DNA repair enzymes recognize and remove damaged bases that are embedded in the duplex. To gain access, most enzymes use nucleotide flipping, whereby the target nucleotide is rotated 180° into the active site. In human alkyladenine DNA glycosylase (AAG), the enzyme that initiates base excision repair of alkylated bases, the flipped-out nucleotide is stabilized by intercalation of the side chain of tyrosine 162 that replaces the lesion nucleobase. Previous kinetic studies provided evidence for the formation of a transient complex that precedes the stable flipped-out complex, but it is not clear how this complex differs from nonspecific complexes. We used site-directed mutagenesis and transient-kinetic approaches to investigate the timing of Tyr162 intercalation for AAG. The tryptophan substitution (Y162W) appeared to be conservative, because the mutant protein retained a highly favorable equilibrium constant for flipping the 1,N6-ethenoadenine (eA) lesion, and the rate of N-glycosidic bond cleavage was identical to that of the wild-type enzyme. We assigned the tryptophan fluorescence signal from Y162W by removing two native tryptophan residues (W270A/W284A). Stopped-flow experiments then demonstrated that a change in tryptophan fluorescence of the Y162W mutant is extremely rapid upon binding to either damaged or undamaged enzyme. We assigned the tryptophan fluorescence signal from Y162W by removing two native tryptophan residues (W270A/W284A). Stopped-flow experiments then demonstrated that a change in tryptophan fluorescence of the Y162W mutant is extremely rapid upon binding to either damaged or undamaged DNA, much faster than the lesion-recognition and nucleotide flipping steps that were independently determined by monitoring the eA fluorescence. These observations suggest that intercalation by this aromatic residue is one of the earliest steps in the search for DNA damage and that this interaction is important for the progression of AAG from nonspecific searching to specific-recognition complexes.

Nucleobases of DNA readily react with intracellular and environmental agents to form damaged base lesions. Failure to repair these base lesions leads to mutations or cell death (1, 2). The base excision repair pathway is the main mechanism by which single base lesions in DNA are repaired (3). The base excision repair pathway is initiated by a DNA glycosylase that is responsible for finding the damaged site and catalyzing the hydrolysis of the N-glycosidic bond. Subsequent action of an abasic-site specific endonuclease, a 5′-deoxyribose phosphate lyase, a DNA repair polymerase, and a DNA ligase are required to restore the correct DNA sequence, using the intact strand as a template. There are 11 known human DNA glycosylases that belong to 4 different structural superfamilies. Despite their structural differences, all have adopted the common strategy of nucleotide flipping to access base lesions in duplex DNA. Nucleotide or base flipping describes the complete 180° rotation of a nucleotide out of the DNA duplex to position the target nucleobase into an enzyme active site, and this general mechanism has been described for many types of DNA modifying enzymes (4). Human alkyladenine DNA glycosylase (AAG)2 is a monomeric DNA glycosylase responsible for recognizing a wide variety of structurally diverse deaminated and alkylated purine lesions (5–8). The minimal kinetic mechanism for the recognition, flipping, and excision of 1,N6-ethenoadenine (eA) was previously determined by following the changes in the intrinsic fluorescence of this lesion (9, 10). Transient-kinetic experiments indicated that an initial recognition complex is rapidly and reversibly formed in which the eA lesion is partially unstacked. Subsequently the eA lesion is flipped out of the duplex into the active site to form a stable specific-recognition complex that positions the N-glycosidic bond for hydrolysis. This kinetic model has been guided by the crystal structures of AAG bound specifically to DNA that implicate a highly conserved β hairpin (β3/β4) in specific DNA recognition (11, 12). The tip of this β hairpin projects into the minor groove, placing the side chain of Tyr162 within the duplex where it occupies the space vacated by the base lesion (Fig. 1).

Recent characterization of a mutant enzyme lacking this tyrosine side chain (Y162A) demonstrated that this mutation destabilizes the flipped-out specific-recognition complex because of an accelerated rate of unflipping (13). This is consistent with the tyrosine serving as a steric plug to prevent the lesion nucleotide from returning to the DNA duplex. In addition, the Y162A mutant of AAG catalyzes nucleotide flipping

1To whom correspondence should be addressed: Dept. of Biological Chemistry, University of Michigan Medical School, 5301 MSRB III, 1150 W. Medical Center Dr., SPC 5606, Ann Arbor, MI 48109-0600. Tel.: 734-647-5821; Fax: 734-763-4581; E-mail: pjobrien@umich.edu.

2The abbreviations used are: AAG, alkyladenine DNA glycosylase, also known as methylpurine DNA glycosylase and 3-methyladenine DNA glycosylase; eA, 1,N6-ethenoadenine; Hx, hypoxanthine; I, deoxyinosine; NaMES, sodium 2-[(N-morpholino)ethanesulfonate.

This work was supported in part by a graduate student award from the National Institutes of Health under Grant 5T32 GM008353 (to J. M. H.) and R01GM108022 (to P. J. O.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Edited by Patrick Sung
50-fold faster than observed for the WT enzyme, and the initial DNA-binding and DNA-searching steps became too fast to measure by stopped flow (13). These observations suggested that tyrosine 162 plays roles beyond serving as a plug. Models for enzyme-catalyzed nucleotide flipping vary from transient capture of extrahelical bases to active destabilization of the duplex. These classes of models can be distinguished by the timing of DNA intercalation, because intercalation happens after nucleotide flipping in the transient capture models and happens earlier than flipping in the active destabilization models. In the current work, we investigated the timing for intercalation by tyrosine 162 in AAG-catalyzed recognition and repair of εA.

We took the approach of mutating the intercalating residue to tryptophan (Y162W), which provides the opportunity to directly probe the changes in environment of the intercalating residue along the reaction coordinate for binding, searching, and flipping out a damaged nucleotide. To unambiguously assign the fluorescence of the introduced tryptophan, we also mutated the native tryptophan residues that are present in the catalytic domain of AAG. We fully characterized the kinetic parameters for Y162W-catalyzed excision of εA to compare it to the WT enzyme and then used steady-state and rapid mixing experiments to monitor the changes in tryptophan fluorescence upon binding to undamaged and damaged DNA.

This work demonstrates that substitution of the highly conserved tyrosine at the tip of the intercalating hairpin of AAG with tryptophan (Y162W) has minimal effects on the overall kinetic parameters. The rate of εA excision is identical for Y162W and WT AAG, and both enzymes have similar highly favorable equilibrium constants for nucleotide flipping. The tryptophan fluorescence of Y162W AAG is rapidly quenched upon binding to DNA and does not change throughout the time scale of nucleotide flipping, which is monitored independently by changes in fluorescence of εA. This suggests that the tip of the β hairpin intercalates early in the search for DNA damage, and it engages with the DNA throughout the process of searching for and subsequently engaging sites of DNA damage.

Results

Binding and excision of εA by Y162W

The Y162W variant of AAG was created via site-directed mutagenesis and purified using the same protocol as used for the WT enzyme. It behaved very similarly to the WT protein throughout the purification. We first monitored steady-state binding of Y162W to εA-DNA to confirm stable substrate binding and calculate the amount of active enzyme. The intrinsic fluorescence of εA, which can be excited at 313 nm and emits at 410 nm, provides a sensitive probe for binding directly to this damaged base (9). The results demonstrate that Y162W behaves very similarly to the WT enzyme in this assay, and 100% of the enzyme is active, which is similar to the value of 86% active that was determined for the WT AAG (Fig. 2A). The strong quenching of εA fluorescence indicates tight binding of the flipped-out εA lesion in the active site (9).

We next tested whether the Y162W mutation affected catalysis of N-glycosidic bond cleavage by performing single-turnover experiments with enzyme in excess over εA-DNA substrate. Under these conditions, the hydrolysis of the N-glycosidic bond is rate-limiting for WT AAG (9, 13). The Y162W mutation does not perturb the transition state for hydrolysis, because the rate constant for εA excision is identical within error to that of WT AAG (Fig. 2B). This suggests that the Y162W mutation may be a very conservative substitution; how-

Figure 1. Structure and mechanism of AAG. A, crystal structure of AAG (12) in complex with εA-DNA (Protein Data Bank code 1EWN) was rendered with PyMOL (26). The intercalating tyrosine (Tyr162) and the three native tryptophan residues are shown in red. B, synthetic oligonucleotide duplexes. C, minimal kinetic mechanism for AAG (13). Searching of nonspecific DNA is very rapid, followed by reversible formation of an initial recognition complex. Nucleotide flipping positions the lesion base in the active site and AAG catalyzes hydrolysis of the N-glycosidic bond. The results described herein suggest that Tyr162 (red circle) intercalates rapidly upon nonspecific DNA binding, in which it plays a role in the microscopic steps associated with DNA searching and nucleotide flipping.
ever, AAG binds quickly and tightly to εA-DNA, and effects on earlier binding steps could be masked by the rate-limiting chemistry (10). Therefore direct binding and nucleotide flipping measurements are needed to evaluate the effects of the mutation on these steps.

**Stopped-flow fluorescence to monitor binding and flipping of εA-DNA**

We used the time-dependent changes in the fluorescence of εA to characterize the microscopic steps involving the binding and flipping of this lesion by Y162W AAG under the same conditions previously used for the WT enzyme (13). When a fixed concentration of εA-DNA duplex was mixed with increasing concentrations of Y162W, an initial decrease in fluorescence was observed that was followed by an increase in fluorescence (Fig. 3A). This was unexpected because WT AAG shows the opposite trend in binding to the εA-DNA substrate under the same conditions (Fig. 3A), and it raises the possibility that the tryptophan side chain of Y162W interacts directly with the εA lesion in the initial recognition complex prior to flipping. By analogy, tyrosine likely interacts with the εA lesion in the WT protein but is less effective than tryptophan at quenching the εA fluorescence.

Although the amplitude of εA fluorescence change is smaller for Y162W AAG than was observed for the WT enzyme, reproducible data were obtained at different concentrations of enzyme (Fig. 3B). These traces were fit by double-exponential fits. As expected, the rate constant for the first phase \( k_{1,\text{obs}} \) is linearly dependent on enzyme concentration (Fig. 3C), and this is assigned to binding and formation of the initial recognition complex (9, 13). The slope corresponds to an observed rate constant of \( 4 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \), and this value is within 3-fold of the value determined for WT AAG (Table 1). The slower rate constant for the second step \( k_{2,\text{obs}} \) was independent of AAG concentration and corresponds to the nucleotide flipping step with formation of the specific lesion-recognition complex (Fig. 3D). The observed rate constant for nucleotide flipping reflects an approach to equilibrium, and therefore it is equal to the sum of the rate constants for flipping and unflipping. To determine the microscopic rate constants for flipping and unflipping, it is necessary to carry out additional experiments such as pulse–chase assays that can measure the partitioning forward and backward from the flipped-out intermediate.

**Pulse–chase experiment to measure unflipping and dissociation of εA-DNA**

We performed a pulse–chase experiment in which either WT or Y162W AAG was mixed with fluorescently labeled εA-DNA and then chased with an excess of pyrrolidine inhibitor DNA (Fig. 4A). Pyrrolidine is a transition state analog of AAG that binds very tightly (11, 14), making it an effective trap. The partitioning between dissociation and base excision can be measured, because the protein that dissociates is immediately bound to the inhibitor. For WT AAG, 70% dissociates from the substrate, and 30% partitions to product (Fig. 4B). This end point can be used for calculating the observed rate constant for dissociation (Equation 6). Assuming fast dissociation of AAG from nonspecific DNA, the observed rate constant is simply the rate constant for unflipping \( k_{\text{unflip}} \). When the same experiment was performed for Y162W AAG, 87% of the substrate dissociated from the bound complex (Fig. 4B), indicating a 3-fold faster value for \( k_{\text{unflip}} \) as compared with the WT enzyme (Table 1).
Although the value of $k_{\text{unflip}}$ is slightly increased by the Y162W mutation, this rate constant remains significantly lower than the observed rate constant for flipping ($k_{\text{obs}}$; Fig. 3D). Therefore the microscopic rate constant for flipping ($k_{\text{flip}}$) is approximately equal to this observed rate constant for formation of the specific-recognition complex. The equilibrium constant for nucleotide flipping is calculated as the ratio of $k_{\text{flip}}$ and $k_{\text{unflip}}$ ($K_{\text{flip}} = k_{\text{flip}}/k_{\text{unflip}}$). The Y162W mutation causes only a 2-fold reduction in the $K_{\text{flip}}$ value relative to the WT enzyme (Table 1), confirming that Y162W is a fairly conservative mutation.

**Table 1**

| Kinetic parameters for recognition and excision of $\varepsilon$A by WT and Y162W AAG |
|---------------------------------------------------------------|
| $k_{\text{on}}$ ($M^{-1}s^{-1}$) | $1.1 \pm 0.3 \times 10^9$ | $0.40 \pm 0.05 \times 10^9$ |
| $k_{\text{flip}}$ ($s^{-1}$) | $3.6 \pm 0.7$ | $7.9 \pm 0.7$ |
| $k_{\text{unflip}}$ ($s^{-1}$) | $1.6 \pm 0.3 \times 10^{-3}$ | $5.5 \pm 0.2 \times 10^{-3}$ |
| $K_{\text{flip}}$ | $2300 \pm 600$ | $1400 \pm 100$ |
| $k_{\text{max, eA}}$ ($s^{-1}$) | $(8.0 \pm 0.6) \times 10^{-4}$ | $(8.3 \pm 0.5) \times 10^{-4}$ |

* The values for WT AAG have been previously published (13).

* The equilibrium constant for flipping is given by the ratio of the flipping and un-flipping rate constants ($K_{\text{flip}} = k_{\text{flip}}/k_{\text{unflip}}$).

Characterization of changes in tryptophan fluorescence

We next investigated the changes in tryptophan fluorescence for binding of Y162W AAG to DNA. AAG has multiple tryptophan residues (Trp$^{243}$, Trp$^{270}$, and Trp$^{284}$) that could complicate the assignment of the observed tryptophan fluorescence; therefore we also sought to mutate each of these residues. Each individual tryptophan could be mutated to alanine, but the W243A mutant was poorly soluble, and the triple mutant W243A/W270A/W284A was completely insoluble (data not shown). However, the double mutant W270A/W284A was soluble and could be purified in good yield. Therefore we also introduced the Y162W mutation into this background to generate a triple mutant Y162W/W270A/W284A, which also behaved well. We confirmed that these additional mutant proteins bound tightly to $\varepsilon$A-DNA and determined the concentration of active AAG as described for the Y162W mutant protein (Fig. 2A). Single-turnover excision of $\varepsilon$A was found to be ~2-fold slower for the mutant proteins with the W270A/W284A mutation, suggesting only a minor perturbation of the
protein structure and ruling out large structural changes (Fig. 2B).

Steady-state titrations were performed with each AAG variant, measuring the tryptophan fluorescence at increasing concentrations of εA-DNA (Fig. 5A). As previously reported, WT AAG exhibits 20% quenching of tryptophan fluorescence upon binding to damaged DNA (10). In contrast, Y162W AAG is quenched by 40%, suggesting that the tryptophan at position 162 is sensitive to DNA binding. The quenching of AAG fluorescence is completely eliminated by the W270A/W284A mutations, suggesting that the fluorescence of Trp243 is not sensitive to DNA binding and demonstrating a clean background for the introduction of the Y162W mutation. The fluorescence quenching of the Y162W/W270A/W284A mutant is also 20%, consistent with the quenching amplitudes of the WT and other mutant enzymes. The stoichiometric quenching of εA fluorescence by each of these AAG variants demonstrates that the specific-recognition complex was formed (Fig. 2A). To test nonspecific DNA binding, these titrations were repeated with undamaged DNA duplex, and the changes in tryptophan fluorescence are summarized in Fig. 5B. In each case, the magnitude of tryptophan quenching was almost identical whether or not the DNA contained an εA site. These results establish that Y162W and one or both of the pair of native tryptophan residues (Trp270/Trp284) are sensitive to nonspecific DNA binding.

We next performed stopped-flow fluorescence experiments to probe the transient changes in tryptophan fluorescence that occur during the early steps associated with finding and flipping out an εA lesion. Y162W AAG showed a rapid quenching of fluorescence that occurred within the dead time of the stopped-flow experiments.
flow (≤2 ms) and no other detectable changes over 2 s (Fig. 6A). Under the same conditions, the εA fluorescence for Y162W binding to εA-DNA demonstrates formation of the initial recognition complex and flipping to form the specific-recognition complex (Fig. 3B). This strongly suggests that the tryptophan fluorescence is quenched upon initial binding to DNA. Consistent with this model, when the experiments were repeated with undamaged DNA, the tryptophan fluorescence was again quenched rapidly upon initial DNA binding (Fig. 6B). Because it was possible that the changes in Trp\textsuperscript{270}/Trp\textsuperscript{284} were masking changes in Y162W fluorescence, the stopped-flow experiments were repeated with Y162W/W270A/W284A AAG. Once again, the tryptophan fluorescence was fully quenched upon initial DNA binding regardless of whether the DNA contained a site of damage (Fig. 6, C and D). The W270A/W284A mutant enzyme was not quenched by binding to DNA, suggesting that the introduced Y162W tryptophan in the triple mutant enzyme is responsible for the rapid quenching of tryptophan fluorescence that occurs on binding to either damaged or undamaged DNA. We did not observe any further changes in the tryptophan fluorescence throughout the entirety of the searching and flipping process. Taken together, these results suggest that tryptophan 162 intercalates rapidly into the DNA upon nonspecific DNA binding. Given the similar overall kinetic parameters of Y162W and WT AAG, we expect a similar intercalation by the native tyrosine at this position.

**Effect of Y162W and Y162A mutations on excision of hypoxanthine (Hx)**

Because AAG recognizes a wide variety of alkylated and deaminated bases, we investigated the effects of mutating Tyr\textsuperscript{162} on the maximal single-turnover rate constant for excision of Hx. We used the gel-based assay to determine glycosylase activity for the natural context for deamination of deoxyadenosine to deoxyinosine (I-T) and for a single nucleotide bulge context that is also efficiently recognized by WT AAG (15). WT AAG removes Hx from an I-T base pair or from a single nucleotide I bulge with similar maximal rate constants (Fig. 7A). As expected, the single-turnover rate constant was independent of the concentration of enzyme under these conditions (\(k_{\text{obs}} = k_{\text{max}}\)), and the rate constants are summarized in Table 2. Y162W AAG exhibits similar rate constants for excision of Hx from these same contexts, providing additional evidence that the Y162W substitution is minimally perturbing
Timing of DNA intercalation by AAG

Figure 7. Single-turnover excision of Hx by AAG. Single-turnover glycosylase reactions catalyzed by WT (A), Y162W (B), or Y162A (C) AAG contained 50 nM of 25-mer oligonucleotide duplex containing a central deoxyinosine in either an I-T pair or an I bulge (see inset). The averaged data from two to four independent experiments are plotted and fit by a single exponential (Equation 3). The rate constants are summarized in Table 2.

Table 2
Single-turnover rate constants for excision of Hx by AAG

|        | I-T bulge | I bulge | \( k_\text{eff} (\text{WT/mutant})^a \) |
|--------|-----------|---------|----------------------------------------|
| WT     | 2.9 ± 0.5 | 2.3 ± 0.3 | (1)                                    |
| Y162W  | 1.2 ± 1.1 | 0.81 ± 0.13 | 2.4                                    |
| Y162A  | 0.0083 ± 0.0014 | 0.0049 ± 0.0006 | 350                                    |

The values of the relative rate constant \( k_\text{rel} \) are for I-T but similar to those calculated for the I bulge.

(Fig. 7B). For comparison, we revisited the previously described Y162A mutation of AAG, which greatly alters the kinetics and thermodynamics of nucleotide flipping for eA-DNA (13). The Y162A mutation strongly reduced the maximal rate of Hx excision from both contexts (Fig. 7C). The 350-fold reduction in Hx excision for Y162A relative to WT AAG demonstrates the importance of an aromatic side chain for efficient engagement of the target site (Table 2).

Discussion

The **Y162W mutation is remarkably well tolerated**

The β3β4 hairpin and the intercalating residue, Tyr\(^{162}\), are highly conserved among AAG homologs, and extensive random mutagenesis failed to identify functional variants at this position (16). Previously this residue was shown to be critical for *in vivo* function, and the Y162A mutant is unable to protect cells against exogenous alkylating agents (12). Biochemical studies suggest that Tyr\(^{162}\) plays multiple roles in the search for DNA damage. It appears to act as a plug to slow the rate of unflipping, thereby stabilizing the specific lesion-recognition complex (13). This result is supported by crystal structures of extrahelical AAG complexes in which the side chain of Tyr\(^{162}\) occupies the position vacated by the flipped-out nucleobase (11, 12, 17). In addition to this expected result, it has been shown that Tyr\(^{162}\) is responsible for slowing the process of nucleotide flipping (13). In the current work, we have characterized the kinetic parameters associated with flipping out an eA lesion.

The kinetic parameters associated with AAG-catalyzed nucleotide flipping that were measured under identical conditions for several different Tyr\(^{162}\) variants, Y162A, Y162F, and Y162W, are summarized in Fig. 8. This kinetic and thermodynamic analysis establishes that both Y162F and Y162W perform roles that are very similar to the native tyrosine 162 in WT, with slight elevation of the flipping and unflipping rate constants and very little change in the equilibrium constant for the flipping of eA. In contrast, the Y162A variant exhibits dramatically increased rate constants for both flipping and unflipping. The much larger effect on the rate constant for unflipping causes a significant destabilization of the specific-recognition complex, indicated by the equilibrium constant for flipping (Fig. 8C). The very similar kinetic parameters of Y162W and WT AAG suggested that the fluorescence of Y162W could report on the timing of intercalation.

**Timing of DNA intercalation**

The tryptophan fluorescence of Y162W and the triple mutant Y162W/W270A/W284A is quenched upon initial binding to nonspecific DNA. It is surprising that the fluorescence of Y162W is not sensitive to the formation of the initial recognition intermediate or to the transition to the specific flipped-out complex that can be monitored by changes in eA fluorescence. There are two general classes of models to explain these observations. The first model is that the Y126W side chain intercalates early in the search for DNA damage and remains in a similar environment, inserted between base pairs, as the enzyme proceeds along the reaction coordinate. This model remains consistent with a transient hopping model for diffusion but would dictate that the microscopic dissociation events are short-lived relative to the intercalated state. The second model is that initial DNA binding coincidentally quenches the tryptophan fluorescence to a similar extent as the base stacking interactions that occur in the specific-recognition complex, and therefore the fluorescence does not probe the conformation dynamics. We cannot absolutely distinguish these models, but in both models the stopped-flow fluorescence indicates that Y162W engages with the DNA upon binding to nonspecific DNA.

Additional insights have come from crystal structures of AAG in complex with noncanonical DNA substrates (18). AAG prefers to act on duplex substrates (15), but the enzyme has
been trapped in crystals bound to a pseudoduplex DNA containing multiple mismatches and also to a nonspecific site near to the DNA end (18). In each of these complexes and in the specific-recognition complex of AAG bound to a flipped-out lesion, the side chain of tyrosine 162 makes similar aromatic stacking interactions with base pairs despite the many other differences in the structure of the DNA and in the ordering of surface loops (11, 12, 18, 19). Therefore the structural evidence is supportive of the model that tyrosine 162 can form intercalating interactions prior to nucleotide flipping.

Early and ubiquitous DNA intercalation is also consistent with the observation that the Y162A mutant has accelerated DNA binding and DNA searching (13). It is intriguing to note that two other families of DNA glycosylases employ DNA intercalating interactions, and mutation of these residues to alanine increases the rate of diffusion (20). These observations suggest that early intercalation may be a common feature of DNA glycosylases, and this would allow the enzyme to perturb the DNA environment and tune this interface for lesion recognition. However, in the case of uracil DNA glycosylase, the rate constant for transient opening of base pairs is not strongly perturbed by flipping of this enzyme (21, 22).

Evidence for an unfavorable equilibrium constant for flipping of Hx

In contrast to the highly favorable equilibrium constant for flipping of eA, there is evidence that AAG exhibits an unfavorable equilibrium constant for flipping of Hx (7, 15). The relationship between the maximal single-turnover rate constant ($k_{\text{max}}$) and the rate constant for N-glycosidic bond cleavage ($k_{\text{chem}}$) is given by Equation 1 (7).

$$k_{\text{max}} = \left( \frac{K_{\text{flip}}}{K_{\text{flip}} + 1} \right) k_{\text{chem}}$$  

(Eq. 1)

WT AAG removes Hx from an I-T base pair or from a single nucleotide I bulge with similar maximal rate constants (Table 2). The small reduction in $k_{\text{max}}$ for the Y162W mutant enzyme, relative to WT AAG, is consistent with the small reduction in the equilibrium constant for flipping measured for the eA-DNA substrate (2.4-fold reduction in $k_{\text{max}}$, compared with 1.6-fold reduction in $K_{\text{flip}}$), suggesting very little perturbation in the transition state for N-glycosidic bond cleavage. We extended this result using the Y162A mutant of AAG, which was previously found to decrease the equilibrium constant for flipping of eA by 140-fold, with only a 2-fold reduction in $k_{\text{max}}$ (13). These data predicted a 280-fold reduction in excision of Hx (2 x 140 = 280) if the Y162A mutation has similar effects on the thermodynamics of binding and flipping with both eA and Hx lesions. We observed that single-turnover excision of Hx by Y162A is 350-fold slower than for WT AAG (Table 2), in remarkably good agreement with this simple prediction. Taken together, the kinetic parameters for the Y162A and Y162W mutants support the model that AAG-catalyzed excision of Hx involves rapid equilibrium flipping with an overall unfavorable equilibrium constant for flipping.

Implications

DNA glycosylases are faced with the task of finding rare DNA damage in a sea of undamaged DNA. These enzymes use nonspecific binding and facilitated diffusion to search for sites of damage. Given that these enzymes have independently evolved on multiple occasions, it is possible that different glycosylases use different mechanisms to gain access to damaged nucleobases. In the case of AAG, the stopped-flow studies suggest that intercalation by the $\beta3\beta4$ hairpin and the tyrosine 162 residue occurs early in the search for DNA damage. We demonstrate that these intercalating interactions control the rates of searching and nucleotide flipping steps. This intercalating mechanism provides the opportunity for AAG to directly probe the intrinsic flexibility and dynamic motions of the DNA and allows for remodeling of a particularly wide range of substrate sites that differ greatly in their surface area and hydrogen-bonding properties.

Experimental procedures

Purification of WT and mutant AAG protein

The catalytic domain of human AAG that lacks the first 79 amino acids was expressed in Escherichia coli and purified as previously described (23). WT and Y162A AAG were previously described (13). Briefly, the AAG proteins were purified by polyethyleneimine precipitation to remove nucleic acids, followed by metal affinity chromatography using an N-terminal
Timing of DNA intercalation by AAG

polyhistidine tag that was subsequently removed by recombinant tobacco etch virus (TEV) protease cleavage. Ion exchange chromatography, dialysis, and concentration yielded protein that was greater than 98% pure as judged by Coomassie-stained gels. The Y162W, W270A/W284A, and W270A/ W284A/Y162W mutants were constructed by site-directed mutagenesis. In the case of the double and triple mutants, these substitutions were introduced sequentially and confirmed by DNA sequencing. The mutant enzymes were purified using the same protocol as for WT AAG. Initial enzyme concentrations were determined by UV absorbance using the theoretical extinction coefficient, and the concentration of active enzyme was determined by fluorescent titration of eA-DNA, as described below.

Synthesis and purification of oligodeoxynucleotides

The 25-mer oligonucleotides were synthesized by Integrated DNA Technologies or by the W. M. Keck Facility at Yale University and purified using denaturing polyacrylamide gel electrophoresis as previously described (24). To form a bulge, a 24-mer was annealed to leave the central position unpaired, 5’-ATGGAGAGAAGGAGATGCATCG. Oligonucleotides for gel-based assays were labeled on the lesion-containing strand with a 5’-fluorescein (6-fluorescein) label. The concentrations of the single-stranded oligonucleotides were determined from the absorbance at 260 nm, using the calculated extinction coefficients. For oligonucleotides containing eA, the extinction coefficient was calculated for the same sequence with an A in place of the eA and corrected by subtracting 9400 M\(^{-1}\) cm\(^{-1}\) to account for the weaker absorbance of eA as compared with A. The lesion-containing oligonucleotides were annealed with a 1.2-fold excess of the complement by heating to 90 °C and cooling slowly to 4 °C.

Steady-state fluorescence measurements

Fluorescence emission spectra were collected with a PTI QuantaMaster fluorometer controlled by FeliX software. For eA fluorescence, an excitation wavelength of 314 nm (6-nm band pass) was used, and the total fluorescence was measured at emission wavelengths from 340 to 480 nm (6-nm band pass). Samples (300 μl) of 400 nM eA-containing DNA were prepared in the standard buffer (50 mM NaMES, pH 6.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT), and spectra were recorded at 25 °C. To determine the steady-state fluorescence of eA-containing DNA bound to AAG, the spectra were recorded within 1 min. No significant excision of eA occurs during this time. Three independent titrations were performed, and the average values were fit to a quadratic equation assuming tight binding by AAG (Equation 2), in which \(F_{\text{rel}}\) is the relative fluorescence, \(A\) is the fractional quenching, \(S\) is the known concentration of eA-DNA, \(E\) is the concentration of enzyme, and \(K_d\) is the dissociation constant.

\[
F_{\text{rel}} = 1 - \frac{A((K_d + E + S) - \sqrt{(K_d + E + S)^2 - 4ES})}{2S} \tag{Eq. 2}
\]

Tryptophan fluorescence was measured as described above for eA fluorescence, except that the excitation wavelength was 295 nm (6-nm band pass), and emission was measured at 356 nm (6-nm band pass). No correction for inner filter effect was needed, because the maximal concentration of DNA had minimal absorbance at the excitation wavelength (\(A_{295} < 0.1\)). The titrations shown in Fig. 5 contained 400 nM of WT or mutant AAG enzymes with the indicated amount of DNA. The observation that only 0.5 equivalents of DNA is needed to quench the protein fluorescence is consistent with the fact that undamaged DNA also quenches AAG fluorescence to the same extent (i.e. two AAG molecules can stably bind to the same 25-mer DNA).

Gel-based glycosylase assay

Single-turnover glycosylase activity was determined by denaturing polyacrylamide gel electrophoresis. Fluorescein-labeled DNA substrates (50 nM) containing eA were prepared in the standard buffer. The reactions were initiated with the addition of 75–1200 nM AAG and incubated at 25 °C. At various time points, a sample from the reaction was removed and quenched in 2 volumes of 0.3 M NaOH, giving a final hydroxide concentration of 0.2 M. Abasic sites were cleaved by heating at 70 °C for 15 min. Samples were mixed with an equal volume of formamide/EDTA loading buffer before loading onto a 15% polyacrylamide gel. Gels were scanned with a Typhoon Imager (GE Trio + Healthcare) to detect the fluorescein label by exciting at 488 nm and measuring emission with a S20BP40 filter. The gel bands were quantified using ImageQuant TL (GE Healthcare). The data were converted to fraction product \([F_{\text{prod}} = \text{product}/(\text{product} + \text{substrate})]\) and then fit by a single exponential using nonlinear least squares regression with Kaleidagraph (Synergy Software), in which \(k_{\text{obs}}\) is the rate constant, \(t\) is the time, and \(A\) is the amplitude (Equation 3). Saturation by AAG was confirmed by demonstrating that the observed rate constant was independent of the concentration of AAG, which was varied by at least 2-fold. The observed rate constant is equal to the maximal single-turnover rate constant \((k_{\text{obs}} = k_{\text{max}})\).

\[
F_{\text{prod}} = A(1 - e^{-k_{\text{obs}}t}) \tag{Eq. 3}
\]

Stopped-flow kinetics

Pre-steady-state kinetic experiments were performed on a Hi-Tech SF-61DSX2, controlled by Kinetic Studio (TgK Scientific). The fluorescence of eA was measured using an excitation wavelength of 313 nm and a W9360 long-pass emission filter as previously described (9). The fluorescence of tryptophan was measured using an excitation wavelength of 296 nm and a 330BP20 band-pass emission filter. At least three traces were averaged together at each concentration. The traces for changes in eA fluorescence upon binding of Y162W AAG were fit by a double exponential (Equation 4), where \(F\) is the fluorescence as a function of time, \(C\) is the fluorescence of free DNA, \(X\) and \(Y\) are the changes in fluorescence of the intermediates, and \(t\) is the time.

\[
F = C - X(1 - e^{-k_1t}) - Y(1 - e^{-k_2t}) \tag{Eq. 4}
\]

The observed rate constants were plotted versus concentration and fit to a straight line. \(k_{1,\text{obs}}\) showed a linear concentra-
tion dependence, and the slope is equal to $k_{\text{on}}$ (M$^{-1}$ s$^{-1}$), and the y intercept is equal to $k_{\text{off}}$ (s$^{-1}$). The value of $k_{\text{obs}}$ was independent of concentration and is equal to $k_{\text{flip}} + k_{\text{unflip}}$.

**Pulse–chase assay to measure substrate dissociation**

The macroscopic rate constant for dissociation of WT and mutant AAG from εA-containing DNA was measured by the pulse–chase method in the standard reaction buffer at 25 °C as previously described for WT AAG (9). Briefly, in 20-μl reactions, 50 nm fluorescein-labeled TEC DNA was mixed with 75–1200 nM AAG for 20 s, and then a chase of 10 μM unlabeled pyrrolidine DNA was added. At various time points, a sample from the reaction was removed and analyzed as described under “Gel-based glycosylase assay.” Base excision catalyzed by AAG results in fluorescein-labeled product, whereas dissociation of AAG from the reaction was removed and analyzed as described previously for WT AAG (9). Briefly, in 20-μl reactions, 50 nM fluorescein-labeled TEC DNA was mixed with 75–1200 nM AAG for 20 s, and then a chase of 10 μM unlabeled pyrrolidine DNA was added. At various time points, a sample from the reaction was removed and analyzed as described under “Gel-based glycosylase assay.”

According to the two-step binding mechanism described in Fig. 1C, two different partitioning equations can be written (25). All labeled substrate is initially bound, and therefore the fraction of product formed is given by the fraction that goes on to react. This is indicated by Equation 5, in which A is the burst amplitude (the fraction of product formed in the burst phase of the experiment), $k_{\text{max}}$ is the maximal single-turnover rate constant for formation of product, and $k_{\text{off,obs}}$ is the macroscopic rate constant for dissociation from the flipped-out complex. This expression can be rearranged to solve for the desired dissociation rate constant (Equation 6). Similarly, for branched pathways, the observed rate constant for the burst phase of the pulse–chase experiment is given by the sum of the rate constants for the competing pathways, formation of product is given by $k_{\text{max}}$, and the macroscopic dissociation of substrate is designated $k_{\text{off,obs}}$ (Equation 7). Solving for $k_{\text{off,obs}}$ gives Equation 8.

$$A = \frac{k_{\text{max}}}{k_{\text{off,obs}}} (\text{Eq. 5})$$

$$k_{\text{off,obs}} = \frac{k_{\text{max}}}{A} - k_{\text{max}} (\text{Eq. 6})$$

$$k_{\text{abs}} = k_{\text{off,obs}} + k_{\text{max}} (\text{Eq. 7})$$

$$k_{\text{off,obs}} = k_{\text{abs}} - k_{\text{max}} (\text{Eq. 8})$$

Control reactions in which no chase was added provided the single-turnover rate constant, $k_{\text{max}}$, and confirmed that these concentrations of AAG were saturating. The value of $A$ is calculated by subtracting the amount of product at the time of chase addition ($t_0$) from the observed end point in the presence of chase and dividing by the end point in the absence of chase. From these values, the dissociation rate constant, $k_{\text{off}}$, for dissociation of AAG from εA-DNA was calculated by two different methods (Equations 6 and 8). Both methods gave similar values for $k_{\text{off,obs}}$ and we report the results obtained from Equation 6.

AAG binds to εA-DNA in two steps; therefore the observed rate constant for dissociation of substrate ($k_{\text{off,obs}}$) could be limited by the unflipping rate ($k_{\text{unflip}}$) or dissociation from non-specific DNA ($k_{\text{off}}$). Assuming that the flipped-out complex is stable (i.e. $k_{\text{flip}} \gg k_{\text{unflip}}$), this observed dissociation rate constant can be expressed in terms of the microscopic rate constants (Equation 9). Stopped-flow fluorescence suggests that dissociation from the initial AAG–DNA complex is rapid, and therefore the observed rate constant for substrate dissociation from the εA-DNA–AAG complex is approximately equal to the reverse rate constant for flipping (Equation 10).

$$k_{\text{off,obs}} = k_{\text{unflip}} \left(\frac{k_{\text{off}}}{k_{\text{off}} + k_{\text{unflip}}}\right) (\text{Eq. 9})$$

$$k_{\text{off,obs}} \approx k_{\text{unflip}} (\text{Eq. 10})$$

**Author contributions**—J. M. H. performed the experiments. J. M. H. and P. J. O. designed the study, analyzed the results, wrote the paper, and approved the final version of the manuscript.

**Acknowledgments**—We thank members of the O’Brien laboratory for helpful discussions and comments on the manuscript. The University of Michigan Comprehensive Cancer Center is supported by NIGMS of the National Institutes of Health Grant P30CA036727.

**References**

1. Lindahl, T. (1993) Instability and decay of the primary structure of DNA. *Nature* **362**, 709–715

2. Robertson, A. B., Kungland, A., Rognes, T., and Leiros, I. (2009) DNA repair in mammalian cells: base excision repair: the long and short of it. *Cell Mol. Life Sci.* **66**, 981–993

3. Krokan, H. E., and Bjørland, M. (2013) Base excision repair. *Cold Spring Harb. Perspect. Biol.* **5**, a012583

4. Roberts, R. J., and Cheng, X. (1998) Base flipping. *Annu. Rev. Biochem.* **67**, 181–198

5. O’Connor, T. R. (1993) Purification and characterization of human 3-methyladenine-DNA glycosylase. *Nucleic Acids Res.* **21**, 5561–5569

6. Engelward, B. P., Weeda, G., Wyatt, M. D., Broekhof, J. L., de Wit, J., Donker, I., Allan, J. M., Gold, B., Hoeijmakers, J. H., and Samson, L. D. (1997) Base excision repair deficient mice lacking the Aag alkyladenine glycosylase. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13087–13092

7. O’Brien, P. J., and Ellenberger, T. (2004) Dissecting the broad substrate specificity of human 3-methyladenine-DNA glycosylase. *J. Biol. Chem.* **279**, 9750–9757

8. Ibeanu, G., Hartenstein, B., Dunn, W. C., Chang, L. Y., Hofmann, E., Coquerelle, T., Mitra, S., and Kaina, B. (1992) Overexpression of human DNA repair protein N-methylpurine-DNA glycosylase results in the increased removal of N-methylpurines in DNA without a concomitant increase in resistance to alkylating agents in Chinese hamster ovaly cells. *Carcinogenesis* **13**, 1989–1995

9. Wolfe, A. E., and O’Brien, P. J. (2009) Kinetic mechanism for the flipping and excision of 1N*-ethenoadenine by human alkyladenine DNA glycosylase. *Biochemistry* **48**, 11357–11369

10. Hendershot, J. M., Wolfe, A. E., and O’Brien, P. J. (2011) Substitution of active site tyrosines with tryptophan alters the free energy for nucleotide flipping by human alkyladenine DNA glycosylase. *Biochemistry* **50**, 1864–1874

11. Lau, A. Y., Schärer, O. D., Samson, L., Verdiene, G. L., and Ellenberger, T. (1998) Crystal structure of a human alkylbase-DNA repair enzyme complexed to DNA: mechanisms for nucleotide flipping and base excision. *Cell* **95**, 249–258

12. Lau, A. Y., Wyatt, M. D., Glassner, B. J., Samson, L. D., and Ellenberger, T. (2000) Molecular basis for discriminating between normal and damaged
Timing of DNA intercalation by AAG

bases by the human alkyladenine glycosylase, AAG. Proc. Natl. Acad. Sci. U.S.A. 97, 13573–13578
13. Hendershot, J. M., and O’Brien, P. J. (2014) Critical role of DNA intercalation in enzyme-catalyzed nucleotide flipping. Nucleic Acids Res. 42, 12681–12690
14. Schärer, O. D., Nash, H. M., Irlan, J., Laval, J., and Verdine, G. L. (1998) Specific binding of a designed pyrroline abasic site analog to multiple DNA glycosylases. J. Biol. Chem. 273, 8592–8597
15. Lyons, D. M., and O’Brien, P. J. (2009) Efficient recognition of an unpaired lesion by a DNA repair glycosylase. J. Am. Chem. Soc. 131, 17742–17743
16. Guo, H. H., Choe, J., and Loeb, L. A. (2004) Protein tolerance to random amino acid change. Proc. Natl. Acad. Sci. U.S.A. 101, 9205–9210
17. Lingaraju, G. M., Davis, C. A., Setser, J. W., Samson, L. D., and Drennan, C. L. (2011) Structural basis for the inhibition of human alkyladenine DNA glycosylase (AAG) by 3,N4-ethenocytosine-containing DNA. J. Biol. Chem. 286, 13205–13213
18. Setser, J. W., Lingaraju, G. M., Davis, C. A., Samson, L. D., and Drennan, C. L. (2012) Searching for DNA lesions: structural evidence for lower- and higher-affinity DNA binding conformations of human alkyladenine DNA glycosylase. Biochemistry 51, 382–390
19. Li, D., Delaney, J. C., Page, C. M., Yang, X., Chen, A. S., Wong, C., Drennan, C. L., and Essigmann, J. M. (2012) Exocyclic carbons adjacent to the N6 of adenine are targets for oxidation by the Escherichia coli adaptive response protein AlkB. J. Am. Chem. Soc. 134, 8896 – 8901
20. Nelson, S. R., Dunn, A. R., Kathe, S. D., Warshaw, D. M., and Wallace, S. S. (2014) Two glycosylase families diffusively scan DNA using a wedge residue to probe for and identify oxidatively damaged bases. Proc. Natl. Acad. Sci. U.S.A. 111, E2091–E2099
21. Cao, C., Jiang, Y. L., Krosky, D. J., and Stivers, J. T. (2006) The catalytic power of uracil DNA glycosylase in the opening of thymine base pairs. J. Am. Chem. Soc. 128, 13034–13035
22. Krosky, D. J., Song, F., and Stivers, J. T. (2005) The origins of high-affinity enzyme binding to an extrahelical DNA base. Biochemistry 44, 5949 – 5959
23. O’Brien, P. J., and Ellenberger, T. (2003) Human alkyladenine DNA glycosylase uses acid-base catalysis for selective excision of damaged purines. Biochemistry 42, 12418 – 12429
24. Hedglin, M., and O’Brien, P. J. (2008) Human alkyladenine DNA glycosylase employs a processive search for DNA damage. Biochemistry 47, 11434 – 11445
25. Hsieh, J., Walker, S. C., Fierke, C. A., and Engelke, D. R. (2009) Pre-tRNA turnover catalyzed by the yeast nuclear RNase P holoenzyme is limited by product release. RNA 15, 224 – 234
26. Delano, W. L. (2012) The PyMOL Molecular Graphics System, version 1.5.0.1, Schrödinger, LLC, New York