A Dimeric Mechanism for Contextual Target Recognition by MutY Glycosylase*

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MutY, an adenine glycosylase, initiates the critical repair of an adenine:8-oxo-guanine base pair in DNA arising from polymerase error at the oxidatively damaged guanine. Here we demonstrate for the first time, using presteady-state active site titrations, that MutY assembles into a dimer upon binding substrate DNA and that the dimer is the functionally active form of the enzyme. Additionally, we observed allosteric inhibition of glycosylase activity in the dimer by the concurrent binding of two lesion mispairs. Active site titration results were independently verified by gel mobility shift assays and quantitative DNA footprint titrations. A model is proposed for the potential functional role of the observed polysteric and allosteric regulation in recruiting and coordinating interactions with the methyl-directed mismatch repair system.

Bases in DNA suffer oxidative damages from normal cellular metabolism even in the absence of exogenous oxidative stress (1, 2). The resulting alterations in base-pairing properties (3) lead to increased mutational frequencies at these damaged bases during subsequent replication (4) with wide-ranging pathological consequences (5). The accumulation of oxidative lesions in DNA have been associated with aging (6–10) and are also strongly correlated to cancers resulting from known exposure to oxidative environmental carcinogens (1, 11–15).

The removal of oxidative lesions and the correction of mutagenic mispairing at these lesions, therefore, represent two essential but distinct lines of defense against genome degradation. The prevalent oxidatively damaged base, 8-oxo-guanine, is excised from DNA when base-paired to cytosine by MutM (16–20) and its eukaryotic homolog, OG11 (21–26). Misincorporation of adenosines by DNA polymerase opposite 8-oxo-guanines that escape MutM-mediated repair are subsequently repaired by the adenine glycosylase, MutY (20, 27–33), and its homolog, MYH (16, 20, 34–36). Additionally, MutT (16, 20, 37–40), a nucleoside triphosphate pyrophosphohydrolase, removes oxidatively damaged dGTPs, thereby preventing their incorporation into DNA.

MutY, therefore, functions physiologically as a mismatch repair enzyme, as originally suggested (41, 42), albeit targeted toward adenosines misincorporated by DNA polymerase at template 8-oxo-guanines. As such, the accuracy of repair depends on the exclusive removal of adenosines from the newly synthesized, daughter strand of DNA. By contrast, MutY repair activity at a mispair where the 8-oxo-guanine has been misincorporated opposite a template adenine would be mutagenic. The observation (43) that A:T → C:G transversion frequencies in MutT/mutY/Esherichia coli is increased relative to MutT'/MutY' strains, whereas the high rate of C:G → A:T mutations in MutY' strains is unaffected by the status of MutT, corroborates the mutagenic effects of such MutY-mediated removal of adenosines from the template strand.

However, the generally accepted view of MutY as a prototypical mono-functional base excision glycosylase provides no mechanistic basis for daughter versus template strand selectivity. Interestingly, the human MutY homolog, hMYH, has recently been shown to interact with the human mismatch repair enzyme, hMSH6 (44), providing the first insight into the mechanistic possibility that nascent strand recognition may result from cooperative recruitment of the mismatch repair system. Even so, such cooperative interaction between different repair systems does not mitigate the need for a regulatory mechanism intrinsic to MutY itself to forestall indiscriminate excision prior to recruitment. Toward this end, we have reexamined the kinetic mechanism of MutY using presteady-state active site titrations to reveal a polysteric and allosteric basis for such a molecular switch.

EXPERIMENTAL PROCEDURES

Buffers and Reagents—Except as noted, all buffers were made with reagent grade chemicals and Milli-Q Plus (Millipore, Bedford, MA) purified distilled-deionized water. MutY Storage Buffer contained 50 mM HEPES-NaOH (pH 7.5), 50 mM NaCl, and 50% spectroscopic grade glycerol. MutY Assay Buffer contained 5 mM HEPES-NaOH (pH 7.5), 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 5% spectroscopic grade glycerol. All buffer stock solutions were filtered through 0.2-μm polystyrene-sulfonate filters (Nalgene, Rochester, NY).

Enzymes and Oligonucleotides—The E. coli MutY was purified from E. coli BL21(DE3) harboring the overproducing plasmid pET24a/MutY-8 as described previously (53). The MutY concentration was determined spectrophotometrically using an extinction coefficient of ε290 = 77,510 M\(^{-1}\) cm\(^{-1}\). Purified MutY had an A\(_{260}\) to A\(_{280}\) ratio between 0.15 and 0.19 and was stored in MutY Storage Buffer at ~80 °C. Synthetic oligodeoxyribonucleotides were synthesized and further purified by urea-polyacrylamide gel electrophoresis and electro-elution as described previously (61). Duplex substrates were constructed by annealing 5\(^{32}\)P-labeled *-AT (5\(^{-32}\)CATGAAACATATCCTGCGTTA) with its complement, b-AOT (5\(^{-32}\)GATGAAATCGTGCTTGAGT, O = 8-oxo-guanine) as described (61), placing a single adenine:8-oxoguanine mismatch at the center (position 11) of the sequence as denoted by the underscore. Concentrations were determined spectrophotometrically using extinction coefficients calculated according to Cantor et al. (62). T4 polynucleotide kinase was obtained from USB (Cleveland, OH), and [γ\(^{32}\)P]ATP (3000 Ci/mmole) was obtained from Amersham Biosciences.

Presteady-State Excision Assays—Single-turnover experiments were carried out using a KinTek RFQ-5 rapid chemical quench (KinTek Instruments, State College, PA) instrument thermostatically maintained at 37 °C with a Neslab RTE-111 refrigerated water bath. Reactions in MutY Assay Buffer were initiated by rapidly mixing 15 μM of MutY with 15 μM of 5\(^{32}\)P-labeled duplex DNA substrate and were...
chemically quenched with 90% of 0.2 M NaOH. Each site-containing excision products were then heated to 90 °C for 8 min to cleave the phospho-ribose backbone of the DNA at the abasic site to generate 10-nucleotide products, which were separated from the 21-nucleotide substrates by electrophoresis on a 20% acrylamide, 8 M urea gel. Following visualization with a PhosphorImager (Amersham Biosciences), the intensities of DNA substrate and product bands were quantified using ImageQuant software (Amersham Biosciences) as described (47, 61). Single-turnover time courses with enzyme present in excess over substrate were fit to a single exponential function, \( |P| = A_1(1 - e^{-k_1t}) \). First turnover time courses in excess substrate were also fit to single exponential functions for time courses less than 2 min because of the undetectable contribution of the linear steady-state phase within this time frame.

First turnover burst amplitudes were determined by performing excision assay using time points in excess of 4 min. Accurate data could be obtained using a minimal set of three time points at 4, 8, and 12 min; however, time courses were typically linear for up to 2 h. Burst amplitudes were obtained as the y-intercept values returned by the linear regression best fit of the data. Active site titrations consisted of a series of first turnover burst amplitude determinations obtained over a serially diluted range of MutY concentrations at fixed DNA concentrations.

Fit to Statistical Thermodynamic Model—The statistical thermodynamic model of Fig. 2 has been described previously in detail (48, 49). Fits to active site titrations were modeled after the species fraction of the Y-D complex as given by Equation 1.

\[
\frac{[YD]}{[D]} = \frac{K_{Y,D} [Y][D]}{1 + K_{Y,D} [Y] + K_{D,Y} [D] + 2K_{Y,D}K_{D,Y}[Y][D]}
\]

Nonlinear fits were obtained for all data sets using an adaptive nonlinear regression algorithm (63) as implemented in the program NLREG (Phillip H. Sherrard, Brentwood, TN). Fractions of DNA found in the Y-Da and YD subpopulations were simulated according to Equations 2 and 3.

\[
\frac{[YD]}{[D]} = \frac{2K_{Y,D} K_{D,Y}[Y][D]}{1 + K_{Y,D} [Y] + K_{D,Y} [D] + 2K_{Y,D}K_{D,Y}[Y][D]} \tag{2}
\]

\[
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\]

Simulated curves shown were generated using KaleidaGraph v.3.51 (Synergy Software, PA) as described (48, 49).

Footprint Titrations—Hydroxyl-radical (Fe-EDTA) footprint titrations were adapted from published protocols (50, 52, 64). Duplex tAA: tAAT, radiolabeled either in the adenine, *tAAT, or 8-oxo-guanine mispair. The base-labile abasic product was cleaved in 0.2 M NaOH at 80 °C to generate a shortened 10-mer, which was separated from the 21-mer substrate by denaturing polyacrylamide gel electrophoresis. In a typical single-turnover assay performed with 4-fold molar excess of MutY (200 nM) over substrate (50 nM), we observed rapid, single-exponential excision of 98 ± 2% of the mispaired adenine at 0.24 ± 0.01 s⁻¹ (Fig. 1A, closed circles). In experiments performed with excess substrate (200 nM) over enzyme (50 nM), however, only 20 ± 0.4% of the substrates were turned over in the exponential phase but with the identical rate constant of 0.22 ± 0.01 s⁻¹ (open circles). Data obtained at longer times (Fig. 1B) showed that a presteady-state burst of product was formed in the first turnover followed by steady-state turnovers at 2 × 10⁻⁶ s⁻¹ corresponding to the slow rate of one or more product release steps that limited the reaction of free active sites. The amplitude of the burst phase, therefore, provided a direct measure of the molar amount of productively bound substrate during the initial turnover (45–47).

In active site titrations, burst amplitudes were measured at different MutY and DNA concentrations. The resulting titration curves, plotted as a function of MutY concentration (Fig. 2A), were sigmoidal at eight different DNA concentrations ranging from 10 to 600 nM. At saturating DNA concentrations (>200 nM), complete binding of substrate required a 2-fold excess of MutY, limiting the reaction stoichiometry to ≤2. However, the nonlinear response at lower MutY concentrations ruled out a monomeric oligomeric species with a single binding site (48, 49).

To further clarify the apparent reaction stoichiometry, we reconstructed the titration data to reflect the fractional occupancy of total available active sites as a function of increasing DNA concentrations at various fixed concentrations of MutY (Fig. 2B). The titration curves were linearly dependent on DNA concentration up to ~45% saturation of active site corresponding to a reaction stoichiometry of two MutY monomers per substrate molecule. Addition of DNA beyond this point, however, resulted in decreased burst amplitudes. As mass action dictated that the addition of DNA ligand must lead to additional total binding, the decline in burst amplitude demonstrated allosteric inhibition of the initial population of two-to-one active complexes by the binding of a second equivalent of substrate. Furthermore, the reduction in burst amplitudes was not accompanied by any change in exponential time course of

\[
\theta = \frac{1 - I_{norm,-}}{1 - I_{norm,-}^*} \tag{5}
\]

where \( I_{norm,-} \) was obtained by averaging normalized intensities at saturation.

**RESULTS**

First Turnover Kinetics and Active Site Titrations—For presteady-state assay of MutY activity, we radiolabeled the adenine-containing strand of a 21-bp duplex substrate, *tAAT: bAO, which contained a centrally positioned adenine:8-oxoguanine mispair. The base-labile abasic product was cleaved in 0.2 M NaOH at 80 °C to generate a shortened 10-mer, which was separated from the 21-mer substrate by denaturing polyacrylamide gel electrophoresis. In a typical single-turnover assay performed with 4-fold molar excess of MutY (200 nM) over substrate (50 nM), we observed rapid, single-exponential excision of 98 ± 2% of the mispaired adenine at 0.24 ± 0.01 s⁻¹ (Fig. 1A, closed circles). In experiments performed with excess substrate (200 nM) over enzyme (50 nM), however, only 20 ± 0.4% of the substrates were turned over in the exponential phase but with the identical rate constant of 0.22 ± 0.01 s⁻¹ (open circles). Data obtained at longer times (Fig. 1B) showed that a presteady-state burst of product was formed in the first turnover followed by steady-state turnovers at 2 × 10⁻⁶ s⁻¹ corresponding to the slow rate of one or more product release steps that limited the reaction of free active sites. The amplitude of the burst phase, therefore, provided a direct measure of the molar amount of productively bound substrate during the initial turnover (45–47).

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the first turnover (Fig. 1A); therefore, the binding of the second substrate abolished, rather than attenuated, the excision activity of the dimer. Our results demonstrate that the active form of MutY is a half-saturated dimeric, Y2D, whose excision activity is allosterically inhibited upon binding a second substrate to form the fully saturated dimer, Y2D2. Identical results were obtained using MutY purified by other protocols including enzyme refolded from guanidine-solubilized inclusion bodies (data not shown).

**Statistical Thermodynamic Model**—To quantitatively model this allosteric regulation, we used a general statistical thermodynamic scheme (Fig. 3A) developed previously to describe the protein assembly and DNA binding properties of dimeric proteins (48, 49). The model required four interaction parameters to describe the dynamic equilibrium between two monomeric states, Y and YD, and three dimeric states, Y2, Y2D, and Y2D2. K0 describes the protein-protein dimerization constant in the absence of substrate, K1 describes the protein-protein dimerization constant in the absence of bound DNA. K11 describes the DNA binding affinity of the monomer for DNA. K1 describes the dimerization constant between a DNA-bound monomer and a free monomer to form the half-saturated dimer, Y2D. Lastly, K22 describes the DNA affinity of Y2D for a second DNA molecule to form the fully saturated dimer, Y2D2. The two remaining equilibrium constants, K2 and K21, were constrained by thermodynamic linkage to the values of K0, K1, K11, and K22. Lastly, the value of K5, the dimerization constant in the absence of substrate, was arbitrarily fixed at 10 mM−1 as gel filtration chromatography experiments showed that MutY was monomeric in the absence of DNA even at micromolar concentrations (data not shown).

Previously, we have shown that titration data obtained at four different DNA concentrations were sufficient to yield satisfactory resolution of the fitted interaction parameters in global nonlinear regression analysis using this model (48).
linear best fit of the active site titrations obtained at eight DNA concentrations to this model showed excellent global agreement between the burst amplitudes and the species population of YD predicted by the model (Fig. 2, solid lines) at all DNA and MutY concentrations, yielding the values for K11, K12, and K22 shown in Table I. Simulations were insensitive to values of K0 < 40 mM⁻¹, confirming our assumption that the unliganded dimer is sparsely populated and validating the value of K0 chosen for the fitting. DNA binding to the monomer was relatively weak (K11 = 6 μM⁻¹) but was strongly linked to protein dimerization with a 5 order of magnitude increase in dimerization constant (K1 = 3 mM⁻¹) upon binding DNA, placing the affinity for the DNA by Y2, the unliganded dimer, at K21 > 450 mM⁻¹ (K21, 21 < 2.2 pm). This positive polysteric linkage between DNA binding and protein oligomerization thermodynamically guaranteed near stoichiometric binding of DNA by a MutY dimer despite the weak affinity of the monomer for DNA. The active half-saturated dimer, YD, however, bound the second equivalent of DNA much more weakly, with K22 = 1.5 μM⁻¹. This negative cooperativity of binding by YD coupled with the negative allosteric inhibition of excision activity by the second bound lesion together accounted for the apparent gradual decline of burst amplitude observed at higher DNA concentrations.

Fig. 3B shows the population distribution of all DNA-bound species for an active site titration at 300 nM DNA. As expected, the data correlated well with the sigmoidal accumulation of YD with increasing MutY added. At low MutY concentrations, however, the high ratio of DNA to MutY favored the formation of species with a one-to-one binding stoichiometry. Accordingly, the fully saturated species, Y2D2, was maximally populated at equimolar concentrations of MutY and DNA (300 nM) but disproportionately to form YD at higher MutY concentrations. Because YD lacked excision activity, the adenine:8-oxo-guanine mismatches of DNA bound in this configuration were silent in active site titrations, thereby giving rise to the sigmoidal behavior observed. Because the DNA-bound monomer, YD, accounted for only ~2% of the DNA bound at this DNA concentration due to the strong positive polysteric linkage, we are less certain that YD also lacked excision activity. However, fits to any model with full excision activity by YD tended to systematically lessen the sigmoidality of the resulting titration curves at all DNA concentrations, compelling us to hypothesize that the monomer is inactive.

Footprint Titrations—Because the model proposes the existence of a silent Y2D2 state, we performed hydroxyl-radical DNA footprint (50) titrations to directly monitor all site-specific DNA interactions (51, 52). Fig. 4A shows sequencing gels of footprint titrations performed in parallel using duplex substrate DNA radiolabeled either on the adenine strand, *aAAT (left), or on the 8-oxo-guanine strand, *bAOT (right). The adventitious lyase activity of MutY resulted in partial strand cleavage at the position of the scissile adenine, which was visible in the *aAAT footprint. Additionally, a distinct, MutY-dependent footprint covering 2–3 nucleotides was observed immediately 5’ of the scissile adenine. The footprint on the 8-oxo-guanine strand, *bAOT, covered 4–5 nucleotides and appeared to be centered about the 8-oxo-guanine.

Quantitation of the extent of protection by MutY (Fig. 4B) revealed different protein concentration dependences for the observed footprints on the two strands of DNA. The protection of the adenine strand was sigmoidal and correlated well with

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**Fig. 3.** Dimeric model. As shown in A, the active site titration data were modeled using the thermodynamic linkage cycle of Wong and Lohman (48). Y, D, and YD represent unbound MutY monomers, unbound adenine:8-oxo-guanine-containing oligonucleotide substrates, and monomeric MutY-substrate complexes, respectively. Y, YD, YD, and Y2D2 represent MutY dimers bound with zero, one, and two substrates. Of the six equilibrium association constants shown, only four (K0, K11, K21, K22) were needed to describe the linkage scheme. Additionally, K0 was constrained at 1 × 10⁴ M⁻¹ based on the absence of dimerization in the absence of DNA. Global best fit of all active site titrations was obtained based on YD being the catalytic species. As shown in B, simulated species fractions of Y, YD, and YD, at low molar ratios of MutY:DNA.

**TABLE I**

| Equilibrium Constants | Reaction |
|-----------------------|----------|
| K0                    | < 4.0 × 10⁴ |
| K11                   | 6.0 ± 0.7 × 10⁶ |
| K1                    | 3.0 ± 0.4 × 10⁹ |
| K22                   | 1.5 ± 0.3 × 10⁶ |
| K2                    | 7.5 × 10⁶ |
| K21                   | > 4.5 × 10¹¹ |

1 Bold face denotes values returned by nonlinear regression analysis. Values shown in italics are either independently constrained (K0) or calculated.
with productive evidence of structurally distinct protein conformations associated with productive versus non-productive binding. The correlation between activity and localized protection immediately adjacent to the scissile base likely reflected the formation of intimate contacts with the active site in the productive complex, Y₂D, that are absent in the inhibited complexes, perhaps suggesting that the adenine base may not be flipped out of the DNA duplex, as is required for excision (53), in the inhibited YₛDₛ state.

**Direct Detection of Inhibited Complexes by Gel Mobility Shift**—Gel mobility shift assays provided an alternate method of separating different binding complexes based on differences in mass and in charge. Previous reports using this technique to measure DNA binding affinity of MutY, however, revealed only a single bound species with bands corresponding to higher order aggregates appearing only at high concentrations of MutY (54–56). Although simple to perform, gel mobility shift assays have been shown by Engler et al. (57) to be sensitive to buffer-dependent artifacts. In our own attempts to reproduce assays under published conditions using TBE buffer in the gel, we observed that the banding patterns as well as the resultant titration curves changed with acrylamide concentration. We, therefore, tested a variety of gel buffers other than TBE and found that MutY Assay Buffer without glycerol reproducibly provided good results.

Fig. 5A shows typical results obtained with 100 nM DNA at 1:1 and 2:1 MutY to DNA molar ratios resolved on 6, 8, 10 and 12% polyacrylamide gels in this buffer. In all cases, three distinct bands were visible. Assays run without MutY (data not shown) verified that the fastest migrating species, band III, corresponded to free DNA. Most of the DNA was unbound at a 1:1 ratio of MutY to DNA. At this ratio, the bound DNA was roughly equally divided between bands I and II with the faster migrating band II showing slightly more DNA than band I. On increasing the MutY:DNA ratio to 2:1, however, most of the DNA became bound as band II. Additionally, the amount of DNA in band I, the slowest migrating species, actually decreased at the higher MutY concentration. Identical results were observed at all four acrylamide concentrations. Fig. 5B, which compares identical samples resolved in 10% polyacrylamide gels in TBE versus MutY Assay Buffer, shows the absence of band I in TBE gels at both MutY:DNA molar ratios, demonstrating that the ability to detect this species was gel buffer-dependent.

The preferential appearance of band I at a low MutY:DNA ratio suggested that band I may correspond to the inactive YₛDₛ state, whereas band II represented the active half-saturated dimer, Y₂D. To verify this assignment, we constructed a Ferguson plot (58) to analyze the relationship between the electrophoretic mobility of the two bands and the acrylamide percentage in the gel. As electrophoresis at all four acrylamide concentrations was carried out in parallel at a constant 70 V for a fixed 40 min, migration distances of each band provided a direct measure of its electrophoretic mobility. We, therefore, plotted the logarithm of migration distance as a function of acrylamide concentration (Fig. 5C), which showed linear dependences for all three bands. Additionally, the line for band I had a steeper slope and a larger y-intercept than the line for band II. As larger complexes are more sensitive to sieving by the gel matrix, the steeper slope, which indicated a greater dependence on acrylamide concentration, showed that band I corresponded to a larger complex than band II. Conversely, the larger y-intercept, which revealed a higher sensitivity to the applied electric field in the absence of sieving by the gel matrix, indicated that band I corresponded to a complex with a higher charge. These results, therefore, confirm our assignment of band I to YₛDₛ, which differed from Y₂D by one DNA substrate.

Lastly, we excised the two bands from the gel and placed them separately into 0.2 N NaOH to inactivate MutY activity.
Contextual Recognition by MutY Dimers

Firstly, DNA binding induced a $10^5$-fold increase in the dimer-port, we have additionally demonstrated that the glycosylase functions as a dimer with several salient mechanistic features. The gel slices were then pulverized and heated to 90°C for 10 min to extract the DNA and to alkali- cleave abasic DNA products. Denaturing PAGE separation of the cleaved DNA extracts from each band (Fig. 5C, inset) revealed that primarily unre acted substrate 21-mers were recovered from band I, whereas the DNA extracted from band II was completely cleaved. These results identified band II as being the active complex, $Y_2D$, and confirmed the lack of excision activity by the inactive $Y_2D_2$ complex found in band I.

DISCUSSION

A New Mechanistic Model—Using UV cross-linking and stopped-flow fluorimetry, we have previously established a double base-flipping reaction sequence requiring a minimum of three distinct structural changes between the substrate binding and excision that provided the mechanistic basis for the efficient screening and accurate selection of both bases in the target adenine:8-oxo-guanine mispair (53). In the current report, we have additionally demonstrated that the glycosylase functions as a dimer with several salient mechanistic features. Firstly, DNA binding induced a $10^5$-fold increase in the dimerization constant of MutY. This strongly positive thermodynamic linkage couples free energies of protein dimerization and substrate binding to provide overall tight binding of a single lesion mispair by the dimer. Secondly, the resulting half-saturated dimer, $Y_2D$, is the functionally active form of the enzyme. Thirdly, binding of a second mispair by the $Y_2D$ dimer is negatively cooperative, i.e. with weak affinity, and allosterically inhibitory such that the resulting fully saturated dimer, $Y_2D_2$, whereas difficult to form, lacks all excision activity.

Porello et al. (30) used active site titrations similar to ours to determine the active concentration of MutY in their enzyme preparations. However, because they assumed a monomeric stoichiometry, their assays utilized only three enzyme concentrations within a limited range and a single fixed DNA concentration to determine the fractional activity of their enzyme preparation. In contrast, we obtained our active site titration data over an extended range of enzyme and DNA concentrations including conditions of high DNA:MutY ratios in which we observed a DNA-dependent decline of excision activity. This result cannot be rationalized by any explanation based on inactive protein in the enzyme preparation. No decline in burst amplitude would have been observed if the 45% maximum burst amplitude had resulted from only 45% active MutY in the enzyme preparation as the binding of DNA by the remaining 55% inactive MutY should have no additional effect on the measured activity unless the two populations of proteins interacted directly. Interestingly, Porello et al. (30) reported typical yields of 25–60% percent active enzyme, a range that is, within statistical error, compatible with our alternative induced dimeric model.

Recruitment of MutS by $Y_2D_2$—The lack of excision activity by the $Y_2D_2$ complex is initially surprising as the inhibition of repair in response to elevated lesion concentrations would appear to be counterproductive. However, the accumulation of 8-oxo-guanines in DNA has been estimated at $2.5 \times 10^{-6}$ per guanine even under oxidative conditions of H$_2$O$_2$ challenge (1, 59), placing these lesions, on average, $0.67\pm1.5 \times 10^6$ base pairs apart within the genome. The probability of forming a $Y_2D_2$ complex between two lesions is, therefore, low in vivo in light of the negative cooperativity of binding the second lesion ($K_{22} = 1.5 \mu M^{-1}$). Consequently, although induced dimerization guarantees recognition, tight binding, and excision of rare lesions by the active $Y_2D$ dimer, the negative cooperativity of binding to its second site ensures that the repair of a single isolated lesion is not impaired.

This negative cooperativity, therefore, imparts a sensitivity to the proximity between pairs of lesions by the dimer, forming a mechanistic basis for detecting catastrophic damages in the DNA. This makes physiological sense if we take into account the fact that the target adenine:8-oxo-guanine mispair results from the accumulation of two separate errors: the oxidative damage of a guanine and a subsequent but independent polymerase error that base-paired it with an adenine instead of cytosine. Thus the localized occurrence of numerous adenine:8-oxoguanine lesions indicates repeated failures by the base excision enzyme MutM to repair the original oxidatively damaged 8-oxo-guanine:cytosine base pairs prior to a replication event, suggesting that the one-base-at-a-time approach of the base excision repair system may not be the most efficient means of removing such clusters of damages. In this context, the bidentate binding of multiple lesions by the $Y_2D_2$ complex may serve the dual function of blocking further rounds of replication (or transcription) at these damage sites while signaling the recruitment of an alternate repair system to ensure more efficient removal of all damages.

Several lines of evidence lead us to favor the recruitment of the methyl-directed mismatch repair system in such instances.
Firstly, Gu et al. (44) have recently identified direct protein-protein interactions between the human mismatch repair homolog, hMSH3, and the human MutY homolog, hMYH. In that report, the interaction site was mapped to conserved amino acid residues 232–254 of hMYH, which is homologous to amino acids 148–170 in E. coli MutY. We have also recently obtained preliminary evidence of a similar interaction between E. coli MutY and MutS.1 Secondly, the recruitment of MutS by MutY in response to locally clustered lesions makes functional sense as MutS-initiated mismatch repair typically results in replacement of hundreds of nucleotides per repair event and would, therefore, provide for a more efficient, single-pass means of eliminating multiple lesions. Thirdly, Mazurek et al. (60), in demonstrating the ability of hMSH3 to recognize and initiate repair of 8-oxo-guanine-containing mismatches, reported a hMSH3 binding affinity for an adenine:8-oxoguanine mispair that is only 2-fold tighter than its affinity for a correctly base-paired homoduplex and 17-fold weaker than its affinity for a thymine:8-oxoguanine mispair. This apparently poor selectivity by hMSH3 for the adenine:8-oxoguanine mispair, therefore, provides for a more efficient, single-pass means of contextual recognition of hundreds of nucleotides per repair event and would, therefore, obviate the need for a mechanism of context-dependent inhibition of MutY activity.

We hypothesize that the allosteric inhibition of excision activity observed in YD2 coupled with the negative cooperativity of binding to the second lesion may together provide such a mechanism. As noted, the negative cooperativity of binding confers a sensitivity toward the proximity of lesions. Additionally, direct random oxidative damages to the DNA genome would position 8-oxo-guanine residues, on average, a million base pairs apart. Consequently, adenosines misincorporated into the nascent strand opposite strand 8-oxo-guanines are likely to be found only in isolation. MutY recognition and binding at these mismatches would, therefore, lead to formation of the active YD2 dimer, resulting in the appropriate removal of the nascent strand adenine.

On the other hand, mismatches with the adenine in the template strand arise from the misinsertion of 8-oxoguanosine-monophosphates by polymerase into the nascent strand. As oxidatively damaged dGTPs are enzymatically eliminated from the dGTP pool by MutT, the accumulation of 8-oxoguanosine-triphosphates available for misincorporation following oxidative challenge would be transient. Replication during such a transient rise in 8-oxo-guanosine-triphosphate concentration would, therefore, lead to the appearance of 8-oxo-guanines in the nascent DNA strand in localized patches. MutY binding within such a patch would lead to formation of the allosterically inhibited YD2 complex with the high local concentration of mismatches. This negative regulation of excision activity by proximity of adenine:8-oxo-guanine mismatches, therefore, effectively constitutes contextual recognition and selection of nascent versus template strand adenosines.

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