Mismatch Uracil Glycosylase from Escherichia coli

A GENERAL MISMATCH OR A SPECIFIC DNA GLYCOSYLASE?*

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The gene for the mismatch-specific uracil glycosylase (MUG) was identified in the Escherichia coli genome as a sequence homolog of the mammalian thymine DNA glycosylase, with activity against uracil in U-G mismatches. Subsequently, 3,N4-ethenocytosine (eC), thymine, 5-hydroxymethyluracil, and 8-(hydroxymethyl)-3,N4-ethenocytosine have been proposed as possible substrates for this enzyme. The evaluation of various DNA adducts as substrates is complicated by the biphasic nature of the kinetics of this enzyme. Our results demonstrate that product release by the enzyme is very slow and hence comparing the “steady-state” parameters of the enzyme for different substrates is of limited use. Consequently, the ability of the enzyme to excise a variety of damage products of purines and pyrimidines was studied under single turnover conditions. Although the enzyme excised both eC and U from DNA, the former adduct was significantly better as a substrate in terms of binding and hydrolysis. Some products of oxidative and alklylation damage are also moderately good substrates for the enzyme, but thymine is a poor substrate. This comparison of different substrates under single turnover conditions provides a rational basis for comparing substrates of MUG and we relate these conclusions to the known crystal structures of the enzyme and its catalytic mechanism.

DNA glycosylases excise damaged bases from DNA and prevent mutations. They act upon a wide range of DNA adducts that result from the action of a number of DNA damaging agents including water, reactive oxygen species, and alkylating agents. The modified bases that are removed in this way include the products of hydrolytic deamination of cytosine (product: uracil (U)) and 5-methylcytosine (T); the oxidative damage products, 8-oxoguanine and 5-hydroxycytosine (5-OHC); and the alkylation products, 3-methyladenine and 3,N4-ethenocytosine (eC). While glycosylases such as uracil DNA glycosylase (UDG) are very specific regarding their substrate (1), others, such as endonuclease III, can excise a wide variety of damaged DNA bases (2).

The Escherichia coli mismatch-specific uracil glycosylase enzyme (MUG), which was identified as a sequence homolog of human thymine DNA glycosylase (TDG), was so named because it was seen to excise U from a U-G pair (3). It was subsequently shown to also excise eC and T (4, 5). The crystal structure of MUG revealed that, despite a lack of sequence homology, there was a significant structural homology to UDG (5). However, the amino acid residues that confer such a high degree of specificity in UDG were altered in MUG in such a way as to remove any specificity from the active site pocket, which provides a rational basis for its broad substrate specificity (6). It appears to gain its specificity from specific interactions with the widowed G in the opposite strand instead (5, 6).

Although MUG has been characterized as being active against U, T, and eC, it is not yet clear whether these are the true substrates for the enzyme. This uncertainty exists partly because a mut mutant does not have a mutator phenotype in dividing E. coli, and in stationary phase cells, where it is expressed well, it is only a modest mutator (7, 8). It is possible that this lack of a strong phenotype is caused by the presence of alternative enzymes in E. coli that process the promutagenic lesion U and the T-G mispair. Uracil is efficiently removed from DNA by UDG (9) and T-G mismatches are repaired by a separate repair pathway called very short patch repair (10, 11). Additionally, there is no evidence, as yet, that E. coli ever generates eC in its DNA (12).

Therefore, it is possible that the biologically important substrate of MUG is something other than U, T, or eC. There have been recent reports suggesting that MUG may process a 5-hydroxymethyluracil (13), 1,N4-ethenoguanine (14), and 8-(hydroxymethyl)-3,N4-ethenocytosine (15). However, these reports either do not contain quantitative comparisons between relative preferences of the enzyme for different substrates or report apparent steady-state rates of reactions. The latter are problematic, since we demonstrate here that MUG is severely inhibited by its dissociation from abasic product DNA. Hence it is difficult to assess whether these are likely in vivo targets for MUG.

If MUG does process a broad diversity of DNA lesions, then it is possible that it acts as a general mismatch DNA glycosylase removing a variety of different lesions that accumulate during the late stationary phase. In this study we have tested this hypothesis by investigating the activity of MUG with a variety of different substrates. We also demonstrate that the use of single turnover kinetics provides a rational means for comparing the rates of nucleotide hydrolysis by MUG.

The bases that we have investigated represent different types of damage from a wide variety of possible sources: these
include the products of redox reactions, alkylation, and deamination. Our results show that, under the right conditions, MUG can process a broad spectrum of DNA adducts.

MATERIALS AND METHODS

**Protein Purification**—The mismatch uracil glycosylase from *E. coli* (MUG) was overexpressed from a construct of the wild type *mug* gene in the vector pTrex99A (supplied by Dr R. Savva (Birkbeck, London)) in *E. coli* strain BL834. An overnight culture of the strain carrying this construct was diluted 1000× into LB broth and grown overnight at 37 °C before induction with 1 μM isopropyl-1-thio-β-D-galactopyranoside. The cultures were grown for a further 5 h at 37 °C before the cells were harvested. The harvested cells were lysed by sonication in buffer A (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1 mM NaCl) over 400 ml. Fractions of the eluate were analyzed by SDS-PAGE and those fractions containing MUG were pooled and the volume reduced by ultrafiltration in a stirred cell. The partially purified MUG protein was loaded onto a Superdex-75 column, equilibrated in buffer C (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 200 mM NaCl), and eluted in the same buffer. The enzyme was first preincubated in the reaction buffer at either 25 or 37 °C for increasing time periods prior to the addition of substrate containing an abasic site. Following the addition of enzyme, the reaction was allowed to proceed for a fixed time period of 30 min before quenching with NaOH. It was first established that this was sufficient to cleave >95% of the particular substrate at either temperature. Preincubation times were varied from 30 min to 72 h. These reactions revealed that, at 37 °C, the activity of MUG decreased rapidly, and after 3 h it was no longer able to convert all of the substrate to product within the time given (Fig. 1A). However, we have also analyzed the aA and inosine substrates as mismatches with a thymine residue.

Since the excision of bases by MUG was seen to be slow, it was essential to first establish conditions under which the enzyme remained active for long time periods so that slow rate constants could be accurately determined. To do this, the enzyme was first preincubated in the reaction buffer at either 25 or 37 °C for increasing time periods prior to the addition of substrate containing a U-G mismatch. Following the addition of substrate, the reaction was allowed to proceed for a fixed time period of 30 min before quenching with NaOH. It was first established that this was sufficient to cleave >95% of the particular substrate at either temperature. Preincubation times were varied from 30 min to 72 h. These reactions revealed that, at 37 °C, the activity of MUG decreased rapidly, and after 3 h it was no longer able to convert all of the substrate to product within the time given (Fig. 1A). However, at 25 °C full enzyme activity was observed up to 48 h, and a reduction in activity was only observed after 72 h (Fig. 1B). All subsequent assays with MUG were therefore performed at 25 °C and with time courses of up to 48 h to ensure that the observed rates of cleavage were as accurate as possible.

**Enzyme Assays**—Oligonucleotide substrates were 5′-labeled with 32P on the modified strand prior to annealing. The 32P-labeled duplex substrates, typically 100 nm, were reacted at 25 °C with MUG in reaction buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, and 0.1 mg ml⁻¹ bovine serum albumin); these are the standard reactions and apply to all assays unless other conditions are stated. At selected time points 10-μl samples were removed and quenched with 10-μl aliquots of 0.1 M NaOH. The quenched samples were then heated to 90 °C for 20 min, to cleave the abasic site products. An equal volume of formamide loading buffer (95% formamide, 0.04% bromphenol blue, 0.04% xylene cyanol, and 20 mM EDTA) was added, and subsequently 38 μl of the heat-denatured samples were separated by denaturing PAGE. The formation of product was quantified using a Fuji phosphorimager and the reaction rate determined by fitting the data to the stated kinetic equations using Grafit 5 (Erithacus Software).

**RESULTS**

Investigation of MUG Assay Conditions—We used a double-stranded substrate in which one strand contained a single modified DNA base. For comparison, all modified bases have been investigated as a mismatch with a guanine residue, since the crystal structure of MUG with DNA shows that the enzyme makes specific contacts with the guanine opposite the lesion (5). However, aA and inosine lesions would normally be paired with thymine under physiological conditions. Therefore, we have also analyzed the aA and inosine substrates as mismatches with a thymine residue.
and cleaves an equal amount of substrate, but where subsequent turnovers are limited by the very slow release of product. Thus the steady-state phase of the reaction is not indicative of the true catalytic abilities of the enzyme. To obtain a better understanding of the substrate specificity of the enzyme, reactions were performed under single turnover conditions where the accumulation of product gives a measure of the actual chemical cleavage step that is not affected by the limited release of product.

**Single Turnover Reactions with MUG**—The rate of substrate cleavage by MUG was investigated directly by performing reactions with an excess of enzyme over substrate. Under these conditions only a single turnover of the reaction will occur and the accumulation of product will thus correspond directly to the rate of $N$-glycosyl bond hydrolysis under the conditions tested. Reactions were initially performed with the U-G, eC-G, and T-G DNAs, which have already been identified as substrates for MUG in vitro (4, 5). The reactions with U-G and eC-G proceeded much faster than the steady-state reaction and a quench flow machine was utilized to accurately perform the quench assay over shorter time scales. Both of these substrates showed a single exponential increase in product with cleavage rates that are at least 2 orders of magnitude faster than the steady-state cleavage rate with U-G (Fig. 3, A and B). However, the T-G substrate was cleaved much more slowly, and after 11 h only $\sim$10% of the substrate had been cleaved (Fig. 3C).

The single turnover reaction with the U-G substrate confirms that the actual cleavage of this substrate by MUG is 100-fold faster than the turnover rate, which will thus be limited by the dissociation of product (cf. Figs. 2 and 3B). The product inhibition observed with this enzyme is so severe that the enzyme is effectively a single turnover enzyme, and it is therefore not possible to determine the classical catalytic constants $k_{cat}$ and $K_m$ for MUG. All subsequent assays with MUG were therefore performed under single turnover conditions.

**Concentration Dependence of Single Turnover Rates**—Even with single turnover reactions, the observed rate of substrate cleavage can vary with enzyme concentration if the initial enzyme-substrate complex is not saturated under the conditions used. To test whether the observed reaction rates were

| Substrate | Modified nucleotide |
|-----------|---------------------|
| UG        | 2'-Deoxyuracil$^a$ |
| T-G       | 2'-Deoxythymine$^a$|
| eG        | 2'-Deoxyethenocytosine$^b$|
| eA-G      | 2'-Deoxyethenoadenine$^c$|
| I-G       | 2'-Deoxyinosine (hypoxanthine)$^a$|
| OHU-G     | 2'-Deoxy-5-hydroxyuracil$^d$|
| OMeUG     | 2'-Deoxy-5-hydroxymethyluracil$^d$|
| OHG       | 2'-Deoxy-5-hydroxyuracil$^d$|
| OMeC-G    | 2'-Deoxy-5-hydroxymethylcytosine$^d$|
| 5,6-Dihydro U-G | 2'-Deoxy-5,6-dihydrouracil$^d$|
| 5,6-Dihydro C-G | 2'-Deoxy-5,6-dihydrothymine$^d$|

$^a$ Phosphoramidite supplied by Cruachem.  
$^b$ Deoxyethenocytidine was synthesized by Tim Waters (University College London) from deoxycytidine using the protocol of Zhang et al. (29), the 5'-dimethoxytrityl protected phosphoramidite was then prepared from this deoxyethenocytidine nucleoside using standard procedures.  
$^c$ Phosphoramidite supplied by Glenn Research.

**FIG. 2.** MUG enzyme (0.1 $\mu$m) was incubated with a 10-fold excess of substrate under standard reaction conditions. The solid line is the best fit to a linear equation for the data points ranging from $1.5 \times 10^3$ to $1.6 \times 10^5$ s, with a rate of $3.5 \times 10^{-4} \pm 7 \times 10^{-6}$ s$^{-1}$.

**FIG. 3.** MUG enzyme (5 $\mu$m) was reacted with double-stranded substrate (0.1 $\mu$m) under standard reaction conditions. A rapid quench flow device was used to take samples at time points ranging from 53 ms to 100 s in A and B. A, data for the eC-G substrate is shown with the best fit to a single exponential with a rate of $0.18 \pm 0.006$ s$^{-1}$. B, data for the U-G substrate is shown with the best fit to a single exponential with a rate of $0.037 \pm 0.002$ s$^{-1}$. C, data for the T-G substrate is shown with the best fit to a linear equation, which gives an initial rate of $2.7 \times 10^{-6} \pm 1 \times 10^{-7}$ s$^{-1}$. 

**TABLE I**

| Substrates                                      |
|------------------------------------------------|
| Table II                                        |

- Phosphoramidite supplied by Cruachem.  
- Deoxyethenocytidine was synthesized by Tim Waters (University College London) from deoxycytidine using the protocol of Zhang et al. (29), the 5'-dimethoxytrityl protected phosphoramidite was then prepared from this deoxyethenocytidine nucleoside using standard procedures.  
- Phosphoramidite supplied by Glenn Research.
sensitive to enzyme concentration, a series of single turnover reactions were performed with substrates U-G and C-G and increasing concentrations of MUG. Reactions with the U-G substrate did indeed show an increase in rate with enzyme concentration, which was found to fit a hyperbolic dependence that gave a saturating cleavage rate \( k_{\text{cat}} \) of 0.04 s\(^{-1}\) and an apparent \( K_d \) of 0.42 \( \mu \)M. B, data for the C-G substrate.

The rate dependence of the C-G substrate by MUG showed a very different behavior to the U-G substrate: all of the rates determined at each concentration were within experimental error of each other and no hyperbolic dependence on the enzyme concentration was observed (Fig. 4B). It was not possible to determine the apparent \( K_d \) for this substrate, which was too low to measure from these data. The conclusion, therefore, is that MUG was saturated with the C-G substrate at the lowest concentration tested. There is thus a significant difference in the affinity of MUG for these two substrates.

**Alternative Substrate Cleavage by MUG**—The activity of MUG was tested with a series of potential substrates to investigate the possible in vivo targets of the enzyme. The potential substrates were incorporated into oligonucleotides with the same sequence context as the U-G substrate. In addition, the ea and inosine bases were also incorporated opposite a T, which is more representative of physiological conditions (see “Materials and Methods”). Reactions were performed under single turnover conditions, and the accumulation of product was monitored over time. Some of the substrates tested were unstable when stored and the reactions did not reach 100% completion. Deviations due to substrate degradation were minimized by performing the reactions with substrate freshly purified by HPLC.

To make valid comparisons between the cleavage rates of MUG with different substrates, it is necessary to perform the reactions under saturating conditions. It was not feasible to do a concentration rate profile with these other substrates, since the rates were too low. Instead, we performed the assays with two enzyme concentrations (5 and 10 \( \mu \)M), which we had shown to be saturating with the U-G and C-G substrates (Fig. 4). If the reaction rates did not change, then we could assume that the reaction was saturating and that the observed rate \( (k_{\text{obs}}) \) was equivalent to the catalytic cleavage rate \( (k_{\text{cat}}) \). But, if the rates were dependent on enzyme concentration, then we could assume that these reactions were not saturating even under these extreme conditions of enzyme excess.

In most instances, the accumulation of product followed a single exponential increase and could be fitted to provide a rate constant for the cleavage reaction. However, the reactions with the T-G (Fig. 3C) OHC-G, eaT, and I-T substrates (data not shown) were so slow that they did not reach a discernable end point. In these cases the data points were fitted to a linear equation and the gradient was used to establish an initial rate.

The cleavage rates of the different substrates at 5 and 10 \( \mu \)M MUG are shown in Table II. Of the substrates that cleaved, all were at saturating rates under the conditions tested, with the exception of ea and inosine. The observed rates of cleavage vary over 6 orders of magnitude, and C was the best substrate for the enzyme. While a wide variety of modified DNA bases were excised by MUG, the enzyme does not cleave all potential base adducts. For three of the substrates tested, no excision activity could be detected.

**Activity against Single-stranded DNA**—MUG is sometimes referred to as double-strand specific uracil glycosylase (17), and the crystal structure suggests a key role for the opposite strand in substrate recognition (5). To verify the importance of this, all of the substrates toward which the enzyme exhibited activity were also tested in their single stranded form. Reactions were performed under the same single turnover conditions that were used for the double stranded substrates. Cleavage activity by MUG was detected only with the three substrates that had the highest activity in double stranded form. When the reaction was performed with 10 \( \mu \)M MUG and 100 nm substrate, C was cleaved from single stranded DNA with a rate of 1.1 \( \times \) \( 10^{-4} \) s\(^{-1}\) (Fig. 5), lowering the enzyme concentration to 5 \( \mu \)M did not alter the rate of reaction (1.2 \( \times \) \( 10^{-4} \) s\(^{-1}\); data not shown), although reducing the enzyme concentration to 2 \( \mu \)M resulted in a halving of the rate (0.47 \( \times \) \( 10^{-4} \) s\(^{-1}\); data not shown). Reactions with uracil and 5-hydroxyuracil did not reach completion after 48 h, but initial cleavage rates with 5 \( \mu \)M MUG were 9.0 \( \times \) \( 10^{-7} \) s\(^{-1}\) and 7.0 \( \times \) \( 10^{-7} \) s\(^{-1}\), respectively (data not shown).

**DISCUSSION**

**Kinetics of Base Excision**—In this study we have examined the kinetics of base excision by MUG. Having first established conditions under which MUG is active for up to 48 h (Fig. 1), we were able to perform reactions over long time courses and accurately determine cleavage rates. Analysis of U-G cleavage under steady-state conditions revealed a burst phase followed by a very slow steady-state phase (Fig. 2). Subsequent analysis of the same substrate under single turnover conditions showed that N-glycosyl hydrolysis proceeds 100-fold faster than the turnover rate (Fig. 4). Similar biphasic behavior has been observed with other DNA glycosylases (18–20), and it is clear that the turnover of MUG is also inhibited by tight binding to its product.
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Rates of substrate cleavage by MUG

All cleavage rates were determined by single turnover reactions under standard assay conditions with 5 and 10 μM MUG with 0.1 μM substrate. ND, no activity detected.

| Substrate       | 5 μM MUG          | 10 μM MUG          | Relative rate |
|-----------------|-------------------|--------------------|---------------|
| C-G             | 1.8 ± 0.65 × 10^{-1} | 1.0                |
| U-G             | 1.6 ± 0.08 × 10^{-4} | 6 ± 1.0 × 10^{-3}   | 21            |
| OHU-G           | 2.1 ± 0.04 × 10^{-3} | 1.5 ± 0.14 × 10^{-3}|
| εA-G            | 2.4 ± 0.10 × 10^{-4} | 2.8 ± 0.13 × 10^{-4}|
| εA-G            | 2.5 ± 0.11 × 10^{-6} | 2.6 ± 0.11 × 10^{-6}|
| OHMeU-G         | 5.2 ± 0.13 × 10^{-5} | 5.1 ± 0.14 × 10^{-5}|
| T-G             | 2.5 ± 0.11 × 10^{-6} | 2.6 ± 0.11 × 10^{-6}|
| εA-T            | 2.1 ± 0.10 × 10^{-6} | 2.1 ± 0.10 × 10^{-6}|
| OHc-G           | 6.0 ± 0.22 × 10^{-7} | 6.0 ± 0.22 × 10^{-7}|
| OHMeo-G         | ND                | ND                |
| 5,6-Dihydro-C-G | ND                | ND                |
| 5,6-Dihydro-U-G | ND                | ND                |

*For the εC-G and U-G substrates only the k_{cl} rate is shown (Fig. 4).

![Graph](https://example.com/graph.png)

**Fig. 5.** MUG enzyme (10 μM) was reacted with single-stranded ethenocytosine substrate (0.1 μM) under standard reaction conditions. The data are shown with the best fit to a single exponential with a rate of 1.1 × 10^{-4} s^{-1}.

\[ E + S \rightarrow ES \rightarrow EP \rightarrow E + P \quad (\text{Eq. 1}) \]

The turnover of MUG is limited by the rate of product dissociation: k_{cl} in Equation 1 above. Under single turnover conditions, when the substrate is saturated with enzyme, the observed rate will be equal to k_{cl}, the chemical rate of hydrolysis. Under non-saturating conditions, the observed rate will be limited by the partial saturation of the precatalytic enzyme-substrate complex (ES). The εC-G substrate saturates at much lower enzyme concentrations than the U-G substrate (Fig. 4). This is a reflection of the different binding characteristics of the two substrates: MUG binds ethenocytosine much more tightly than uracil.

The tighter binding of the εC containing substrate by MUG probably also accounts for its activity against single-stranded DNA containing εC. Cleavage of the single-stranded substrate is 1500-fold slower than the double-stranded substrate (cf. Figs. 3 and 5), although MUG must have a higher K_f for εC in the single-stranded substrate, since it does not saturate at the lowest enzyme concentrations used. This demonstrates the importance of interactions with the second DNA strand, although interactions with the ethenocytosine are sufficient to produce some excision activity when it is present in a single DNA strand. However, with the weaker binding uracil, the enzyme activity against the single DNA strand was so low that it is not likely to be of any significance.

The activity of MUG in vivo may well be modulated by the action of other enzymes. It has been shown that E. coli endonuclease IV stimulates MUG reactions in vitro (8, 17), presumably by accelerating product release. Furthermore, the MutY glycosylase is also stimulated by endonuclease IV (21), and the human glycosylase TDG and OGG1 have been shown to be stimulated by the human AP endonuclease (22, 23). In vivo, where the process of base excision is expected to be coupled with abasic endonucleolytic cleavage, product release may not be the rate-limiting step. When considering the activity of MUG with different substrates in vitro, it is thus more informative to compare single turnover rates, as this gives a clearer indication of the relative activity of MUG with different substrates.

Of the different substrates tested here, a duplex containing the εC-G pair had the highest affinity for the enzyme and was cleaved with k_{cl} of 0.18 s^{-1} (Table II). While U-G was the next best substrate, substantially higher amounts of enzyme were required to saturate it (Fig. 4), and k_{cl} was ~5-fold lower than for εC-G (k_{cl} 0.037 s^{-1}; Table II). Of the other substrates tested, 5-hydroxycytosine (5-OHU), εA, and inosine (hypoxanthine) had observed cleavage rates 2–3 orders of magnitude slower than εC-G (Table II); thymine, 5-hydroxymethyluracil, and 5-hydroxycytosine were cleaved to some extent, although extremely slowly; and the enzyme did not demonstrate any detectable activity toward 5-hydroxymethylcytosine, 5,6-dihydouracil, and 5,6-dihydroxytocine. Inosine and εA were the only substrates tested that did not reach saturation under the conditions tested and, notably, when paired with the more biologically relevant T, the observed rate dropped a further 2 orders of magnitude. It is possible that, in vivo, the enzyme may have different substrate selectivity, act on other sequence contexts, or be modified by the presence of accessory factors. However, based on the results presented here, these other base modifications cannot be considered to be the preferred substrates for the enzyme.

Previous studies have reported that a variety of other nucleotide lesions are also substrates for MUG, including 5-hydroxymethyluracil (13), 1,N²-ethenoguanine (14), and 5-(hydroxyethyl)-3,N²-ethenocytosine (15). However, these reports either do not contain quantitative data or report apparent steady-state rates of reactions. The use of steady-state conditions to study MUG and other product inhibited DNA glycosylases leads to an underestimation of reaction rates of the best substrates and consequently makes the poorer substrates appear better than they really are. The reported k_{cl} value for εC is only ~2200-fold higher than that for T (4), while the k_{cl} value
for εC•G is –65,000 times the observed cleavage rate for T•G (Table II). This is in large part due the fact that the reported $k_{\text{cat}}$ value for εC is lower than the $k_{\text{cat}}$ value by a factor of –11.

It is thus difficult from these reports to assess the relative efficiencies with which MUG is able to act upon these different substrates and hence whether they are likely in vivo targets for the enzyme.

**Accommodation of Modified Nucleotides**—There are notable trends that can be gleaned from the observed activities of MUG toward different substrates. The bulkiness at the 5-position has a dramatic effect on the activity of MUG. This trend can be clearly seen when comparing U•G with OHU•G, which has 10-fold lower activity and OHMeU•G, which has four orders of magnitude lower activity. However, OHMeU•G is a better substrate than T•G (5-methyl-U), even though it is larger. It has recently been reported that MUG activity is dependent on the electron withdrawing capability of the 5-substituent, rather than simple steric exclusion (24). This is consistent with a dissociative mechanism of cleavage with a uracil anion leaving group, as proposed for UDG (25, 26), and the uncatalyzed cleavage of deoxyuridine derivatives (27). Our data support the view that there is a balance between the size of any 5-substituent and its electron withdrawing capability on the activity of MUG.

In contrast to the poor activity of 5-substituted nucleotides, substrates that have bulky substituents on the 3- and 4-positions are, in many cases, good substrates. Most notable in this respect is ethenocytosine, which is the best substrate tested. But, MUG is also able to cleave the purines inosine and εA. Modeling of εC into the active site of MUG has demonstrated that the active site has considerable space to accommodate bulky adducts on this face of the nucleotide (6). The results presented here, and elsewhere (14, 15), demonstrate that these interactions are not completely specific to εC and that even more bulky adducts can be accommodated by MUG.

It is also of note that, with the exception of ethenocytosine, all other cytosine derivatives are poor substrates: a comparison of OHU•G and OHεC•G reveals a difference of 4 orders of magnitude in the cleavage rate. In addition, N4-methyl and N4-ethyl cytosine containing DNAs were not substrates for MUG. This trend can be clearly seen when comparing U•G with OHU•G, which has 10-fold lower activity and OHMeU•G, which has four orders of magnitude lower activity. However, OHMeU•G is a better substrate than T•G (5-methyl-U), even though it is larger. It has recently been reported that MUG activity is dependent on the electron withdrawing capability of the 5-substituent, rather than simple steric exclusion (24). This is consistent with a dissociative mechanism of cleavage with a uracil anion leaving group, as proposed for UDG (25, 26), and the uncatalyzed cleavage of deoxyuridine derivatives (27). Our data support the view that there is a balance between the size of any 5-substituent and its electron withdrawing capability on the activity of MUG.

In the active site pocket of UDG, an Asn residue is thought to form specific interactions with the Watson-Crick base paring functions of the target uracil (1). In MUG, however, there are no specific interactions to discriminate between the N4 of cytosine or the O4 of uracil (6). Alternatively, the electronic inductive properties of the 4-substituent may provide discrimination: a 4-carbonyl group would stabilize a departing anion, whereas a 4-amino group would destabilize the anion, reducing cleavage rate (Fig. 6). This is presumably why cytosine is inherently a more stable base than thymine: hydrolysis of unprotonated T will occur, but hydrolysis of unprotonated C will not (28). The relative rates of cleavage of different substrates by MUG are consistent with the hydrolysis reaction proceeding via a dissociative mechanism with a departing uracil anion. This type of mechanism has been attributed to the UDG enzyme (25, 26), and it seems likely that it also applies to MUG.

**CONCLUSIONS**

In this study we have used a kinetic approach to investigate the action of MUG with a broad range of DNA damage lesions. We have shown that the use of single turnover kinetics provides a rational basis for comparing the cleavage rates of different substrates and avoids misinterpretations due to the slow release of MUG from its product. The best substrate for MUG is ethenocytosine and it may be that this is the biologically relevant substrate. The biology of MUG suggests that it may be active in stationary phase (8), and hence it is possible that *E. coli* DNA accumulates εC during this stage of cell growth.

However, previous studies have failed to isolate εC from the DNA of *E. coli* (12). But, the mutator phenotype of *mug* was only apparent after 2 days of the cells being in stationary phase (8), and it is unlikely that, when attempting to isolate εC from *E. coli*, the cells were starved for several days prior to the preparation of chromosomal DNA. Alternatively, it could be that MUG efficiently repairs εC lesions in *vivo*, so that it is difficult to detect any residual εC in the DNA from *mug* cells used for that work. Thus, the question of whether εC is the biologically relevant substrate for MUG can only be resolved by the careful quantitative of DNA adducts in late stationary phase *mug* *E. coli* cells.

The other DNA damage lesions that we have tested here have a significantly lower activity against MUG than εC and, to a lesser extent, uracil. It therefore seems unlikely that MUG acts as a general X-G mismatch glycosylase *in vivo*. However, since MUG appears to be active only in the late stationary phase (8), the rate of reaction may not be critical to the biological relevance of this enzyme. If εC is not found to accumulate in late stationary phase *E. coli*, then it remains possible that MUG acts as a general X-G glycosylase and repairs a wide range of DNA damage lesions, caused by oxidation and other cellular processes, such as those characterized here. Alternatively, MUG may be predominantly active against another DNA lesion that we have not investigated.

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Mismatch Uracil Glycosylase from *Escherichia coli*: A GENERAL MISMATCH OR A SPECIFIC DNA GLYCOSYLASE?

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