnover experiments, allow for determination of k_2 and k_3 rate constants for the chemical reaction step(s) and product release step(s), respectively (Scheme 1). MutY, Stop 225, and Fpg exhibited rate constants for base removal (Table 1) with the 25-nt duplex substrate that were comparable to those obtained with duplex substrates that have been used previously (*16*, *22*, *23*). The high affinity of MutY for the abasic product is reflected in the small values for k_3 in Table 1. Analogous to previous work, Stop 225 displayed a faster rate of product release (larger k_3) for OG·A-containing substrates but similar rates for G·A-containing substrates compared to full-length MutY (*22*). Fpg is kinetically different from MutY in that product release is not rate-limiting; therefore, a value for k_3 cannot be directly obtained from multiple-turnover experiments. As illustrated in Table 1 and Figure 2, the 25-nt monomeric duplexes were shown to be competent substrates for both enzymes.

EcoRI Assay. To characterize the concatemeric DNA as potential substrates for processivity experiments, experiments

Table 1: Rate Constants for Glycosylase Activity of Fpg and MutY on Various DNA Substrates at 37 °C

	OG·A[a]		G·A[a]	
	k_2[b] (min^{-1})	k_3[c] (min^{-1})	k_2[b] (min^{-1})	k_3[c] (min^{-1})
		MutY		
25-mer[d]	>10	0.002 ± 0.001	1.3 ± 0.2	0.02 ± 0.01
30-mer[e]	>10[f]	0.004 ± 0.002[f]	1.2 ± 0.4[f]	0.03 ± 0.01[f]
		Stop 225		
25-mer[d]	0.6 ± 0.2	0.010 ± .005	0.20 ± 0.04	0.02 ± 0.01
30-mer[e]	0.4 ± 0.1[g]	0.010 ± 0.003[g]	0.15 ± 0.01[g]	0.03 ± 0.01[g]

	k_2[b] (min^{-1})	
Fpg[h]	OG·C[a]	OG·A[a]
25-mer[d]	>7[i]	0.2 ± 0.01
30-mer[e]	>7[i]	0.3 ± 0.01

[a] Designates centrally located base pair within the duplex. [b] The kinetic rate constant, k_2, is obtained from single-turnover experiments. [c] The rate constant k_3 is obtained from multiple-turnover experiments. [d] Designates 25-nt duplex with overhanging ends used in formation of concatemeric substrates. [e] Designates 30-bp duplex with blunt ends, a standard substrate used in this laboratory. [f] MutY data with the 30-bp duplex were reported previously in ref *16*. [g] Stop 225 data with the 30-bp duplex were previously reported in ref *22*. [h] The value of k_3 for Fpg is not shown, given that product release with Fpg is not rate-limiting and therefore not as readily determined. [i] Estimated lower-limit of rate based on first time-point. Rate is too fast for manual method used in this work.

with the *Eco*RI restriction endonuclease were performed. The scanning capabilities of *Eco*RI have been well established and, therefore, were exploited to provide a model for the production of various fragment lengths as a function of time with a processive enzyme (*40*, *41*). Similar to what was reported by Bennett and Sanderson (*34*), the substrates were engineered to contain regularly spaced *Eco*RI restriction sites that would properly form only if ligation occurred correctly. Therefore, proper ligation would result in *Eco*RI restriction sites every 25 base pairs.

The digestion products of *Eco*RI treatment upon each substrate were analyzed by denaturing PAGE. As shown in Figure 3, a rapid accumulation of 25-nt fragments occurred and continued to be the major product over the course of time. Thus, the observation of 25-nt fragments confirms correct ligation of the 25-nt monomers. Moreover, this behavior is consistent with what was expected with the known high processivity of *Eco*RI and provides a benchmark for relative processivity of MutY and Fpg with these substrates.

Processivity of E. coli MutY. The MutY glycosylase catalyzes hydrolytic removal of adenine from OG·A- and G·A-containing substrates, leaving a duplex containing an AP site as one of its products. Further treatment with NaOH catalyzes β- and δ-elimination at the AP site, producing strand scission. Hence, to test whether MutY locates these two distinct target base pairs via a processive or distributive mechanism, concatemeric substrates containing OG·A and G·A mismatches every 25 bp were used in reactions with MutY.

Experiments were performed under conditions where [MutY] = 4 nM and [substrate] = 5 μM to ensure that, on average, no more than one enzyme would be present per DNA molecule. The difference in the reaction of MutY with OG·A- and G·A-containing concatemers is strikingly dif-

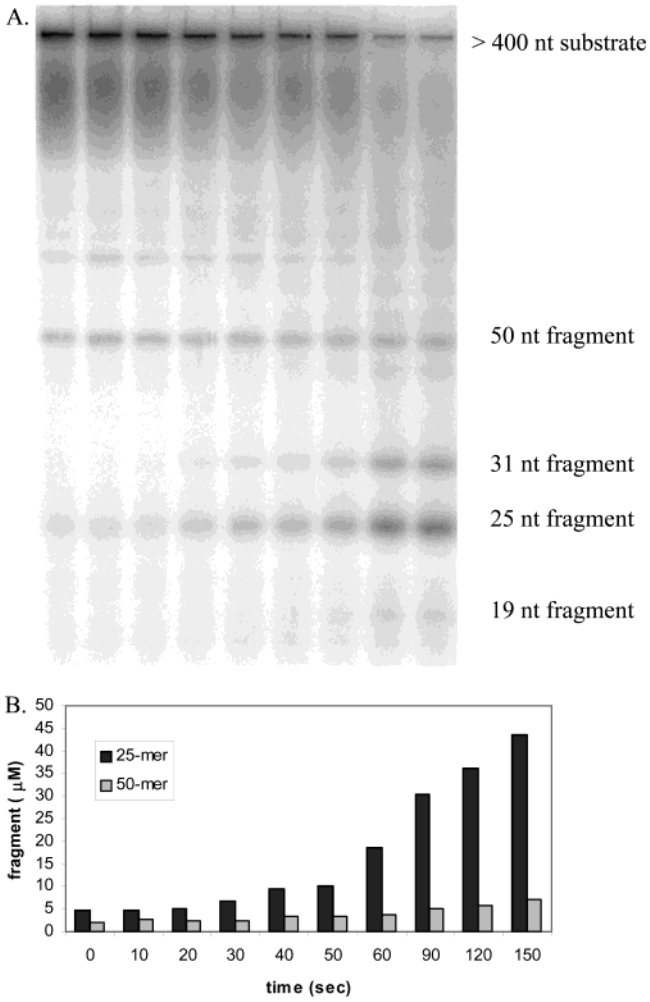

FIGURE 3: *Eco*RI processivity assays. Autoradiogram depicting *Eco*RI processing of a concatemeric substrate containing an *Eco*RI restriction site at 25-nucleotide intervals. As expected, the 25-nt fragment predominates and increases with time (panel A). The 31 nt fragment is formed upon the cleavage of the restriction site at the 3′ end of the concatamer, whereas a 19-nt fragment is formed upon cleavage of the restriction site at the 5′ terminus. Other odd fragments pertaining to intermediate fragments are also present. Experiments were conducted with 4 units of *Eco*RI and 100 μM substrate. A graph obtained from the *Eco*RI assay is shown in panel B.

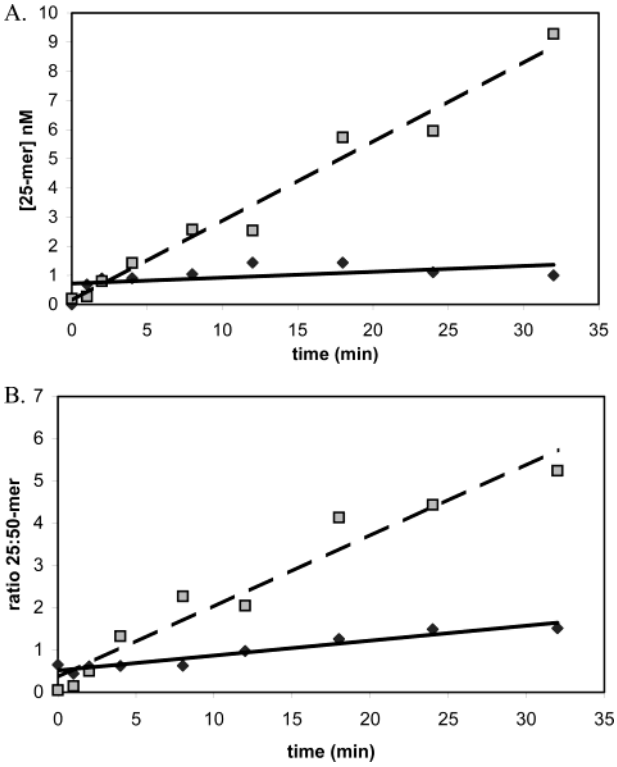

FIGURE 4: Processivity experiments of the reaction of MutY with concatemers containing OG·A (black diamond) and G·A (gray squares). Shown are representative plots of (A) the accumulation of 25-nt fragments and (B) the ratio of 25-nt:50-nt fragments as a function of time. The 50-nt fragment represents intermediate-length fragments since it is assumed the enzyme would not translocate past a recognition site without acting upon it. Conditions: [enzyme] = 4 nM, [substrate] = 5 μM without added NaCl.

ferent. The amount of 25-mer produced in the reaction of MutY with OG·A- and G·A-containing concatemers is shown in Figure 4A. In the experiment with the OG·A-containing concatemers (Supporting Information), small amounts of reaction products with varying lengths (19, 25, 31, 50, etc.) were observed at the first time point; however, product formation did not increase with time, as illustrated by the amount of 25-nt product (Figure 4A). In addition, with time, essentially no increase in the ratio of 25-nt:50-nt fragments was observed (Figure 4B). Thus, in this case, behavior consistent with a processive mechanism is not observed; however, the reaction cannot be described as distributive since the amount of product formed does not significantly increase with time, suggesting that there is no significant turnover of the enzyme. In contrast, adenine removal by MutY upon substrates containing multiple G·A mismatches results in production of a continuously increasing amount of 25-nt fragments. At 1 min the ratio of 25-nt:50-nt fragments is 0.94, supporting the assumption that initial binding events are diffusion-controlled and thus random. By the end of the 32-min time course, the ratio of 25-nt:50-nt fragments reaches 2.9, suggesting that MutY acts in a more processive manner with the concatemeric G·A-containing substrates. Interestingly, the rate of production of 25-nt fragments (i.e., the rate of processivity, k_{proc}) from the concatemeric substrates derived from the linear portion of the plots of the data in Figure 4A are 0.0005 ± 0.003 and 0.07 ± 0.02 min^{-1} for the OG·A and G·A substrates, respectively. These values are similar to rate constant values ascribed to the product release rate (k_3), listed in Table 1, determined in multiple-turnover experiments with single-mismatch-containing substrates (Figure 2). Thus, this illustrates that the rate of processivity with MutY is dominated by the rate of release of MutY from the OG/G·AP site product.

Processivity of Stop 225. The N-terminal domain of MutY (Stop 225) that contains only the first 225 amino acids has been shown to have a reduced efficiency for adenine removal from mismatched susbstrates as well as a loss of specificity for OG (*20*, *22*). The C-terminal domain of MutY is structurally homologous to the d(OG)TPase MutT and thus has been implicated as the OG-binding domain. Therefore, processivity experiments were conducted with Stop 225 on the concatemeric substrates containing OG·A and G·A mismatches to determine the correlation between substrate specificity and mechanism of target location. The same reaction conditions used for the previous studies with full-length MutY were used with Stop 225 and the results are

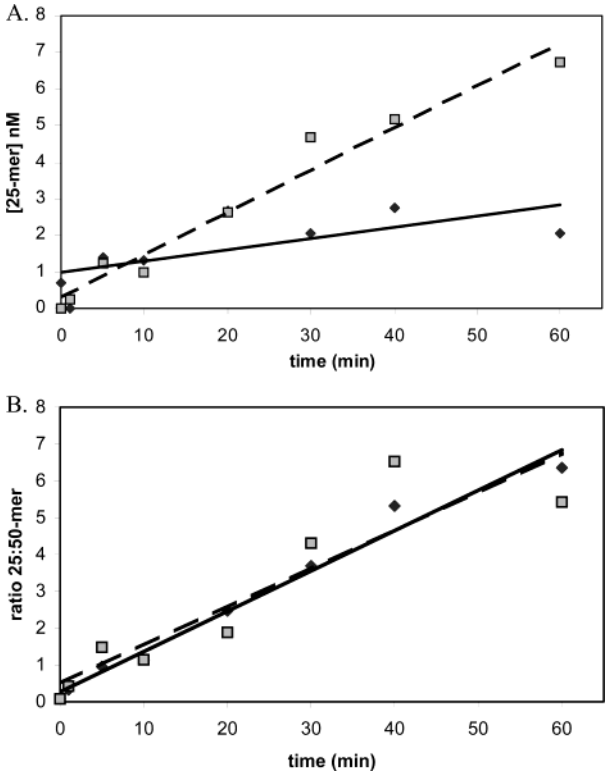

FIGURE 5: Processivity experiments of the reaction of Stop225 with concatemers containing OG·A (black diamond) and G·A (gray squares). Shown are representative plots of (A) the accumulation of 25-nt fragments and (B) the ratio of 25-nt:50-nt fragments as a function of time. The 50-nt fragment represents intermediate-length fragments since it is assumed the enzyme would not translocate past a recognition site without acting upon it. Conditions: [enzyme] = 4 nM, [substrate] = 5 μM without added NaCl.

depicted in Figure 5. In contrast to full-length MutY, the ratio of 25-nt:50-nt fragments over time indicates that Stop 225 behaves similarly with OG·A- and G·A-containing substrates. With Stop 225, accumulation of 25-nucleotide monomers occurs at a slightly faster rate with concatemers containing G·A (k_{proc} 0.03 ± 0.01 min^{-1}) versus OG·A (k_{proc} 0.006 ± 0.003 min^{-1}); however, both retain similar 25-mer:50-mer ratios. This behavior appears to be in contrast to the reaction with the monomer substrate, in which the intrinsic rate of adenine removal (k_2) is faster with OG·A substrates. However, enzyme turnover (k_3) is slightly faster with G·A substrates, and the rates of processivity with the concatemeric substrates correlate well with the rate of product release (k_3) determined with the monomeric substrates. Thus, the increased rate of 25-nt fragment production (i.e., processivity) with the G·A substrates is likely due to faster substrate *turnover* due to more efficient release of the enzyme from the product AP site. This result further demonstrates the role of the C-terminal domain in OG recognition and, furthermore, suggests that the high affinity of MutY for OG hinders processive scanning of DNA.

Processivity of Fpg. Fpg catalyzes the removal of a wide variety of lesions resulting from oxidative damage from duplex DNA (*10*). It has been previously shown that Fpg removes OG from OG·C-containing substrates much faster than from OG·A-containing substrates (*23*). This activity is consistent with the biological role of Fpg in that removal of OG from substrates containing OG·A would be promutagenic, leading to a permanent G·C to T·A transversion.

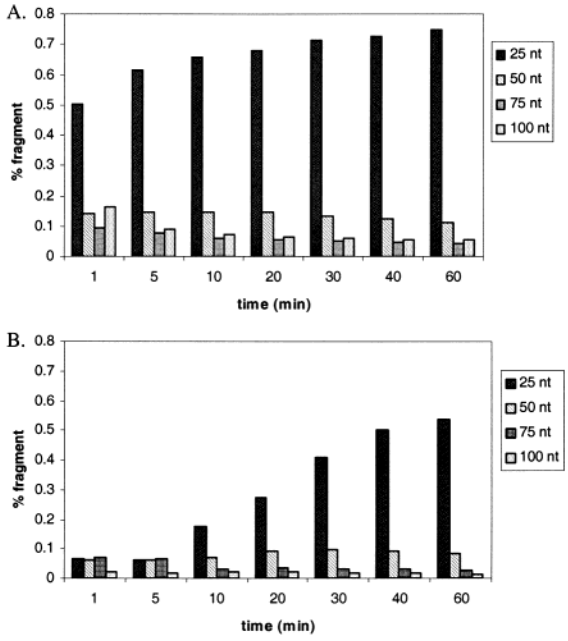

FIGURE 6: Processivity results of Fpg with concatemers containing (A) OG·C and (B) OG·A. Shown are representative plots of fragment percentages as a function of time, depicting both 25-nt and intermediate-length fragments. Conditions: [Fpg] = 4 nM, [substrate] = 5 μM, without added NaCl.

Therefore, in experiments analogous to those with MutY, we tested whether Fpg would scan more efficiently with substrates containing OG·C versus OG·A. The main alteration to the experimental design was lack of alkali treatment used for strand scission, since Fpg also catalyzes strand scission via β- and δ-elimination concomitant with its glycosylase activity (*24*).

Results from Fpg activity on polymeric substrates are shown in Figure 6. With OG·C-containing concatemeric substrates and Fpg, a rapid and sustained burst of 25-nt fragments is observed compared to the intermediate length fragments (Figure 6A). This swift accumulation of 25-nt fragments suggests that Fpg uses a processive mechanism for target location. The results with substrates containing OG·A mispairs also suggest a processive mechanism. As shown in Figure 6B, the rate of monomer production with substrates containing OG·A mispairs (k_{proc} 0.17 ± 0.03 min^{-1}) is much slower than with the OG·C-containing concatemer (k_{proc} 2.5 ± 0.6 min^{-1}), even though the buildup of intermediate fragments is similar with the two substrates. This indicates that Fpg remains processive with the OG·A concatemer, albeit at a much slower rate. At 10 min the ratio of 25-nt:50-nt fragments is 4.5 with substrates containing OG·C, versus a ratio of 2.5 with substrates containing OG·A. This is consistent with the intrinsic rate of OG removal (i.e., steps including chemistry) from the two substrates dominating the rate of processivity, rather than the rate of product release. Indeed, the ratio of the steady-state rates (k_{proc}) for the OG·C- versus OG·A-containing concatemeric substrates is similar to the ratio of the rate constants that characterize chemistry (k_2) derived from single-turnover experiments on the monomeric substrates (Table 1).

Effect of NaCl on Processivity. Enzymes that exhibit processive behavior have been shown to be highly responsive to variations in salt concentration due to the electrostatic nature of the scanning process (*42*). Thus, experiments were

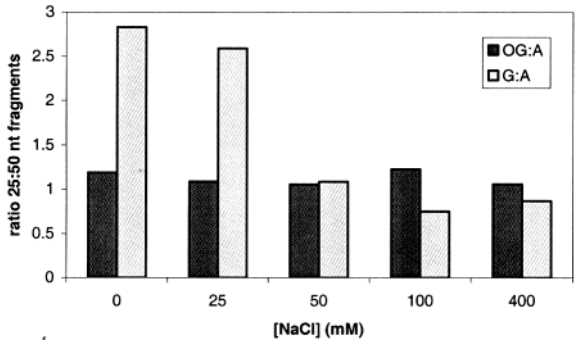

FIGURE 7: Effect of sodium chloride on processivity of MutY with concatemers containing OG·A and G·A. Shown are representative plots of the ratio of 25-nt:50-nt fragment ratios as a function of NaCl concentration at 32 min time point. Conditions: [MutY] = 4 nM, [substrate] = 5 μM.

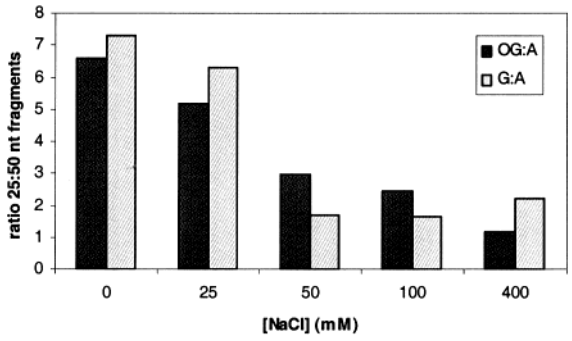

FIGURE 8: Effect of sodium chloride on processivity of Stop 225 with concatemers containing OG·A and G·A. Shown are representative plots of 25-nt:50-nt fragment ratios as a function of NaCl concentration at 32 min time point. [Stop 225] = 4 nM, [substrate] = 5 μM.

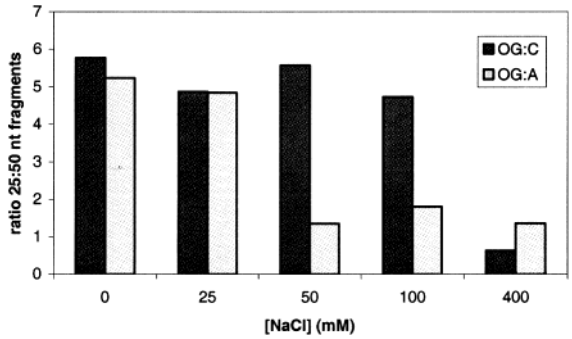

FIGURE 9: Effect of sodium chloride concentration of processivity of Fpg with concatemers containing OG·C and OG·A. Shown are representative plots of 25-nt:50-nt fragment ratios as a function of NaCl concentration at 32 min time point. [Fpg] = 4 nM, [substrate] = 5 μM.

carried out to investigate whether the target location's mechanism of MutY, Stop 225, and Fpg could be shifted from processive to distributive by increasing the sodium chloride concentrations. The same reaction conditions were carried out as described above with the exception of the addition of various amounts of sodium chloride.

The ratio of 25-nt:50-nt fragments was monitored at five different sodium chloride concentrations (0, 25, 50, 100, and 400 mM) for MutY with both OG·A- and G·A-containing concatemeric substrates (Figure 7). MutY retains a 25-nt:50-nt fragment ratio of about 1.2 with OG·A-containing polymeric substrate over the entire range of sodium chloride concentrations. We have previously observed that increased monovalent salt concentration can slightly increase the rate of product release in reaction of MutY with OG·A substrates (*43*), and therefore the increased salt concentration may have been expected to enhance turnover and therefore allow for observation of processive behavior with the OG·A concatemer. However, the lack of an observable change in the product distribution with the OG·A concatemeric substrate in the presence of increasing sodium chloride concentrations suggests either that these salt concentrations are not high enough to facilitate turnover or that any enhancement of product release is counterbalanced by a negative effect on the electrostatic association of the enzyme with the DNA required for scanning. In marked contrast, with G·A-containing concatemers, at low salt concentrations (0 and 25 mM) the 25-nt:50-nt fragment ratio remains high; however, at sodium chloride concentrations of 50 mM and above, the ratio of 25-nt:50-nt fragments is considerably reduced. This is consistent with a shift of MutY with the G·A concatemer from a more processive mechanism to one predominantly distributive at 50 mM NaCl. At these higher salt concentrations, the observed behavior of the G·A substrate is similar to that of OG·A. It should be noted that at 100 mM sodium chloride a decrease in the total amount of cleavage was observed, and at 400 mM the total cleavage is significantly diminished. Previously, we have observed that at KCl concentrations above 100 mM there is a decrease in the rate of adenine removal with 30-bp substrates containing a single G·A mismatch, under single-turnover conditions (*43*).

The effect of increasing sodium chloride concentration on experiments with Stop 225 with the OG·A and G·A concatemeric substrates are shown in Figure 8. The observed behavior with Stop 225, unlike full-length MutY, was consistent with a processive mechanism with both OG·A- and G·A-containing substrates at low ionic strength. However, at higher sodium chloride concentrations, the observed distribution of fragments remains constant with time, indicating the same mechanistic shift, from processive to distributive, for both concatemeric substrates. In the case of Stop 225, the adenine glycosylase activity with 30-bp duplexes containing G·A and OG·A mismatches was affected by concentrations of NaCl above 100 mM (N. H. Chmiel and S. S. David, unpublished results). Thus, at 50 mM NaCl, the observed shift in the distribution of fragments is due to an effect on scanning, rather than an intrinsic effect on the ability to remove adenine from mismatched substrates.

The effects of increasing the salt concentration in the processivity experiments with Fpg are shown in Figure 9. Similar to the results obtained with full-length MutY and Stop 225, the observed distribution of fragments produced by the catalytic activity of Fpg suggests a distributive mechanism at high sodium chloride concentrations. There is, however, a notable difference between the reaction of Fpg with OG·C- and OG·A-containing concatemers. A salt concentration above 100 mM NaCl was required to make the mechanistic shift for the substrates containing OG·C, whereas substrates containing OG·A mispairs assumed distributive behavior around 50 mM NaCl. Notably, above 100 mM NaCl, the total amount of product formation is decreased, consistent with previous reports by Castaing et al. (*44*) for a duplex containing a single OG·C site.

DISCUSSION

In this study, we have demonstrated that *E. coli* MutY and Fpg, two enzymes in the 8-oxoguanine repair pathway, utilize a processive mechanism in target location. In addition, we have shown that the C-terminal domain of MutY, thought to be responsible for substrate specificity, also plays an important role in target location. We have also observed that increasing monovalent salt concentrations shifts the damage location mechanism to one that is predominantly distributive, consistent with the expected electrostatic nature of the scanning process.

Several concatemeric polynucleotide substrates were synthesized with repeating damaged/mismatched base pairs and *Eco*RI restriction sites at 25-nt intervals. The analysis of the processivity of *Eco*RI endonuclease, an enzyme known to scan DNA efficiently, served as a benchmark for the relative distribution of 25-nt versus longer fragments as a function of time. A rapid accumulation of 25-nt *Eco*RI restriction products was observed with each substrate as expected for a processive enzyme. This also illustrates that the abnormal OG nucleotide does not interfere with the ability of *Eco*RI to translocate along the DNA or catalyze its endonuclease reaction.

For these concatemeric substrates, the reaction with *E. coli* MutY resulted in predominantly 25-nt fragments, in a manner similar to *Eco*RI, consistent with the use of a processive mechanism for target location. However, the nature of the recognition site was shown to strongly affect the mechanism of target location by MutY. Indeed, MutY clearly uses a processive mechanism with concatemeric G·A-containing substrates. In the case of the OG·A concatemer, the target location mechanism is less clear. Previous work has shown that MutY has a higher affinity for its OG·AP site product over its G·AP site product (*16*, *37*). Hence, we suggest that the strong affinity for the OG·AP site stalls the enzyme and therefore disrupts the ability to obseve the translocation process. One possible scenario may be that MutY protects the OG·AP site from the action of OG glycosylases, to prevent a double strand break, until the proper enzymes are recruited to continue the repair of the lesion. Though it may be important to protect the G·AP site product due to the inherent toxicity of AP sites, the presence of a normal base, G, may make this site less in need of ardent protection relative to an OG·AP site. Indeed, we have recently shown that *E. coli* AP endonucleases enhance the turnover of MutY with G·A substrates but not OG·A substrates (*45*). Obviously MutY cannot predetermine what type of substrate it will encounter. Therefore, this would suggest that MutY utilizes a scanning mechanism to find the first damaged base pair and, depending on the substrate found, would continue to translocate in search of another mismatch or remain bound to the product for an extended time. In the case of the OG·AP product, the residence time for MutY is much longer (half-life $\sim$ 3 h) (*16*) than the time course analyzed (32 min); thus, this prevents observation of translocation to another OG·A site in a reasonable time frame for enzymatic experiments.

Experiments were also conducted with various ionic strengths to observe whether a processive mechanism can be modulated to a distributive mechanism. In the case of MutY with the OG·A substrate, the high salt conditions did not affect the observed distribution of fragments. In contrast with the G·A concatemeric substrate, the presence of higher salt concentrations decreased the observed ratio of 25-nt:50-nt fragments. This is consistent with a switch to a predominantly distributive mechanism when acting upon G·A-containing substrates at salt concentrations around 50 mM and higher. Notably, the same sodium chloride concentration was reported to produce a similar shift to a distributive mechanism for the BER glycosylases *E. coli* UDG (*35*), and T4 endonuclease V (*32*). The sensitivity of target location mechanism to high salt concentration supports the conclusion that MutY utilizes a processive mechanism with substrates containing G·A mispairs at low salt concentrations.

On the basis of kinetic and binding experiments of the N-terminal domain (Stop 225), and the structural and sequence homology of the C-terminal domain to the d(OG)-Tpase, MutT, the C-terminal domain of MutY has been implicated as a primary determinant for discrimination between OG·A-containing substrates relative to G·A-containing substrates. Hence, processivity assays were carried out to test the effect of substrate specificity upon target location mechanisms. Results show that, like full-length MutY, Stop 225 behaves processively with G·A-containing substrates. In contrast to what is observed with the full-length protein, Stop 225 also exhibited processive behavior with OG·A-containing substrates. The processive nature of Stop 225 with both substrates was further corroborated by the effect of increased ionic strength that resulted in a shift to a distributive pattern. The similarity in behavior of Stop 225 with G·A and OG·A substrates is completely consistent with the proposed role of the C-terminal domain in OG recognition. This result further suggests that the tight binding of full-length MutY to the OG·AP site product may be responsible for the inability to observe processive behavior with OG·A substrates.

Determination of the mechanism of target location for Fpg, the bifunctional glycosylase responsible for 8-oxoguanine removal from OG·C mispairs, was conducted in a similar manner. Though a primary substrate for Fpg is likely to be OG·C mispairs, Fpg can also catalyze removal of 8-oxoguanine from OG·A mispairs, albeit at a much reduced rate of catalysis (*23*, *44*). The inefficient removal of OG from OG·A base pairs by Fpg is consistent with the enzyme's biological role; indeed, Fpg-catalyzed removal of 8-oxoguanine from an OG·A mispair would be promutagenic, leading to a permanent G to T transversion. Experiments with Fpg were performed on both OG·A and OG·C substrates to determine whether, as in the case of MutY, specific substrates can modulate the mechanism of target location. The results with the concatemeric substrates suggest that Fpg behaves in a processive manner toward substrates containing either target base pairs. In addition, the accumulation of monomer fragments with the OG·C-containing concatemeric substrate is much more rapid than with the concatemers containing OG·A mispairs. This is consistent with previous data with duplex substrates containing a single OG·C or OG·A base pair (*23*, *44*). Thus, these results suggest that the mechanism of target location is unaffected by the rate of catalysis. That is, the rate of enzymatic catalysis is distinct from the rate at which the enzyme translocates along the DNA. Therefore, we suggest that the difference in the rate of scanning, as determined by the rate of accumulation of 25-nt fragments,

is due to a difference in the rate of catalysis and not the rate of translocation. This suggestion is also consistent with the observation that the rate of scanning with MutY correlates with the differences in rate of product release of MutY with OG- and G-containing substrates. In the case of Fpg, the rate for formation of 25-nt fragments is dictated by the intrinsic rate of base removal since product release is not rate-limiting, while with MutY, product release is rate-limiting and therefore dominates the observed scanning speed.

The reaction of Fpg on the concatemeric substrates was also modulated by the presence of salt, consistent with a shift from a processive to distributive mechanism with both OG·A- and OG·C-containing substrates. However, with the OG·C substrates, the reaction was more tolerant of higher salt concentrations, remaining processive up to a NaCl concentration of 100 mM. With OG·A-containing concatemers, Fpg switched to a distributive mechanism at 50 mM NaCl. This difference in ionic strength could be largely due to the differences in binding affinity of Fpg for the two substrates. It has been shown that Fpg has a significantly lower affinity (approximately 40-fold lower) for OG·A sites versus OG·C sites (*46*). The degree of processivity is intimately related to the ability of the enzyme to remain associated with the DNA duplex. Thus, the decreased affinity of Fpg toward substrates containing OG·A mispairs would be expected to result in a greater susceptibility of dissociation with higher ionic strength.

Though enzymes are usually categorized as utilizing a processive or distributive mechanism, it is important to appreciate that a continuum exists between the two extremes. The *relative* processivity must be taken into account. Replicative polymerases may be cited as an example of extreme processivity, wherein they catalyze their reactions for thousands of nucleotides before dissociation (*47*). In this study, using the relative amount of 25-nt to 50-nt fragments indicates that the MutY enzyme excises approximately four adenine residues from the G·A-concatemer prior to dissociation and this corresponds to traversing approximately 100 bp per association event. Interestingly, removal of the C-terminal domain increases the observed scanning to approximately seven sites and 175 bp per association event. Fpg appears to be slightly more processive, with removal of 11 OG residues from the OG·C substrate while traveling 275 bp. These data correpond well to those reported for other base-excision repair enzymes. Indeed, the human AP endonuclease (*48*) and UDG (*34*) were reported to scan 175−200 and 200−225 nt, respectively, per association event. These values also place MutY and Fpg into the "quasi-processive" category in that hundreds, rather than thousands, of base pairs are traversed at a time. Using a similar method to calculate the processivity of *Eco*RI at similar extents of conversion of substrate to product indicates that *Eco*RI travels approximately 275−300 bp per association event. This value corresponds exceedingly well to previous estimates of processivity of *Eco*RI (*41*). In the previous studies *Eco*RI was shown to act processively over distances of up to several hundred base pairs, with 300 bp estimated as the upper limit (*41*). Interestingly, the rate of formation of 25-nt fragments, which is the rate of processivity, is much faster with *Eco*RI than Fpg and MutY. Thus, the rate of processivity may differ considerably, even though the extent of association with the DNA double helix is comparable. However, there does seem to be a correlation with a higher rate and a larger number of base pairs traversed. Indeed, *Eco*RI has a higher rate of processivity and a greater number of base pairs are traveled per association event relative to MutY, which has a lower rate of processivity and remains associated with a smaller number of nucleotides during a given binding event.

In comparing MutY and Fpg, Fpg is slightly more processive than its MutY cousin. Also, Fpg was shown to sustain its scanning ability into higher salt concentrations, whereas MutY is more sensitive. This evidence is consistent with the kinetic data, which suggest that the affinity of the enzyme for its product is inversely proportional to its degree of processivity. That is, MutY has a strong affinity for its product, which results in a lower capacity to scan. This relationship between product affinity and scanning is also supported in the experiments with Stop 225; the product release rate for Stop 225 with OG- and G-containing substrates is similar, resulting in a similar ability to catalyze adenine removal from multiple mismatches opposite OG or G on the same target duplex.

The strong attraction of MutY for its product, resulting in diminished processivity, could have important physiological implications. The slow product release of MutY might be advantageous in that the OG·AP site would be protected until the proper enzymes are recruited to continue repair of the lesion. Therefore, other enzymes could be involved in displacing MutY from the product, in effect increasing the rate of processivity. In addition, the significance of the activity of MutY with G·A substrates remains enigmatic. Indeed, removal of A from G·A mismatches may be promutagenic unless there is coordination with other proteins, such as enzymes involved in mismatch repair or DNA replication. Thus, with both substrates, the relative processivity may be modulated in vivo. Alternatively, the amount of MutY in *E. coli*, estimated at 30 copies per cell (*49*), may be sufficient for the number of OG·A base pairs ($\ll$1 per 10^5 Gs)[2] (*2*, *50*) within the cell, such that processing of several mismatches by a single enzyme is not necessary. However, one might expect that the rarity of these mismatches would make a facilitated diffusion mechanism of target location appealing. The majority of OG would most likely be found within an OG·C context, which would correlate well with the increased rate of Fpg processivity and increased amount of this enzyme in *E. coli* (200 copies/cell) (*49*). Thus, the relative processivity may be directly related to the type and number of damaged/mismatched base pairs located within the cell.

ACKNOWLEDGMENT

We thank Mike Leipold and Nik Chmiel for purifying the Fpg and Stop 225 enzymes used in this work.

SUPPORTING INFORMATION AVAILABLE

Storage phosphor autoradiograms for processivity experiments of MutY with OG·A- and G·A-containing concate-

[2] A variety of estimates of the number of OG lesions in cells have been reported varying from 1 per 10^5 to 10^7 G_5. Thus, the maximum number of OG·A lesions will be dependent on the amount of OG and would be expected to be significantly lower.

mers. This material is available free of charge via the Internet at http::/pubs.acs.org.

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BI026375+