

Recognition of 1,N²-ethenoguanine by alkyladenine DNA glycosylase is restricted by a conserved active site residue

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Running title: *Recognition of 1,N²-ethenoguanine by AAG*

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ABSTRACT

The adenine, cytosine, and guanine bases of DNA are susceptible to alkylation by the aldehyde products of lipid peroxidation and by the metabolic byproducts of vinyl chloride pollutants. The resulting adducts spontaneously cyclize to form harmful etheno lesions. Cells employ a variety of DNA repair pathways to protect themselves from these pro-mutagenic modifications. Human alkyladenine DNA glycosylase (AAG) is thought to initiate base excision repair of both 1,N⁶-ethenoadenine (ϵ A) and 1,N²-ethenoguanine (ϵ G). However, it is not clear how AAG might accommodate ϵ G in an active site that is complementary to ϵ A. This prompted a thorough investigation of AAG-catalyzed excision of ϵ G from several relevant contexts. Using single-turnover and multiple-turnover kinetic analyses, we found that ϵ G in its natural ϵ G•C context is very poorly recognized relative to ϵ A•T. Bulged and mispaired ϵ G contexts, which can form during DNA replication, were similarly poor substrates for AAG. Furthermore, AAG could not recognize an ϵ G site in competition with excess undamaged DNA sites. Guided by previous structural studies, we hypothesized that Asn-169, a conserved residue in the AAG active site pocket, contributes to discrimination against ϵ G. Consistent with this model, the N169S variant of AAG was 7-fold more active for excision of ϵ G as compared to the wild-type (WT) enzyme. Taken together, these findings suggest that ϵ G is not a primary substrate of AAG, and that current models for etheno lesion repair in humans should be revised. We propose that other

repair and tolerance mechanisms operate in the case of ϵ G lesions.

All cellular life contends with the challenge of DNA alkylation damage. DNA bases are alkylated by both endogenous and exogenous compounds, and the failure to repair these base lesions results in a variety of deleterious consequences ranging from point mutations to the stalling of DNA replication or transcription (1). Etheno lesions represent a subset of exocyclic alkylation adducts that can arise through alkylation of purine and pyrimidine bases of genomic DNA (Fig. 1) (2). The etheno lesions 1,N⁶-ethenoadenine (ϵ A), N²,3-ethenocytosine (ϵ C), 1,N²-ethenoguanine (ϵ G), and N²,3-ethenoguanine (N²,3- ϵ G) are naturally formed through exposure to the reactive aldehyde products of lipid peroxidation and subsequent ring closure (3,4). Notably, etheno lesions have also been shown to arise due to reactions with chloroacetaldehyde and other metabolic byproducts of the common industrial compound vinyl chloride (5). *In vitro* DNA replication assays have demonstrated the miscoding properties of etheno lesions as well as the propensity for them to halt replication (6-8). Consistent with these deleterious effects on DNA replication, etheno lesions have cytotoxic effects on mammalian cells (9).

The base excision repair (BER) pathway exists in all domains of life, and it is responsible for removing and replacing diverse single nucleotide lesions such as those which arise through DNA alkylation, oxidation, and deamination (10). BER is initiated by a DNA glycosylase that searches DNA

to locate specific sites of DNA damage and catalyzes the excision of the base lesion to generate an abasic site. Subsequent action by an AP endonuclease, dRP lyase, DNA polymerase and DNA ligase nicks the backbone at the abasic site, removes the deoxy sugar, inserts an undamaged base to complement the opposing strand, and ligates the nick.

Alkyladenine DNA glycosylase (AAG) utilizes a nucleotide flipping mechanism to target a broad range of alkylated DNA lesions (11-13). Upon specific binding of the enzyme to a target lesion, the lesion is rotated out of the duplex and into the enzyme active site for cleavage. AAG was initially proposed to repair all of the etheno adducts which can arise in human cells (14). ϵ A is a well-characterized substrate of human AAG, and it is recognized with high affinity by the enzyme *in vitro* (11,15). AAG also recognizes and binds to sites of ϵ C damage, however it is unable to catalyze cleavage of the *N*-glycosidic bond (16). Rather, the human glycosylases TDG and SMUG1, along with the direct reversal protein AlkBH2, have been shown to repair ϵ C (17-19).

Compared to ϵ C and ϵ A, less is known about the repair of ϵ G. *In vitro* reactions between DNA and aldehydes derived from lipid peroxidation favor the production of ϵ G over $N^2,3$ - ϵ G, indicating that ϵ G might be the more relevant natural lesion (20-22). The opposite appears to be true of damage originating from exposure to vinyl chloride and its byproducts (20,23). ϵ G causes both replication blocks and a mixture of $G \rightarrow T$ and $G \rightarrow C$ transversion mutations in mammalian cells (6,7). The lesion also blocks transcription by enzymes such as human RNA polymerase II (24). The exact frequency of ϵ G lesions in the human genome is not firmly established, however the harmful effects of unrepaired ϵ G combined with the natural origin of the lesion suggest that there must be a means of repairing the lesion.

AAG was previously investigated for glycosylase activity toward ϵ G (14,25,26). While these studies conclude that AAG catalyzes the excision of ϵ G, the kinetics of that excision vary widely between reports (25,26). The relevance of the N-terminal domain of AAG for ϵ G recognition is also contentious. The N-terminal 79 residues of human AAG form a flexible region that is not conserved among different species. One study concluded that truncation of the N-terminus of

human AAG eliminated activity toward ϵ G (25), whereas a subsequent study reported similar activity of full-length and N-terminally truncated AAG toward ϵ G (26). However, this second study reported a very low percentage (6%) of ϵ G could be excised. Studies employing different base lesion substrates concluded that the truncation of the N-terminus reduces the searching efficiency of AAG, but does not significantly affect the rate constant for *N*-glycosidic bond cleavage *in vitro* (27,28).

In this work we characterized the single- and multiple-turnover kinetics of the AAG-catalyzed excision of ϵ G from a variety of DNA duplexes. Comparison of the kinetic constants for excision of ϵ G and ϵ A, and the results of direct competition experiments, together demonstrate that ϵ G is a very poor substrate of AAG. We investigated the structural origins for this substrate specificity and found that mutation of N169 to a smaller side chain allows significantly increased activity toward ϵ G, presumably because the bulky etheno adduct can be better accommodated in the active site. Although we have verified the claims that AAG is capable of catalyzing excision of ϵ G *in vitro*, our work argues against this being a *bona fide* physiological substrate and leads to the prediction that other DNA repair pathways are responsible for ϵ G repair *in vivo*.

Results and Discussion

Multiple turnover kinetics of AAG-catalyzed excision of ϵ G

To examine the kinetics of ϵ G excision by human AAG, we expressed and purified $\Delta 80$ AAG from *E. coli* for *in vitro* glycosylase assays. We used steady-state kinetics to characterize the efficiency of AAG-catalyzed excision of ϵ G from a 25mer DNA duplex containing a central ϵ G•C target site (see Experimental Procedures). Reaction conditions were selected to maximize the activity of AAG while maintaining enzyme stability over a long time-course, and linear initial rates were observed up to 10% product formation with no evidence of a pre-steady state burst (Fig. 2A). Despite the optimized conditions for AAG activity, the reaction proceeded slowly with a k_{cat} of 0.0048 min^{-1} (Fig. 2B). The catalytic efficiency of the reaction, given by k_{cat}/K_M , was measured to be $1.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. In comparison, the catalytic efficiency of AAG for excision of ϵ A, a well-

characterized substrate, has been estimated to be 3 orders of magnitude higher under the same conditions (11). This discrepancy is conspicuous given the similar origin and the mutagenicity of both lesions.

To more directly compare the efficiencies for the excision of ϵ G and ϵ A, the lesion-containing oligonucleotides were competed in the same glycosylase assay mixture. The ϵ G lesion was paired with a complementary C, whereas the ϵ A lesion was paired with a complementary G. The ϵ A•G pairing is the least efficient base pairing for ϵ A excision, potentially allowing for the relatively slow ϵ G excision to compete (29). To distinguish the ϵ A and ϵ G DNA substrates and products on a gel, the ϵ G lesion was incorporated into a 25mer DNA sequence while the ϵ A lesion was incorporated in a 19mer DNA sequence. We controlled for the effect of DNA length on the kinetics of glycosylase activity by performing a competition assay between ϵ A incorporated into the 19mer and 25mer sequence. No substantial preference between the 19mer and 25mer was observed (Fig. S1). The formation of the fluorescently labeled 12mer and 9mer products from excision of ϵ G and ϵ A, respectively, was monitored to obtain initial rates for the excision of both substrates. At a ratio of 10:1 ϵ G to ϵ A, no detectable ϵ G product was formed during the initial period of the ϵ A reaction (Fig. 3). As 0.5% of the ϵ G excision product could have been reliably detected, we conservatively estimate that ϵ A•G is preferred over ϵ G•C by at least 200-fold. These data further support the conclusion that ϵ G is poorly recognized relative to primary substrates of AAG.

AAG-catalyzed single turnover excision of ϵ G

Single turnover glycosylase reactions were performed to measure the rate of ϵ G and ϵ A excision catalyzed by AAG. These reactions report on all the steps preceding and including N-glycosidic bond cleavage, but subsequent steps such as product release are excluded. To directly compare to previous results, an optimal pH of 6.1 was used (28), and AAG was up to 3-fold more active for excision of ϵ G at pH 6.1 as compared to pH 7.5 (Fig. S2). Each single turnover reaction of ϵ G excision proceeded to ~85% completion, indicating the presence of a small quantity of nonreactive species in the ϵ G-DNA (Fig. 4A & B). As observed in the multiple turnover experiments,

AAG-catalyzed excision of ϵ G proceeded more slowly than excision of ϵ A. Furthermore, the concentration dependence of Δ 80 AAG exhibited biphasic behavior (Fig. 4C; red squares).

In the classic model for single turnover kinetics, the relationship between k_{obs} and the concentration of enzyme should fit to a hyperbola. However, a reduction in the rate constant for excision of ϵ G was observed at elevated concentrations of AAG (Fig. 4C). A similar inhibitory effect was previously reported for the *E. coli* 3-methyladenine DNA glycosylase (AlkA), but this is the first instance of such behavior from AAG (30). Using the inhibition model developed for AlkA, described in Fig. S4, a K_i value of 680 nM was determined for Δ 80 AAG. These concentrations of AAG far exceed cellular conditions, making such enzyme crowding unlikely in a cellular context. However, the inability of AAG to distinguish ϵ G from undamaged sites could have serious physiological implications.

To assess the relative affinity of AAG for undamaged and damaged sites, single turnover reactions of AAG with ϵ G and ϵ A were challenged with varying concentrations of undamaged 25mer DNA oligonucleotides containing a central A•T pair. The excision of ϵ A was unaffected by the presence of undamaged DNA, up to at least 80-fold excess over the damaged DNA, which was the highest concentration tested (Fig. 5). In contrast, the excision of ϵ G was strongly inhibited by the presence of undamaged DNA, with an IC_{50} of 400 nM. This experiment indicates that ϵ G•C is preferred by a factor of 300-fold relative to binding to undamaged sites, which is much less than the lower limit of >30,000 preference for ϵ A•T. The 300-fold preference for ϵ G•C, relative to a typical undamaged DNA site, is unlikely to be sufficient for repair in the cell where there is a vast excess of undamaged relative to damaged sites.

The N-terminal region of AAG is not necessary for the excision of ϵ G

Previous studies have demonstrated that the catalytic domain of AAG is more stable than the full-length protein and has similar rates of N-glycosidic bond cleavage with many different substrates (27,28). Despite this, it has been reported that ϵ G in particular cannot be excised by N-terminal truncations of AAG (25). Our multiple turnover and single turnover glycosylase assays

indicate the contrary, that $\Delta 80$ AAG is active toward ϵ G under a variety of conditions. However, differences may still exist between the two protein variants. To compare the activity of full length and truncated AAG, we repeated the single turnover glycosylase assays with full-length AAG (Fig. 4C; black circles). Both full-length and $\Delta 80$ AAG were able to reach similar endpoints with comparable k_{\max} values. Similar to the truncated protein, the $K_{1/2}$ for ϵ G excision by full-length AAG was too low to accurately measure. The full-length protein also displayed inhibition at high enzyme concentrations, although to a lesser degree than the truncated protein. These results support the model that the N-terminal region of AAG is unnecessary for catalytic activity.

AAG recognizes ϵ G poorly in other relevant DNA contexts

The oligonucleotides used in the preceding assays represent the expected context for the alkylation of a G•C pair to form an ϵ G lesion. However, other pairings can occur during replication of the damaged template (6,7). We characterized the single turnover kinetics of ϵ G•T excision and found rates similar to those of ϵ G•C excision (Table 1). AAG exhibited similar enzyme concentration-dependent inhibition for excision of ϵ G from ϵ G•T as was observed for ϵ G•C (Fig. S3). The similar glycosylase activity with either opposing pyrimidine is consistent with the similar multiple turnover rates of excision that were previously reported (25). These results demonstrate that the inhibition of ϵ G excision by excess AAG molecules is not exclusive to a single base complement.

During replication of ϵ G, DNA polymerases can slip to generate a -1 frameshift (31,32). The slippage event places the ϵ G lesion into a bulged context without a complementary base. Previously it was shown that AAG excises ϵ A and Hx from a bulged context at comparable rates to base-paired contexts (33,34). To assess the ability of AAG to recognize ϵ G from a bulged conformation, single turnover glycosylase assays were performed with a 25mer bulged substrate (Fig. 6). Notably, the bulged lesion appeared to lack the concentration-dependent inhibitory effect observed with ϵ G base pairs and the data could be readily fit to a typical hyperbolic concentration dependence. It is not clear why there was not an inhibitory effect at high

concentrations of AAG, but it is possible that the presence of the bulge structure disrupts the competing nonspecific DNA binding sites which allows for better equilibration between the specific lesion site and the competing nonspecific sites. However, the bulged ϵ G substrate was excised with a 10-fold lower k_{\max} value than was observed for the duplex ϵ G•C substrate (Table 1). This observation suggests that ϵ G is inefficiently recognized in the bulged context, whereas AAG readily recognizes other base lesions in the same bulged context (33,34). An NMR structure of the ϵ G bulge DNA (35) shows that the unpaired ϵ G can be accommodated within the DNA duplex. It appears that this stable structure limits the ability of AAG to gain access and flip out the lesion, as compared to ϵ G mispairs. Although AAG is capable of excising ϵ G from a bulged context *in vitro*, it is clear that this is not a favorable context for AAG-initiated repair of ϵ G.

Asn169 of AAG limits the rate of ϵ G excision

Crystal structures of AAG bound to a flipped-out ϵ A lesion revealed how this active site pocket can accommodate base lesions and exclude undamaged bases (13). The side chain of N169 defines one surface of the active site, closely contacting the N¹ face of the ϵ A lesion (Fig. 7A). This residue plays a role in blocking the binding of undamaged guanine with its N²-amino group (11,36), leading us to consider whether this side chain might also make contact with the 1,N²-etheno ring of ϵ G. We used site-directed mutagenesis to generate variants of $\Delta 80$ AAG with either a serine or an alanine residue in place of N169, and subsequently determined the impact of these mutations on the single turnover excision kinetics of ϵ A and ϵ G.

The N169A mutation caused an increase in the k_{\max} value for ϵ G excision from 0.034 min⁻¹ to 0.041 min⁻¹ and the inhibition at high concentration of enzyme was much less prominent as compared to the behavior of the WT enzyme (Fig. 7B; Table 2). This increase in the excision rate of the N169A mutant suggests that N169 interferes with the excision of ϵ G to some degree. Surprisingly, the k_{\max} for ϵ A excision was also substantially increased by the N169A mutation (Fig. 7C). This likely reflects a removal of a deleterious interaction between the Asn169 side chain and the ϵ A lesion or could result from rearrangements in the Ala variant

that create more favorable interactions with the substrate.

The N169S mutation is a more conservative change to the active site structure, as it maintains hydrogen bonding capability while shortening the side chain length to expand the binding pocket. The N169S variant displayed a dramatic elevation in the k_{\max} values for excision of both ϵ G and ϵ A (Fig. 7C). However, the N169S mutant enhanced ϵ G excision by more than 7-fold, while only increasing ϵ A excision by 2-fold (Fig. 7D). The greater activity of N169S relative to N169A suggests the possibility of positive hydrogen bonding interactions for the serine side chain which were lacking in the alanine substitution. The N169S mutant also showed little to no enzyme concentration-dependent inhibition of ϵ G excision, even up to concentrations several times higher than those tested for the WT enzyme. This absence of detectable inhibition can be explained by the previously proposed inhibition model, whereby the stronger recognition of ϵ G by the N169S variant would enable the lesion to compete more favorably with undamaged sites for binding and excision. The observation that ϵ G excision by AAG is improved by mutation of N169 is consistent with the model that this side chain clashes with the ϵ G lesion and contributes to its inefficient excision by AAG.

Conclusions

The exocyclic ring structure of the mutagenic and cytotoxic lesion ϵ G presents a unique challenge for recognition by the DNA base excision repair pathway. It has previously been postulated that base excision repair initiated by AAG is the preferred mechanism for repair of both ϵ A and ϵ G (14,25,26). Herein, we demonstrated that ϵ G is excised with much lower efficiency than other primary substrates of AAG under both single- and multiple-turnover conditions. We also provide the first examination of competition between undamaged and ϵ G-containing DNA, demonstrating that AAG has a difficult time recognizing ϵ G sites.

Our findings highlight some of the limitations of single-turnover kinetic approach to studying DNA repair glycosylases with defined homogenous substrates. Whereas experiments with simple substrates are indispensable for quantitative analysis and the dissection of individual reaction

steps, these assays neglect the impact of relevant cellular factors such as excess undamaged DNA. We infer that inhibition of ϵ G excision at higher concentration of AAG protein is indicative of relatively poor lesion recognition, such that nonspecific binding modes compete with the lesion-specific binding mode, and this has now been observed for both AAG and *E. coli* AlkA (30). We demonstrated that nonspecific competitor DNA competes effectively for binding of AAG to the ϵ G lesion, suggesting that it would be difficult for AAG to effectively locate these lesions in the nucleus. The packaging of DNA into nucleosome core particles presents another potential challenge for the repair of ϵ G in cells, as nucleosomes have been shown to restrict AAG and other glycosylases from accessing sites of DNA damage (37,38).

To provide a physical explanation for the discrimination by AAG against ϵ G, we have shown that residue Asn169 limits the ability of AAG to excise ϵ G from DNA. This is consistent with the model that Asn169 plays a crucial role in governing the selectivity of the enzyme against substrates with a functional group at the C2 position (13,36). Mutation of Asn169 results in a substantial increase in the excision of undamaged G from mispairs, a promutagenic change that could offset the benefit of a more versatile active site (36,39,40). The inability of AAG to efficiently catalyze the excision of ϵ G may reflect a tradeoff for greater specificity and discrimination against undamaged G nucleotides that are present in great excess within the genome (11,36).

In light of the dangers posed to the cell by unrepaired ϵ G lesions, the inability of AAG to efficiently excise ϵ G suggests that other repair pathways are likely to bear primary responsibility for protecting the genome against this particular lesion. Previous *in vitro* studies of the human homologs of AlkB, which catalyze oxidative dealkylation of certain alkylated bases, demonstrates that ALKBH2, but not ALKBH3, is capable of recognizing and repairing ϵ G in duplex context (41). Although ALKBH2 is a strong candidate for physiological repair of ϵ G•C, it is not known if this enzyme is able to capture rare ϵ G lesions from amongst the excess of undamaged sites and it is not known how ϵ G might be recognized in post-replicative repair. Although ϵ G has not been specifically investigated, genetic

studies of mice lacking either AAG, ALKBH2, or ALKBH3 show increased sensitivity to induced colitis (42). Strong synergy was observed when all three genes were knocked out, demonstrating redundancy in the repair of DNA alkylation damage *in vivo* (42). More research is needed to decipher the complexities of substrate specificity in mammalian alkylation repair.

Experimental Procedures

Preparation of DNA

Undamaged oligonucleotides were synthesized by Integrated DNA Technologies, and lesion-containing oligonucleotides were synthesized by the W.M. Keck Facility at Yale University. The lesion-containing strand of each oligonucleotide was labeled at the 5' end with 6-fluorescein (FAM). Oligonucleotides were purified via denaturing polyacrylamide gel electrophoresis, and the concentrations were determined by the theoretical extinction coefficient at 260 nm as described previously (27).

| | |
|-------------|--------------------------------------|
| 25mer | 5' - (FAM) CGATAGCATCCTXCCTTCTCTCCAT |
| | 3' - GCTATCGTAGGAYGGAAGAGAGGTA |
| 19mer | 5' - (FAM) TAGCATCCTXCCTTCTCTC |
| | 3' - ATCGTAGGAYGGAAGAGAG |
| 25mer bulge | 5' - (FAM) CGATAGCATCCTXCCTTCTCTCCAT |
| | 3' - GCTATCGTAGGA-GGAAGAGAGGTA |

X: εG, εA, Hx Y: C, T

Scheme 1

Preparation of enzymes

The catalytic domain of AAG, (Δ80 AAG) was expressed and purified from *E. coli* C41(DE3) as described previously (28). The construct for N169S was previously described (11) and the N169A mutation was generated by site-directed mutagenesis and confirmed by sequencing both strands of the open reading frame. These two variant AAG proteins were expressed and purified using the same methods. Full-length AAG was expressed and purified as described previously (43). The concentration of each AAG variant was initially estimated using the UV absorbance and the active concentration of each enzyme was established through analysis of the burst kinetics for excision of hypoxanthine (Hx) as described previously (27).

Multiple turnover glycosylase assay

Reactions were performed at 37°C in reaction buffer containing 50 mM NaHEPES (pH 7.5), 10% glycerol, 0.1 mg/mL BSA, 1 mM DTT, 1 mM EDTA, and 100 mM ionic strength adjusted with NaCl. The DNA concentration was kept at a 50:1 ratio relative to the AAG concentration to ensure multiple turnover conditions. Aliquots were removed from the reactions at various time points and were quenched in an equal volume of 0.4 M NaOH to reach 0.2 M NaOH final concentration. The quenched aliquots were heated at 70°C for 12 minutes to cleave abasic sites, and then were mixed 1:2 with loading buffer (90% formamide, 10 mM EDTA, 0.025% bromphenol blue, 0.025% xylene cyanol). For time courses lasting longer than 24 hours, quenched samples were stored at 4 °C for no more than 12 hours before being heated and mixed with loading buffer. The samples were run out on 20% polyacrylamide gels containing 6.6 M urea and were scanned with an Amersham Typhoon 5 Biomolecular Imager (GE Healthcare Life Sciences). The samples were excited at 488 nm and the emission of fluorescein was measured with a 525BP20 filter. The bands on the gel were quantified using ImageQuant TL (GE Healthcare). The fraction product [product / (substrate + product)] was calculated for each lane, and the steady state formation of product was fit with linear regression. The change in observed reaction velocity at varying DNA concentrations was fit to the Michaelis-Menten equation (1).

$$V_{obs} = \frac{k_{cat}[E][S]}{K_M + [S]} \quad (1)$$

V_{obs} represents the observed initial reaction velocity, k_{cat} the steady state rate constant, $[E]$ the concentration of enzyme, $[S]$ the concentration of substrate, and K_M the Michaelis constant, equal to the concentration of DNA at the half-maximal velocity.

Single turnover glycosylase assay

To achieve single turnover conditions, glycosylase assays were performed with 10–20 nM DNA and 50 nM to 6 μM enzyme in reaction buffer containing 50 mM NaMES (pH 6.1), 10% glycerol, 0.1 mg/mL BSA, 1 mM DTT, 1 mM EDTA, and 100 mM ionic strength adjusted with NaCl. All

reactions were performed at 37 °C. Aliquots were quenched and quantified as described above. Reactions were fit to a single exponential according to equation (2).

$$\text{Fraction Product} = A(1 - e^{-k_{obs}t}) + c \quad (2)$$

A represents the amplitude, k_{obs} the observed single turnover rate constant, t the reaction time, and c the starting amount of abasic DNA. The dependence of the single turnover rate constant, k_{obs} , on enzyme concentration was fit by a hyperbola according to equation (3) in which k_{max} represents the maximum k_{obs} value and $K_{1/2}$ represents the concentration at which enzyme is half saturating.

$$k_{obs} = \frac{k_{max}[E]}{K_{1/2} + [E]} \quad (3)$$

For reactions demonstrating enzyme-dependent inhibition, a multivalent inhibitory model was applied, in which K_i is the binding constant for the inhibitory complex (equation 4).

$$k_{obs} = \frac{k_{max}[E]}{K_{1/2} + [E](1 + \frac{[E]}{K_i})} \quad (4)$$

This model has been used previously for the nonspecific binding of another DNA glycosylase to multiple DNA sites (30). For the titration of undamaged DNA, the IC_{50} was calculated using equation (5), where k_{obs} is the observed rate constant, k_{unin} is the rate constant without undamaged DNA inhibitor, and I is the concentration of undamaged inhibitor DNA.

$$k_{obs} = \frac{k_{unin}[I]}{IC_{50} + [I]} \quad (5)$$

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FOOTNOTES

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The abbreviations used are: AAG, alkyladenine DNA glycosylase (also known as MPG, methylpurine DNA glycosylase); BER, base excision repair; ϵ A, 1, N^6 -ethenoadenine; ϵ C, 3, N^4 -ethenocytosine; ϵ G, 1, N^2 -ethenoguanine.

Table 1**Maximum rate constants for the AAG-catalyzed single turnover excision of ϵ G^a**

| Lesion | k_{\max} (min ⁻¹) |
|--------------------|---------------------------------|
| ϵ G•C | 0.034 ± 0.005 |
| ϵ G•T | 0.032^b |
| ϵ G bulge | 0.0057 ± 0.0003 |
| ϵ A•T | 0.23 ± 0.015 |

^aValues are reported for Δ 80 AAG at 37 °C in a buffer containing 50 mM NaMES (pH 6.1), 10% glycerol, 0.1 mg/mL BSA, 1 mM DTT, 1 mM EDTA, and 100 mM ionic strength adjusted with NaCl. Each value represents the average of ≥ 3 replicates. Reactions were fit to the biphasic model detailed in Figure S4 and the maximum single turnover rate constant k_{\max} for the first phase in each reaction are listed. ^bThe maximum rate constant was calculated from the average of 2 replicates (Fig. S3).

Table 2**Maximum rate constants for the single turnover excision of ϵ G and ϵ A by WT and mutant AAG ^a**

| AAG Variant | k_{\max} (min ⁻¹) | |
|-------------|---------------------------------|-----------------------------|
| | ϵ G•C ^a | ϵ A•T ^b |
| WT | 0.034 ± 0.005 | 0.23 ± 0.015 |
| N169S | 0.25 ± 0.004 | 0.58 ± 0.034 |
| N169A | 0.041 ± 0.006 | 1.4 ± 0.11 |

^aValues are reported for Δ 80 AAG at 37°C in a buffer containing 50 mM NaMES (pH 6.1), 10% glycerol, 0.1 mg/mL BSA, 1 mM DTT, 1 mM EDTA, and 100 mM ionic strength adjusted with NaCl. Each value represents the average of ≥ 3 replicates. ^aReactions with ϵ G•C were fit to the biphasic model detailed in Figure S4 and the maximum single turnover rate constant k_{\max} for the first phase in each reaction are listed. ^bReactions with ϵ A•T were determined from exponential fits at saturating enzyme concentration of 1 μ M.

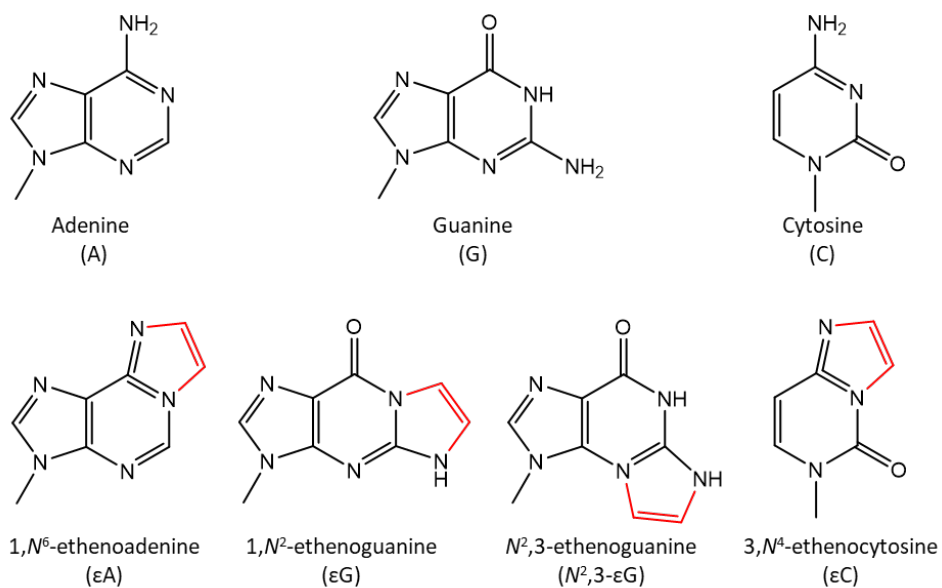


Figure 1. Diverse structures of the etheno DNA lesions. The four etheno lesions are depicted below their undamaged forms.

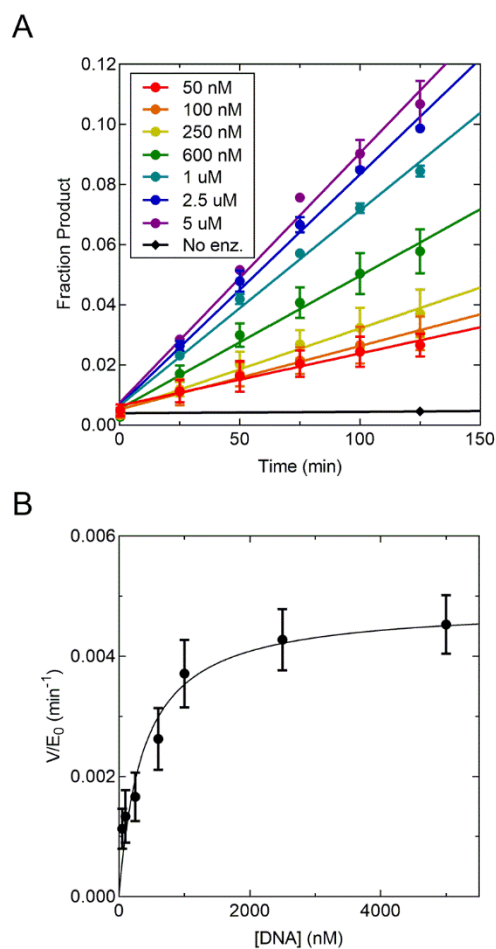


Figure 2. Multiple turnover excision of ϵ G by AAG. (A) Time course of the $\Delta 80$ AAG-catalyzed excision of ϵ G from a 25mer DNA substrate with an opposing C base. Concentrations of DNA are listed in the legend, with DNA in 50-fold excess of enzyme for each reaction. Error bars represent the S.D. of 2 trials. (B) Dependence of ϵ G excision rate on DNA concentration, fit to a hyperbola with $k_{\text{cat}} = 0.0048 \text{ min}^{-1}$ and $K_M = 370 \text{ nM}^{-1}$. Points represent the average of 4 replicates, and error bars represent the S.D.

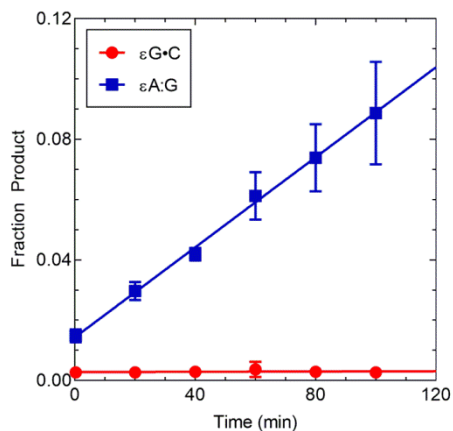


Figure 3. Competition of ϵ G and ϵ A for AAG-catalyzed excision. Reactions contained 2 nM Δ 80 AAG, 2 μ M ϵ G in the 25mer DNA sequence with an opposing C, and 200 nM ϵ A in the 19mer DNA sequence with an opposing G. Lines of best fit were calculated using linear regression. Points represent the average of 4 replicates, and error bars represent the S.D.

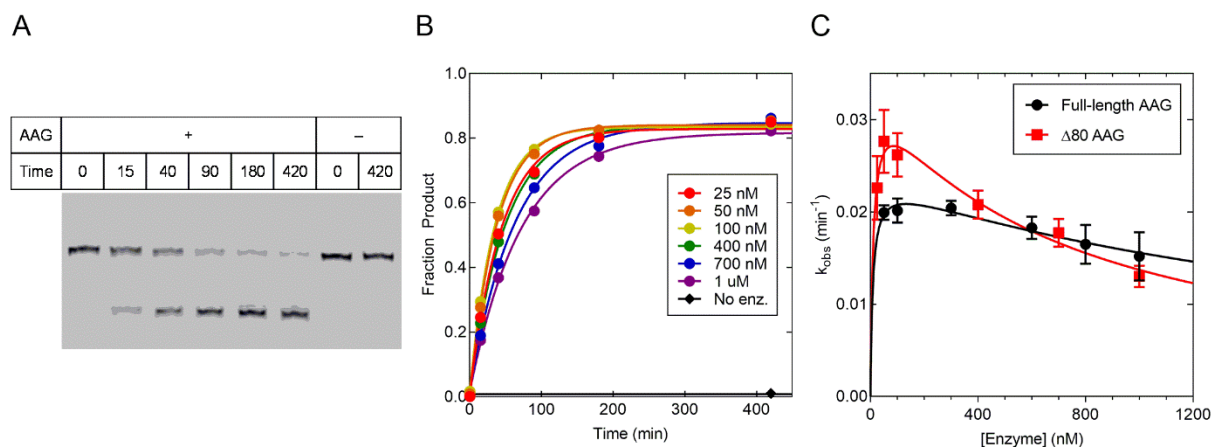


Figure 4. Single turnover excision of ϵ G by AAG. (A) Representative gel for the AAG-catalyzed single turnover excision of ϵ G. Reactions contained 20 nM 25mer DNA with ϵ G complemented by a C. Enzyme concentration was varied between reactions. Pictured are reactions with or without 100 nM Δ 80 AAG. (B) Time courses for ϵ G excision by Δ 80 AAG. Enzyme concentrations are indicated in the legend. (C) The single turnover rate constants for ϵ G excision catalyzed by both full-length and Δ 80 AAG are shown plotted by enzyme concentration. Reactions contained 20 nM ϵ G in the 25mer DNA sequence. The values were fit to the multivalent interference model detailed in Fig S4. Points for full-length AAG represent the mean of 4 replicates, while points for 80 AAG represent the mean of 6 replicates. Error bars represent the S.D. For Δ 80 AAG the best fit values are $k_{\max} = 0.034 \text{ min}^{-1}$, $K_{1/2} = 11 \text{ nM}$, and $K_i = 680 \text{ nM}$. For full-length AAG the values are $k_{\max} = 0.024 \text{ min}^{-1}$, $K_{1/2} = 9 \text{ nM}$, and $K_i = 2.0 \text{ } \mu\text{M}$.

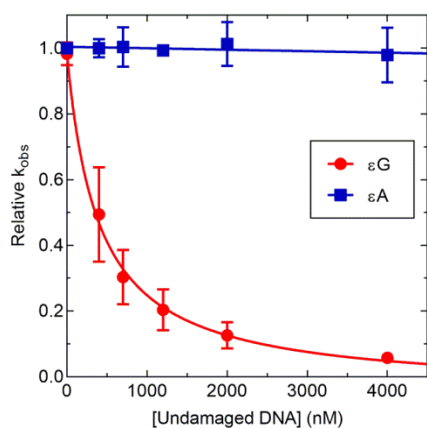


Figure 5. Competition of AAG-catalyzed excision of ϵ G and ϵ A by undamaged DNA. Reactions containing 100 nM Δ 80 AAG and 50 nM of lesion-containing substrate were incubated with increasing concentrations of undamaged 25mer DNA. The loss of ϵ G excision activity was fit with Equation 5, producing an IC_{50} of 400 nM. Points represent the mean of 4 replicates, and error bars represent the S.D.

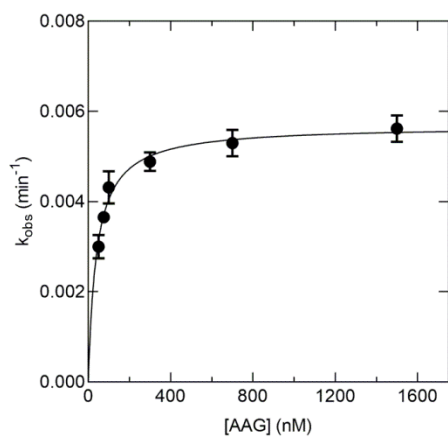


Figure 6. Single turnover excision of ϵ G from a bulged substrate. The observed single turnover rate constants for the excision of bulged ϵ G were plotted against varying concentrations of Δ 80 AAG. A k_{max} value of 0.0057 min^{-1} was determined, with a $K_{1/2}$ value of 40 nM. Reactions contained 20 nM 25mer bulge DNA with the ϵ G lesion. Points represent the mean of 4 replicates, and error bars indicate the S.D.

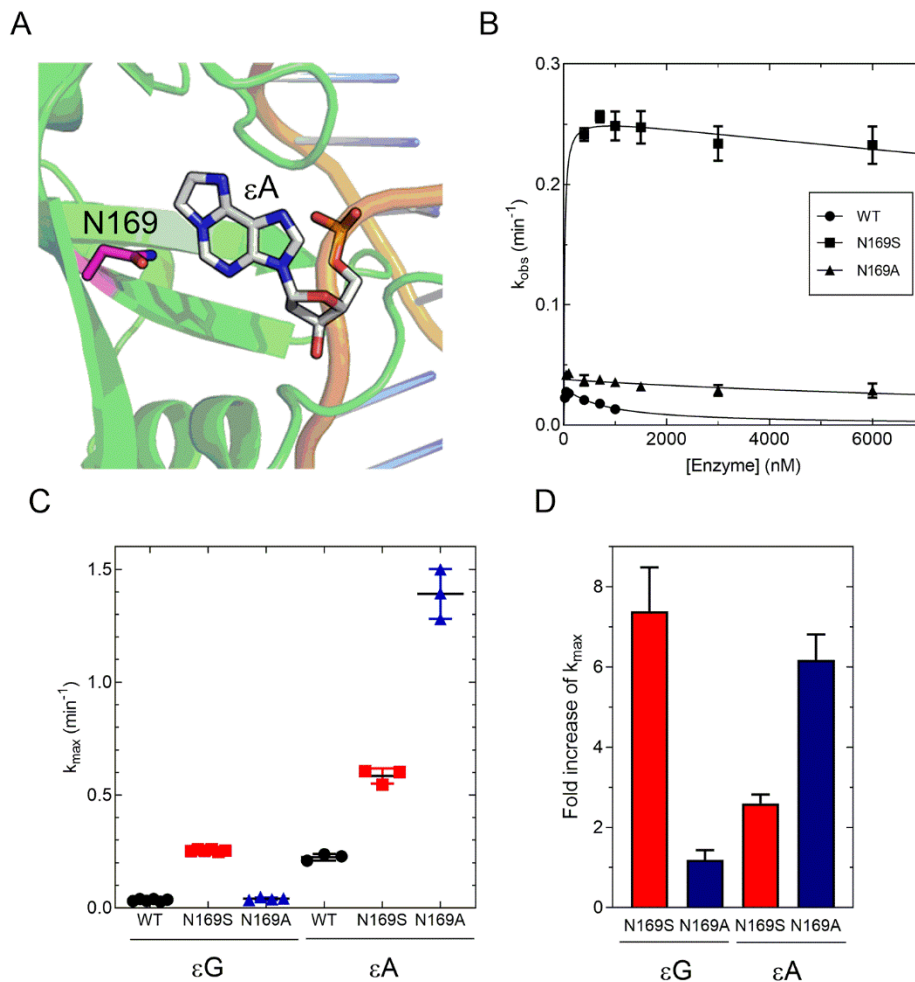


Figure 7. Asn169 of AAG restricts excision of ϵG . (A) In crystal structures of AAG bound to ϵA , the side chain of N169 is in close proximity to N^1 and C^2 of the nucleobase. If ϵG were to bind in the same conformation its exocyclic ring is predicted to clash with the side chain of Asn169. (B) Single turnover rate constants for the excision of ϵG , catalyzed by variants of $\Delta 80$ AAG. Reactions contained 20 nM ϵG in the 25mer DNA sequence with a C complement. Rate constants for WT are replotted from Fig. 4C, while data for N169S and N169A represent the average of 4 replicates. Error bars represent the standard deviation. (C) The maximum single turnover rate constants from panel B are plotted alongside values for ϵA excision. (D) The fold increase in the maximum single turnover rate constant due to each AAG mutation. Each value is calculated by dividing the values of k_{max} for the variant enzyme by the k_{max} for the WT enzyme. Error bars represent the propagated error of both measured values.