The Escherichia coli MutY adenine glycosylase plays a critical role in repairing mismatches in DNA between adenine and the oxidatively damaged guanine base 8-oxoguanine. Crystallographic studies of the catalytic core domain of MutY show that the scissile adenine is extruded from the DNA helix to be bound in the active site of the enzyme (Guan, Y., Manuel, R. C., Arvai, A. S., Parikh, S. S., Mol, C. D., Miller, J. H., Lloyd, S., and Tainer, J. A. (1998) Nat. Struct. Biol. 5, 1058–1064). However, the structural and mechanistic bases for the recognition of the 8-oxoguanine remain poorly understood.

In experiments using a single-stranded 8-bromoguanine-containing synthetic oligodeoxyribonucleotide alone and in a duplex construct mismatched to an adenine, we observed UV cross-linking between MutY and the 8-bromoguanine probe. We further observed enhanced cross-linking in the single strand experiments, suggesting that neither the duplex context nor the mismatch with adenine is required for recognition of the 8-oxoguanine moiety. Stopped-flow fluorescence studies using 2-aminopurine-containing oligodeoxyribonucleotides further revealed the sequential extrusion of the 8-oxoguanine at 108 s⁻¹ followed by the adenine at 16 s⁻¹. A protein isomerization step following base flipping at 1.9 s⁻¹ was also observed and is postulated to provide additional stabilization of the extruded adenine thereby facilitating its capture by the active site for excision.

The effect of cellular damages by reactive oxygen species in carcinogenesis and aging is well documented (1–3). Even in the absence of external oxidative stress, normal metabolic processes produce oxidative damages to DNA (4–6) requiring repair. Oxidative damage of DNA bases alters their base pairing properties (5, 7) thereby interfering with replication and transcription (8). A predominant lesion found in DNA exposed to reactive oxygen species is 8-oxoguanine, which is especially deleterious due to its ability to form a stable Hoogsteen base pair with adenine in addition to the canonical Watson-Crick base pair with cytosine (9, 10). The facile by DNA polymerase misincorporation of an adenine across the 8-oxoguanine (11) results in a mutagenic adenine:8-oxoguanine mismatch, a site where further replication prior to repair would lead to C → A or G → T transversions. In Escherichia coli, the MutY adenine glycosylase (MutY)¹ plays a critical role in preventing mutations stemming from oxidative damages to DNA by excising the adenine from the adenine:8-oxoguanine mismatch.

Like all DNA-nucleotide-modifying enzymes, including DNA methylases, base-excision repair glycosylases, and endonucleases (12–15), MutY faces the 2-fold task of recognizing and accessing chemical moieties on DNA bases hidden within the double helix of duplex DNA. These enzymes have evolved an elegant and simple strategy for exposing their targets by rotating the phosphodiester bonds surrounding the nucleotide, causing the target base to be flipped out of the DNA helix (16–21). Using the E. coli uracil-DNA glycosylase, we have recently reported the first kinetic mechanism of a base-flipping enzyme, defining for the first time the correct temporal sequence of events at the enzyme active site during catalysis (22). Following initial nonspecific binding, the DNA backbone is scanned to locate the site. A protein-induced distortion of the DNA helix at the target site causes the target nucleotide to become mobile, capable of rapid and reversible extrusion from the DNA duplex. The efficient capture of the extruded base, however, requires a protein conformation change, which inserts the side chain of Leu¹⁹¹ into the DNA duplex at the site vacated by the extruded base. This ratchet-like movement by the protein prevents the flipped-out base from returning into the DNA duplex, thereby driving it into the active site of the enzyme to be excised.

Crystallographic studies on the catalytic core domain of MutY reveals an active site binding pocket for an extruded adenine (23), suggesting that MutY also uses a similar base-flipping reaction mechanism. However, MutY is unique among base-excision repair enzymes in recognizing a mismatch between a damaged 8-oxoguanine and a normal adenine while exclusively catalyzing the removal of the undamaged base (24–26). Unlike other base-flipping enzymes, the recognition target of MutY must extend beyond the adenine being excised; thus, the characteristic base-flipping reaction mechanism where the extruded base is also the site of the chemical reactions cannot fully explain the mode of target recognition by MutY.

Biochemical evidence shows that the C-terminal domain missing from the crystal structure of the catalytic core domain is required for 8-oxoguanine recognition (27–29). NMR studies of this missing domain reveal significant structural homologies with the known structure of MutT (29–31), an enzyme that hydrolyzes deoxy-8-oxoguanosine 5ʹ-triphosphate. As MutT uses a binding pocket to recognize the 8-oxoguanine portion of its substrate (32), the presence of an analogous pocket has been postulated for the C-terminal domain of MutY (29, 30). If such a cleft were to exist, then the recognition of an 8-oxoguanine by MutY would likely require the flipping of the non-scissile 8-oxo-
Double Base Flipping by MutY

Oligodeoxyribonucleotides

| 1° (top) strand | Oligodeoxyribonucleotide substrates |
|-----------------|----------------------------------|
| tXYZ | 5' CAT CAG AAC XYZ TCA TCG TTA |
| tAA T | 5' --- --- --- --- --- --- |
| tPAT | 5' --- --- --- --- --- --- |
| tACT | 5' --- --- --- --- --- --- |
| b (bottom) strand | |
| aXYZ | 3' GTA GTC TTT ZYX AGC AAT |
| hAOT | 3' --- --- --- --- --- --- |
| hPOT | 3' --- --- --- --- --- --- |
| hABT | 3' --- --- --- --- --- --- |

Oligodeoxyribonucleotides are designated as either t for the adenine-bearing top strand or b for the 8-oxoguanine-containing bottom strand followed by the 5'-3' sequence of the central triplet. Duplex constructs are designated by their component top and bottom strands conjoined by a colon. 5'-32P-Labeled sequences are denoted by an asterisk (*) in the text.

RESULTS AND DISCUSSION

UV Cross-linking via 8-Bromoguanine—To directly detect structural contacts between MutY and 8-oxoguanine, we synthesized the 21-nucleotide hABT with a photoreactive 8-bromoguanine (B) substituted for the 8-oxoguanine (see Table I). Cross-linking experiments were carried out with either 5'-32P-labeled single-stranded *bABT or the duplex tAAT:*bABT containing an adenine:8-bromoguanine mismatch. Single-turnover adenine excision assays performed with tAAT:hABT showed only a ~2-fold reduction in the rate of excision (data not shown) relative to a “normal” adenine:8-oxoguanine mismatched duplex, tAAT:hAOT, validating the suitability of the 8-bromoguanine substitution in these cross-linking studies.

Cross-linked products formed after irradiation at 302 nm for 40 min were resolved by SDS-polyacrylamide gel electrophoresis. Fig. 1 shows the results of experiments carried out at 1 μM MutY and 0.5 μM DNA, conditions under which active site titration experiments indicated near maximal productive binding of substrate DNA (data not shown). A distinct radiolabeled band consistent with DNA-cross-linked MutY appeared with either single-stranded *hABT (Lane 4) or duplex tAAT:*hABT (Lane 8) with the former showing moderately higher efficiency of cross-linking. The overall extent of cross-linking (<10%) was poorly due to the non-optimal wavelength of irradiation. Significantly better cross-linking was observed with irradiation at 254 nm; however, we chose to cross-link at the longer wavelength to avoid unnecessary damage to the DNA and protein as well as to avoid non-8-bromoguanine-induced, nonspecific DNA cross-linking. Control experiments with 1 μM bovine serum albumin (Lanes 1 and 5) or no MutY (Lanes 2 and 6) did not
Double Base Flipping by MutY

**Fig. 1.** UV cross-linking with 8-bromoguanine-containing oligodeoxyribonucleotides. 8-Bromoguanine-containing oligodeoxyribonucleotide *bABT (0.5 μM) was 5'-32P-labeled and irradiated either as single-stranded DNA, *bABT (Lanes 1–4), or duplex DNA, tAAT:*bABT (Lanes 5–8). Lanes 1 and 5 are controls performed with bovine serum albumin (BSA) (1 μM) instead of MutY to indicate the absence of nonspecific cross-linking. Lanes 2 and 6 are controls where DNA alone was irradiated for 40 min in the absence of MutY. Lanes 3 and 7 represent samples withdrawn and quenched prior to irradiation containing both DNA and MutY (1 μM). Lanes 4 and 8 show cross-linked products as indicated by the arrow after irradiation. Cross-linking was performed at 302 nm for 40 min at 20 °C in Buffer Y. Cross-linked products were separated from un-cross-linked DNA by SDS-PAGE on a 12% acrylamide gel.

**Fig. 2.** Single-turnover adenine excision time courses. The suitability of the two fluorescent duplex substrates, tPAT:bAOT (C) and tAAT:bPOT (D), were assayed in single-turnover experiments and compared with results from a normal non-fluorescent substrate, tAAT:*bABT (*). All three substrates were hydrolyzed identically by MutY, demonstrating that the inclusion of the fluorophore did not alter the kinetic properties of the substrates. The solid line represents a best fit of the data to a single exponential function with an apparent rate constant of 0.25 ± 0.03 s⁻¹.

show cross-linking nor did samples quenched prior to irradiation² (Lanes 3 and 7).

UV cross-linking demonstrated direct contact between MutY and the 8-bromoguanine during catalysis. In a duplex DNA substrate where this base is hidden in the DNA helix, such contact could occur either from the insertion of a part of the enzyme into the helical space or by the extraction of the base from the helix into a binding pocket on the enzyme surface.

² Additional controls not shown also showed that cross-linking to the single-stranded *bABT was not inhibited by the addition of non-8-bromoguanine-containing DNA, while the cross-linking of duplex tAAT: *bABT was inhibited by the addition of unlabeled bABT. These results show specific binding of the single-stranded bABT.

Therefore, the observation of cross-linking with the duplex construct alone does not constitute compelling evidence of flipping of the 8-substituted guanine. However, the observation of cross-linking with the single-stranded DNA, where the rigid structural determinants of the duplex and the scissile adenine are absent, implies that the recognition of the 8-substituted guanine requires neither the prior binding of duplex DNA nor the extrusion of adenine. In addition, the increased cross-linking observed with single-stranded DNA suggests that the site of cross-linking on MutY is more readily accessible to a “free” 8-bromoguanine residue than one hidden within the helical space of duplex DNA. Conversely if contact were to occur with a protein side chain inserted into the DNA duplex to probe for the 8-substituted-guanine, then less cross-linking would be expected with the single-stranded construct where the “binding
site" provided by the helical cage of the duplex is absent.\(^3\) Consequently these results support the hypothesis of a preexisting 8-oxoguanine binding pocket on the enzyme surface as proposed based on the structural homology to MutT (29–31).

**Stopped-flow Detection of Double Base Flipping**—To directly monitor the extrusion of 8-oxoguanine from the DNA duplex, we synthesized an 8-oxoguanine:adenine mismatched substrate with the fluorescent base analog 2-aminopurine (P), positioned 5’-adjacent to the 8-oxoguanine (O), tAAT:bPOT. The reduction of quantum yield of 2-aminopurine from base-stacking interactions (37) has been widely exploited in studies of DNA-metabolizing enzymes (22, 38–41). Therefore, we expected to observe an enhancement of the fluorescence intensity of the 2-aminopurine probe in response to the loss of base-stacking interaction upon extrahelical extrusion of its neighboring 8-oxoguanine. Similarly a homologous duplex substrate, tPAT: bAOT, with the 2-aminopurine probe adjacent to the scissile adenine was also synthesized to monitor adenine flipping. Single-turnover adenine excision assays at 500 nM MutY and 250 nM DNA showed identical time courses for the fluorescent base analog 2-aminopurine (P) and 2-aminopurine-containing duplex substrates tAAT: bPOT and tPAT: bAOT and the non-fluorescent, normal substrate tAAT: bAOT (Fig. 2). Nonlinear regression best fit of the data yielded a rate constant for excision for all three substrates at the active site of 0.25 ± 0.03 s\(^{-1}\) in agreement with reported values (26, 29), validating the suitability of these fluorescent duplexes as substrate analogs in quantitative kinetic measurements.

Fig. 3a shows the real time fluorescence emission of the 2-aminopurine-containing tAAT:bPOT (250 nM) within 40 ms of mixing with 500 nM MutY in a stopped-flow fluorimeter. On this time scale, we observed a rapid, single-exponential increase in fluorescence with a rate constant of 108 ± 13 s\(^{-1}\). With the 2-aminopurine probe positioned next to the 8-oxoguanine, this experiment directly demonstrates 8-oxoguanine base flipping at 108 s\(^{-1}\) under these reaction conditions. In a similar experiment performed under identical conditions using a substrate where the fluorophore was positioned to monitor adenine flipping (tPAT: bAOT), the observed fluorescence enhancement appeared later, slower, and with multiple exponential phases. Fig. 3b shows split time-based stopped-flow traces for adenine flipping (top trace), 8-oxoguanine flipping (middle trace), and a negative control (bottom trace). The time course for tPAT: bAOT corresponding to adenine flipping occurred with an initial 30-ms lag followed by two distinct exponential increases. Comparison with the initial 30 ms of the tAAT: bPOT time course shows that the initial lag observed with adenine flipping coincided with 8-oxoguanine flipping, indicating that the extrusion of the adenine follows 8-oxoguanine flipping. The subsequent biphasic increase in fluorescence further reflects a two-step process for adenine extrusion with an initial extrusion of the adenine with an apparent rate constant of 16 ± 1.2 s\(^{-1}\) followed by a subsequent step at 1.9 s\(^{-1}\). In a negative control experiment using a duplex containing a non-cognate 8-oxoguanine: cytosine base pair, tACT: bPOT, no fluorescence change was detected (Fig. 3b, bottom trace).

The large amplitude of the 1.9 s\(^{-1}\) exponential phase implicated a large favorable forward equilibrium for this step. However, an 8-fold smaller excision rate constant of 0.25 s\(^{-1}\) would rule out this step as being excision. In the paradigmatic base-flipping kinetic mechanism of the E. coli uracil-DNA glycosylase, a conformational change step following base flipping corresponds structurally to the insertion of a leucine side chain of the enzyme into the DNA helix to occupy the space vacated by the extruded base (22). The leucine insertion isomerization conferred additional stabilization for the "flipped-out" conformation of the extruded base by preventing its return into the DNA double helix. By analogy, the 1.9 s\(^{-1}\) exponential phase likely reflects a similar isomerization step in the reaction mechanism of MutY to facilitate the capture of the extruded adenine in preparation for excision.

**Double Base-flipping Model for Target Selection**—Identical rate constants were observed irrespective of the placement of the 2-aminopurine to detect 8-oxoguanine or adenine flipping consistent with the sequential three-step mechanism shown in Fig. 4: 8-oxoguanine extrusion (108 s\(^{-1}\)), adenine flipping (16 s\(^{-1}\)), and isomerization (1.9 s\(^{-1}\)). Each step along this pathway thus provides incremental energetic stabilization toward the formation of the final doubly flipped conformation where the scissile adenine is captured by the active site of the enzyme.

---

\(^3\) While it is possible that the cross-linking with single-stranded bABT could in principle have occurred via binding of the 8-bromoguanine by the adenine binding pocket, we consider this to be highly improbable due to the presence of six adenines and five guanines elsewhere in the sequence of bABT.
requiring the 8-oxoguanine to be flipped before the adenine, the enzyme ensures that only those adenines mispaired to it are targeted for excision. Similarly the stabilization provided by the isomerization step following the extrusion of both bases of the mismatch ensures that only base pairs containing both 8-oxoguanine and adenine bases become captured with commitment toward catalysis. Accuracy in target selection is thereby achieved as each step of the mechanism functions to trigger the forward progress toward catalysis while coordinately stabilizing the previous steps.

In addition, increased efficiency is achieved in searching for a scissile adenine. By sequentially coordinating the selection of the 8-oxoguanine and the adenine bases, the double base-flipping mechanism establishes a hierarchical order for the search process where the much rarer 8-oxoguanine constituent is first targeted. Interestingly the 8-oxoguanine of this mismatched base pair is readily discernable by the syn conformation of its glycosidic bond as illustrated in structural studies (42), and biochemical evidence further suggests that MutY can recognize the syn conformation of the 8-oxoguanine base in this context (43). MutY therefore likely takes advantage of this conformational feature in its scan along the DNA backbone to trigger the double base-flipping mechanism upon location of an 8-oxoguanine:adenine lesion. Such a hierarchically ordered search model would greatly enhance the efficacy of the search by mitigating the need to extrude and examine each individual base in the duplex DNA.

The reaction rate constants reported here represent macroscopic rate constants observed at 250 nM DNA and 500 nM MutY. The resolution of the microscopic forward and reverse rate constants for each elementary step must await the completion of concentration dependence studies currently underway, although preliminary results suggest that the reaction conditions used were near saturation. However, the design and use of the 2-aminopurine-containing base-flipping-sensitive substrate pair tAAT:bPOT and tPAT:bAOT unambiguously demonstrates 8-oxoguanine flipping. In addition, by establishing the temporal sequence of the substrate recognition steps leading up to catalysis, we were able to observe the coordination of the mechanistic interplay between steps along the catalytic pathway responsible for substrate recognition. These stopped-flow studies not only provide direct kinetic detection of key structural intermediates, but more importantly, by placing these structures in their proper temporal order, they allow us to deduce the dynamic mechanistic basis by which this class of diverse DNA-metabolizing enzymes (13) recognizes and gains access to a naturally hidden target.

Acknowledgments—We thank Jacqueline A. Wirz for help in purifying the oligodeoxynucleosides and Dr. Chris Mathews, Jacqueline A. Wirz, and Andrea Mahr for critical reading of the manuscript and helpful discussions.

REFERENCES

1. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1995) Biochim. Biophys. Acta 1271, 165–170
2. Shigenaga, M. K., Hagen, T. M., and Ames, B. N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10771–10778
3. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7915–7922
4. Beckman, K. B., and Ames, B. N. (1997) J. Biol. Chem. 272, 19633–19636
5. Lindahl, T. (1993) Nature 362, 709–715
6. Demple, B., and Harrison, L. (1994) Annu. Rev. Biochem. 63, 915–948
7. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, pp. 159–160, ASM Press, Washington, D. C.
8. Verdine, G. L., and Bruner, S. D. (1997) Chem. Biol. 4, 329–334
9. Lemos, L. A., Poek, M. E., Morningstar, M. L., Verghis, S. M., Miller, K. M., Rich, A., Eissmann, J. M., and Williams, L. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 719–723
10. Gannett, P. M., and Sura, T. P. (1993) Chem. Rev. Toxicol. 6, 699–700
11. Hargreaves, L., Yu, S. L., Johnson, R. E., Prakash, L., and Prakash, S. (2000) Nat. Genet. 25, 458–461
12. Cheng, X., and Blumenthal, R. M. (1996) Structure 4, 639–645
13. Lloyd, R. S., and Cheng, X. (1997) Biopolymer 44, 139–151
14. Bandwar, R. P., and Patel, S. S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 181–198
15. Horbury, D. P., and Ford, G. C. (1998) Curr. Opin. Biotechnol. 9, 354–358
16. Lau, A. Y., Schaar, O. D., Samson, L., Verdine, G. L., and Ellenberger, T. (1998) Cell 86, 249–258
17. Morikawa, K., and Shirakawa, M. (2000) Mutat. Res. 466, 257–275
18. O’Gara, M., Horton, J. R., Roberts, R. J., and Cheng, X. (1998) Nat. Struct. Biol. 5, 872–877
19. Parikh, S. S., Mol, C. D., Slupphaug, G., Bharati, S., Krokan, H. E., and Tainer, J. A. (1998) EMBO J. 17, 5214–5226
20. Hosfield, D. J., Guan, Y., Haas, B. J., Cunningham, R. P., and Tainer, J. A. (1999) Cell 97, 397–408
21. Slupphaug, G., Mol, C. D., Kavli, B., Arvai, A. S., Krokan, H. E., and Tainer, J. A. (1996) Nature 384, 87–92
22. Wong, I., Lundquist, A. J., Bernards, A. S., and Mosbaugh, D. W. (2002) J. Biol. Chem. 277, in press
23. Guan, Y., Manuel, R. C., Arvai, A. S., Parikh, S. S., Mol, C. D., Miller, J. H., Lloyd, S., and Tainer, J. A. (1998) Nat. Struct. Biol. 5, 1058–1064
24. Chmiel, N. H., Golzelnii, M. P., Francis, A. W., and David, S. S. (2001) Nucleic Acids Res. 29, 553–564
25. Lu, A. L. (2000) Methods Mol. Biol. 152, 3–16
26. Porell, S. L., Leyes, A. E., and David, S. S. (1998) Biochemistry 37, 14756–14764
27. Gogos, A., Cille, J., Clarke, N. D., and Lu, A. L. (1996) Biochemistry 35, 16665–16671
28. Li, X., and Lu, A. L. (2000) Nucleic Acids Res. 28, 4593–4603
29. Noll, D. G., Gogos, A., Granek, J. A., and Clarke, N. D. (1999) Biochemistry 38, 6174–6179
30. House, P. G., Volk, D. E., Thrivinathan, V., Manuel, R. C., Luxon, B. A., Gorenstein, D. G., and Lloyd, R. S. (2001) Prog. Nucleic Acids Res. Mol. Biol. 69, 1476–1478
31. Volk, D. E., House, P. G., Thrivinathan, V., Luxon, B. A., Zhang, S., Lloyd, R. S., and Gorenstein, D. G. (2000) Biochemistry 39, 7331–7336
32. Abu-Elwahab, M., and Loh, A. J. (2002) Biopolymers 67, 1077–1084
33. Loh, A. J., and Loh, A. J. (2002) Biochemistry 41, 16226–16236
34. Bao, R. R., Xu, Z. A., and Wang, W. (2000) Biopolymers 51, 1209–1216
35. Cantor, C. R., Warshaw, M. M., and Shapiro, H. (1970) Biopolymers 9, 1059–1077
36. Porell, S. L., Williams, S. D., Kuhn, H., Michaels, M. L., and David, S. (1996) J. Am. Chem. Soc. 118, 10684–10692
37. Lomberg, L. A., Poek, M. E., Morningstar, M. L., Verghis, S. M., Miller, K. M., Rich, A., Eissmann, J. M., and Williams, L. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1709–1717
38. Bandwar, R. P., and Patel, S. S. (2001) J. Biol. Chem. 276, 14080–14082
39. McAuley-Hecht, K. E., Leonard, G. A., Gibson, N. J., Thomson, J. B., Watson, W. P., Hunter, W. N., and Brown, T. (1994) Biochemistry 33, 10266–10277
40. Bulychev, N. V., Varaprasad, C. V., Dorman, G., Miller, J. H., Eisenberg, M., Grollman, A. P., and Johnson, F. (1996) Biochemistry 35, 13147–13156