A Repair System for 8-Oxo-7,8-dihydrodeoxyguanine†

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ABSTRACT: Active oxygen species can damage DNA and may play a role in aging and carcinogenesis. We have tested MutY glycosylase for activity on undamaged mispairs as well as mispairs formed with the oxidatively damaged substrates, 8-oxo-7,8-dihydrodeoxyguanine (GO) or 8-oxo-7,8-dihydrodeoxyadenine (AO). MutY acts as a glycosylase on four of the heteroduplexes tested, A/G, A/GO, A/C, and A/AO, removing the undamaged adenine from each substrate. Genetic data suggest that the primary substrate for MutY glycosylase in vivo is the A/GO mispair. We present biochemical evidence demonstrating that MutY glycosylase is an important part of a repair system that includes the MutM and MutT proteins. The GO repair system is dedicated to the repair of the oxidatively damaged guanine and the mutations it can induce.

Oxidative stress is emerging as one of the most important causative agents of mutagenesis, carcinogenesis, aging, and a number of diseases (Farr & Kogoma, 1991). DNA and deoxynucleotides are potential targets for attack by active oxygen species, and virtually all aerobic organisms have developed complex defense and repair mechanisms to mitigate the deleterious effects of active oxygen species.

The first line of defense seeks to neutralize the active oxygen species themselves. This line of defense includes various enzymatic and nonenzymatic defense mechanisms such as superoxide dismutase, catalase, β-carotene, and ascorbic acid. Active oxygen species that escape the primary defenses can damage nucleic acids. 8-Oxo-7,8-dihydrodeoxyguanine (GO, also known as 8-hydroxydeoxyguanine) is one of the most stable products of oxygen radical attack on DNA (Dizdaroglu, 1985). The second line of defense removes damage caused by active oxygen species that escape the primary defense mechanisms. Several proteins have been demonstrated to be involved in the repair of GO lesions. The MutT protein hydrolyzes the oxidatively damaged deoxynucleotide 8-oxo-dGTP to 8-oxo-dGMP, thus eliminating a potentially mutagenic substrate from the deoxynucleotide pool (Maki & Sekiguchi, 1992). In the absence of MutT protein, 8-oxo-dGTP is frequently misincorporated opposite a template dA (Maki & Sekiguchi, 1992). This activity provides a potential explanation for the specific increase in A:T → C:G transversions in a *mutT* strain (Yanofsky et al., 1966). The MutM protein (Michaels et al., 1991) [also known as FPG protein (Boiteux et al., 1990) or 8-oxoguanine DNA glycosylase (Tchou et al., 1991)] removes GO lesions from double-stranded DNA (Tchou et al., 1991). Inactivation of this gene leads specifically to G:C → T:A transversions (Cabrera et al., 1988) because synthesis past GO lesions leads to the misincorporation of dAMP opposite the damaged guanines (Wood et al., 1990; Shibutani et al., 1991; Moriya et al., 1991; Cheng et al., 1992).

We have presented genetic and biochemical evidence that MutY glycosylase is also involved in the repair of oxidatively damaged DNA and represents a third level of defense against the mutagenic effects of oxygen damage (Michaels et al., 1992). Unlike the other lines of defense, which target the active oxygen species themselves or the lesions they form upon reaction with DNA or deoxynucleotides, MutY glycosylase removes the A from A/GO mispairs that arise in DNA when error-prone tranlesion synthesis occurs past GO lesions in DNA (Michaels et al., 1992). Thus, at least three genes are involved in defending *Escherichia coli* from the mutagenic effects of GO lesions.

In this study we have examined the activity of MutY on heteroduplexes containing the GO lesion or an analogous oxidatively damaged adenine derivative, 8-oxo-7,8-dihydrodeoxyadenine (AO), as well as all possible undamaged mispairs. We show that MutY is active on four of the heteroduplexes tested (A/G, A/GO, A/AO, A/C), removing the undamaged dA from each mispair. We also present biochemical evidence that MutY is critical to the correct repair of A/GO mispairs in DNA. MutY removes the undamaged A from the A/GO mispair and remains bound to the site to prevent MutM from removing the GO lesion, which could lead to the loss of one base, and a strand break.

MATERIALS AND METHODS

Materials

Oligodeoxynucleotides. The 23-mer oligodeoxynucleotides, including the ones containing the site-specific GO lesion or site-specific AO lesion, were prepared by solid-state synthesis as previously described (Bodepudi et al., 1991; Shibutani et al., 1991).

Methods

Preparation of Radiolabeled Duplexes. The purified oligomers were ^{32}P-labeled as previously described and annealed with an excess of unlabeled oligomer to form heteroduplexes (Michaels et al., 1992). The oligodeoxynucleotides used in this study are listed in Table I.

MutY Glycosylase Assay. Glycosylase reactions were carried out in a solution (10 μL) containing 20 mM Tris–HCl

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[1] Abbreviations: GO, 8-oxo-7,8-dihydro-2′-deoxyguanine or 8-hydroxydeoxyguanine; AO, 8-oxo-7,8-dihydro-2′-deoxyguanine or 8-hydroxydeoxyadenine; Rif^r, rifampicin resistant.

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(pH 7.6), 50 μg/mL bovine serum albumin, 10 mM EDTA, 45 ng of purified MutY glycosylase, and 20 fmol of 23-mer duplex with the indicated mispair and were incubated at 37 °C for 30 min. MutY glycosylase was purified essentially as previously described (Au et al., 1989). Blank reactions contained everything except the glycosylase. Reactions were terminated by the addition of 2 μL of 1 M NaOH and incubation at 90 °C for 4 min. This also served to cleave any apurinic/apyrimidinic sites generated by the glycosylase. The progress of the glycosylase reaction can therefore be monitored by a change in migration of the cleaved products. A 4-μL aliquot of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cylanol FF) was added to the mix, and aliquots were run on 15% denaturing (7 M urea) polyacrylamide gels. Time course reactions were run as above except that the volume of the reaction was scaled up 7-fold and 10-μL aliquots were removed at various time points. The relative activity of MutY on its four substrates was determined by scanning autoradiographs of reactions done in triplicate and averaging the results.

Preparation of the Apurinic Substrate. The apurinic substrate used in the MutY protection assay described below was prepared by incubating 1.16 pmol of an A/GO duplex which was 5′-^{32}P-labeled in the strand containing the GO lesion (oligomers 7/2* from Table I) with 0.45 μg of purified MutY glycosylase in the above buffer for 1 h at 37 °C. The reaction was then phenol–chloroform extracted and purified using the Mermaid kit (Bio101, Richmond, CA) as described by the manufacturer. The mixture was resuspended in 10 mM Tris–HCl (pH 7.6) and 50 mM NaCl and reannealed as described above.

MutY Protection Assays. The protection assays were run the same as the MutY glycosylase assays except that 10 ng of purified MutY and/or MutM protein was added to the reaction. The substrate was added last. Blank reactions contained everything except enzyme. Reactions were terminated by adding 3 μL of loading dye and heating at 90 °C for 2 min. Aliquots were run on 15% denaturing (7 M urea) polyacrylamide gels.

Gel Shift Assays. The gel shift assays were run in 25 mM Hepes–KOH (pH 7.9), 5 mM EDTA, 0.5 mM DTT, 150 mM NaCl, 10% glycerol, and 100 μg/mL poly(dA-dT) with 10 ng of purified MutY and/or MutM in a volume of 10 μL. Fifty femtomoles of labeled duplex, prepared as previously described (Rydberg et al., 1991), was present in each reaction and 100–500 fmol of unlabeled competitor oligonucleotides was added when necessary. After 30 min at 25 °C, aliquots were loaded onto a 6% nondenaturing polyacrylamide gel using TBE buffer (0.067 M Tris, 0.04 M boric acid, 1 mM EDTA, pH 8.8) and run at 5 V/cm for about 2 h.

RESULTS

Substrate Specificity of MutY Glycosylase. Table I lists the nucleotide sequences of the oligonucleotides used in these studies. Heteroduplexes were formed between a 5′-^{32}P-labeled oligomer and an unlabeled oligomer that was complementary to the labeled oligomer except for a single mismatch. All of the possible undamaged mispairs were formed as well as mispairs with the oxidatively damaged oligomers containing the GO or AO lesions.

Figure 1 shows the results of the substrate specificity tests. As previously determined, MutY glycosylase was active on heteroduplexes containing either an A/G mispair (Au et al., 1989) or an A/GO mispair (Michaels et al., 1992) (lanes 10 and 11). Among the undamaged mispairs, we found that

Table I: Sequences of Modified and Unmodified Oligodeoxynucleotides Used To Test the Substrate Specificity of MutY Glycosylase[a]

sequence no.	sequence
1	5′-CTCTCCCTTC*AO*CTCCTTTCCTCT-3′
2	5′-CTCTCCCTTC*GO*CTCCTTTCCTCT-3′
3	5′-CTCTCCCTTC*A*CTCCTTTCCTCT-3′
4	5′-CTCTCCCTTCCCTCCTTTCCTCT-3′
5	5′-CTCTCCCTTC*G*CTCCTTTCCTCT-3′
6	5′CTCTCCCTTC*T*CTCCTTTCCTCT-3′
7	3′-GAGAGGGAAG*A*GAGGAAAGGAGA-5′
8	3′-GAGAGGGAAG*C*GAGGAAAGGAGA-5′
9	3′-GAGAGGGAAG*G*GAGGAAAGGAGA-5′
10	3′-GAGAGGGAAG*T*GAGGAAAGGAGA-5′
11	5′-GTTTTCCC*A*GTCACGAC-3′
12	3′-CAAAAGGG*C*CAGTGCTG-5′

[a] Nucleotides involved in mispair formation are in italics. *AO*, 8-oxodA; *GO*, 8-oxo-dG.

MutY was also active on the A/C mispair but reacted at a reduced rate compared to the other substrates (lane 12). While MutY did not remove an AO lesion from any of the four possible duplexes, including the AO/G duplex (lanes 15–18), it did remove the undamaged A from an A/AO heteroduplex (lane 14). Thus, MutY glycosylase is active on A/G, A/GO, A/C, and A/AO heteroduplexes and removes the undamaged A from each of the mispairs. MutY was not active on any of the other possible mispairs formed by the oligomers in Table I.

Since MutY glycosylase was less active on the A/C heteroduplex relative to the other substrates, we tested two additional A/C heteroduplexes as substrates for MutY in a time course reaction. Figure 2 shows that all three A/C mispairs were substrates for MutY and that products increased with time. The specific activity on the A/C mispairs ranged from 5 to 0.08 nmol·mg^{-1}·h^{-1}, which is 5–35-fold lower than the activity on the other substrates (Michaels et al., 1992). Thus, although the reaction rate on A/C mispairs is lower, this mispair is a substrate for MutY glycosylase.

MutY Protection of A/GO Mispairs from MutM Protein. Although MutY glycosylase is active on four different mispairs, we have presented genetic evidence indicating that the primary substrate in vivo is the A/GO mispair and that MutY and MutM are involved in the GO repair system (Michaels et al., 1992). In order to better characterize this repair system, we conducted further biochemical analysis. The purpose of these studies was to determine how MutY and MutM would interact on a heteroduplex containing an A/GO mispair. The MutM protein can remove GO lesions from DNA, and as shown in Figure 3 (lane 3), it can remove the lesion from the A/GO mispair. If both MutY and MutM acted on the heteroduplex containing the A/GO mispair, it could lead to the loss of one base and a strand break. However, our studies show that when both MutY glycosylase and MutM protein are present in the same reaction, MutY prevents MutM from removing the GO lesion and cleaving the DNA strand (Figure 3, lane 4).

We then tested MutM activity on a heteroduplex containing a GO lesion opposite an apurinic/apyrimidinic (AP) site. The purpose of the experiment was to determine if MutY prevents MutM from acting on the GO lesion by forming an AP/GO heteroduplex, which might not be a substrate for MutM. To create an AP site opposite the GO lesion, MutY was incubated with an A/GO heteroduplex to form an AP/GO mispair. The heteroduplex was extracted and purified to remove MutY protein. MutM protein removed the GO lesion from an AP/GO heteroduplex (Figure 3, lane 7). However, if both MutY

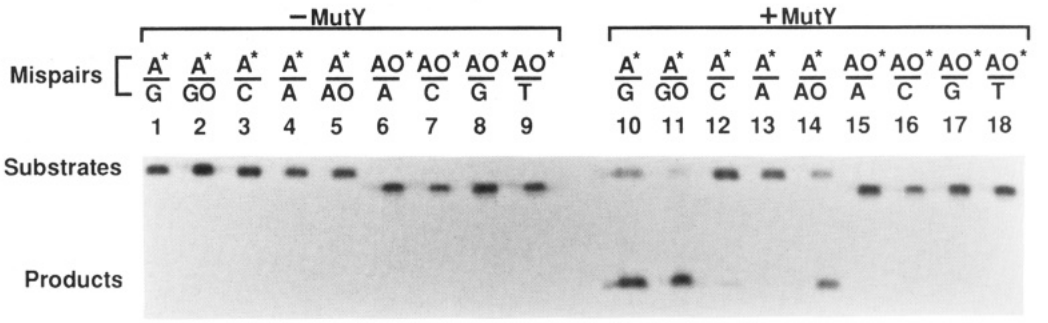

FIGURE 1: Substrate specificity of MutY glycosylase. 23-Mer heteroduplexes containing the indicated mismatch were tested as substrates for MutY glycosylase in a standard reaction. Lanes 1–9 are blank reactions that were treated identically to the reactions in lanes 10–18 except that they did not contain MutY glycosylase. MutY removes the undamaged dA from the A/G, A/GO, A/C, and A/AO heteroduplexes (lanes 10–12 and 14, respectively). MutY was not active on any of the other possible heteroduplexes that could be formed between sequences 1–10 in Table I (data not shown). The asterisk indicates which strand was ^{32}P-labeled.

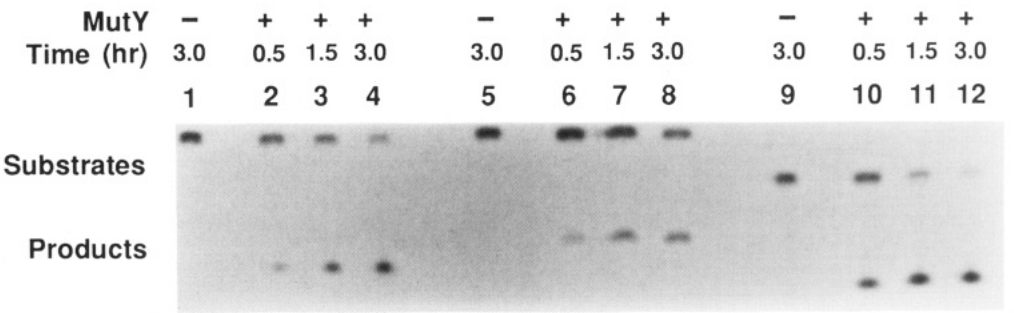

FIGURE 2: MutY activity on A/C mispairs. Three different A/C mispairs were tested as substrates for MutY glycosylase in a standard reaction mix in a time course reaction. Lanes 1, 5, and 9 are blanks. Lanes 2–4, 6–8, and 10–12 are aliquots taken during the time course reaction for each of the three different substrates. The substrates in lanes 1–4, 5–8, and 9–12 are heteroduplexes formed between sequences 3*/8, 7*/4, and 11*/12 from Table I, respectively. The ^{32}P label was at the 5′ end of the oligomer that carried the mismatched dA.

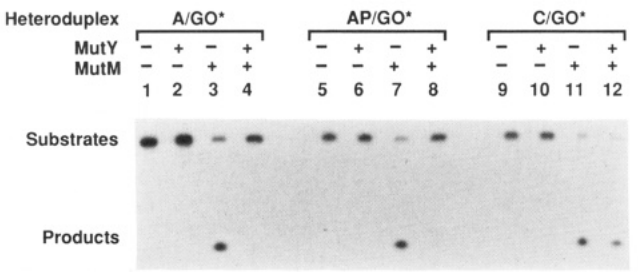

FIGURE 3: MutY protection of A/GO mispairs and AP/GO sites from attack by MutM protein. Protection assays were run much the same as the standard glycosylase reactions except that MutY and/or MutM protein was added as indicated. The AP/GO substrate used in lanes 5–8 was prepared as described in Materials and Methods. The heteroduplex in lanes 1–8 and 9–12 were composed of sequences 7/2* and 8/2*, respectively, from Table I. The asterisk indicates which strand was ^{32}P-labeled.

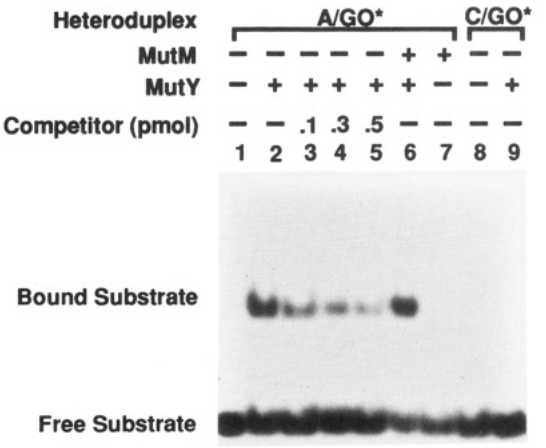

FIGURE 4: MutY binding to A/GO mispairs. Gel shift analysis was carried out on the indicated mispairs and included purified MutY glycosylase and/or MutM protein as indicated. Lanes 3–5 show that the MutY gel shift band for the A/GO 23-mer heteroduplex can be competitively eliminated by using a 2-, 6-, or 10-fold excess of unlabeled A/GO heteroduplex. The substrates in lanes 1–7 and lanes 8 and 9 are heteroduplexes formed between sequences 7/2* and 8/2* from Table I. The asterisk indicates which strand was, respectively, ^{32}P-labeled.

and MutM were added to the AP/GO substrate, MutY once again prevented MutM from removing the GO lesion (Figure 3, lane 8). This result demonstrates that MutM can remove the GO lesion from an AP/GO heteroduplex and suggests that MutY protein prevents the reaction by remaining bound to the AP/GO site. As shown in Figure 3, MutY does not react with the natural substrate for MutM, C/GO (lane 10), nor does it interfere with the reaction of MutM on this substrate (lane 12).

Gel Shift Studies. Gel shift analysis confirms that MutY glycosylase binds to A/GO heteroduplexes (Figure 4, lane 2). The gel shift band can be competitively eliminated with unlabeled A/GO substrate (Figure 4, lanes 3–5). When MutY and MutM are both added to the A/GO heteroduplex, no detectable change in the migration is observed, indicating that MutY and MutM do not form a complex at the site of the lesion or that the dissociation rate of MutM is significantly faster than the time course of the experiment (Figure 4, lane 6). Under the conditions of the assay, no gel shift band is observed for the MutM protein alone on the A/GO substrate. This suggests that MutM does not remain bound to the duplex after removal of the lesion (Figure 4, lane 7). As expected, MutY does not bind to the C/GO heteroduplex (Figure 4, lane 9).

DISCUSSION

In this paper we have identified two additional substrates for MutY glycosylase, A/C and A/AO heteroduplexes. As

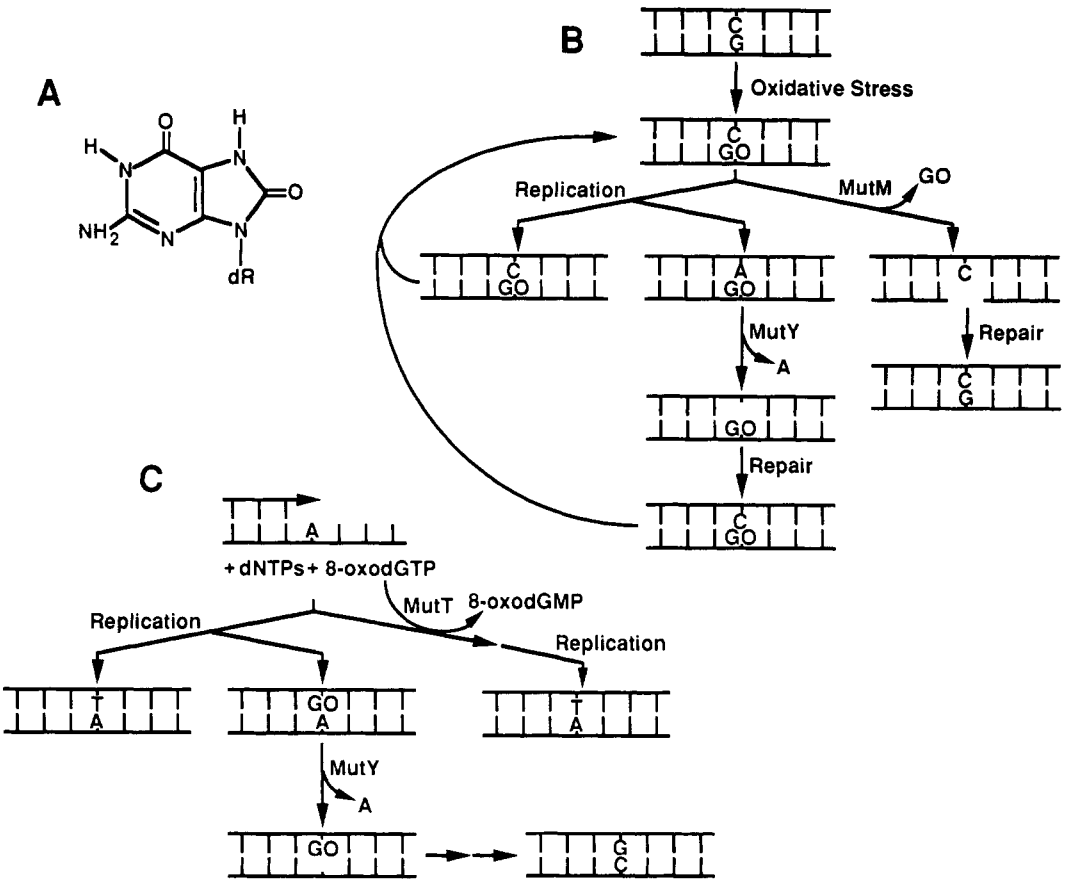

FIGURE 5: GO repair system. (A) 8-Oxo-7,8-dihydrodeoxyguanine (GO). This is the structure of the predominant tautomeric form of the GO lesion. (B) Oxidative stress can lead to GO lesions in DNA. The GO lesions can be removed by MutM protein and subsequent repair can restore the original G:C base pair. If the GO lesion is not removed before replication, translesion synthesis can be accurate, leading to a C/GO pair, which is a substrate for MutM protein. However, translesion synthesis by replicative DNA polymerases is frequently inaccurate, leading to the misincorporation of dAMP opposite the GO lesion. MutY glycosylase removes the misincorporated dA from the A/GO mispairs that result from error-prone replication past the GO lesion. Repair polymerases are much less error-prone during translesion synthesis and can lead to a C/GO pair—a substrate for MutM. (C) Oxidative stress can also lead to damaged deoxynucleotides, such as 8-oxo-dGTP. MutT is active on 8-oxo-dGTP and hydrolyzes it to 8-oxo-dGMP, effectively removing it from the deoxynucleotide pool. If MutT were not active and replication occurred with 8-oxo-dGTP in the deoxynucleotide pool, replication could be accurate or inaccurate, with 8-oxo-dGTP being misincorporated opposite template adenines to form A/GO mispairs. MutY could be involved in fixing these mutations because it is active on the A/GO substrate and would remove the template dA, leading to the A:T → C:G transversions that are characteristic of a *mutT* strain. Although not shown, 8-oxo-dGTP could also be incorporated opposite template cytosines, resulting in a substrate for MutM.

for the previously identified substrates, A/G and A/GO heteroduplexes, MutY glycosylase removes the undamaged A from the mispairs.

Previous experiments have not detected MutY activity on A/C mispairs in phage f-1-derived heteroduplexes (Au et al., 1989). The inability to detect glycosylase activity in those studies may have been due to the lower specific activity of MutY glycosylase on the A/C mispair and/or the effect of surrounding sequence upon glycosylase activity. MutY may be active on only one of several possible conformations of the A/C mispair in DNA (Hunter et al., 1987). We found that MutY was active on the three A/C heteroduplexes tested but that the specific activity on this substrate was 5–35-fold lower than for the other substrates. The in vitro activity we observe does correlate with previous in vivo repair analysis. In a mismatch repair deficient background, the repair of an A/C mismatch to a G/C base pair is reduced if the strain also lacks active MutY glycosylase (Radicella et al., 1988). Thus, both the in vitro and in vivo evidence suggest that under certain conditions MutY glycosylase can affect A/C mispair correction.

The observation that MutY glycosylase is active on the undamaged A in an A/AO mispair was surprising. The in vivo mutation spectrum of a *mutY* strain does not suggest that MutY would have a role in A:T → T:A transversions. Further, in vitro replication studies have indicated that translesion synthesis past a site-specific AO lesion is not error-prone (Shibutani & Grollman, unpublished). However, the in vivo processing of the AO lesion has not been determined and our in vitro evidence at least suggests MutY could contribute to the repair of mutations induced by the oxidatively damaged adenine.

One can also make a prediction about the structure of the A/AO mispair based upon the activity of MutY on this substrate. MutY is equally active on the A/G, A/GO, and A/AO substrates. The conformation most commonly observed in the A/G and A/GO duplexes pairs A(anti) with G(syn) (Leonard et al., 1990) or GO(syn) (Kouchakdjian et al., 1990). One would therefore predict that the conformation of the A/AO duplex would be A(anti)/AO(syn).

The fact that MutY cleaves the glycosylic bond of a normal deoxynucleoside, dA, from four different mispairs (A/G, A/GO, A/AO, A/C) leads one to consider how the enzyme recognizes its substrates. The interpretation that MutY glycosylase recognizes non-Watson–Crick base pairing cannot be entirely correct since MutY is not active on either A in an A/A mispair. In terms of replication fidelity, it is noteworthy that removal of an A from an A/A mispair would be mutagenic 50% of the time. On the other hand, removal of the A from an A/AO mispair would probably help preserve the fidelity

of replication since the damaged base would likely be from the parental strand.

We have reported several convincing lines of genetic evidence that the primary role of MutY glycosylase in vivo is the removal of A from A/GO mispairs in DNA and that MutY and MutM are part of a related repair pathway (Michaels et al., 1992). First, the mutation spectra of *mutY* (Nghiem et al., 1988) and *mutM* (Cabrera et al., 1988) in the *lacI* forward mutation system are strikingly similar—both specifically stimulate G:C → T:A transversions. Second, overproduction of the MutM protein completely complements a *mutY* strain (Michaels et al., 1992). Similarly, a chromosomal suppressor of the mutator phenotype of *mutY* has a 15-fold elevation in MutM activity (Michaels et al., 1992). Finally, a *mutM–mutY* double mutant has a G:C → T:A transversion rate that is 20-fold higher than would be expected if the genes were involved in unrelated repair pathways (Michaels et al., 1992).

In this study we have used protection assays and gel shift analysis to more precisely define the role of MutY in the repair of A/GO mispairs in DNA. We find that MutY is critical to the correct processing of this oxidatively damaged mispair. MutY removes the misincorporated A from the A/GO mispair and remains bound to the site, thus preventing MutM from removing the GO lesion and cleaving the DNA strand.

The repair system that has evolved to prevent GO lesions from causing mutations in the *E. coli* chromosome is quite elaborate (Figure 5). The misincorporation of A opposite GO lesions in DNA can occur if MutM protein (FPG protein) fails to remove all of the GO lesions before replication. Interestingly, replicative DNA polymerases are more likely to misinsert an A opposite the template GO adducts that are repair polymerases (Shibutani et al., 1991). Error-prone translesion synthesis past GO lesions can lead to A/GO mispairs—substrates for the MutY glycosylase. MutY removes the misincorporated A and remains bound to the site, perhaps as a signal to other proteins that can advance the repair process.

Oxidative damage to the nucleotide pool is also a potentially mutagenic event. The MutT protein hydrolyzes the oxidatively damaged deoxynucleotide 8-oxo-dGTP to 8-oxo-dGMP, thereby eliminating it from the nucleotide pool (Maki & Sekiguchi, 1992). If the 8-oxo-dGTP is not removed, it can be misincorporated opposite template adenines and form A/GO mispairs. Ironically, in a strain deficient in MutT, MutY glycosylase could play a role in fixing these mutations by removing the template adenine (Figure 5C). However, if MutT has functioned properly, the A/GO mispairs present in the cell should be the result of the misincorporation of dAMP opposite a template GO lesion.

The GO repair system is critical to the maintenance of replication fidelity. A *mutM–mutY* double mutant has a mutation rate that rivals the mutation rate observed when the polymerase III proofreading function is disabled and is significantly higher than the mutation rate of a strain lacking the methyl-directed mismatch repair system (Michaels et al., 1992). The mutation rate of a strain lacking the GO repair system provides convincing evidence that active oxygen species pose a significant threat to the cell. As demonstrated by the role of MutY protein in the GO repair system, the cell goes to great lengths to prevent mutations due to the oxidatively damaged guanine in DNA.

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