# Kinetic Mechanism for the Excision of Hypoxanthine by *Escherichia coli* AlkA and Evidence for Binding to DNA Ends

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Ⓢ *Supporting Information*

ABSTRACT: The *Escherichia coli* 3-methyladenine DNA glycosylase II protein (AlkA) recognizes a broad range of oxidized and alkylated base lesions and catalyzes the hydrolysis of the N-glycosidic bond to initiate the base excision repair pathway. Although the enzyme was one of the first DNA repair glycosylases to be discovered more than 25 years ago and there are multiple crystal structures, the mechanism is poorly understood. Therefore, we have characterized the kinetic mechanism for the AlkA-catalyzed excision of the deaminated purine, hypoxanthine. The multiple-turnover glycosylase assays are consistent with Michaelis—Menten kinetics. However, under single-turnover conditions that are commonly employed for studying other

DNA glycosylases, we observe an unusual biphasic protein saturation curve. Initially, the observed rate constant for excision increases with an increasing level of AlkA protein, but at higher protein concentrations, the rate constant decreases. This behavior can be most easily explained by tight binding to DNA ends and by crowding of multiple AlkA protamers on the DNA. Consistent with this model, crystal structures have shown the preferential binding of AlkA to DNA ends. By varying the position of the lesion, we identified an asymmetric substrate that does not show inhibition at higher concentrations of AlkA, and we performed pre-steady state and steady state kinetic analysis. Unlike the situation in other glycosylases, release of the abasic product is faster than N-glycosidic bond cleavage. Nevertheless, AlkA exhibits significant product inhibition under multiple-turnover conditions, and it binds approximately 10-fold more tightly to an abasic site than to a hypoxanthine lesion site. This tight binding could help protect abasic sites when the adaptive response to DNA alkylation is activated and very high levels of AlkA protein are present.
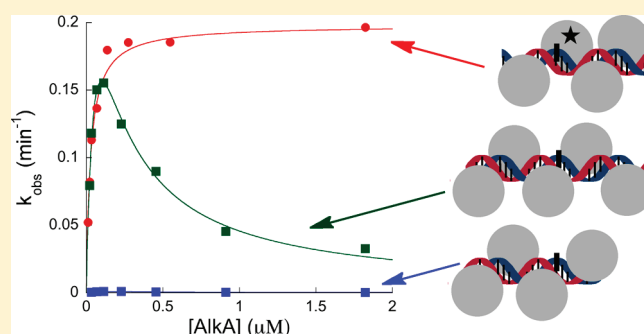
The integrity of the genome is challenged by spontaneous reactions that modify the nucleobases of DNA. These deleterious reactions include oxidation, deamination, and a variety of alkylation reactions involving endogenous metabolites and exogenous reactive compounds. Failure to repair these many forms of DNA damage can block DNA-templated activities such as transcription and replication and lead to permanent mutations or cell death.[1,2] The base excision repair (BER) pathway corrects the majority of damaged bases. This pathway is initiated by a DNA glycosylase that searches the genome to locate the sites of damage and then catalyzes the hydrolysis of the N-glycosidic bond to release the base lesion and create an abasic site. Downstream enzymes subsequently process this abasic site and use the information about the undamaged strand to restore the original coding sequence.

Escherichia coli contains two 3-methyladenine DNA glycosylases that are encoded by the *tag* and *alkA* genes.[3] Tag (3-methyladenine DNA glycosylase I) is constitutively expressed and has high specificity for 3-methyladenine. In contrast, AlkA (3-methyladenine DNA glycosylase II) is induced as part of the adaptive response to alkylation stress and recognizes a diverse group of damaged bases.[3,4] Studies exploring potential substrates

for AlkA have established glycosylase activity toward more than a dozen lesions, including the alkylation adducts 3mA, 7mG, and 1, N^6-ethenoadenine (εA), and the deaminated bases hypoxanthine (Hx) and xanthine.[5−9] Many of the alkylated nucleotides have unstable N-glycosidic bonds and are difficult to work with, but previous work suggests that AlkA has similar rate enhancements for a wide range of disparate lesions.[10] We have focused on the excision of Hx, because the homogeneous substrate can be prepared and it has a stable N-glycosidic bond.

A wealth of structural information is available for the interaction of AlkA with DNA. AlkA has been crystallized in complex with a 1-azaribose transition state analogue,[11] and there are several structures of AlkA bound to the ends of oligonucleotides.[12,13] Most recently, engineered protein—DNA cross-links were used to obtain structures of AlkA interacting with undamaged DNA.[14] Remarkably, all of these structures exhibit a very similar overall set of protein—DNA interactions

Table 1. Sequences of Oligonucleotide Substrates[a]

25	5'-(FAM)CGATAGCATCCT**X**CCTTCTCTCCAT-3' 3'-GCTATCGTAGGATGGAAGAGAGGTA-5'
19u	5'-(FAM)CATCCT**X**CCTTCTCTCCAT-3' 3'-GTAGGATGGAAGAGAGGTA-5'
19d	5'-(FAM)CGATAGCATCCT**X**CCTTCT-3' 3'-GCTATCGTAGGATGGAAGA-5'

[a] Oligonucleotides were annealed to give the indicated duplex substrates containing a single lesion site (X). The DNA tested includes undamaged adenosine (A), the lesions inosine (I) and 1,N^6-ethenoadenosine (εA), the abasic product, and the transition state analogue pyrrolidine (Y).

with only subtle rearrangements that are localized to the active site pocket and neighboring residues.[14]

Mechanistic insights into the AlkA reaction have lagged behind the structural studies. In this work, we have characterized the single- and multiple-turnover kinetics for excision of Hx from oligonucleotides in which the lesion is located different distances from the ends. Comparison of these substrates provides evidence that AlkA binds to DNA ends more tightly than to Hx lesions, and this restricts the binding of subsequent AlkA protamers. We identified a well-behaved substrate that is amenable to kinetic studies and describe the minimal kinetic mechanism for recognition and excision of Hx.

■ MATERIALS AND METHODS

Preparation of Proteins. Full-length AlkA and truncated human alkyladenine DNA glycosylase (AAG) lacking the first 79 amino acids (Δ80 AAG) were expressed in *E. coli* and purified as described previously.[10,15] Initial estimates of the protein concentration were made on the basis of the UV absorbance and the calculated extinction coefficients. The concentration of active Δ80 AAG was determined from the burst amplitude for the excision of Hx, as previously described.[16] The concentration of active AlkA was determined by titration with a DNA inhibitor (see below).

Preparation of Oligonucleotides. DNA substrates were obtained from Integrated DNA Technologies or the Keck Center at Yale University and purified by denaturing polyacrylamide gel electrophoresis. The DNA was then extracted and desalted by reverse phase (C18 Sep-pak, Waters). The concentrations of single-stranded DNA were determined by UV absorbance at 260 nm using the calculated extinction coefficients. For the εA-containing oligonucleotide, the extinction coefficient was calculated on the basis of an identical sequence containing A instead of εA and a value of 9400 M^{-1} cm^{-1} was subtracted to account for the weaker absorbance of εA relative to that of A. Prior to glycosylase assays, the oligonucleotides were annealed with a 2-fold excess of the complementary strand by being heated to 90 °C and cooled to 4 °C over a period of 15 min. Control experiments have shown that the excess single strand does not affect the observed rate constants (data not shown). The sequences of the oligonucleotide duplexes are listed in Table 1.

The abasic DNA product was formed by the complete hydrolysis of I·T DNA with AAG, followed by phenol/chloroform extraction, and buffer exchange using Sephadex G-25 that had been equilibrated with annealing buffer [10 mM NaMES (pH 6.5) and 50 mM NaCl]. The fraction abasic was determined to be >90%, with a small amount of nicked DNA and a small

amount of intact substrate.[17] The concentration of DNA after buffer exchange was determined from the fluorescence of the fluorescein label after purification.

General Glycosylase Activity Assay. Reactions were performed at 37 °C with the enzyme and DNA substrates mixed in a solution containing 50 mM NaMES (pH 6.1), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 0.1 mg/mL BSA. The ionic strength was adjusted to 100 mM with NaCl. The pH value of 6.1 is the optimal pH for AlkA-catalyzed excision of neutral lesions.[10] Reactions were initiated with the addition of a small volume of enzyme that produced a total reaction volume of 20 μL. Aliquots were withdrawn at the desired times and reactions quenched with an equal volume of 0.4 M sodium hydroxide to attain a final concentration of 0.2 M. For long time courses, the quenched samples were stored at 4 °C until all aliquots were taken to prevent the base-catalyzed ring opening and subsequent depurination of εA lesions. The abasic sites were quantitatively cleaved by heating samples at 70 °C for 15 min. For the εA-containing substrate, control experiments showed negligible cleavage of εA sites by this heating process [$\leq$1% (data not shown)]. Samples were then mixed with 2 volumes of loading buffer (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol FF) and resolved by 20% (w/v) polyacrylamide sequencing gels containing 6.6 M urea. Gels were scanned using a Typhoon Trio fluorescence imager (GE Healthcare) with 532 nm excitation and a 526 nm short-pass filter to detect fluorescein. The fluorescence signal was quantified using Image Quant TL (GE Healthcare), and the fraction product for each time point was calculated [fraction product = intensity of product band/(intensity of product band + intensity of substrate band)].

To evaluate the stability of AlkA under these standard reaction conditions, we incubated AlkA in the absence of DNA for up to 50 h, and then the steady state glycosylase activity was measured. The half-life for AlkA inactivation under these conditions was $\geq$100 h (see the Supporting Information).

Determination of the Active AlkA Concentration. We titrated AlkA with a 25mer pyrrolidine-containing oligonucleotide duplex (from 0 to 400 nM) to determine the concentration of active AlkA. The enzyme was preincubated at 37 °C with the inhibitor for 10 min before the reactions were initiated with the addition of 19mer Hx-containing substrate (19u). Reactions were performed under our standard conditions with 1 μM DNA substrate and at two different concentrations of AlkA (100 and 200 nM). The relative AlkA activity in the presence of inhibitor was determined by the ratio of the observed velocity in the presence of inhibitor to the velocity in the absence of inhibitor (V_{obs}/V_{max}). The fraction of active AlkA was

determined from this titration plot, using the quadratic binding equation (eq 1)

$$V_{\text{obs}}/V_{\text{max}} = 1 - \frac{K_{\text{d}} + E + I - \sqrt{(K_{\text{d}} + E + I)^2 - 4EI}}{2E} \quad (1)$$

where E is the concentration of AlkA and I is the concentration of pyrrolidine-containing DNA inhibitor.

Single-Turnover Glycosylase Activity. DNA substrates were incubated with excess enzyme to ensure single-turnover conditions. Unless otherwise indicated, the concentration of substrate was 20 nM and the concentration of AlkA was varied from 40 to 3000 nM. Samples were removed and quenched at appropriate intervals, and the extent of the glycosylase reaction was analyzed as described above. The fraction of base excised was fit by a single exponential according to eq 2

$$F = A[1 - \exp(-k_{\text{obs}}t)] + c \quad (2)$$

where F is the fraction product, A is the amplitude, k_{obs} is the observed single-turnover rate constant, t is the reaction time, and c is the amount of preexisting abasic DNA.

The hyperbolic dependence of the single-turnover rate constant was fit by eq 3

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

where k_{max} is the maximal single-turnover rate constant when AlkA is fully saturated and $K_{1/2}$ is the concentration at which the enzyme is 50% saturated. The $K_{1/2}$ value is analogous to a K_{M} determined in steady state experiments and is equal to the dissociation constant (K_{d}) if substrate binding is readily reversible. Given the low values of k_{max} determined here, this is likely to be the case.

The single-turnover excision of Hx from 19d by AlkA was too slow to cover the entire time course without concern for the loss of stability of AlkA. Therefore, time points were restricted to the first 21 h, which corresponds to between 40 and 65% completion. The experimental data were fit by eq 2 with c fixed to 0.03 (based on quantification of the product band from control reactions with no enzyme) and A was fixed to a value of 0.95 (based on fraction product at completion from the reaction with the same substrate and a saturating concentration of AAG).

For substrates in which inhibition was observed at high enzyme concentrations, we used a simple noncompetitive inhibition model using eq 4

$$k_{\text{obs}} = \frac{k_{\text{max}}E}{K_{\text{d}} + E\left(1 + \dfrac{E}{K_{\text{i}}}\right)} \quad (4)$$

where K_{d} is the binding constant for the stimulatory site and K_{i} is the binding constant for the inhibitory site on the DNA substrate. This is analogous to the previous treatment for substrate inhibition in which a second substrate molecule can bind to an enzyme.[18]

Steady State Kinetics. Steady state studies were performed with the DNA substrate in 100-fold excess over enzyme. To investigate the possibility that AlkA exhibits burst kinetics, we lowered the ratio of substrate to enzyme (50:1, 25:1, and 10:1) to observe both pre-steady state and multiple-turnover kinetics in a reasonable time frame. Because significant product inhibition was observed under steady state conditions, we used only the first
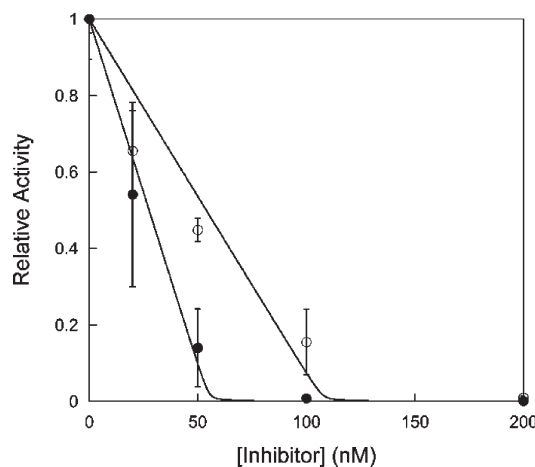

Figure 1. Titration with a tight-binding pyrrolidine inhibitor to determine the concentration of active AlkA. Experiments were performed using 1 μM 19mer I·T substrate (19u) with varying concentrations (from 0 to 400 nM) of 25mer pyrrolidine inhibitor (Y·T). The concentration of AlkA was 100 (●) or 200 nM (○). The fraction of active AlkA was determined by measuring the initial rate of product formation and plotting the relative rate $(V_{\text{obs}}/V_{\text{max}})$ vs inhibitor concentration. The average and standard deviation of two to four replicates are shown. This titration gives an average value of 0.57 ± 0.03 for the fraction of active AlkA, assuming a single monomer binds to each DNA.[11]

8% of the reaction to calculate the initial velocities (V_{obs}). Plots of V_{obs}/E versus substrate concentration were fit by the Michaelis—Menten equation (eq 5).

$$V_{\text{obs}} = \frac{k_{\text{cat}}ES}{K_{\text{M}} + S} \quad (5)$$

Measurement of Inhibition Using Steady State Kinetics. To determine the relative affinity of AlkA for undamaged DNA and for the abasic DNA product, we measured the competitive inhibition by these species under multiple-turnover conditions. The advantage of this approach over direct binding assays is that all DNA species are in excess over enzyme, and therefore, it is unlikely that multiple enzymes can bind to the same DNA. These experiments used 2 nM AlkA, 100 nM I·T 19u substrate, and an amount of either undamaged (A·T) or abasic 25mer DNA product that varied between 10 and 1000 nM. Initial rates were measured as described above, and the resulting inhibition was fit by the equation for competitive inhibition (eq 6). Under these conditions, the inhibition constant (K_{I}) is equal to the dissociation constant (K_{d}) for the inhibitor.

$$V_{\text{obs}} = \frac{k_{\text{cat}}ES}{S + K_{\text{M}}(1 + I/K_{\text{I}})} \quad (6)$$

■ RESULTS

Determination of the Concentration of Active AlkA. To compare single-turnover and multiple-turnover kinetics, it is critical to know the concentration of active enzyme. The concentration of active sites can be determined by measuring the amplitude of a pre-steady state burst or by titrating the enzyme with a tight binding inhibitor. For the AlkA-catalyzed excision of

Figure 2. Single-turnover excision of Hx by AlkA. (A) Representative time course for AlkA-catalyzed excision from the I·T 19u substrate (20 nM) at a saturating AlkA concentration (1.8 μM). Reactions were performed in triplicate, and the average value is shown (error bars indicate the standard deviation). The reaction progress curve was fit by the equation for a single exponential (see Materials and Methods for details). (B) Dependence of the single-turnover rate constant on the concentration of AlkA for I·T DNA. The symmetric 25mer ($\square$), the upstream shortened 19u ($\bullet$), and the downstream shortened 19d ($\bigcirc$) substrates are shown. Each data point is the average of at least three independent determinations, and the error bars indicate the standard deviation. (C) The data for the 19d substrate are replotted from panel B to show that this substrate also shows inhibition at high AlkA concentrations. The equations for a simple hyperbolic dependence (eq 3) and for the inhibitory site model (eq 4) are given in Materials and Methods, and the values obtained from these fits are summarized in Table 2.

εA and Hx, no burst was observed (see below); therefore, we took advantage of a previously identified tight binding inhibitor of AlkA, a pyrrolidine-containing oligonucleotide. The pyrrolidine bears a positive charge at pH <7 and therefore mimics the oxocarbenium ion transition state of a glycosylase reaction.[19,20]

Scheme 1. Model for Explaining the Inhibition by Multiple Molecules of AlkA

A binding affinity of ∼20 pM has previously been reported for a 25mer oligonucleotide containing a pyrrolidine site.[20] AlkA was incubated with various concentrations of pyrrolidine DNA inhibitor under conditions far above the dissociation constant for inhibitor binding, and the steady state rate of N-glycosidic bond cleavage was determined by measuring the initial rates. As expected for a tight binding inhibitor, the concentration dependence of the inhibition followed a quadratic function that is linear at low inhibitor concentrations (Figure 1). Titration of a 2-fold higher concentration of AlkA increased the x-intercept by 2-fold, confirming that stoichiometric binding of inhibitor occurred under these conditions. These data indicate that the actual concentration of active AlkA is ∼57% of the value estimated from the absorbance at 280 nm. For the remainder of the experiments that are presented, we use the concentration of active AlkA that was determined from this titration.

Single-Turnover Excision of Hx. The crystallographic footprint of AlkA binding to DNA is ∼8 bp.[11] Therefore, we used a 25mer symmetric oligonucleotide substrate that was expected to be sufficiently large to support AlkA binding (Table 1). Single-turnover reactions were performed with AlkA in excess of the 25mer substrate. In all cases, the reactions followed first-order kinetics and the reaction progress curves were fit with single exponentials. The single-turnover reaction monitors all of the steps up to and including the first irreversible step, N-glycosidic bond cleavage, and is not affected by product release or product inhibition steps. It is expected that varying the concentration of enzyme under these conditions would result in a hyperbolic dependence upon the concentration of enzyme according to eq 3. However, for the 25mer I·T substrate, we observed a reproducible reduction in rate at high AlkA concentrations [Figure 2B ($\square$)]. These data are consistent with AlkA binding to another site on the DNA that inhibits the binding or activity of AlkA at the lesion site (Scheme 1). According to this model, binding of two or more AlkA monomers could severely reduce or even completely block the activity of one monomer. As several crystal structures have been reported in which AlkA is found bound to the ends of short oligonucleotides, instead of at a central damaged site, we considered the possibility that the inhibition at high AlkA concentrations could be attributed to end binding by AlkA. Similar inhibition was observed for the AlkA-catalyzed excision of the alkyl adduct, 1,N^6-ethenoadenine (εA), suggesting that the inhibition was not exclusive to the I·T form of damage (see the Supporting Information). These observations suggest that AlkA has roughly comparable affinity for DNA ends and for internal sites of DNA damage.

We next tested the simple idea that changing the position of the lesion relative to the end might alter the inhibition. We

Table 2. Kinetic Parameters for AlkA-Catalyzed Excision of Hypoxanthine[a]

substrate	k_{max} (min^{-1})[b]	K_d (nM)	K_i[c] (nM)	k_{cat} (min^{-1})	K_M (nM)	k_{cat}/K_M ($M^{-1} min^{-1}$)
19u	0.21 ± 0.01	32 ± 3	–	0.092 ± 0.003	52 ± 10	1.7×10^6
19d	0.007 ± 0.001^d	500 ± 50^d	47 ± 5^d	0.00054 ± 0.00004	ND[e]	ND[e]
25	0.28 ± 0.01^f	$(42)^f$	220 ± 30^f	0.075 ± 0.003	42 ± 9	1.8×10^6

[a] The sequences of these I·T substrates are listed in Table 1. All reactions were conducted at 37 °C, and the reaction buffer contained 50 mM NaMES (pH 6.1), 10% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA, and sufficient NaCl to produce an ionic strength of 100 mM. [b] The maximal single-turnover rate constants were determined with a saturating concentration of AlkA. [c] K_d for the inhibitory site. The values were determined using eq 4 (Figure 2). No inhibition was observed for the 19u substrate. [d] At suboptimal concentrations of AlkA, the single-turnover excision was too slow to follow all of the reactions to completion (see Materials and Methods for details). The errors were estimated by setting each variable to the optimal value and monitoring the uncertainty in the values that were not fixed. [e] Not determined. [f] As the inhibitory model contains three variables, we fixed the K_d value for this substrate to be the K_M value determined under multiple-turnover conditions. This constrains the inhibitory K_i value and the k_{max} value as reported. Letting all parameters float gave a k_{max} value of 0.4 min^{-1}, a K_d value of 140 nM, and a K_i value of 230 nM.

prepared oligonucleotides that lacked 6 bp from either the upstream (5′ from the lesion) or downstream (3′ from the lesion) portion of the duplex (Table 1) and measured the AlkA concentration dependence for the single-turnover reaction. The results are shown in Figure 2B. Removing 6 bp from upstream (19u) did not result in a significant decrease in AlkA glycosylase activity but rather eliminated the inhibition that was observed at high concentrations of AlkA for the 25mer substrate. In contrast, removal of 6 bp downstream from the lesion (19d) resulted in a profound decrease in AlkA activity. The decreased activity in this orientation is consistent with the asymmetric footprint of AlkA, whereby more extensive protein–DNA contacts are made downstream from the lesion site.[11] Fraying of the ends is likely to exacerbate this effect.[21] Nevertheless, the AlkA-catalyzed glycosylase activity on the 19d substrate showed the same inhibition at high concentrations of AlkA that was observed for the 25mer (Figure 2C).

To gain additional insight into the binding affinities of AlkA for the lesions and DNA ends, we fit the inhibition to a model in which the DNA contains multiple binding sites, with at least one inhibitory site in addition to the lesion site (eq 4 in Materials and Methods). This provided estimates for the maximal single-turnover rate constant (k_{max}) and the dissociation constants for the lesion site and for the inhibitory site, and these are summarized in Table 2. For the 25mer substrate, AlkA has modestly higher affinity for the lesion site than for the DNA ends. For the 19d substrate, AlkA had much tighter affinity for the ends than for this suboptimal lesion site.

Multiple-Turnover Glycosylase Activity of AlkA. To obtain a comprehensive view of the entire AlkA-catalyzed reaction, we also performed multiple-turnover glycosylase assays. Under conditions with excess DNA over protein, it is unlikely that multiple AlkA molecules would be present on a single DNA. However, if AlkA binds to DNA ends, then the steady state reaction rate is expected to be slower than the theoretical rate on a DNA without ends, because end binding is competitive with lesion binding. We found that even at a saturating DNA substrate concentration, the rate of AlkA-catalyzed multiple-turnover reactions decreased significantly above 10% reaction (see the Supporting Information). In principle, this lack of linearity could be due to instability of AlkA or product inhibition. Control experiments demonstrated that AlkA is stable under the conditions used (see the Supporting Information), suggesting that AlkA is extremely sensitive to product inhibition. Below we test this hypothesis directly and show that the abasic DNA product is a potent inhibitor of AlkA.

To avoid the problem of product inhibition, we monitored initial rates under conditions in which <8% product was formed.

At <8% product, the reaction progress curves were linear (Figure 3A). Confident that initial rates were measured, we varied the concentration of both 25 and 19u substrates. The substrate concentration dependence is very similar for both substrates and obeys Michaelis–Menten kinetics (Figure 3B). The K_M value of 52 nM that was determined for the 19u substrate is very similar to the $K_{1/2}$ value of 32 nM that was determined for the single-turnover reaction on this same substrate (Table 2). However, the k_{cat} value of 0.09 min^{-1} is approximately half the value of k_{max} that was determined for the single-turnover reaction. This raises the possibility that another step after N-glycosidic bond cleavage is rate-limiting for multiple-turnover excision. Most previously characterized glyocosylases are limited by release of the abasic DNA product; therefore, this seemed like a possible candidate.[17,22–24] We performed kinetic experiments in which the concentration of AlkA was 2–10% of the concentration of DNA. If product release was rate-limiting, then a pre-steady state burst would be expected between 2 and 10%. However, no burst was detected under these conditions (Figure 4A). Furthermore, the initial rates measured for this first turnover by AlkA were almost identical to those determined for the first eight turnovers (steady state conditions), corresponding to a k_{cat} value of ~0.1 min^{-1} for the 19u substrate (Figure 4B). Thus, the absence of a burst confirms that product release is much faster than N-glycosidic bond cleavage.

The calculated value of k_{cat} depends upon the concentration of active AlkA and the concentration of DNA substrate; therefore, it is difficult to completely rule out the possibility that the 2-fold difference between single-turnover and multiple-turnover excision is due to experimental error. However, the decreased reaction rate under multiple-turnover conditions is consistent with the model in which AlkA binds to DNA ends and base lesions with similar affinities. A 2-fold slower k_{cat} value could result from an equal probability that AlkA would be nonproductively bound to the ends or productively bound to the lesion.

Measurement of the Dissociation Constant for Undamaged and Abasic-Containing DNA. We next used steady state kinetics to measure the inhibition of AlkA by a 25mer undamaged DNA (A·T 25) and by the abasic product. Under these conditions, the inhibition constant is simply the dissociation constant (K_d) for AlkA binding. The inhibition by added DNA followed the expected equation for competitive inhibition, yielding K_d values of 61 nM for the undamaged DNA and 3 nM for the abasic DNA product (Figure 5). It is striking that the undamaged DNA binds to AlkA with an affinity very similar to that of the I·T substrates [30–50 nM (Table 2)]. This suggests that features of a DNA end closely mimic features important for
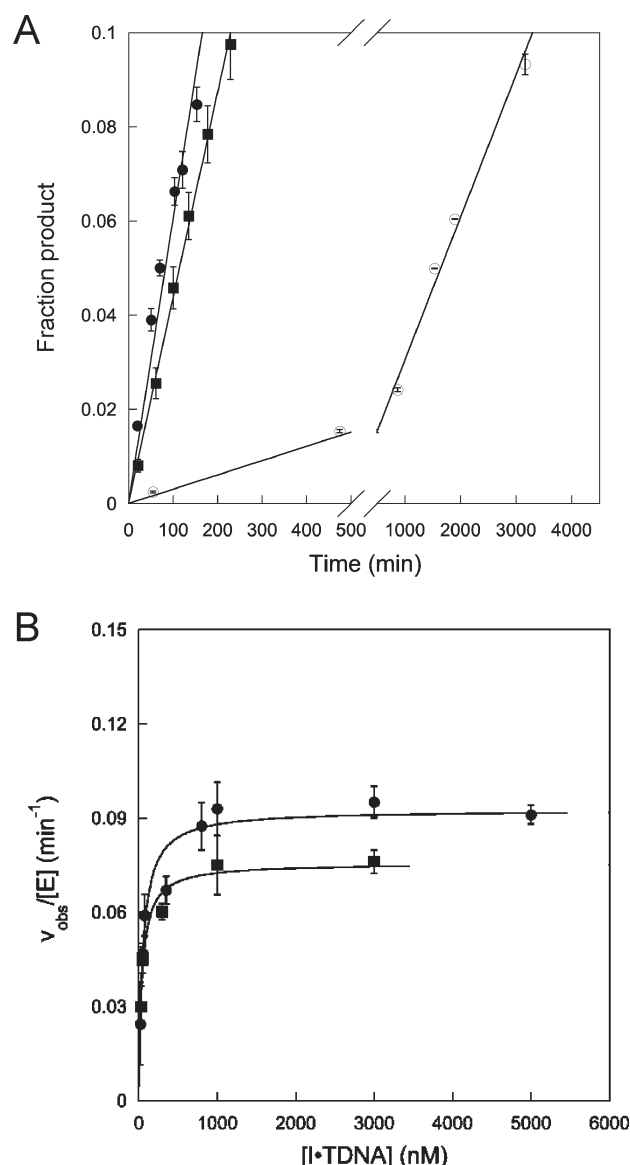
Figure 3. Multiple-turnover glycosylase activity of AlkA. (A) Representative data for AlkA-catalyzed excision of Hx from 19u (●), 19d (○), and the symmetric 25mer (■) with 10 nM AlkA and 1000 nM DNA. (B) Concentration dependence for 25mer (■) and 19u (●) I·T substrates. Each data point is the average of at least three independent determinations, and the error bars indicate one standard deviation. The lines indicate the best fit to the Michaelis—Menten equation (eq 5). The k_{cat} and K_M values are listed in Table 2.

binding to damaged DNA and is consistent with the very similar structures observed for binding of AlkA to DNA ends and to an internal 1-azaribose site.[11−13]

■ DISCUSSION

AlkA has been extensively studied, and there has been recent progress in elucidating the structure of the protein in complex with DNA.[11−14] Previous work has focused on identifying the mechanism of transcriptional regulation[25] and in identifying new substrates of the enzyme. These studies identify AlkA as having one of the most broad substrate ranges of any DNA repair glycosylase.[5−7,9,10] Despite the identification of dozens of
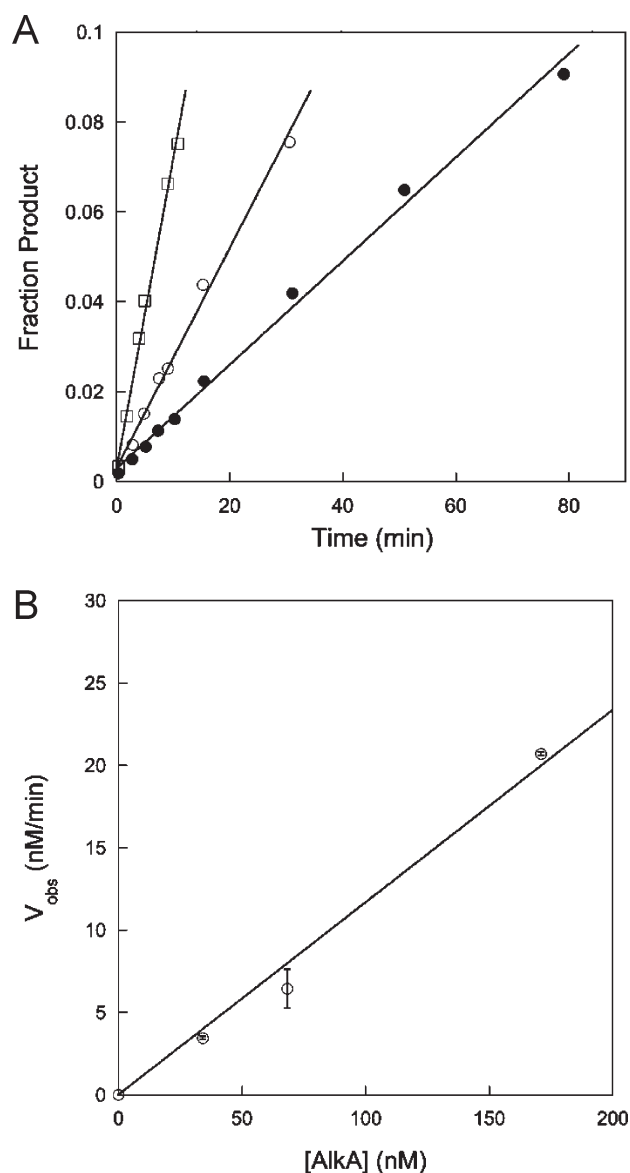
Figure 4. AlkA-catalyzed excision of Hx under conditions suitable for detecting a burst. (A) Reactions were performed with a saturating concentration of I·T 19u DNA (3 μM) and 34 (●), 68 (○), and 140 nM AlkA (□). Reaction rates were linear, indicating the absence of a burst. (B) Initial rates from panel A plotted as a function of AlkA concentration. The line shows a linear fit and yields a k_{cat} value of 0.12 ± 0.01 min^{-1} that is in reasonable agreement with the k_{cat} value of 0.092 min^{-1} that was determined from a wider range of substrate concentrations under steady state conditions (Table 2).

deaminated and alkylated substrates, the kinetic mechanism has not previously been reported for any substrate. Here we have used single-turnover and multiple-turnover kinetics and DNA binding assays to determine the minimal kinetic mechanism for the recognition and excision of Hx from I·T lesion sites. These studies provide kinetic evidence that multiple AlkA molecules can bind close together and block repair of damaged DNA.

Multiple AlkA Molecules Bind to DNA and Interfere with Repair. Single-turnover kinetic analysis of AlkA revealed an unusual dependence upon the concentration of protein, in which it reached an optimal concentration and then declined beyond that [25mer I·T (Figure 2B)]. This type of behavior could be
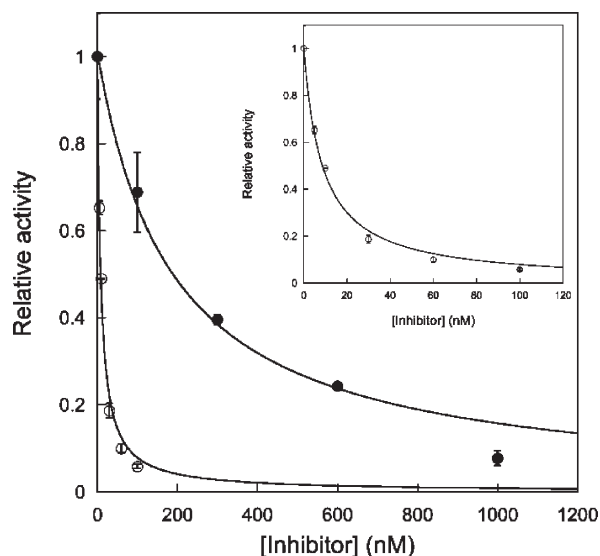
Figure 5. Inhibition of AlkA by undamaged and abasic-containing DNA. Reaction mixtures contained 100 nM I·T 19u DNA, 2 nM AlkA, and the indicated concentration of abasic or undamaged (A·T) 25mer DNA inhibitor. Lines indicate the best fits to the equation for competitive inhibition and give K_d values of 61 ± 9 nM for undamaged DNA (●) and 2.8 ± 0.3 nM for abasic product [○ (see inset)].

explained by protein aggregation or by binding of multiple molecules to the oligonucleotide substrate. Numerous lines of evidence argue against a general aggregation effect, including the ability to purify and crystallize AlkA at a much higher protein concentration and the finding that even concentrated stocks of protein are stable for months at 4 °C (data not shown). However, the most convincing evidence is that the effect is completely absent for a related substrate that bears the identical local sequence context but is shortened asymmetrically so that the lesion is located 7 bp from the end, instead of 13 bp from the end (Figure 2B). The inhibition was retained when the opposite (downstream) end of the DNA was shortened (Figure 2C). These observations, and the finding that AlkA prefers to crystallize in an end-binding conformation (Figure 6 and refs 12 and 13), suggest that binding by AlkA to the end of the DNA affects the binding register of additional protamers (Figure 7). We are forced to invoke multiple binding of AlkA molecules to the interior of the duplex, because the physical distance between the central position of a 25mer substrate and an AlkA molecule bound at the end of the DNA is too great to allow a direct interaction (Figure 6).

Consistent with this hypothesis, AlkA obeys normal Michaelis—Menten kinetics under conditions of excess substrate (Figure 3). The pattern of inhibition under single-turnover conditions requires that AlkA bind with greater or equal affinity to the DNA end and to internal adjacent sites, as it binds to an I·T lesion. This tight binding was confirmed by measuring the binding of DNA that contains ends, but no sites of damage. Under these conditions, the K_M value for the I·T reaction of 30−50 nM (Table 2) is similar to the K_d for binding to undamaged DNA of 61 nM (Figure 5).

AlkA is induced in response to DNA damage and expressed at very high levels. In light of these findings, it is worth considering whether the ability of AlkA to bind to broken DNA ends, and to undamaged DNA at very high density, could have physiological
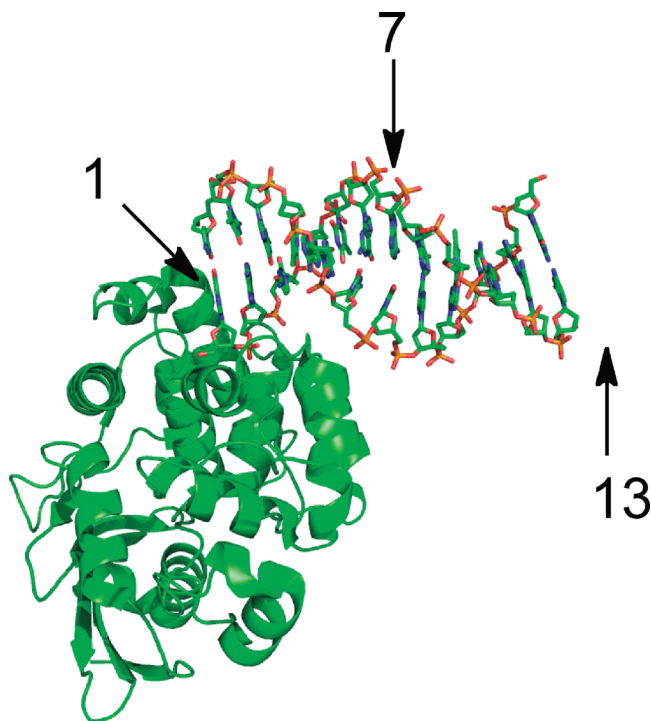

Figure 6. Structure of AlkA bound to the end of a duplex. The figure was rendered with Pymol, and the coordinates are from the Protein Data Bank (entry 3CWT[12]). This crystal form contains two oligonucleotides and four AlkA monomers per asymmetric unit, but only one AlkA—DNA interaction is shown. AlkA interacts predominantly with the strand that donates the 5′ end of the DNA. Arrows indicate the nucleotide position relative to the 5′ end. Position 7 corresponds to the position of the lesion in the 19u substrate, and this lies on the opposite face of the DNA from a protein bound to the 5′ end. Position 13 (not present in the crystallized 12mer oligonucleotide) is on the same face as the end-bound AlkA molecule but is separated by approximately one turn of the helix. This suggests that more than one AlkA molecule would need to bind to the 25mer symmetric substrate before the lesion site (position 13) would experience interference.

consequences beyond its DNA glycosylase activity. Along these lines, it is notable that several other DNA repair proteins have been shown to bind to DNA ends. For example, the single-strand selective uracil DNA glycosylase (SMUG1) and the mismatch-specific uracil DNA glycosylase (MUG) have both been crystallized in complex with DNA ends.[26,27] For MUG, it has recently been noted that multiple enzyme molecules are required for binding to DNA and for the excision of uracil from synthetic oligonucleotides.[28] Other repair proteins have been reported to bind in the proximity on DNA. For example, thymine DNA glycosylase (TDG) has been found to form a dimer with one molecule bound to the site of damage and the other bound in the proximity.[29,30] Biochemical characterization does not reveal any functional effect on the glycosylase reaction in this case.[30] It is also intriguing that O6-alkylguanine-DNA alkyltransferase has been shown to bind at a very high density to undamaged DNA.[31] In all of these cases, it is not clear whether these properties are a byproduct of strong nonspecific DNA binding that would facilitate a genome-wide search for DNA damage or play some role in DNA damage signaling or in shielding a site from DNA repair.

Kinetic Mechanism for AlkA-Catalyzed Excision of Hypoxanthine. We took advantage of the fact that the glycosylase

Substrate	[AlkA] ≫ [DNA]	[AlkA] ≪ [DNA]
I·T 25		
I·T 19u		
I·T 19d		

Figure 7. Models for inhibition of repair by binding of multiple AlkA molecules. If more than one AlkA (gray spheres) is bound ([AlkA] ≫ [DNA]), then not all sites can be sampled by the active site and the position relative to the end determines whether a damaged base (rectangle) is engaged productively (★). If there is a limiting amount of AlkA ([AlkA] ≪ [DNA]), then there will be an ensemble of DNA with AlkA bound at different binding sites across the DNA and every site can be sampled. For the sake of simplicity, only a few possible bound states are shown.

reaction catalyzed by AlkA on the 19u I·T substrate follows a simple concentration dependence to describe the minimal kinetic mechanism of AlkA (Figure 8). We infer that this asymmetric substrate is sufficiently long to support AlkA binding, because very similar steady state kinetic parameters were observed for this substrate and for a symmetrical 25mer substrate (Table 2). We assume that DNA binding and nucleotide flipping are rapid, because this has been the case for every glycosylase that has been previously studied.[32] Regardless of whether this assumption is valid, the single-turnover glycosylase reaction places a lower limit on the rate of N-glycosidic bond cleavage of 0.2 min^{-1}. In contrast, the steady state rate constant (k_{cat}) is approximately 2-fold lower. This could indicate a slow step after N-glycosidic bond cleavage such as product release. This was an attractive hypothesis, because slow product release has been observed for many DNA glycosylases.[33] However, this model predicts the existence of a pre-steady state burst, which was not observed (Figure 4). This suggests that the rate constant for product release is greater than 0.2 min^{-1}. It should be noted that

$$E + S \underset{K_d = 40\ \text{nM}}{\overset{\text{fast}}{\rightleftharpoons}} E{\cdot}S \overset{K_{flip}}{\rightleftharpoons} E{\cdot}S' \overset{k_{chem} \geq 0.2\ \text{min}^{-1}}{\longrightarrow} E{\cdot}AP{\cdot}B \underset{K_d = 3\ \text{nM}}{\overset{k_{off} > 0.2\ \text{min}^{-1}}{\rightleftharpoons}} E + AB + P$$
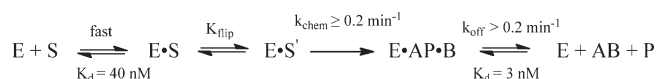
Figure 8. Minimal kinetic mechanism for the recognition and excision of hypoxanthine by AlkA. The binding and flipping steps are assumed to be in rapid equilibrium, which has been seen for other glycosylases.[32] The absence of a burst indicates that the release of Hx (B) and abasic DNA (AP) products is not rate-limiting. However, AlkA shows potent inhibition by abasic sites. The rate of N-glycosidic bond cleavage must be at least as fast as the observed rate constant for the single-turnover reaction; however, it could be significantly faster if the flipping equilibrium is unfavorable.[37]

it is difficult to exclude experimental error in this comparison, because the single-turnover k_{max} value does not require knowledge of the enzyme and DNA concentration whereas the calculated k_{cat} value is dependent upon the concentration of enzyme and DNA substrate. However, we prefer an alternative explanation. It is expected that competitive binding sites on the DNA will weaken the binding of AlkA to the lesion site. The quantitative analysis of this level of competition is model-specific (i.e., how many binding sites and what their affinities are relative to the lesion site), but qualitatively, a 2-fold lower rate constant could be explained by AlkA spending ∼50% of its time bound to nondamaged sites and 50% of its time bound to the damaged site. Consistent with this model, the affinity for undamaged DNA [K_d = 61 nM (Figure 5)] is similar to the K_d value for excision of I·T from the 19u substrate [K_M = 32 nM (Table 2)]. This indicates many alternative sites with much weaker affinity or a few alternative sites that are bound with an affinity similar to that of damaged DNA. Crystal structures suggest that these competitive sites are simply the 5′ ends of the duplex.[12,13]

Although AlkA appears to dissociate from the Hx and abasic DNA products quickly, product inhibition is observed at points in the reaction where 10% conversion to product had occurred (see the Supporting Information). We could find no evidence of inhibition of AlkA by 100 μM Hx, and limited solubility precluded going to higher concentrations (data not shown). In contrast, we observed potent inhibition by the abasic site DNA product, with a K_d value of ∼3 nM that is at least 10-fold higher than the affinity of AlkA for an I·T lesion (Figure 5). This suggests that AlkA will bind tightly to any abasic sites that are present, even in the presence of broken DNA or weak-binding lesions such as I·T. Similar tight binding has been observed by many DNA glycosylases, and this is expected to protect abasic sites from deleterious reactions and to provide better coordination of the downstream reactions in the BER pathway.

Significance. In summary, we have provided biochemical evidence that AlkA binds tightly to DNA ends, supporting crystal structures of AlkA bound to DNA ends. Furthermore, we have identified a binding register that permits simple kinetic analysis under single-turnover conditions. As most studies of AlkA have previously used symmetric substrates, it is likely that the activity of AlkA toward some substrates has been underestimated. It should be noted that *E. coli* has an alternative, faster pathway for repairing deaminated adenosine sites that is initiated by endonuclease V (Endo V).[34−36] Therefore, it is not clear if the activity of AlkA toward Hx lesions is under selective pressure in *E. coli*. Nevertheless, the rate enhancement for excision of Hx by AlkA is similar to that reported for both positively charged and neutral classes of substrates,[10] suggesting that AlkA is not highly specialized for any of the known forms of DNA damage. The

minimal kinetic mechanism presented here will serve as a framework for evaluating the activity of AlkA toward the exceptionally diverse group of damaged nucleotides that are substrates of AlkA.

■ ASSOCIATED CONTENT

ⓢ Supporting Information. Preincubation controls confirming the stability of AlkA, evidence of product inhibition, and additional data for the single-turnover excision of εA and Hx. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

AlkA, *E. coli* 3-methyladenine DNA glycosylase II; BER, base excision repair; BSA, bovine serum albumin; εA, $1,N^6$-etheno-adenine; FAM, 6-aminofluorescein; Hx, hypoxanthine (base moiety of inosine); I, 2′-deoxyinosine; NaHEPES, sodium N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonate; NaMES, sodium 2-(N-morpholino)ethanesulfonate; PAGE, polyacrylamide gel electrophoresis.

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