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

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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,N²-etheno adenine (eA) and 1,N²-etheno guanine (eG). However, it is not clear how AAG might accommodate eG in an active site that is complementary to eA. This prompted a thorough investigation of AAG-catalyzed excision of eG from several relevant contexts. Using single-turnover and multiple-turnover kinetic analyses, we found that eG in its natural eG-C context is very poorly recognized relative to eA-T. Bulged and mispaired eG contexts, which can form during DNA replication, were similarly poor substrates for AAG. Furthermore, AAG could not recognize an eG 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 eG. Consistent with this model, the N169S variant of AAG was 7-fold more active for excision of eG compared with the wildtype (WT) enzyme. Taken together, these findings suggest that eG 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 eG 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-etheno adenine (eA),² N²,3-ethenocytosine (eC), 1,N⁶-etheno guanine (eG), and N²,3-ethenoguanine (N⁵,3-eG) 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 mis-coding 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 that 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 that can arise in human cells (14). eA 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 eC damage, however, it is unable to catalyze cleavage of the N⁵-glycosidic bond (16). Rather, the human glycosylases TDG and SMUG1, along with the direct reversal protein AlkBH2, have been shown to repair eC (17–19).

Compared with eC and eA, less is known about the repair of eG. In vitro reactions between DNA and aldehydes derived from lipid peroxidation favor the production of eG over N²,3-eG, indicating that eG 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). eG

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,N²-etheno adenine (eA) and 1,N²-etheno guanine (eG). However, it is not clear how AAG might accommodate eG in an active site that is complementary to eA. This prompted a thorough investigation of AAG-catalyzed excision of eG from several relevant contexts. Using single-turnover and multiple-turnover kinetic analyses, we found that eG in its natural eG-C context is very poorly recognized relative to eA-T. Bulged and mispaired eG contexts, which can form during DNA replication, were similarly poor substrates for AAG. Furthermore, AAG could not recognize an eG 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 eG. Consistent with this model, the N169S variant of AAG was 7-fold more active for excision of eG compared with the wildtype (WT) enzyme. Taken together, these findings suggest that eG 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 eG lesions.
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![Figure 1. Diverse structures of the etheno DNA lesions. The four etheno lesions are depicted below their undamaged forms.](image)

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 eG lesions in the human genome is not firmly established, however, the harmful effects of unrepaired eG 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 eG (14, 25, 26). Although these studies conclude that AAG catalyzes the excision of eG, the kinetics of that excision vary widely between reports (25, 26). The relevance of the N-terminal domain of AAG for eG 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 eG (25), whereas a subsequent study reported similar activity of full-length and N terminally truncated AAG toward eG (26). However, this second study reported a very low percentage (6%) of eG 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 eG from a variety of DNA duplexes. Comparison of the kinetic constants for excision of eG and eA, and the results of direct competition experiments, together demonstrate that eG is a very poor substrate of AAG. We investigated the structural origins for this substrate specificity and found that mutation of Asn-169 to a smaller side chain allows significantly increased activity toward eG, 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 eG 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 eG repair in vivo.

Results and discussion

Multiple-turnover kinetics of AAG-catalyzed excision of eG

To examine the kinetics of eG excision by human AAG, we expressed and purified Δ80 AAG from Escherichia coli for in vitro glycosylase assays. We used steady-state kinetics to characterize the efficiency of AAG-catalyzed excision of eG from a 25-mer DNA duplex containing a central eG-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 220 M⁻¹ s⁻¹. In comparison, the catalytic efficiency of AAG for excision of eA, a well-characterized substrate, has been estimated to be 4 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 eG and eA, the lesion-containing oligonucleotides were competed in the same glycosylase assay mixture. The eG lesion was paired with a complementary C, whereas the eA lesion was paired with a complementary G. The eA-G pairing is the least efficient base pairing for eA excision, potentially allowing for the relatively slow eG excision to compete (29). To distinguish the eA and eG DNA substrates and products on a gel, the eG lesion was incorporated into a 25-mer DNA sequence, whereas the eA lesion was incorporated in a 19-mer DNA sequence. We controlled for the effect of DNA length on the kinetics of glycosylase activity by performing a competition assay between eA incorporate into the 19- and 25-mer sequences. No substantial preference between the 19- and 25-mer was observed (Fig. S1). The formation of the fluorescently labeled 12- and 9-mer products from excision of eG and eA, respectively, was monitored to obtain initial rates for the excision of both substrates.
At a ratio of 10:1 εG to εA, no detectable εG product was formed during the initial period of the εA reaction (Fig. 3). As 0.5% of the εG excision product could have been reliably detected, we conservatively estimate that εG is preferred over εA by at least 200-fold. These data further support the conclusion that εG is poorly recognized relative to primary substrates of AAG.

**AAG-catalyzed single-turnover excision of εG**

Single-turnover glycosylase reactions were performed to measure the rate of εG and εA 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 with previous results, an optimal pH of 6.1 was used (28), and AAG was up to 3-fold more active for excision of εG at pH 6.1 as compared with pH 7.5 (Fig. S2). Each single-turnover reaction of εG excision proceeded to ~85% completion, indicating the presence of a small quantity of nonreactive species in the εG-DNA (Fig. 4, A and B). As observed in the multiple-turnover experiments, AAG-catalyzed excision of εG proceeded more slowly than excision of εA. Furthermore, the concentration dependence of Δ80 AAG exhibited biphasic behavior (Fig. 4C, red squares).

In the classic model for single-turnover kinetics, the relationship between $k_{\text{obs}}$ and the concentration of enzyme should fit to a hyperbola. However, a reduction in the rate constant for excision of εG 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 εG. These concentrations of AAG far exceed cellular conditions, making such enzyme crowding unlikely in a cellular context. However, the inability of AAG to distinguish εG 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 εG and εA were challenged with varying concentrations of undamaged 25-mer DNA oligonucleotides containing a central A:T pair. The excision of εA 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 εG was strongly inhibited by the presence of undamaged DNA, with an $IC_{50}$ of 400 nM. This experiment indicates that εG-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 εA-T. The 300-fold preference for εG-C, relative to a typical...
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Figure 4. Single-turnover excision of eG by AAG. A, representative gel for the AAG-catalyzed single-turnover excision of eG. Reactions contained 20 nM 25-mer DNA with eG complemented by a C. Enzyme concentration was varied between reactions. Pictured are reactions with or without 400 nM Δ80 AAG. B, time courses for eG excision by Δ80 AAG. Enzyme concentrations are indicated in the legend. C, the single-turnover rate constants for eG excision catalyzed by both full-length and Δ80 AAG are shown plotted by enzyme concentration. Reactions contained 20 nM eG in the 25-mer 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, whereas points for 80 AAG represent the mean of 6 replicates. Error bars represent the S.D. For Δ80 AAG the best fit values are $k_{\text{max}} = 0.034 \text{ min}^{-1}$, $K_{\text{dJ}} = 11 \text{ nM}$, and $K_{\text{J}} = 680 \text{ nM}$. For full-length AAG the values are $k_{\text{max}} = 0.024 \text{ min}^{-1}$, $K_{\text{dJ}} = 9 \text{ nM}$, and $K_{\text{J}} = 2.0 \text{ \mu M}$.

Figure 5. Competition of AAG-catalyzed excision of eG and eA by undamaged DNA. Reactions containing 100 nM Δ80 AAG and 50 nM of lesion-containing substrate were incubated with increasing concentrations of undamaged 25-mer DNA. The loss of eG excision activity was fit with Equation 5, producing an IC₅₀ of 400 nM. Points represent the mean of 4 replicates, and error bars represent the S.D.

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 eG

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 eG in particular cannot be excised by N-terminal truncations of AAG (25). Our multiple-turnover and single-turnover glycosylase assays indicate the contrary, that Δ80 AAG is active toward eG 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 Δ80 AAG were able to reach similar end points with comparable $k_{\text{max}}$ values. Similar to the truncated protein, the $K_{\text{dJ}}$ for eG 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 eG 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 eG lesion. However, other pairings can occur during replication of the damaged template (6, 7). We characterized the single-turnover kinetics of eG-T excision and found rates similar to those of eG-C excision (Table 1). AAG exhibited similar enzyme concentration-dependent inhibition for excision of eG from eG-C as was observed for eG-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 eG excision by excess AAG molecules is not exclusive to a single base complement.

During replication of eG, DNA polymerases can slip to generate a −1 frameshift (31, 32). The slippage event places the eG lesion into a bulged context without a complementary base. Previously it was shown that AAG excises eA and Hx from a bulged context at comparable rates to base-paired contexts (33, 34). To assess the ability of AAG to recognize eG from a bulged conformation, single-turnover glycosylase assays were performed with a 25-mer bulged substrate (Fig. 6). Notably, the bulged lesion appeared to lack the concentration-dependent inhibitory effect observed with eG 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 that allows for better equilibration between the specific lesion site and the competing nonspecific sites. However, the bulged eG substrate was excised with a 10-fold lower \( k_{\text{max}} \) value than was observed for the duplex eG:C substrate (Table 1). This observation suggests that eG 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 eG bulge DNA (35) shows that the unpaired eG 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 with eG mismatches. Although AAG is capable of excising eG from a bulged context in vitro, it is clear that this is not a favorable context for AAG-initiated repair of eG.

### Table 1

| Lesion       | \( k_{\text{max}} \) (min\(^{-1}\)) |
|--------------|-------------------------------------|
| eG:C        | 0.034 ± 0.005                      |
| eG:T        | 0.032 ± 0.002                      |
| eG bulge    | 0.0057 ± 0.0003                    |
| eA:T        | 0.23 ± 0.015                       |

* The maximum rate constant was calculated from the average of 2 replicates (Fig. S3).

### Figure 6. Single-turnover excision of eG from a bulged substrate.

The observed single-turnover rate constants for the excision of bulged eG were plotted against varying concentrations of \( \Delta 80 \) AAG. A \( k_{\text{max}} \) value of 0.0057 min\(^{-1}\) was determined, with a \( K_{\text{m}} \) value of 40 nM. Reactions contained 20 nM 25-mer bulge DNA with the eG lesion. Points represent the mean of 4 replicates, and error bars indicate the S.D.

### Asn-169 of AAG limits the rate of eG excision

Crystal structures of AAG bound to a flipped-out eA lesion revealed how this active site pocket can accommodate base lesions and exclude undamaged bases (13). The side chain of Asn-169 defines one surface of the active site, closely contacting the N1 face of the eA lesion (Fig. 7A). This residue plays a role in blocking the binding of undamaged guanine with its N\(^2\)-amino group (11, 36), leading us to consider whether this side chain might also make contact with the 1,N\(^2\)-etheno ring of eG. We used site-directed mutagenesis to generate variants of \( \Delta 80 \) AAG with either a serine or an alanine residue in place of Asn-169, and subsequently determined the impact of these mutations on the single-turnover excision kinetics of eA and eG.

The N169A mutation caused an increase in the \( k_{\text{max}} \) value for eG excision from 0.034 to 0.041 min\(^{-1}\) and the inhibition at high concentrations of enzyme was much less prominent as compared with the behavior of the WT enzyme (Fig. 7B; Table 2). This increase in the excision rate of the N169A mutant suggests that Asn-169 interferes with the excision of eG to some degree. Surprisingly, the \( k_{\text{max}} \) for eA excision was also substantially increased by the N169A mutation (Fig. 7C). This likely reflects a removal of a deleterious interaction between the Asn-169 side chain and the eA lesion or could result from rearrangements in the Ala variant that create more favorable interactions with the substrate.

The N169S mutant 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 \( k_{\text{max}} \) values for excision of both eG and eA (Fig. 7C). However, the N169S mutant enhanced eG excision by more than 7-fold, whereas only increasing eA 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 that were lacking in the alanine substitution. The N169S mutant also showed little to no enzyme concentration-dependent inhibition of eG 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 eG by the N169S variant would enable the lesion to compete more favorably with undamaged sites for binding and excision. The observation that eG excision by AAG is improved by mutation of Asn-169 is consistent with the model that this side chain clashes with the eG lesion and contributes to its inefficient excision by AAG.

### Conclusions

The exocyclic ring structure of the mutagenic and cytotoxic lesion eG 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 eA and eG (14, 25, 26). Herein, we demonstrated that eG 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 1, N²-ethenoguanine containing DNA, demonstrating that AAG has a difficult time recognizing 1, N²-ethenoguanine sites.

Our findings highlight some of the limitations of single-turnover kinetic approaches 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 and bound histone proteins. We infer that inhibition of 1, N²-ethenoguanine 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 1, N²-ethenoguanine 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 1, N²-ethenoguanine 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 1, N²-ethenoguanine, we have shown that residue Asn-169 limits the ability of AAG to excise 1, N²-ethenoguanine from DNA. This is consistent with the model that Asn-169 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 Asn-169 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 eG 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 eG lesions, the inability of AAG to efficiently excise eG 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, demonstrate that ALKBH2, but not ALKBH3, is capable of recognizing and repairing eG in duplex context (41). Although ALKBH2 is a strong candidate for physiological repair of eG-C, it is not known if this enzyme is able to capture rare eG lesions from among the excess of undamaged sites and it is not known how eG might be recognized in post-replicative repair. Although eG has not been specifically investigated, genetic studies of mice lacking 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 (Scheme 1). The lesion-containing strand of each oligonucleotide was labeled at the 5’ end with 6-fluorescein (FAM). Oligonucleotides were purified via denaturing PAGE, and the concentrations were determined by the theoretical extinction coefficient at 260 nm as described previously (27).

**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 ORF. 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 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 of 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 min 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 h, quenched samples were stored at 4 °C for no more than 12 h before being heated and mixed with loading buffer. The samples were run out on 20% polyacrylamide gels containing 6.6 M urea and scanned with an Amersham Biosciences 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 (Equation 1).

\[
V_{obs} = \frac{k_{cat}[E][S]}{K_m + [S]} \quad (Eq. 1)
\]

\(V_{obs}\) 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 of 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.

\[
\text{Fraction product} = A(1-e^{-k_{obs}t}) + c \quad (Eq. 2)
\]

\(A\) 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.
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\[ k_{\text{obs}} = \frac{k_{\text{max}}[E]}{K_{I/2} + [E]} \quad (\text{Eq. 3}) \]

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).

\[ k_{\text{obs}} = \frac{k_{\text{max}}[E]}{K_i^{1/2} + [E]} \quad (\text{Eq. 4}) \]

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 IC50 was calculated using Equation 5, where \( k_{\text{obs}} \) is the observed rate constant, \( k_{\text{unin}} \) is the rate constant without undamaged DNA inhibitor, and \( I \) is the concentration of undamaged inhibitor DNA.

\[ k_{\text{obs}} = \frac{k_{\text{unin}}[I]}{IC50 + [I]} \quad (\text{Eq. 5}) \]

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