The human alkyladenine DNA glycosylase has a broad substrate specificity, excising a structurally diverse group of damaged purines from DNA. To more clearly define the structural and mechanistic bases for substrate specificity of human alkyladenine DNA glycosylase, kinetics of excision and DNA binding activities were measured for several different damaged and undamaged purines within identical DNA sequence contexts. We found that 1,N6-ethenoadenine (eA) and hypoxanthine (Hx) were excised relatively efficiently, whereas 7,8-dihydro-8-oxoguanine, O6-methylguanine, adenine, and guanine were not. Single-turnover kinetics of excision of Hx and eA paired with T showed that excision of Hx was about four times faster than eA, whereas binding assays showed that the binding affinity was about five times greater for eA than for Hx. The opposing pyrimidine base had a significant effect on the kinetics of excision and DNA binding affinity of Hx but a small effect on those for eA. Surprisingly, replacing a T with a U opposite Hx dramatically reduced the excision rate by a factor of 15 and increased the affinity by a factor of 7–8. The binding affinity of human alkyladenine DNA glycosylase to a DNA product containing an abasic site was similar to that for an Hx lesion.

The base excision repair pathway provides the cell with a major line of defense against damage to DNA bases by excising damaged bases and resynthesizing DNA. Base excision repair is initiated by the activity of DNA glycosylases, which function to identify and excise damaged bases. Because these enzymes recognize DNA base damage, they are key to the overall effectiveness of the pathway. Monofunctional DNA glycosylases such as human alkyladenine DNA glycosylase (hAAG) excise damaged DNA bases by hydrolysis of the C1'-N glycosylic bond, forming a free DNA base and an abasic sugar residue. Once the damaged base is removed, other enzymes in the pathway remove the remaining sugar residue and resynthesize DNA to fill in the gap.

DNA glycosylases are damage-specific; different enzymes are responsible for excising different types of damaged DNA bases. Some glycosylases such as uracil DNA glycosylase are very specific and excise only a single damaged base, uracil, in this case. Other DNA glycosylases have broader substrate specificities. For example, formamidopyrimidine DNA glycosylase (FpPy) recognizes oxidative damage to DNA bases and excises 7,8-dihydro-8-oxoguanine (8-oxoG), FpPy, and 5-hydroxycytosine. Based on both structural (for recent reviews, Refs. 1–3) and spectroscopic (4) data, DNA glycosylases are believed to “flip” damaged nucleotides out of the DNA helix and into an enzyme active site, where catalysis takes place. Given this type of flipping mechanism, it is easy to imagine how a DNA glycosylase may recognize a specific damaged DNA base through interactions in the active site that provide a “tight” fit and align the glycosylic bond for chemistry. For DNA glycosylases with broader substrate specificities, the nature of the structural interactions and mechanisms that provide specificity are less clear. We are interested in the mechanisms by which DNA glycosylases are able to efficiently identify and excise damaged DNA bases.

Alkyladenine DNA glycosylase (also referred to as 3-methyladenine DNA glycosylase and N-methylpurine DNA glycosylase) is the only glycosylase identified to date in human cells that excises alkylation-damaged bases. This glycosylase has been shown to have a broad substrate specificity and has been reported to excise at least 12 different damaged bases including 3-methyladenine (5–10), 7-methylguanine (5–7, 10, 11), 1,N6-ethenoadenine (8, 9, 11, 12), etheno adducts of guanine (12), 7,8-dihydro-8-oxoguanine (13), hypoxanthine (11, 14, 15), and undamaged purines (16, 17). These damaged purine bases are structurally diverse and contain modifications to both the major and minor groove sides of base pairs as well as to groups involved in base pairing. For example, the methyl group of 3-methyladenine projects into the minor groove, whereas that of 7-methylguanine projects into the major groove, but neither of these methyl groups interrupts hydrogen bonding interactions with its base pairing partner. On the other hand, hydrogen-bonding interactions are disrupted for ethenoadenine and the etheno adducts of guanine. Deamination of adenine to form hypoxanthine alters base pairing and converts a Watson-Crick base pair with T to a wobble base pair, whereas 8-oxoG is still capable of forming a Watson-Crick base pair with C. Given these different base and base pair structures, it is difficult to formulate one model for the structural and mechanistic bases of recognition and excision of these chemically diverse substrates.

To begin to define the mechanistic basis for substrate recognition and excision by hAAG, kinetics of excision and DNA binding affinities were measured for DNA containing different damaged DNA bases within the same sequence context. In addition, the effects on excision rates and binding constants of...
Base Pair Specificity of hAAG

Fig. 1. Structures for some of the DNA base pairs tested as substrates for hAAG. When paired opposite T, A and Hx were excised most rapidly. Changing the base opposite A to C and U had only modest effects on base excision rates and DNA binding. In contrast, changing the base opposite Hx had a dramatic effect on base excision and DNA binding. Replacing T opposite Hx resulted in no excision and DNA binding. The effects of the base opposing a lesion on DNA binding and base excision activity of hAAG demonstrate that recognition and excision of a damaged base is not simply a function of the structure of the damaged base alone but is also a function of the structure of a damaged base pair.

varying the base paired with a damaged DNA base were examined. Although the alkylated DNAs processed by hAAG appear to have few characteristics in common, the goal of our experiments is to determine whether there are common underlying structural features that are recognized by hAAG. Base excision and DNA binding by hAAG were measured for more than 20 different base pair combinations. Fig. 1 illustrates structures of some of the base pairs that were incorporated into DNA substrates. We found ethenoadenine (Ea) and hypoxanthine (Hx) to be the most efficiently excised; however, excision of Hx was affected dramatically by its base-pairing partner.

EXPERIMENTAL PROCEDURES

Oligonucleotides—Synthetic oligonucleotides were either purchased from Fisher-Genosys or made on an Applied Biosystems, Inc. 392 DNA synthesizer using standard β-cyanoethylphosphoramidite chemistry and reagents from Glen Research. Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. Concentrations of purified single-stranded oligonucleotides were determined from absorbances measured at 260 nm using extinction coefficients calculated for each oligonucleotide at 260 nm (18). The extinction coefficient used for Ea was 5000 M⁻¹ cm⁻¹ (extinction coefficient for 1,N⁶-ethenoadenosine (19)), and extinction coefficients for Ea dinucleotides were estimated to be the average of mononucleotide extinction coefficients. For Hx, extinction coefficients for A and A dinucleotides were used, and for O⁶-methylguanine (O⁶-MeG) and 7,8-dihydro-8-oxoguanine (8-oxoG), extinction coefficients for G and G dinucleotides were used. The overall error in oligonucleotide extinction coefficients contributed by using estimated values for Hx, O⁶-MeG, and 8-oxoG is small because the damaged base is only 1 out of 25 total nucleotides. All oligonucleotides were 25 nucleotides in length and of identical sequence (5'-GGGTGGTGGATTTTCGATG-3') except for the central damaged base (D). Duplex DNA substrates were made by annealing labeled oligonucleotides to an equal concentration of unlabeled complementary oligonucleotides. Annealed duplexes were typically prepared at 20 times greater concentrations than used in excision or binding assays and then diluted directly into assay mixtures without further purification.

Human 3-Methyladenine DNA Glycosylase (hAAG)—A deletion mutant of hAAG that is missing the first 79 amino acids from the N terminus (hAAG79) was used in all assays. Deletion of this conserved N-terminal region has been shown to have no effect on either base excision or DNA binding activities of the enzyme (7, 20, 21), but the truncated enzyme is more soluble at low ionic strength. A catalytically inactive mutant, hAAG79E125Q, containing a single point mutation, Glu-125→Gln, was used in DNA binding assays. Both hAAG79 and hAAG79E125Q were overexpressed in Escherichia coli and purified as previously described (21).

Excision Assays—Base excision was measured using a chemical cleavage/gel assay. DNA strands containing a damaged DNA base were 5' end-labeled with ³²P and annealed to a complementary strand. Excision reactions were performed by incubating hAAG79 with a DNA substrate at 37 °C in 50 mM HEPES, pH 8.0, 100 mM NaCl, 10 mM EDTA, 1 mM DTT, and 9.5% v/v glycerol. Typical reaction mixtures contained 400 nM hAAG79 and 50 nM duplex DNA. At several time points during the course of excision reactions, an aliquot of the reaction mixture was quenched in 0.2 M NaOH (final concentration) and heated at 90 °C for 5 min to cleave DNA products containing apurinic sites. After heating, samples were diluted with 2 volumes of loading buffer containing 95% formamide and 20 mM EDTA. Unreacted substrates were separated from cleaved products by electrophoresis on 16% denaturing polyacrylamide gels and quantitated using a Molecular Dynamics Storm PhosphorImager and ImageQuant software.

DNA Binding Assays—DNA binding was measured in electrophoretic mobility shift assays (EMSAs). The DNA strand containing the damaged base was 5' end-labeled with³²P and annealed to a complementary strand. DNA binding was measured in electrophoretic mobility shift assays (EMSAs). The DNA strand containing the damaged base was 5' end-labeled with³²P and annealed to a complementary strand containing either T, C, or U opposite the damaged base. Changing the base opposite A to C and U had only modest effects on base excision rates and DNA binding. The effects of the base opposing a lesion on DNA binding and base excision activity of hAAG demonstrate that recognition and excision of a damaged base is not simply a function of the structure of the damaged base alone but is also a function of the structure of a damaged base pair.
was measured in time course assays by incubating these \( ^{32} \text{P} \)-labeled DNA substrates (50 nM) with hAAG\( \Delta 79 \) (400 nM) at 37 °C for periods up to 160 min. A chemical cleavage/gel assay was used to measure the amount of excision of each damaged base at several times during the course of the excision reaction. In this assay, DNA products containing apurinic sites were chemically cleaved by heating in 0.2 M NaOH at 90 °C for 5 min. Cleaved DNA products were then separated from uncleaved substrates by denaturing polyacrylamide gel electrophoresis and quantitated by phosphorimaging. Assay buffer consisted of 50 mM HEPES, pH 8.0, 100 mM NaCl, 10 mM EDTA, 9.5% glycerol, and 1 mM DTT.

Individual time course reactions at each enzyme concentration fit to an exponential rise (Equation 2) to determine values for \( k_{\text{obs}} \).

\[
y = a(1 - e^{-k_{\text{obs}}t}) \quad \text{(Eq. 2)}
\]

For \( \varepsilon \text{A} \), \( k_{\text{obs}} \) values were 0.080 ± 0.003, 0.075 ± 0.001, and 0.076 ± 0.001 min\(^{-1}\) for duplicate measurements at concentrations of 400, 600, and 800 nM enzyme, respectively. Calculated values of \( k_{\text{obs}} \) were 4–5 times greater for \( \varepsilon \text{A} \) and were 0.31 ± 0.01, 0.32 ± 0.01, 0.37 ± 0.01 min\(^{-1}\) for triplicate measurements at 400, 600, and 800 nM enzyme, respectively.

Effects of Base-pairing Partners on Excision of \( \varepsilon \text{A} \) and \( Hx \)—To determine whether the base paired opposite \( \varepsilon \text{A} \) or \( Hx \) had any effect on the efficiency of hAAG-catalyzed excision, thymidine (T) was replaced with both 2′-deoxycytidine (C) and 2′-deoxyuridine (U). The pyrimidine base paired opposite the damaged base had a larger effect on excision of \( Hx \) than on excision of \( \varepsilon \text{A} \). Time course assays (Fig. 4) were done in duplicate using 400 nM hAAG and 50 nM “damaged” DNA as above. Replacing T with a C resulted in little if any effect on the efficiency of hAAG-catalyzed excision, and replacing T with a U resulted in a decrease in the observed rate of excision (Fig. 4).

For each reaction, quenched with 0.2 M NaOH, and analyzed by the chemical cleavage/gel assay method described above. For each concentration of hAAG\( \Delta 79 \), two or three separate time course reactions were performed. The averages and S.D. for time courses at 400, 600, and 800 nM concentrations of hAAG\( \Delta 79 \) are plotted in Fig. 3. For both damaged base pairs, \( \varepsilon \text{T} \) and \( Hx \), reaction time courses are essentially the same at these three enzyme concentrations, demonstrating that the concentration of enzyme is saturating, and reaction kinetics are not a function of enzyme-substrate binding rates.

Single Turnover Kinetics of Excision of 1,N⁶-Ethenoadenine and Hypoxanthine—The kinetics of excision of \( \varepsilon \text{A} \) and \( Hx \) were examined in more detail. Because excision kinetics were extremely slow under steady-state kinetic conditions and the enzyme loses activity with prolonged incubation at 37 °C, single turnover kinetics of excision were measured (note: when hAAG is incubated with DNA under conditions where DNA binding occurs, the enzyme is protected from inactivation at 37 °C). For these assays, the 25-nucleotide DNA duplex substrates above containing a central \( \varepsilon \text{T} \) or \( Hx \)-T base pair were used. In these experiments, 50 nM DNA was incubated with increasing concentrations of hAAG\( \Delta 79 \) up to 800 nM in separate reactions. Aliquots were withdrawn at several time points during each reaction, quenched with 0.2 M NaOH, and analyzed by the chemical cleavage/gel assay method described above. For each concentration of hAAG\( \Delta 79 \), two or three separate time course reactions were performed. The averages and S.D. for time courses at 400, 600, and 800 nM concentrations of hAAG\( \Delta 79 \) are plotted in Fig. 3. For both damaged base pairs, \( \varepsilon \text{T} \) and \( Hx \), reaction time courses are essentially the same at these three enzyme concentrations, demonstrating that the concentration of enzyme is saturating, and reaction kinetics are not a function of enzyme-substrate binding rates.

For each reaction, quenched with 0.2 M NaOH, and analyzed by the chemical cleavage/gel assay method described above. For each concentration of hAAG\( \Delta 79 \), two or three separate time course reactions were performed. The averages and S.D. for time courses at 400, 600, and 800 nM concentrations of hAAG\( \Delta 79 \) are plotted in Fig. 3. For both damaged base pairs, \( \varepsilon \text{T} \) and \( Hx \), reaction time courses are essentially the same at these three enzyme concentrations, demonstrating that the concentration of enzyme is saturating, and reaction kinetics are not a function of enzyme-substrate binding rates.

For each reaction, quenched with 0.2 M NaOH, and analyzed by the chemical cleavage/gel assay method described above. For each concentration of hAAG\( \Delta 79 \), two or three separate time course reactions were performed. The averages and S.D. for time courses at 400, 600, and 800 nM concentrations of hAAG\( \Delta 79 \) are plotted in Fig. 3. For both damaged base pairs, \( \varepsilon \text{T} \) and \( Hx \), reaction time courses are essentially the same at these three enzyme concentrations, demonstrating that the concentration of enzyme is saturating, and reaction kinetics are not a function of enzyme-substrate binding rates.

Individual time course reactions at each enzyme concentration fit to an exponential rise (Equation 2) to determine values for \( k_{\text{obs}} \).

\[
y = a(1 - e^{-k_{\text{obs}}t}) \quad \text{(Eq. 2)}
\]

For \( \varepsilon \text{A} \), \( k_{\text{obs}} \) values were 0.080 ± 0.003, 0.075 ± 0.001, and 0.076 ± 0.001 min\(^{-1}\) for duplicate measurements at concentrations of 400, 600, and 800 nM enzyme, respectively. Calculated values of \( k_{\text{obs}} \) were 4–5 times greater for \( \varepsilon \text{A} \) and were 0.31 ± 0.01, 0.32 ± 0.01, 0.37 ± 0.01 min\(^{-1}\) for triplicate measurements at 400, 600, and 800 nM enzyme, respectively.

Effects of Base-pairing Partners on Excision of \( \varepsilon \text{A} \) and \( Hx \)—To determine whether the base paired opposite \( \varepsilon \text{A} \) or \( Hx \) had any effect on the efficiency of hAAG-catalyzed excision, thymidine (T) was replaced with both 2′-deoxycytidine (C) and 2′-deoxyuridine (U). The pyrimidine base paired opposite the damaged base had a larger effect on excision of \( Hx \) than on excision of \( \varepsilon \text{A} \). Time course assays (Fig. 4) were done in duplicate using 400 nM hAAG and 50 nM “damaged” DNA as above. Replacing T with a C resulted in little if any effect on the efficiency of hAAG-catalyzed excision, and replacing T with a U resulted in a decrease in the observed rate of excision (Fig. 4).

Surprisingly, replacing T with a U resulted in a decrease in the rates of excision of both \( \varepsilon \text{A} \) and \( Hx \). Again, the effect on the rate of excision of \( \varepsilon \text{A} \) was smaller and was reduced by a factor of 1.7 to 0.045 min\(^{-1}\). Excision of \( Hx \) was reduced by a factor of about 15 to 0.022 min\(^{-1}\) when T was replaced with U. As a control, the DNA strand that contained U was labeled, and excision was measured in assays using both hAAG\( \Delta 79 \) and \( E. \text{coli} \) uracil DNA glycosylase. No uracil DNA glycosylase activity was observed in reactions with hAAG\( \Delta 79 \), whereas quantitative excision of U was seen in reactions with uracil DNA glycosylase (data not shown). The effect of replacing T with U is striking because U differs from T in that it simply lacks the 5-methyl
group, which extends into the major groove. To determine whether a 5-methyl group affects base excision by hAAG, C was replaced with 5-methylcytosine (5-MeC) in base pairs with eA and Hx. In this case, the 5-methyl group had no effect, and excision was the same for base pairs with C and 5-MeC (data not shown).

**Binding of hAAG79 and a Catalytically Inactive Mutant to DNA Containing an eAzT Base Pair—**To better define the interactions between hAAG and different damaged DNA bases, the binding affinity of hAAG to DNA duplexes containing different damaged DNA bases was measured. For these experiments, a catalytically inactive mutant of hAAG, hAAG\(_{D79E125Q}\), was used so that binding to DNA substrates could be measured in the absence of excision. In this mutant, glutamic acid 125 was replaced by glutamine. This Glu residue has been proposed to act as a general base to activate water for hydrolysis of the glycosylic bond (21). In excision assays with eA paired opposite T, hAAG\(_{D79}\) was unable to excise either eA or Hx over a period of 80 min under conditions as in Fig. 2 (data not shown).

To determine whether the mutation of Glu-125 to Gln affected binding activity, binding of “wild type” hAAG\(_{D79}\) and hAAG\(_{D79E125Q}\) to DNA duplexes containing an eAT base pair was measured. Incubation of hAAG\(_{D79}\) with eA-containing DNA at 4 °C significantly reduces the rate of excision of eA, so that binding to this substrate can be measured in the absence of significant product formation. The same 25-nucleotide duplex DNA substrates used in excision assays were used in binding assays, and the DNA strand containing the damaged base was 5'-end-labeled with\(^{32}\)P. Binding experiments were done by incubating the eAT-containing DNA duplex with different concentrations of enzyme at 4 °C for 10 min. After 10 min, an aliquot of these reaction mixtures was removed and analyzed using an EMSA. Two additional aliquots of each reaction mixture were removed: the first, when the EMSA gel was loaded, and the second, after the gel was completed. These additional aliquots were immediately quenched with 0.2M NaOH and analyzed to determine the amount of excision of eA that occurred during the time course of the EMSA.

Results from EMSAs are shown in Fig. 5 for hAAG\(_{D79}\) and Fig. 6 (upper panel) for hAAG\(_{D79E125Q}\). Binding isotherms for the wild type and catalytically inactive mutant are virtually identical (Fig. 5B), indicating that the point mutation reduces excision activity but has little if any effect on DNA substrate binding. For wild type hAAG\(_{D79}\), only about 16% of the substrates were converted to abasic DNA products during the time course of the EMSA at the highest enzyme concentration.
mutant, hAAGΔ79E125Q, was used to measure the binding affinity of the enzyme to DNA substrates containing different damaged DNA bases. Electrophoretic mobility shift assays were done as above using the same damaged duplex DNA substrates used in excision assays. Representative phosphor-imager scans of binding data are shown in Fig. 6A for DNA duplexes containing εA and Hx base pairs with T, C, and U. In general, hAAG binds with greater affinity to DNA containing εA base pairs than Hx base pairs. Each DNA substrate, three separate EMSA experiments were performed and quantitated. Binding isotherms showing the average and S.D. of these three independent experiments are shown in Fig. 6B. Data were fit to a simple two-state binding model shown in Equation 3 using a quadratic equation (see “Experimental Procedures,” Equation 1) to determine an apparent dissociation constant ($K_{d,app}$) where $ED^{total}$ represents all species of $ED$ complexes formed (i.e. both complexes where the damaged nucleotide are flipped ($ED^{flip}$) and not flipped ($ED^{non}$)).

\[
E + D \rightarrow ED^{non} \quad (Eq \ 3)
\]

Apparent dissociation constants calculated for εA base pairs were $20 \pm 2$, $23 \pm 2$, and $6.3 \pm 1.0$ nM for base pairs with T, C, and U, respectively. Dissociation constants for DNA duplexes containing Hx base pairs were affected to a much greater extend by changing the base-pairing partner. Apparent dissociation constants were $92 \pm 2$ and $12 \pm 2$ nM for Hx:T and Hx:U base pairs, respectively. For the duplex containing Hx:C base pairs, $K_{d,app}$ is $\sim 600$ nM and is too great to accurately determine because at high enzyme concentrations, bands “smear” on EMSA gels, probably due to nonspecific binding of the enzyme to undamaged DNA.

In addition to measuring binding to DNA containing εA and Hx lesions, hAAG binding was measured to DNA containing each of the base pairs that were used in excision assays. These base pairs included O6-MeG opposite C and T, 8-oxoG opposite C and T, G opposite T, C, and U, and A opposite T, C, U, and 5-MeC. Significant binding to these DNA substrates was not observed (data not shown). Although U opposite a lesion increased binding to DNA containing εA and Hx, it had no effect when placed opposite G or A.

**Binding of hAAGΔ79 to DNA Duplexes Containing Abasic Sites**—Several DNA glycosylases including E. coli MutY (22), human thymine DNA glycosylase (23), and human methyl-CpG-binding endonuclease 1 (24) have been shown to bind very tightly to the products of their excision reactions. To determine whether hAAG has a high affinity for apurinic DNA products, binding of hAAGΔ79 to duplex DNA substrates containing a synthetic abasic site was measured. A synthetic “reduced” abasic site was used in place of the natural abasic site because this substrate is more stable and can be incorporated at a specific site using standard synthetic chemistry. As a control, binding of hAAGΔ79 to DNA containing a natural abasic site was measured (data not shown) and found to be similar to binding to a reduced abasic site, as has been observed by others (15). Results from EMSA experiments with DNA substrates containing abasic sites are shown in Fig. 7. As with damaged bases, the affinity of the enzyme for abasic sites is affected by the base opposite the abasic site. The enzyme only binds duplexes that contain pyrimidines opposite the abasic site. Apparent binding constants calculated for substrates with pyrimidines opposite the abasic site were 140, 300, and 56 nM for T, C, and U, respectively. Interestingly, when either εA or Hx was placed opposite the abasic site, the enzyme did not bind DNA duplexes, suggesting that the enzyme cannot recognize these damaged bases unless a base is paired opposite them.

**DISCUSSION**

The human alkyladenine DNA glycosylase has been shown to have a broad substrate specificity excising damaged purines, particularly alkylated purines. Various studies have shown that hAAG is capable of excising 3-methyladenine (5–10), 7-methylguanine (5–7, 10, 11), 1,N6-ethenoadenine (8, 9, 11, 12), etheno adducts of guanine (12), 7,8-dihydro-8-oxoguanine (13), hypoxanthine (11, 14, 15), and undamaged purines (16, 17). However, the relative efficiencies of excision of all of these damaged bases have not been firmly established by direct comparison of excision kinetics for each within the same DNA sequence context. This study examines the structural and mechanistic principles for recognition and excision of damaged DNA bases by hAAG. In essence, our approach was to perform “site-directed mutagenesis” on damaged base pairs to determine which structural features of a base pair were important in binding and excision. Much of the previous work in this field has focussed largely on the damaged base alone, but more recent evidence suggests that its base-pairing partner plays a role (9, 11, 14, 25). Our results demonstrate that for some damaged bases, the opposing base can have a dramatic effect on binding and excision. This result is surprising based on the structural data available for the enzyme-DNA complex, which shows no
specific contacts between the enzyme and opposing base.

The crystal structure of hAAG complexed with DNA containing a pyrrolidine abasic site analog (21) and a more recent structure of hAAG bound to eA-containing DNA (26) have revealed that this enzyme, like other DNA glycosylases, flips a damaged nucleotide out of the DNA helix and into an enzyme binding pocket where hydrolysis takes place. A hairpin projects into the minor groove and widens the minor groove at the site of damage and at base pairs immediately 3' to the pyrrolidine, suggesting that the enzyme may scan DNA from the minor groove to detect damage. A tyrosine residue (Tyr-162) projects from this hairpin and intercalates in the DNA helix in the "hole" where the damaged base would have been. In contrast to cocrystal structures of uracil DNA glycosylase with DNA, little compression of the sugar-phosphate backbone is seen in the hAAG-DNA complexes (27, 28). For hAAG, "pushing" the damaged nucleotide out of the helix may be accomplished by the action of Tyr-162 along with other residues of the hairpin without the assistance of "pinching" due to backbone compression that is seen for uracil DNA glycosylase. Although these structures have provided significant insights into the mechanism of recognition and excision by hAAG, questions about substrate specificity remain to be answered.

To gain further insight into the structural and mechanistic principles for excision of damaged DNA bases, excision and binding activities of hAAG were measured for different damaged substrates within an identical DNA sequence context. Initial assays were done to qualitatively compare the excision of four damaged bases, 1,N6-ethenoadenine, hypoxanthine, 7,8-dihydro-8-oxoguanine, and O6-methylguanine, as well as undamaged purines both correctly paired and mispaired with pyrimidines. Hypoxanthine and 1,N6-ethenoadenine, both paired opposite T, were the only bases excised during the 160-min time courses of these assays. Another study using full-length His-tagged hAAG also found that 8-oxoG was not excised (11). It is possible that undamaged purines, 8-oxoG, and O6-MeG may be excised after much longer times, but since excision of these bases was so inefficient, further characterization was not done. Neither 3-methyladenine nor 7-methylguanine were examined in this study because 3-MeA cannot be incorporated site-specifically into DNA, and 7-MeG is relatively labile.

A comparison of the structures of these base pairs, shown in
purine was found to be opposite a purine base, even if the observed base-pairing interactions were observed for DNAs containing an abasic site opposite a pyrimidine, T, C, or U. B, no binding of hAAGΔ79 was observed for DNAs containing an abasic site opposite a pyrimidine base, even if the base was eA or Hx.

The binding of hAAGΔ79 to DNA containing abasic sites was measured by EMSA under conditions identical to excision assays and binding assays with damaged bases (Figs. 2 and 5). A reduced abasic site analog was used in place of the natural abasic site in these assays. A, hAAGΔ79 binds DNA containing an abasic site opposite a pyrimidine, T, C, or U. B, no binding of hAAGΔ79 was observed for DNAs containing an abasic site opposite a pyrimidine base, even if the base was eA or Hx.

Fig. 1, highlights structural similarities and differences that may be important in the excision reaction. Both eA and Hx have two hydrogen bond acceptors that project into the major groove, N7 for both and an exocyclic nitrogen at the 6 position for eA and an exocyclic oxygen at the 6 position for Hx. However, the base pair that each forms with T is different. The exocyclic aetheno group of eA creates a more bulky base and prevents hydrogen-bonding interactions with T. NMR studies show that to accommodate the larger size of eA, an eA/T pair adopts a conformation where both bases are stacked in the helix but skewed relative to one another so that they do not form a planar base pair (35). Hypoxanthine hydrogen bonds with T but forms a wobble pair rather than a Watson-Crick-type pair. This wobble pair differs from a Watson-Crick pair in that the purine is shifted into the minor groove, and the pyrimidine is shifted into the major groove. If hAAG scans the minor groove, it may detect either of these distortions. The fact that Hx forms hydrogen bonds with T and eA does not make eA easier to flip, whereas the smaller size of Hx may increase the rate of excision by a better fit in the binding pocket.

It is interesting that excision of G was not observed when placed opposite T because a G/T pair forms a wobble base pair very similar in structure to Hx/T, the major difference being the 2-amino group that is present on G but not on Hx. Perhaps the 2-amino group is not accommodated within the enzyme active site or it misaligns the nucleotide in the active site so that hydrolysis of the glycosyl bond is not efficient (26). In contrast, 7-MeG also has a 2-amino group but is excised by hAAG (5–7, 10, 11). The 7-methyl group acts to increase the lability of the purine base and may also serve to enhance the efficiency of hydrolysis of the glycosyl bond in the enzyme active site even though alignment of the nucleotide may not be optimal.

To further characterize the excision of eA and Hx, single turnover kinetics were performed to establish the maximal rate for excision of each base. When paired opposite T, the observed rate constant (kobs) for excision of Hx (0.33 min⁻¹) was about 4-fold greater than that for eA (0.077 min⁻¹). Since observed rates in these experiments are not limited by the rate of enzyme-DNA binding and the assay measures both enzyme-bound products and free products, kobs values reflect the rate of conversion of an enzyme-substrate complex to an enzyme-product complex. Depending on the kinetic mechanism, this rate could be limited by the rate of nucleotide flipping or the actual rate of hydrolysis of the glycosyl bond but in any case reflects the rate of conversion of enzyme-bound substrates to products.

The pyrimidine base opposing the lesion has a much greater effect on excision of Hx than eA. For both lesions, excision rates decreased in the order T > C > U. For Hx, changing from T to C and T to U reduced the observed rates by factors of 5 and 15, respectively. Replacing T with U resulted in a more modest 1.7-fold decrease in excision of eA. A similar study done by Aasaeda et al. (11) using full-length His-tagged hAAG also found that excision of Hx was affected to a much greater extent by its base-pairing partner than excision of eA. The fact that the base-pairing partner has a much larger effect on excision of Hx than eA may be due to differences in hydrogen-bonding interactions in the base pairs. Because the etheno group bridges N1 and the exocyclic amino group of adenine, eA is prevented from making hydrogen-bonding interactions with T, C, and U (Fig. 1). If nucleotide flipping is important in the mechanism of excision, the lack of hydrogen-bonding interactions may simply make eA relatively easy to flip regardless of which base opposes it. It is important to note that although excision of eA by hAAG is relatively insensitive to the base opposite eA, a base is required. The fact that hAAG does not excise either eA or Hx when placed opposite an abasic site further suggests that hAAG does not simply capture damaged bases that transiently assume extrahelical positions but instead actively finds and flips damaged bases. The lack of base-pairing interactions at an abasic site is likely to increase the frequency of transient spontaneous flipping of a damaged DNA base.

The effects that the pyrimidine base-pairing partner has on excision rates for Hx is somewhat surprising, particularly
when a T is replaced by a U. The major difference between a T and U base-pairing partner is the presence or absence of a 5-methyl group that extends into the major groove (Fig. 1). The fact that the enzyme discriminates between and Hx:T and Hx:U suggests that the structure of the base pair rather than simply the damaged DNA base plays a role in the mechanism of recognition and excision by hAAG. Two possible explanations are that the enzyme either initially interacts with both the damaged base and its partner or that the base-pairing partner affects the interaction of the enzyme with the damaged base in some way. Although it is possible that the enzyme could interact with both the damaged base and its partner by binding DNA at the major groove before flipping the damaged base from the minor groove, it seems unlikely. Instead, the crystal structure suggests that the presence of uracil opposite the lesion could affect the alignment of the flipped base in the enzyme active site. Intercalation of Tyr-162 into the space formerly occupied by the damaged base may help push the nucleotide into the enzyme active site so that the glycosyl bond is aligned properly for hydrolysis. Uracil opposite hypoxanthine may reduce the rate of catalysis by preventing Tyr-162 from intercalating into the DNA far enough to push the damaged nucleotide into the enzyme active site in the proper alignment. The unpaired uracil may shift back into the helix toward the minor groove to maximize base stacking interactions, and this shift may prevent Tyr-162 from intercalating into the DNA far enough to push the damaged base into its proper orientation. The 5-methyl group on T may prevent T from shifting as far back into the helix due to its bulkiness and unfavorable steric interactions with the bases above and below T. In the crystal structure, the T opposite the damaged base is also pushed into the major groove by about 1.5 Å (21).

The effect of the base-pairing partner on excision efficiency may have an important biological role in helping to ensure that the damage is repaired correctly. Hypoxanthine is excised more efficiently when placed opposite T than opposite C. Initially, Hx would be formed in DNA from deamination of A opposite T. Once in DNA, Hx is mutagenic, miscoding for C so that if replication occurs before repair, then an HxC pair may be formed. Once an HxC pair is formed, excision of Hx and repair by base excision repair would create a G:C mutation. The structural basis for this difference may be due to the fact that HxC forms a Watson-Crick-like base pair, whereas Hx:T or Hx:U form wobble base pairs (Fig. 1). The addition of a 5-methyl group to C does not enhance the excision of Hx as does replacing U with T. Perhaps, the normal Watson-Crick-type structure of the base pair masks the presence of the Hx.

To further characterize the interaction of hAAG with damaged DNA bases, binding to DNA containing damaged bases was also measured with a catalytically inactive mutant, hAAG379E125Q. In general, DNA binding and base excision activities were correlated. For damaged bases that were poorly excised by hAAG such as 8-oxoG, O6-MeG, and undamaged G and A, significant binding was not observed. There was one exception to this rule; binding to εA-U and Hx:U pairs was relatively strong. Overall, hAAG had a greater affinity for DNA containing εA (K_a = 20 nM for εA:T, as also reported by Kartalou et al. (34)) than for DNA containing Hx (K_a = 92 nM for Hx:T). Binding affinities decreased with the base opposing the lesion in the order U > T > C, and the effect was much greater for Hx than εA. Binding to DNA containing Hx pairs was 7.6-fold greater for Hx:U than Hx:T and at least 50-fold greater for Hx:U than Hx:C, whereas the affinity of hAAG for DNA containing εA-U was 3.2- and 3.6-fold greater than for DNA containing εA:T and εA:C, respectively. The same trend was seen for hAAG binding to DNA containing abasic sites. hAAG does not bind to abasic site DNA containing purine residues opposite the abasic site even if these purine residues are damaged εA and Hx. The magnitude of the binding interactions to DNA products containing an abasic site is on the same order of magnitude as binding to a DNA substrate containing an Hx:T base pair and about 7-fold weaker than binding to DNA containing an εA:T base pair. This result seems to imply that hAAG may not remain tightly bound to DNA products after excision, as has been found for some other DNA glycosylases including E. coli MutY (22), human thymine DNA glycosylase (23), and human methyl-CpG-binding endonuclease 1 (24).

Which is the better substrate for hAAG, εA or Hx? To some extent this depends on the base opposite the lesion, because for Hx both DNA binding and excision are affected significantly by the opposing base. Both damaged bases are likely to be initially formed opposite a T since they arise from damage to A. Hx opposite T is excised about 4 times more rapidly than εA opposite T; however, this rapid excision rate is balanced by a greater binding affinity of hAAG for DNA containing εA. hAAG binds DNA containing an εA:T pair about five times better than DNA containing an Hx:T. So when εA and Hx are paired with T, they are about equally good substrates for hAAG.

Based on these initial experiments, we have developed a working model for damaged base recognition and excision by hAAG. In this model, there are two important criteria for efficient base excision, initial identification of the damaged DNA base and proper alignment of this damaged nucleotide in the enzyme active site for cleavage of the glycosyl bond. Initially, the enzyme must find the damaged base amid the vast excess of undamaged DNA bases. Initial recognition of damage may depend on recognition of structural distortions in DNA induced by the damage followed by nucleotide flipping, which checks for fit of the damaged base in the enzyme active site. Alternatively, damaged base recognition may occur solely through flipping the damaged base into the active site. If the damaged base does not fit properly into the enzyme active site then it will not attain the proper geometry for hydrolysis to take place and excision will be inefficient. The base opposite a damaged base might affect excision by influencing either the initial recognition of damage and/or substrate alignment in the enzyme active site. For example, a C opposite Hx may mask Hx from efficient recognition because it forms a Watson-Crick-type base pair, whereas U opposite Hx may affect how Hx is aligned in the enzyme active site. This model will be tested further with more extensive kinetic and mechanistic experiments.

Acknowledgments—We thank Dr. Joyce Feller for helpful discussions and for proofreading this manuscript.

REFERENCES
1. Mol, C. D., Parikh, S. S., Putnam, C. D., Lo, T. P., and Tainer, J. A. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 101–129
2. McCullough, A. K., Dodson, M. L., and Lloyd, R. S. (1999) Annu. Rev. Biochem. 68, 255–285
3. Hollis, T., Lau, A., and Ellenberger, T. (2000) Mutat. Res. 460, 201–210
4. Stivers, J. T., Pankiewicz, K. W., and Watanabe, K. A. (1999) Biochemistry 38, 952–963
5. Chakravarti, D., Beenan, G. C., Tano, K., and Mitra, S. (1991) J. Biol. Chem. 266, 15710–15715
6. Samson, L., Derfier, B., Boosalis, M., and Call, K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9127–9131
7. O’Connor, T. R. (1993) Nucleic Acids Res. 21, 5561–5569
8. Dosanjh, M. K., Roy, R., Mitra, S., and Singer, B. (1994) Biochemistry 33, 1624–1628
9. Saparbaev, M., Kleib, K., and Laval, J. (1995) Nucleic Acids Res. 23, 3750–3755
10. Roy, R., Kennel, S. J., and Mitra, S. (1996) Carcinogenesis 17, 2177–2182
11. Asada, A., Ide, H., Asagohi, K., Matsuyma, S., Tano, K., Murakami, A., Takamori, Y., and Kubo, K. (2000) Biochemistry 39, 1959–1965
12. Dosanjh, M. K., Chenna, A., Kim, E., Fraenkel-Conrat, H., Samson, L., and Singer, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1024–1028
13. Bessho, T., Roy, R., Yamamoto, K., Kasai, H., Nishimura, S., Tano, K., and Mitra, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8901–8904
14. Saparbaev, M., and Laval, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5873–5877
Base Pair Specificity of hAAG

15. Miao, F., Bouziane, M., and O’Connor, T. R. (1998) *Nucleic Acids Res.* **26**, 4034–4041
16. Berdal, K. G., Johansen, R. F., and Seeberg, E. (1998) *EMBO J.* **17**, 363–367
17. Wyatt, M. D., Allan, J. M., Lau, A. Y., Ellenberger, T. E., and Samson, L. D. (1999) *Bioessays* **21**, 668–676
18. Fasman, G. (ed). (1975) *Handbook of Biochemistry: Nucleic Acids*, Vol. 1, p. 589, CRC Press, Inc., Boca Raton, FL
19. Secrist, J. A., III, Barrio, J. R., Leonard, N. J., and Weber, G. (1972) *Biochemistry* **11**, 3499–3506
20. Roy, R., Biswas, T., Hazra, T. K., Roy, G., Grabowski, D. T., Izumi, T., Srinivasan, G., and Mitra, S. (1998) *Biochemistry* **37**, 580–589
21. Lau, A. Y., Schärer, O. D., Samson, L., Verdine, G. L., and Ellenberger, T. (1998) *Cell* **85**, 249–258
22. Porello, S. L., Leyes, A. E., and David, S. S. (1998) *Biochemistry* **37**, 14756–14764
23. Waters, T. R., and Swann, P. F. (1998) *J. Biol. Chem.* **273**, 20007–20014
24. Petronzelli, F., Riccio, A., Markham, G. D., Seeholzer, S. H., Stoerker, J., Gennauro, M., Yeung, A. T., Matsumoto, Y., and Bellacosa, A. (2000) *J. Biol. Chem.* **275**, 32422–32429
25. Wyatt, M. D., and Samson, L. D. (2000) *Carcinogenesis* **21**, 901–908
26. Lau, A. Y., Wyatt, M. D., Glassner, B. J., Samson, L. D., and Ellenberger, T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 13573–13578
27. Parikh, S. S., Mol, C. D., Slupphaug, G., Bharati, S., Krokan, H. E., and Tainer, J. A. (1998) *EMBO J.* **17**, 5214–5226
28. Parikh, S. S., Walcher, G., Jones, G. D., Slupphaug, G., Krokan, H. E., Blackburn, G. M., and Tainer, J. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 5083–5088
29. Savva, R., McAuley-Hecht, K., Brown, T., and Pearl, L. (1995) *Nature* **373**, 487–493
30. Mol, C. D., Arvai, A. S., Slupphaug, G., Kavli, B., Alseth, I., Krokan, H. E., and Tainer, J. A. (1995) *Cell* **86**, 869–878
31. Labahn, J., Schärer, O. D., Long, A., Ezaz-Nikpay, K., Verdone, G. L., and Ellenberger, T. E. (1996) *Cell* **86**, 321–329
32. Yamagata, Y., Kato, M., Odawara, K., Tokuno, Y., Nakashima, Y., Matsushima, M., Yasumura, K., Tomita, K., Ibara, K., Fujii, Y., Naka-heppu, Y., Sekiguchi, M., and Fujii, S. (1996) *Cell* **86**, 311–319
33. Kouchakdjian, M., Riemen, M., Yarema, K., Basu, A., Essigmann, J., and Patel, D. J. (1991) *Biochemistry* **30**, 1820–1828
34. Kartalou, M., Samson, L. D., and Essigmann, J. M. (2000) *Biochemistry* **39**, 8032–8038

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
