Effects of Hydrogen Bonding within a Damaged Base Pair on the Activity of Wild Type and DNA-intercalating Mutants of Human Alkyladenine DNA Glycosylase*

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Aarthry C. Vallur‡, Joyce A. Feller‡§, Clint W. Abner‡¶, Robert K. Tran‡, and Linda B. Bloom‡**

From the ‡Department of Biochemistry and Molecular Biology and the ¶Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida 32610-0245

Human alkyladenine DNA glycosylase “flips” damaged DNA bases into its active site where excision occurs. Tyrosine 162 is inserted into the DNA helix in place of the damaged base and may assist in nucleotide flipping by “pushing” it. Mutating this DNA-intercalating Tyr to Ser reduces the DNA binding and base excision activities of alkyladenine DNA glycosylase to undetectable levels demonstrating that Tyr-162 is critical for both activities. Mutation of Tyr-162 to Phe reduces the single turnover excision rate of hypoxanthine by a factor of 4 when paired with thymine. Interestingly, when the base pairing partner for hypoxanthine is changed to difluorotoluene, which cannot hydrogen bond to hypoxanthine, single turnover excision rates increase by a factor of 2 for the wild type enzyme and about 3 to 4 for the Phe mutant. In assays with DNA substrates containing 1,N7-ethenoadenine, which does not form hydrogen bonds with either thymine or difluorotoluene, base excision rates for both the wild type and Phe mutant were unaffected. These results are consistent with a role for Tyr-162 in pushing the damaged base to assist in nucleotide flipping and indicate that a nucleotide flipping step may be rate-limiting for excision of hypoxanthine.

Human alkyladenine DNA glycosylase (AAG)† is one of several damage-specific DNA glycosylases that function in the base excision repair pathway (reviewed in Refs. 1–4). These DNA glycosylases initiate repair by identifying and removing damaged bases from DNA. Monofunctional DNA glycosylases, including AAG, hydrolyze the glycosylic bond between the base and sugar to leave an abasic sugar residue in DNA. Other enzymes in the pathway remove this apurinic/apyrimidinic lesion and resynthesize DNA to complete repair. The ability of DNA glycosylases to identify and excise damaged DNA bases is key to the overall success of base excision repair.

Structural studies of AAG (5, 6) and other DNA glycosylases have revealed that they use a nucleotide “flipping” mechanism for damaged base recognition and excision where the damaged base is flipped out of the DNA helix and bound in an enzyme active site. In these nucleotide-flipped DNA glycosylase-DNA complexes, an enzyme amino acid side chain is inserted into the base stack at the site vacated by the flipped base and may assist in nucleotide flipping by pushing the damaged base from the helix. It is believed that DNA glycosylases actively flip damaged bases out of the helix rather than passively capturing bases that have transiently adopted extrahelical conformations. This active nucleotide flipping mechanism is supported by detailed kinetic studies of *Escherichia coli* uracil DNA glycosylase which show a two-step binding mechanism where UDG initially binds DNA to form an unflipped protein-DNA complex prior to flipping uracil from the helix (7).

Many questions remain about how nucleotide flipping enables DNA glycosylases to discriminate between damaged and undamaged bases. For DNA glycosylases that have a narrow substrate specificity, a mechanism where a “tight fit” of the damaged base in the enzyme active site allows the DNA glycosylase to discriminate between damaged and undamaged bases seems probable. For example, UDG excises only uracil from DNA, and mutation of enzyme residues that form specific interactions with U alters the specificity of the enzyme so that it can excise C and T (8, 9). On the other hand, for DNA glycosylases that excise a structurally diverse group of damaged bases such as AAG, a mechanism for damaged base recognition and excision that depends solely on specific interactions between enzyme binding pocket residues and a damaged base seems unlikely. Damaged bases excised by AAG, including 3-methyladenine, 1,N7-ethenoadenine (eA), hypoxanthine (Hx), and 7-methylguanine, have no obvious structural features in common that would allow the enzyme to distinguish between damaged and undamaged bases (10–18). In addition, the efficiency of excision by AAG is dependent on the base pairing partner for some damaged bases (15–17, 19, 20) even though the enzyme makes no specific contacts with the base pairing partner in the crystal structures (5, 6). This base pair specificity of AAG further suggests that substrate specificity is governed by a mechanism that involves more than the fit of the damaged base in the enzyme binding pocket.

To define further the mechanisms of damaged base recognition and excision by AAG, the question of how nucleotide flipping contributes to the efficiency of base excision by AAG was addressed using two general approaches. First, site-directed mutations that were predicted to reduce the efficiency of nucleotide flipping were made to the DNA intercalating Tyr-162 residue of AAG. Second, hydrogen bonding interactions within the damaged base pair were removed by substitution of the
**AAG DNA-intercalating Mutants**

**Experimental Procedures**

Oligonucleotides—Synthetic oligonucleotides were made on an Applied Biosystems 392 DNA synthesizer using standard β-cyanoethylphosphoramidite chemistry and reagents from Glen Research (Sterling, VA). Oligonucleotides were purified by denaturing PAGE. Concentrations of purified single-stranded oligonucleotides were determined from absorbances measured at 260 nm using extinction coefficients calculated for each oligonucleotide at 260 nm (25). The extinction coefficient used for 4A was 50000 x 1 cm⁻¹ (extinction coefficient for 1N'-ethenoadenosine (24)), and extinction coefficients for 4A dinucleotides were estimated to be the average of mononucleotide extinction coefficients. Extinction coefficients for A and A dinucleotides were used for Hx-containing oligonucleotides. All oligonucleotides were 25 nt in length and of identical sequence (5'-GGGCAAAATGCTGCGATATTCATG-3') except for the central damaged base (D). Duplex DNA substrates were made by annealing labeled oligonucleotides to an equal concentration of unlabeled complementary oligonucleotide. 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.

Cloning AAG cDNA—cDNA was isolated from near-confluent monolayers of human foreskin fibroblast cells (American Type Culture Collection) as described by Jarman et al. (25). This RNA was used to prepare cDNA using the cdNA cycle kit (Invitrogen). The cDNA encoding AAG was amplified by PCR in reactions containing human foreskin fibroblast cDNA, 1.25 mm dNTPs, 600 ng of each primer (LD1, 5' CGA ATT CGT GGT GTT GCC TCA TAA CCC ACA 3'; LD2, 5' CGA ATT CAA TTC TGT CGG CAC GCC CTT TG 3'), and 2.5 units of Ampli-Taq polymerase (Applied Biosystems, Foster City, CA) in a total volume of 50 μl of buffer containing 1.5 mm Tris-HCl, pH 8.8, 16.6 mm ammonium sulfate, 0.17 mg/ml bovine serum albumin, and 5 mm MgCl₂. Following this initial amplification, products were purified using a QiAquick PCR purification kit (Qiagen Inc., Valencia, CA) and PCR-amplified a second time. Products from several amplification reactions were made in the coding sequence of this truncated gene using the Transformer Site-directed mutagenesis kit (BD PharMingen and CLONTECH, Palo Alto, CA). The primers used to generate the desired mutations were also engineered to contain silent mutations that created restriction sites to facilitate screening of clones.

**Enzyme Expression and Purification**—The coding sequence for each AAG mutant was cloned into a pET-14b expression vector (Novagen, Madison, WI). Proteins were expressed in E. coli BL21(DE3) cells and purified as described previously (5).

**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 AAG379 or mutants with a DNA substrate at 37°C in 50 mm HEPES, pH 8.0, 100 mm NaCl, 10 mm EDTA, 0.25 mm DTT, and 9.5% v/v glycerol. Typical reaction mixtures contained 400–1600 mm AAG379 and 50 mm duplex DNA. At several time points during the course of excision reactions, an aliquot of the reaction mixture was quenched in 0.2 n 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 consisting of 95% formamide and 20 mm EDTA. Unreacted substrates were separated from cleaved products by electrophoresis on 12% denaturing polyacrylamide gels and quantitated using an Amersham Biosciences 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 with ³²P and annealed to a complementary strand containing either T or F (difluorotoluene) opposite the damaged base. Labeled oligonucleotides (50 nM) were incubated with increasing concentrations of AAG379 for 10 min at 4°C, diluted with loading buffer, and loaded directly onto a 6% nondenaturing polyacrylamide gel. PAGE was performed at 4°C for 180 min at 8 V/cm. The EMSA buffer was identical to the buffer used in excision assays and contained 50 mm HEPES, pH 8.0, 100 mm NaCl, 10 mm EDTA, 0.25 mm DTT, and 9.5% v/v glycerol. The fraction of DNA bound by AAG was quantitated using an Amersham Biosciences Storm PhosphorImager and ImageQuant software.

**Results**

**Wild Type AAG and Mutants**—The 298-amino acid coding sequence for AAG was amplified by PCR from cDNA made from human foreskin fibroblast cells as described under the “Experimental Procedures.” To improve yields of soluble protein when expressed in E. coli, a deletion mutant, AAGΔ79, missing the first 79 amino acids from the N terminus was constructed (5). Deletion of this unconserved N-terminal domain does not affect the base excision activity of the enzyme (5, 26), and all site-directed mutations were made in the AAGΔ79 protein. Mutation of Glu-125 to Gln creates a catalytically inactive mutant (E125Q) with undetectable base excision activity but has no effect on DNA binding activity (20). To assess the contribution of the DNA-intercalating Tyr-162 residue to the base excision activity of AAG, Tyr-162 was converted to Ser and Phe by site-directed mutagenesis to generate two mutant proteins, Y162S and Y162F, respectively. A catalytically inactive double mutant, Y162F/E125Q, was made for DNA binding experiments.

**Base Excision and DNA Binding Activities of the Y162S Mutant**—Converting the Tyr-162 residue to Ser removes the aromatic ring generating a smaller amino acid side chain that should not be able to penetrate the DNA helix as deeply when intercalated. Base excision activity for the Y162S mutant was measured in a chemical cleavage/gel assay for DNA substrates where damaged bases were located at nucleotide 13 of oligonucleotides 25 nucleotides in length. The strand containing the damage was end-labeled with ³²P prior to annealing to its complementary strand to create duplexes of otherwise identical sequences that contained Hx-T and eA-T base pairs. In 60-min assays using 1600 nM Y162S and 50 nM DNA substrate, no detectable base excision was observed for either DNA sub-

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strate. We estimate that the Y162S mutant is at least 1000-fold less active than the AAGΔ79 enzyme based on this result and using the conservative assumption that 1 ns product (2% reaction) would have been detected if formed in these assays.

The DNA binding activity of the Y162S mutant was measured in EMSAs with the same damaged duplexes as used in excision assays, where the damage-containing DNA strand was 5′-end-labeled with 32P. A damage-specific protein-DNA complex was not observed for the Y162S mutant with DNA substrates containing Hx or εA opposite T (Fig. 2). At high Y162S concentrations in EMSAs, a general smearing of the DNA band was observed in a pattern similar to that for AAGΔ79 with undamaged DNA (not shown). This smearing may represent weaker damage-independent DNA binding.

**Base Excision by the Y162F Mutant**—Mutation of Tyr-162 to Phe removes the hydroxyl group but leaves the aromatic ring intact to intercalate into the DNA base stack. Single turnover kinetics of excision of Hx when paired with T were measured in a chemical cleavage/gel assay for both AAGΔ79 and the Y162F mutant. Enzymes, at concentrations of 400, 800, and 1600 nM, in two separate experiments at each concentration were incubated with 50 nM 32P-labeled 25-nt duplex DNA substrates containing Hx or εA opposite T (Fig. 2). At high Y162S concentrations in EMSAs, a general smearing of the DNA band was observed in a pattern similar to that for AAGΔ79 with undamaged DNA (not shown). This smearing may represent weaker damage-independent DNA binding.

**Table 1**

| Enzyme | Base pair | k_{obs} | k_{rel} |
|--------|-----------|---------|---------|
| AAGΔ79 | Hx-T | 0.62 ± 0.19 | 4.1 |
| Hx-F  | 1.3 ± 0.1 | 2.5 |
| εA-T  | 0.062 ± 0.003 | 1.7 |
| εA-F  | 0.052 ± 0.005 | 1.2 |
| Y162F  | Hx-T | 0.15 ± 0.03 | 1.5 |
| Hx-F  | 0.52 ± 0.13 | 2.0 |
| εA-T  | 0.037 ± 0.001 | 1.7 |
| εA-F  | 0.042 ± 0.002 | 1.7 |

Values for k_{obs} were calculated from single exponential fits to individual experiments. For Hx base pairs, two independent experiments were done at enzyme concentrations of 400, 800, and 1600 nM, and average k_{obs} values and S.D. are reported for all six experiments. For εA pairs, two independent experiments were done at 400 and 800 nM enzyme, and average k_{obs} values and S.D. are reported for the four experiments.

Relative values, k_{rel}, were calculated from the ratio of the k_{obs} for wt to Y162F for each base pair.

Because AAG catalyzes excision of a structurally diverse group of damaged purine bases, the possibility that the Y162F mutation may have differential effects on excision of different damaged bases was tested. Kinetics of excision of the structurally dissimilar 1,N4-ethenoadenine placed opposite T were measured in single turnover assays containing 400 and 800 nM enzyme. For each enzyme, observed rates were the same at both enzyme concentrations. The Y162F mutation had a smaller effect on the single turnover excision rate for εA where AAGΔ79 was 1.7-fold faster than the Y162F mutant (Table 1).

**DNA Binding Activity of the Y162F Mutant**—Mutation of the Tyr-162 residue to Phe also reduces the DNA binding activity measured in EMSAs. For EMSAs, 50 nM 32P-labeled duplex DNA substrates, identical to those used in excision assays, were incubated with increasing concentrations of the Y162F mutant for DNA containing a Hx-T pair (Fig. 3). The ratio of the k_{obs} for wt to Y162F was reduced relative to E125Q (Fig. 3, A and B, upper panels). A concentration of 50 nM Y162F/E125Q was needed to form a similar fraction of enzyme-DNA complex as seen with 20 nM E125Q. At concentrations of 400 nM enzyme, about 70% of the DNA is bound by E125Q whereas about 25% is bound by Y162F. As reported previously (20), E125Q binds to DNA containing an εA-T pair with greater affinity than an Hx-T pair (Fig. 3A and Fig. 4A, upper panels). This is also true for the Y162F/E125Q mutant (Fig. 3B and Fig. 4B, upper panels). The Y162F/E125Q mutant binds DNA containing an εA-T pair more weakly than E125Q as it takes 20 nM Y162F/E125Q to form about the same concentration of enzyme-DNA complex as 10 nM E125Q.

**Effects of Hydrogen Bonding within a Base Pair on Excision**—To determine whether rates of excision of Hx would increase by making the base easier to displace from the helix,
the T opposite Hx was replaced by difluorotoluene (F), which does not form hydrogen bonds with Hx (Fig. 1). Single turnover kinetics of excision of Hx opposite F were measured in the chemical cleavage/gel assay with 50 nM DNA and 400, 800, and 1600 nM enzyme (Table I). Excision rates were not dependent on enzyme concentration for either AAG/H900479 or Y162F. Excision activities for both AAG/H900479 and the Y162F mutant increased on the Hx/H18528F DNA substrate relative to the Hx/H18528T duplex (data for 400 nM enzyme are shown in Fig. 5, A and B). The magnitude of the increase was greater for the Y162F mutant (3.5-fold) than for AAG/H900479 (2-fold).

It is possible that the increased excision activity could be due to some effect of replacing T with F other than removing hydrogen bonding interactions. To rule out this possibility, excision was also measured for DNA substrates containing eA-T and eA-F base pairs. eA does not form Watson-Crick-type hydrogen bonding interactions with either T or F. Single turnover kinetics of excision of eA opposite F were measured in chemical cleavage/gel assays using 50 nM DNA and 400 and 800 nM enzyme in separate experiments (Table I). There was not a significant effect on excision rates of eA, as a 1.2-fold decrease in the excision rate for AAG/H900479 and a 1.1-fold increase for the Y162F mutant were observed (Fig. 5C).

Effects of Hydrogen Bonding within a Base Pair on DNA Binding—To determine what effect substitution of T with F would have on the DNA binding activity of AAG, EMSAs were...
done for DNA substrates containing HxF and eA-F pairs. Binding assays contained 50 nM 32P-labeled duplex DNA and increasing concentrations of AAG E125Q or Y162F/E125Q (10–800 nM). For both enzymes, binding was similar for DNA duplexes containing HxT and HxF pairs, and binding was slightly enhanced on duplexes containing eA-F in comparison with eA-T (Figs. 3 and 4).

**DISCUSSION**

The ability of DNA glycosylases to identify and excise damaged DNA bases is key to the overall success of base excision repair. Structural studies of AAG and other DNA glycosylases have revealed that DNA glycosylases use a base or nucleotide flipping mechanism for damaged base recognition and excision. Even though structural data for DNA glycosylases bound to damaged DNA indicate that they use a nucleotide flipping mechanism, many questions remain about how nucleotide flipping enables DNA glycosylases to discriminate between damaged and undamaged bases. This is particularly true for AAG, which is capable of exciting a structurally diverse group of damaged bases including 3-methyladenine, 1-N6-ethenoadenine, hypoxanthine, and 7-methylguanine (10–18). A mechanism for damaged base specificity that depends solely on specific interactions between enzyme binding pocket residues and functional groups on a damaged base seems unlikely for AAG. It is possible that the substrate specificity of AAG depends at least in part on the ability of AAG to flip damaged nucleotides out of the helix. If this were true then mutations to AAG that impaired its ability to flip damaged nucleotides would decrease the efficiency of base excision by AAG and changes in a DNA substrate that decreased the stability of a damaged base in the helix would increase the efficiency of base excision.

In this study, the DNA-intercalating Tyr-162 residue of AAG was converted to serine (Y162S) and phenylalanine (Y162F) by site-directed mutagenesis. A decrease in the base excision activities of both mutants was observed as expected if the Tyr-162 residue contributed to nucleotide flipping by helping to push the damaged base from the helix. Base excision and DNA binding activities of the Y162S mutant were reduced to undetectable levels for DNA substrates containing Hx-T and eA-T pairs, indicating that this mutant must be at least 1000-fold less active than AAGΔ79. The fact that DNA binding activity of the Y162S mutant was not detectable by EMSA suggests that the enzyme-DNA complex seen for AAGΔ79 is a nucleotide flipped complex.

A similar mutation in UDG converting the DNA-intercalating Leu residue to Ala resulted in an 8–80-fold decrease in excision activity, and mutation of Leu to Gly reduced UDG’s excision activity by a factor of 100–600 (27, 28). The comparatively large effect of the Y162S mutation on AAG’s activity may reflect a greater contribution of the DNA-intercalating residue to the activity of AAG than UDG. It has been proposed that UDG uses steric compression or “pinching” of the sugar-phosphate backbone to destabilize the damaged base within the helix and assist in nucleotide flipping (29, 30). This pinching may not make as great a contribution to the activity of AAG as AAG-DNA structures do not show the degree of backbone compression seen in UDG-DNA structures.

Mutation of Tyr-162 to Phe leaves the aromatic ring to intercalate in DNA but removes the hydroxyl group from the aromatic ring. This mutation decreases the size of the DNA-intercalating residue much less than the Ser mutation but still affects the excision activity of the enzyme. Excision of Hx when paired with T by the Y162F mutant is 4 times slower than excision by AAGΔ79, and excision of eA paired with T is 1.7 times slower. Interestingly, the activity of the Y162F mutant is “rescued” on a DNA substrate where Hx is paired with F. The excision rate for the Y162F mutant increases to the rate measured for AAGΔ79 excision of Hx paired with T. It is possible that making Hx easier to flip in the context of an HxF pair counterbalances a deficiency in the flipping ability of the Y162F mutant. An alternative explanation for the effect of the Y162F mutation on the excision activity of AAG is that the slightly smaller Phe residue is not able to “push” the displaced base as far into the enzyme binding pocket to align it properly for catalysis. If this were true then no difference in excision rates for Hx when paired with T and F would have been seen because the Phe mutant would have “pushed” Hx the same distance in both cases.

The rationale for replacing T with F in Hx base pairs was that F is isosteric with T having the same overall shape but will not form hydrogen bonds with Hx. The expectation was that the lack of hydrogen bonding will increase the ease of flipping
Hx by decreasing the stability of the base pair. To rule out the possibility that F could have some other unanticipated effect on excision activity, excision of εA was measured when paired with T and F where neither pair forms hydrogen bonding interactions. Substitution of T with F had no significant effect on excision rates of εA for either AAGΔ79 or the Y162F mutant, whereas it increased the excision rate of Hx by a factor of 2 for AAGΔ79 and about 3 to 4 for the Y162F mutant. Thus, the increase in Hx excision rates is likely to be due to changes in hydrogen bonding interactions in the Hx pair. These results are consistent with a model where the ease of flipping a damaged base contributes to the base pair specificity of AAG; however, the ease of flipping is not the only criterion by which AAG selects damaged bases for excision as neither A nor G were excised when paired with F (data not shown).

The kinetic mechanism for base excision by AAG is likely to contain a nucleotide flipping step in addition to the chemistry step where base excision occurs. Changing the ease of nucleotide flipping either by mutations to the enzyme or by changes to the stability of a damaged base within the helix would not affect single turnover excision rates unless nucleotide flipping were rate-limiting. The observation that substitution of T with F increases single turnover excision rates of Hx for both AAGΔ79 and the Y162F mutant suggests that nucleotide flipping is rate-limiting for Hx excision. A rate-limiting nucleotide flipping step for Hx excision would also explain the base pair specificity observed previously (16, 20). Hypoxanthine is excised more slowly from a more stable Hx-C Watson-Crick type pair than an Hx-T wobble pair (31–33). Two explanations are possible to explain why excision of εA is not affected to a great degree by its base pairing partner. Either the nucleotide flipping step may not be rate-limiting for εA excision or nucleotide flipping may be rate-limiting, but is not affected by the base pairing partner because εA lacks hydrogen bonding interactions with its partner. For UDG, the chemistry step, not the flipping step, was found to be rate-limiting for excision of uracil under single turnover conditions (7, 34).

Based on the results of this paper and previous work, we have developed a working model that explains the damaged base and base pair specificity of AAG. We propose that the specificity of base excision by AAG is governed by two important selection steps, nucleotide flipping and proper fit of the damaged base in the enzyme active site. The enzyme may use the ease of flipping a damaged base as the initial criterion for discriminating between damaged and undamaged bases and then use fit of the damaged base in the active site as a final check. The first nucleotide flipping selection step would be affected by changes in local DNA sequence or structure that affect the stability of a damaged base within the helix. Once a damaged base is flipped, it still must be aligned properly in the active site for hydrolysis of the glycosyl bond to occur. This second criterion, proper fit in the active site, would explain why Hx but not G is excised from a wobble-type base pair with T (20). The 2-amino group may prevent G from fitting in the active site properly (6). An implication of this two-step selection is that the overall efficiency of base excision repair may be a function of local DNA sequence and structure which affect the stability of damaged bases in the helix. A dependence of the efficiency of base excision on DNA sequence and structure could contribute to the formation of mutational “hot spots” and “cold spots.” Both AAG DNA-intercalating or pushing mutants and the difluoroalene base pairing partner will be useful tools for testing this model further.

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