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DNA-*N*-Glycosylases Process Novel *O*-Glycosidic Sites in DNA†

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Abstract

After hydrolyzing the *N*-glycosyl bond between a damaged base and C1' of a deoxyribosyl moiety of DNA, human alkyladenine DNA glycosylase (AAG) and *E. coli* 3-methyladenine DNA glycosylase II (AlkA) bind tightly to their abasic DNA products, potentially protecting these reactive species. Here we show that both AAG and AlkA catalyze reactions between bound abasic DNA and small, primary alcohols to form novel DNA-*O*-glycosides. The synthesis reactions are reversible, as the DNA-*O*-glycosides are converted back into abasic DNA when incubated with AAG or AlkA in the absence of alcohol. AAG and AlkA are therefore able to hydrolyze *O*-glycosidic bonds in addition to *N*-glycosyl bonds. The newly discovered DNA-*O*-glycosidase activities of both enzymes compare favorably with their known DNA-*N*-glycosylase activities: AAG removes both methanol and 1,N⁶-ethenoadenine (εA) from DNA with single turnover rate constants that are 2.9×10⁵-fold greater than the corresponding uncatalyzed rates, whereas the rate enhancement of 3.7×10⁷ for removal of methanol from DNA by AlkA is 300-fold greater than its rate enhancement for removal of εA from DNA. Although the biological significance of the DNA-*O*-glycosidase reactions is not known, the evolution of new DNA repair pathways may be aided by enzymes that practice catalytic promiscuity, such as these two unrelated DNA glycosylases.

An abasic site occurs within DNA when the *N*-glycosyl bond between a nucleobase and deoxyribose is hydrolyzed. This scission is promoted when the *N*-glycosyl bond is destabilized by nearby chemical modifications, such as alkylation of the nucleobase by alkylating agents.¹ Abasic sites are formed enzymatically by DNA glycosylases that remove specific damaged bases, and subsequent enzymes of the base excision repair (BER) pathway ultimately restore the original DNA sequence by replacing the deoxyribosyl phosphate moiety with the nucleotide that is complementary to the unpaired opposing nucleotide. Unrepaired abasic lesions can interfere with DNA replication, transcription, and topoisomerase activity, resulting in mutagenesis or toxicity.² The abasic site can also access an unstable open-chain aldehyde form that readily cross-links with amines or undergoes β-elimination accompanied by DNA strand breakage.³⁻⁵

Many DNA glycosylases, including AAG and AlkA, bind tightly to their abasic DNA products.⁶⁻¹² Such tight binding has been proposed to shield this reactive DNA intermediate from side reactions until it is further processed by downstream enzymes of the BER pathway.² We explored the effect of binding to AAG and AlkA on the reactivity of abasic DNA by exposing glycosylase-abasic DNA complexes to small alcohols. Remarkably, both

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Supporting Information Available

Figures S1-S6 and a calculation to estimate the frequency of formation of DNA-*O*-glycosides in human cells, as described in the text, are available free of charge via the Internet at <http://pubs.acs.org>.

DNA glycosylases catalyzed the formation of alcohol-DNA adducts. The adducts contain an *O*-glycosidic linkage between a hydroxyl group of the alcohol and C1' of the deoxyribose, based on their resistance to alkaline hydrolysis. These results reveal that the reactive environments of their active sites provide AAG and AlkA with the capacity for *O*-glycosidic bond synthesis and highlight the susceptibility of abasic DNA to damage, even when glycosylase-bound.

The DNA-*O*-glycosides synthesized by AAG and AlkA were tested as substrates for breakdown by AAG and AlkA. Alcohols were efficiently excised from the DNA-*O*-glycosides by both enzymes, extending the known substrate range of DNA glycosylases to include sites of DNA damage with alcohol substitutions at C1' of deoxyribose.

Experimental Procedures

Chemicals

Glycerol, ethanol, methanol, 2-propanol, 1-propanol, ethylene glycol, 1,2-propanediol, 1,3-propanediol, and 1,N⁶-ethenoadenine (eA) were from Sigma-Aldrich.

Purification of recombinant proteins

Wild-type and E125Q mutant forms of truncated human AAG lacking the first 79 amino acids were expressed in *E. coli* and purified as previously described.¹³ Full-length *E. coli* AlkA was produced as a C-terminal 6×His-tagged protein from a modified pET24 vector that encoded a TEV cleavage site. D238N AlkA was generated by site-directed mutagenesis. Both wild-type and D238N mutant AlkA proteins were purified with NTA-Ni²⁺, the tags were removed with TEV protease, and the proteins were further purified using source S cation exchange. Peak fractions were dialyzed into storage buffer containing 50 mM NaHEPES (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, and 1 mM DTT and stored at -80 °C.

Preparation of oligonucleotides

The 25mer duplex ab-DNA substrate was prepared by combining equal concentrations of 5'-(FAM)-CGATAGCATCCT**ab**CCTTCTCTCCAT-3', an oligonucleotide with a centrally located abasic site (**ab**) and a 5'-fluorescein (**FAM**) label, and its complement, 5'-ATGGAGAGAAGGTAGGATGCTATCG-3'. The unlabeled oligonucleotide was synthesized by IDT and the labeled oligonucleotide by the Keck Center at Yale University. Standard protecting groups were used in both syntheses, and deprotection was carried out according to the manufacturer's recommendations (Glen Research), with one exception. The lesion-containing oligonucleotide was initially supplied with an *O*-tert-butyldimethylsilyl (*O*-TBDMS) protecting group on C1' of its central deoxyribosyl group. Both oligonucleotides were desalted using Sephadex G-25 and purified using denaturing polyacrylamide gel electrophoresis as previously described.¹⁴ The *O*-TBDMS protecting group was subsequently removed in 80% acetic acid:20% water; after neutralization the deprotected oligonucleotide was desalted using Sephadex G-25. Denaturing polyacrylamide gel electrophoresis of the deprotected oligonucleotide that had been subjected to alkaline hydrolysis at 70 °C showed that ~98% of the 25mer was cleaved into 12mer, consistent with deprotection of the central deoxyribosyl group to an abasic site. The concentrations of the oligonucleotide containing an abasic site and its complement were determined from their absorbances at 260 nm using calculated extinction coefficients prior to annealing. Preliminary experiments with ab-DNA prepared conventionally, via treatment of a uracil-containing oligonucleotide with uracil DNA glycosylase,¹⁵ gave the same results as those performed with the ab-DNA described above. The authentic 25mer duplex edA-DNA was prepared as previously described.¹⁵

Preparation and isolation of alcohol-DNA adducts for study of their breakdown by AlkA and AAG

Preparative reactions contained 50 mM NaMES (pH 6.5), 100 mM NaCl, 0.1 mg/mL BSA, 1 mM EDTA, 1 mM TCEP, 2.5 M alcohol, 0.5 μ M ab-DNA, and 2 μ M AlkA or AAG in 100 μ L volumes. Reactions to prepare propanol-DNA contained 1 M propanol, since higher concentrations of propanol inactivated both AlkA and AAG. Control reactions without alcohol were performed and processed alongside reactions that contained alcohol. Reactions were incubated at 37 °C for 4 hrs (AlkA reactions) or 20-48 hrs (AAG reactions). DNA was isolated from the remaining reaction components by phenol-chloroform extraction followed by gel filtration on MicroSpin G-25 columns (GE Healthcare). The alcohol-DNA adducts are also stable to standard ethanol precipitation conditions and can be isolated using this method.¹⁶ Since the alcohol reactions proceeded to concentration-dependent end points rather than to completion, each final preparation consisted of 5-20% alcohol-DNA and 80-95% unreacted ab-DNA.

Synthesis of N-glycosyl and O-glycosidic linkages within DNA by AAG (for Figure 1)

Reactions containing 50 mM NaMES (pH 6.5), 100 mM NaCl, 0.1 mg/mL BSA, 1 mM EDTA, 1 mM TCEP, 2 μ M AAG, and 0.5 μ M ab-DNA were supplemented with 5 mM eA, 2.5 M glycerol, or no additive, and incubated at 37 °C for 20 hrs. A control reaction containing 2.5 M glycerol and all other reaction components except AAG was also performed. Samples were quenched with sodium hydroxide to give a final concentration of 0.2 M. The quenched samples were heated at 70 °C for 10 min to quantitatively cleave abasic sites and subsequently mixed with an equal volume of formamide/EDTA loading buffer. All samples were loaded and separated on a 20% (w/v) polyacrylamide sequencing gel containing 6.6 M urea. The gel was scanned using a Typhoon Trio imager (GE Healthcare), and emission was measured with a 520BP40 filter following excitation of the fluorescein label at 488 nm.

Single turnover alcohol-DNA synthesis and breakdown assays for AAG and AlkA

Reactions were carried out at 37 °C in 50 mM NaMES (pH 6.5), 100 mM NaCl, 0.1 mg/mL BSA, 1 mM EDTA, and 1 mM TCEP. Typical reactions contained 10-100 nM DNA (ab-DNA or an alcohol-DNA adduct), 0.2-1 μ M enzyme (AAG or AlkA), and 0-2.5 M alcohol; the observed rate constants did not change when the enzyme concentration was increased, confirming that single turnover kinetics were monitored under these conditions. Reactions were initiated by adding a small volume of enzyme to the remaining reaction components in a final volume of 20-40 μ L. Aliquots were withdrawn at various times and quenched with sodium hydroxide to give a final concentration of 0.2 M. The quenched samples were heated at 70 °C for 10 min to quantitatively cleave abasic sites. Samples were mixed with an equal volume of formamide/EDTA loading buffer and run on 15% (w/v) polyacrylamide sequencing gels containing 6.6 M urea. Gels were scanned using a Typhoon Trio imager (GE Healthcare), and emission was measured with a 520BP40 filter following excitation of the fluorescein label at 488 nm. Fluorescence intensities of gel bands were quantified using ImageQuant TL (GE Healthcare) and corrected for the amount of background signal. The data were converted to fraction alcohol-DNA (fraction alcohol-DNA = [alcohol-DNA]/([alcohol-DNA] + [ab-DNA])) and then fit by a single exponential. For reactions of E•ab-DNA and alcohol, the single exponential included a zero point of 0.02, to reflect the amount of nonhydrolyzable DNA in reactions without alcohol, and an end point term: fraction alcohol-DNA = end point(1-exp(- k_{obs} t) + 0.02, where k_{obs} is the observed rate constant and t is time. For reactions of E•alcohol-DNA, the single exponential included a term representing the initial fraction of alcohol-DNA in each preparation, and an end point term, to reflect the amount of nonhydrolyzable DNA: fraction alcohol-DNA = (fraction alcohol-DNA)_{init}(exp(-

$k_{\text{obs}}t$)) + end point, where k_{obs} is the observed rate constant and t is time. In all cases, the nonlinear least-squares fit was good ($R > 0.98$).

Results

Glycosylase-catalyzed reactions of abasic DNA with alcohols

In previous work we showed that AAG catalyzes formation of an *N*-glycosyl linkage between ϵ A and DNA containing an abasic site.¹⁵ The 25mer ab-DNA undergoes β,δ -elimination at its abasic position and is converted to a 12mer when subjected to alkaline hydrolysis, whereas DNA that has covalently incorporated ϵ A (ϵ dA-DNA) remains stable (Figure 1, lanes 1-3). By analogy, we reasoned that it may be possible to identify novel substrates for AAG by incubating it with ab-DNA and small molecules that are not known to be excised from DNA by AAG; the accumulation of DNA adducts that are stable to alkaline hydrolysis would provide evidence for AAG-dependent covalent incorporation of such molecules into DNA at C1' of the base-free deoxyribosyl moiety. When AAG was incubated with ab-DNA in the presence of glycerol, a base-stable adduct was observed (Figure 1, lane 4). The adduct did not form when AAG was omitted from the reaction (Figure 1, lane 5), and the amount of adduct that formed increased as the concentration of glycerol in the synthesis reaction was increased (Figure S1).

The simplest model for formation of the glycerol-DNA species is that glycerol can occupy the nucleobase binding pocket of AAG when ab-DNA is bound in the active site. Although the anomeric form of ab-DNA that binds to AAG is unknown, it is widely assumed that the *N*-glycosylase activity of AAG entails direct displacement of a damaged base by water to initially form the α -anomer. Nucleophilic attack by glycerol on the α -anomer of ab-DNA would result in a β -anomeric cyclic acetal, which contains an *O*-glycosidic linkage between C1' of the deoxyribosyl moiety and a hydroxyl group of glycerol (Figure 2). The stability of the glycerol-DNA adduct to alkaline hydrolysis (Figure 1, lane 4) is consistent with this chemical linkage, since model conjugates of ADP-ribose containing a similar acetal linkage between C1' of the ribosyl moiety and hydroxyl groups of small alcohols are known to be base-stable.^{17, 18} The attacking hydroxyl group is likely to be one of the two equivalent primary hydroxyl groups of glycerol, since 1-propanol but not 2-propanol reacts similarly to glycerol (see below). To the best of our knowledge, the conversion of ab-DNA and glycerol to RO-DNA by AAG represents the first example of *O*-glycosidic bond synthesis by a DNA-*N*-glycosylase. The AAG-catalyzed reaction shown in Figure 2 is reminiscent of the *N*-glycosyl bond synthesis reaction that AAG catalyzes between ab-DNA and the nucleobase ϵ A to form ϵ dA-DNA.¹⁵

To explore the generality of *O*-glycosidic bond synthesis by DNA-*N*-glycosylases, we surveyed the effects of various alcohols in reactions of AAG or the structurally unrelated AlkA. Both glycosylases formed DNA adducts between ab-DNA and methanol, ethanol, 1-propanol, ethylene glycol, 1,2-propanediol, 1,3-propanediol or glycerol (alcohol structures shown in Figure 2). The DNA adduct made in reactions containing methanol, the smallest alcohol, could be resolved from the DNA adduct made in reactions containing glycerol, the largest alcohol, by denaturing polyacrylamide gel electrophoresis, directly demonstrating that AAG and AlkA can incorporate different alcohols into abasic DNA (Figure S2). The amount of alcohol-DNA increased with time for each combination of enzyme and alcohol, as shown for the AlkA-catalyzed reaction between glycerol and ab-DNA in Figure 3A. Neither enzyme formed a DNA adduct between ab-DNA and the secondary alcohol 2-propanol, even though its size and intrinsic reactivity are comparable to the other alcohols, suggesting that the flexibility of primary alcohols is an important factor for the synthesis reactions.

The AlkA-catalyzed syntheses were significantly faster and resulted in higher product yields than the AAG-catalyzed syntheses, so the dependence of each AlkA reaction on alcohol concentration was investigated. As shown for the glycerol reaction in Figure 3B and for the other alcohols in Figure S3, reactions of AlkA•ab-DNA and alcohols proceeded to higher end points as the concentration of alcohol increased. These end points appear to represent internal equilibria¹⁹ between the AlkA-bound ab-DNA (AlkA•ab-DNA) and the AlkA-bound alcohol-DNA (AlkA•alcohol-DNA), since addition of more AlkA after the apparent internal equilibrium is established has no effect on the amount of alcohol-DNA present (Figure S4), whereas dilution of alcohol decreases the amount of alcohol-DNA present (Figure S5).

The ratios of alcohol-DNA to ab-DNA at the end points of reactions of AlkA•ab-DNA and alcohols are plotted as a function of the alcohol to water ratios in Figure 3C, where the identity reaction of water is shown as a dashed line with a slope of 1. Saturation of the alcohol reactions with respect to the end point is not observed, suggesting that AlkA lacks well-defined binding sites for the alcohols, and preventing the values for K_{int} , the internal equilibrium constants, from being obtained. Therefore, the maximal ratio of alcohol-DNA to ab-DNA shown in Figure 3C for each alcohol is a lower limit for the value of K_{int} for reaction of that alcohol. Interestingly, there is an apparent clustering of the slopes for the end point concentration dependencies that correlates with the structures of the alcohols. Slopes close to the identity slope of 1 are observed for methanol and ethanol, which are small, monohydroxylic alcohols with pK_{a} values similar to the pK_{a} of water. The propanol and 1,2-propanediol reactions result in a slope near 2, suggesting that these slightly larger molecules may be more favorably positioned for reaction with ab-DNA than are the small nucleophiles. The even steeper slopes of ~3 for the reactions of ethylene glycol, 1,3-propanediol, and glycerol may reflect both a size advantage and a statistical advantage arising from their equivalent primary hydroxyl groups.

Alcohol-DNA adducts are substrates for AAG and AlkA

The alcohol-DNA adducts synthesized by AlkA and AAG are novel DNA-*O*-glycosides and their reactions are expected to be reversible. Therefore, they were tested as substrates for these DNA-*N*-glycosylases. The complete set of seven alcohol-DNA adducts was synthesized preparatively in reactions containing AlkA, ab-DNA, and alcohols. AlkA and the alcohols were subsequently removed from the preparations by phenol-chloroform extraction and gel filtration, respectively. Glycerol-DNA was also prepared using AAG, in order to address the question of whether or not the adducts made by AlkA and AAG are identical.

The amount of alcohol-DNA decreased with time for each combination of wild-type AAG and alcohol-DNA adduct, as shown for a representative reaction of glycerol-DNA (Figure 4A, left). However, no conversion of glycerol-DNA into ab-DNA by the active site mutant E125Q AAG, which lacks the general base that is required for the *N*-glycosylase reaction,^{13, 20, 21} could be detected under the same conditions (Figure 4A, right). This result confirms that wild-type AAG, not a contaminating enzyme, is responsible for the DNA-*O*-glycosidase activity, since E125Q AAG prepared and tested in the same way has no activity. The representative reactions of glycerol-DNA were quantified, and the resulting data points were combined with data from equivalent reactions in Figure 4B. The kinetics of glycerol-DNA breakdown are monophasic, suggesting that the adduct is a single species, and wild-type AAG converts it into ab-DNA with a rate constant of 0.0029 min^{-1} . The presence of multiple species that react with identical rate constants is unlikely but cannot be ruled out. Analogous results were obtained for the other alcohol-DNA adducts (Figure S6), and the single turnover rate constants for their breakdown by AAG are summarized in Table 1.

Like AAG, wild-type AlkA excises alcohols from glycerol-DNA (Figure 5) and the other alcohol-DNA adducts (Figure S6). The active site mutant D238N AlkA, which lacks the general base that is important for the *N*-glycosylase reaction,^{22, 23} has little or no activity (Figure 5A, right), indicating that the observed DNA-*O*-glycosidase activity belongs to wild-type AlkA, not to a contaminant. The kinetics of alcohol-DNA breakdown by AlkA are monophasic (Figures 5B, S6), as observed for AAG (see above), providing additional evidence that each alcohol adduct consists of a single species. The presence of multiple species that react with identical rate constants is unlikely but cannot be ruled out. The single turnover rate constants for the breakdown of alcohol-DNA substrates by AlkA range from 0.21-0.83 min⁻¹ and are 30-150-fold larger than the corresponding rate constants for the AAG-catalyzed reactions (Table 1).

Glycerol-DNA prepared using AlkA was the substrate for the reactions shown in Figures 4 and 5. For comparison, glycerol-DNA was also prepared using AAG. The breakdown of glycerol-DNA from the two independent preparations was investigated with both AAG (Figure 6A) and AlkA (Figure 6B). The reaction of each enzyme with either preparation is indistinguishable, after normalization to account for the different initial percentages of glycerol-DNA in the two preparations (Figure 6 insets). This is strong evidence that AlkA and AAG synthesize identical glycerol-DNA species.

Discussion

Glycosylases accelerate the reaction of abasic DNA with alcohols

Our results reveal that binding to AAG or AlkA promotes the reaction of abasic DNA with alcohols to form DNA-*O*-glycosides. High concentrations of alcohols were used to facilitate detection of alcohol-DNA adducts using our gel-based assay, but analogous reactions to form and process related DNA-*O*-glycosides may be biologically relevant at much lower levels. Simple estimates indicate that formation of ~1 DNA-*O*-glycoside lesion per human cell per day is possible (see Supporting Information). Although DNA-*O*-glycosides are predicted to be rare in human cells, it is important to note that use of even a single such site as a template during replication of the genome has the potential to introduce a harmful mutation. This is because DNA-*O*-glycosides are expected to be miscoding, as are fragmented nucleotides in DNA.²⁴⁻²⁶

The reactions that AAG and AlkA catalyze between alcohols and C1' of the deoxyribosyl moiety of abasic DNA could yield DNA-*O*-glycosides that are β -anomeric cyclic acetals, α -anomeric cyclic acetals, or a combination of both configurations. However, both the synthesis and the breakdown reactions of the DNA-*O*-glycosides are monophasic, suggesting that only one of the two anomeric forms predominates. Attack of an alcohol at C1' of bound α -anomeric abasic DNA from within the nucleobase binding pocket would produce a β -anomeric DNA-*O*-glycoside, as depicted in Figure 2, whereas attack of an alcohol at C1' of bound β -anomeric abasic DNA would produce the α -anomeric alternative. We favor the former model, that AAG and AlkA synthesize the β -anomeric DNA-*O*-glycosides, because the *O*-glycosidic bonds of the predominant species are readily hydrolyzed by AAG and AlkA, which are both known to hydrolyze the *N*-glycosyl bonds of damaged β -anomeric nucleotides, but not those of α -anomeric nucleotides.

The AAG- and AlkA-catalyzed reactions of small alcohols with abasic DNA could be detected and characterized in this study because of the stability of the cyclic acetal products. It is highly likely that the same reaction that occurs between alcohols and abasic DNA within the glycosylase active sites also occurs with water, whereby water reacts with α -anomeric abasic DNA to form β -anomeric abasic DNA. However, this water reaction has

not been verified because the α - and β -anomeric cyclic hemiacetals are in equilibrium in solution (α : β , 60:40),²⁷ where they interconvert via a reactive aldehyde form.³

Although ours is the first report of *O*-glycosidic bond synthesis by DNA-*N*-glycosylases and is notable because the products of these reactions are potentially mutagenic DNA adducts, similar enzymatic reactions between alcohols and small molecule substrates have previously been observed. Several enzymes that hydrolyze the *N*-glycosyl bond between a nitrogenous base and a simple ribosyl- or deoxyribosyl-containing moiety can incorporate methanol instead of water at C1 of the sugar-containing product.²⁸⁻³⁷

Practical implications for DNA repair studies

The discovery that glycerol and other alcohols react with abasic DNA bound to AAG and AlkA has practical implications. AAG and AlkA are structurally unrelated, yet both enzymes catalyze the same reaction to form DNA-*O*-glycosides, so it seems likely that this reaction is also catalyzed by other DNA-*N*-glycosylases. Glycerol is frequently added to biochemical solutions to stabilize proteins, and glycerol or diols are often present at high concentrations during protein crystallography as precipitants or cryoprotectants. Indeed, several crystal structures of the DNA-*N*-glycosylase UDG show glycerol in the uracil binding site.³⁸⁻⁴⁰ Small alcohols should be used cautiously in studies of the BER pathway, given their potential to react with abasic DNA and thus complicate the interpretation of experimental results. Alcohol-DNA adducts are likely to be overlooked in routine assays because of their similarities to normal oligonucleotides that contain nitrogenous bases. For example, they are stable to treatments that cause strand cleavage at abasic sites, as are normal oligonucleotides, and they co-migrate with normal oligonucleotides on denaturing gels run under standard conditions.

Novel DNA-*O*-glycoside substrates for AlkA and AAG

Our results demonstrate that alcohols can be removed from DNA by the DNA glycosylases AlkA and AAG. The rate enhancements for *O*-glycoside hydrolysis by AlkA and AAG can be estimated by comparing the enzyme-catalyzed rate constants for release of methanol from methanol-DNA to the nonenzymatic rate constant for release of methanol from the model substrate 1-*O*-methyl-2-deoxy-D-ribose (Table 2).⁴¹ For AlkA, the rate enhancement of 3.7×10^7 is 300-fold greater than the rate enhancement for release of the damaged base *eA* from DNA. For AAG, the same rate enhancement of 2.9×10^5 is obtained for *O*-glycoside hydrolysis of methanol-DNA and for *N*-glycosyl hydrolysis of *edA*-DNA. These robust rate enhancements for *O*-glycoside hydrolysis likely reflect the fact that both AAG and AlkA have binding pockets that allow them to excise damaged nucleobases of varying sizes.^{23, 42} Therefore, they are able to accommodate the replacement of nucleobase leaving groups with alcohol leaving groups.

The E125Q AAG and D238N AlkA active site mutants lack the carboxylate group that their wild-type counterparts use as general bases to activate a water nucleophile, so the abilities of these mutants to hydrolyze the *N*-glycosyl bonds of damaged nucleotides are substantially reduced.^{13, 20-23} As described above, little or no conversion of glycerol-DNA into ab-DNA by E125Q AAG (Figure 4) or by D238N AlkA (Figure 5) could be detected under the same conditions in which the corresponding reactions catalyzed by the wild-type glycosylases had proceeded to completion. These results suggest that the same carboxylate side chains that activate water for AAG- and AlkA-catalyzed hydrolysis of *N*-glycosyl bonds also activate water for AAG- and AlkA-catalyzed hydrolysis of *O*-glycosidic bonds.

In addition to the hydrolysis of alcohol-DNA adducts by the monofunctional DNA glycosylases AAG and AlkA reported herein, slow cleavage of the β -anomer of a methanol-

DNA adduct by the bifunctional pyrimidine dimer DNA glycosylase from T4 bacteriophage has been detected.⁴³ Although the biological significance of this class of DNA-*O*-glycoside substrates is unknown, their breakdown by three unrelated DNA glycosylases highlights the capacity to develop novel DNA repair mechanisms in response to a changing environment. Many other DNA repair enzymes also exhibit catalytic promiscuity, as would be expected if new enzymatic activities have been recruited for DNA repair throughout evolution.⁴⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AAG	human alkyladenine DNA glycosylase (also known as MPG, methylpurine DNA glycosylase)
AlkA	3-methyladenine DNA glycosylase II
eA	1, <i>N</i> ⁶ -ethenoadenine
BER	base excision repair
BSA	bovine serum albumin

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Figure 1.

AAG synthesizes *N*-glycosyl and *O*-glycosidic linkages within DNA. The image shows a fluorescence scan of samples that were subjected to alkaline hydrolysis prior to separation on a 20% denaturing polyacrylamide gel. The original reactions were incubated at 37 °C for 20 hrs and contained 50 mM NaMES (pH 6.5), 100 mM NaCl, 0.1 mg/mL BSA, 1 mM EDTA, 1 mM TCEP, and 0.5 μM ab-DNA; the presence or absence of 2 μM AAG, 5 mM εA, and 2.5 M glycerol in the original reactions is indicated in the chart for samples in lanes 2-5. Lane 1 contains an authentic sample of the edA-DNA 25mer, which is slightly less mobile than the glycerol-DNA 25mer (lane 4) under these conditions. In the scheme, ab represents the abasic site, x is εA or glycerol, and * denotes the location of the 5'-fluorescein label. The fraction edA-DNA in lane 3 is 0.40, and the fraction glycerol-DNA in lane 4 is 0.08.

Figure 2.

Model for the reversible reaction of $\text{E}\cdot\text{ab-DNA}$ with an alcohol molecule (ROH) under single-turnover conditions, where all of the DNA present is bound to E, and E represents AAG or AlkA. Alcohols that have been shown to react in this study are boxed.

Figure 3.

Alcohol-DNA synthesis by AlkA. (A) The amount of glycerol-DNA 25mer increases with time in a representative reaction that contained 1 μ M AlkA, 100 nM ab-DNA, and 2.5 M glycerol. The reaction was performed at 37 °C in 50 mM NaMES (pH 6.5), 100 mM NaCl, 0.1 mg/mL BSA, 1 mM EDTA, and 1 mM TCEP, and samples were quenched and subjected to alkaline hydrolysis prior to separation on a 15% denaturing polyacrylamide gel. The fraction of glycerol-DNA at each time point was determined, and these data are included with data from duplicate 2.5 M glycerol reactions in Figure 3B (●). (B) The amount of glycerol-DNA at the reaction end point increases as the concentration of glycerol increases in reactions containing 1 μ M AlkA, 100 nM ab-DNA, and 0.8 M (■), 1.7 M (▼), or 2.5 M

(●) glycerol. A no enzyme control reaction containing 100 nM ab-DNA and 2.5 M glycerol (○) is also shown. All reactions were incubated at 37 °C and contained 50 mM NaMES (pH 6.5), 100 mM NaCl, 0.1 mg/mL BSA, 1 mM EDTA, and 1 mM TCEP. Exponential fits to the data are shown, but we have not interpreted rate constants for the synthesis reactions due to presumed solvent effects at high concentrations of glycerol (see Figure S3 legend). (C) The ratio of alcohol-DNA (RO-DNA) to ab-DNA (HO-DNA) at the end point of reactions of AlkA•ab-DNA and alcohol increases linearly as a function of the ratio of alcohol (ROH) to water (HOH) present. Concentrations of methanol (●), ethanol (■), propanol (○), 1,2-propanediol (□), ethylene glycol (◆), 1,3-propanediol (▲), or glycerol (▼) ranged from 0.5-2.5 M in the various reactions (Figures 3B, S3). Water concentration does not remain constant when alcohol concentration is varied over this wide range, so the ratio of alcohol concentration to water concentration ($[ROH]/[HOH]$) is plotted on the x-axis rather than simply alcohol concentration. The identity reaction of water is shown as a dashed line with a slope of 1.

Figure 4.

Glycerol-DNA is a substrate for wild-type AAG. (A) The amount of glycerol-DNA 25mer decreases with time in a representative reaction that contained 250 nM wild-type AAG and 50 nM of a glycerol-DNA preparation (from AlkA synthesis: 16% glycerol-DNA, 84% ab-DNA), but it stays constant in an analogous reaction that contained 250 nM E125Q AAG. Samples were quenched and subjected to alkaline hydrolysis prior to separation on a 15% denaturing polyacrylamide gel. The fraction of glycerol-DNA at each time point was determined, and these data are included with data from equivalent reactions in B. (B) Glycerol-DNA decreases with a single turnover rate constant of 0.0029 min^{-1} in the presence of wild-type AAG (●), but no breakdown of glycerol-DNA was detected in analogous reactions containing E125Q AAG (■). Reactions were performed at 37 °C in 50 mM NaMES (pH 6.5), 100 mM NaCl, 0.1 mg/mL BSA, 1 mM EDTA, and 1 mM TCEP.

Figure 5.

Glycerol-DNA is a substrate for wild-type AlkA. (A) The amount of glycerol-DNA 25mer decreases with time in a representative reaction that contained 250 nM wild-type AlkA and 50 nM of a glycerol-DNA preparation (from AlkA synthesis: 16% glycerol-DNA, 84% ab-DNA), but glycerol-DNA is at least 100-fold less reactive in an analogous reaction that contained 250 nM D238N AlkA. Samples were quenched and subjected to alkaline hydrolysis prior to separation on a 15% denaturing polyacrylamide gel. The fraction of glycerol-DNA at each time point was determined, and these data are included with data from equivalent reactions in 5B. (B) Glycerol-DNA decreases with a single turnover rate constant of 0.21 min^{-1} in the presence of wild-type AlkA (●), but little breakdown of glycerol-DNA was detected in analogous reactions containing D238N AlkA (■). Reactions were performed at 37 °C in 50 mM NaMES (pH 6.5), 100 mM NaCl, 0.1 mg/mL BSA, 1 mM EDTA, and 1 mM TCEP.

Figure 6.

Glycerol-DNA adducts synthesized by AlkA or by AAG react identically. (A) Breakdown of glycerol-DNA adducts synthesized by AlkA (●) or by AAG (○) by wild-type AAG. The two preparations have different initial percentages of glycerol-DNA, since each synthesis reaction proceeded to a concentration-dependent end point rather than to completion. To more easily compare glycerol-DNA from the two preparations, the inset shows the same data after the fraction of glycerol-DNA at each time point was normalized relative to the fraction of glycerol-DNA present at time zero for that preparation [0.16 for glycerol-DNA synthesized by AlkA; 0.08 for glycerol-DNA synthesized by AAG]. The time courses were indistinguishable after normalization, so data from both preparations were combined to calculate the rate constant of 0.0029 min^{-1} for conversion of glycerol-DNA to ab-DNA by AAG (Table 1). (B) Breakdown of glycerol-DNA adducts synthesized by AlkA (●) or by AAG (○) by wild-type AlkA. The inset shows the normalized data, as described in A above. The time courses were indistinguishable after normalization, so data from both preparations were combined to calculate the rate constant of 0.21 min^{-1} for conversion of glycerol-DNA to ab-DNA by AlkA (Table 1). Reactions were performed at 37°C in 50 mM NaMES (pH 6.5), 100 mM NaCl, 0.1 mg/mL BSA, 1 mM EDTA, and 1 mM TCEP.

Table 1

Rate constants for hydrolysis of alcohol-DNA adducts by DNA glycosylases.

Substrate	$k_{st} \text{ (min}^{-1}\text{)}^{\dagger}$	
	AAG	AlkA
ethanol-DNA	0.0041	0.59
ethylene glycol-DNA	0.0027	0.31
glycerol-DNA	0.0029	0.21
methanol-DNA	0.0044	0.56
1,2-propanediol-DNA	0.0026	0.22
1,3-propanediol-DNA	0.012	0.39
propanol-DNA	0.0089	0.83

[†] Single turnover rate constants for release of alcohols opposite T in 25mer duplex DNA with saturating enzyme were measured at 37 °C in NaMES, pH 6.5. Measurements are the average of 2 independent determinations, and the S.D. is 25% of the value.

Table 2

Rate enhancement for release of damaged moieties from DNA by AlkA and AAG.

Molecule released	Enzymatic		Nonenzymatic		Rate enhancement ^a	
	k_{st} (min ⁻¹), AlkA	k_{st} (min ⁻¹), AAG	k_{non} (min ⁻¹)		AlkA	AAG
methanol	5.6×10^{-1b}	4.4×10^{-3b}	1.5×10^{-8c}		3.7×10^7	2.9×10^5
eA	7.5×10^{-2d}	2.0×10^{-1e}	7.0×10^{-7e}		1.1×10^5	2.9×10^5

^aRate enhancement is defined as k_{st}/k_{non} .

^bFor release of methanol opposite T in 25mer duplex DNA at 37 °C, pH 6.5; from Table 1.

^cFor release of methanol from 1-*O*-methyl-2-deoxy-D-ribose, extrapolated to 37 °C; from Ref. 41.

^dFor release of eA opposite T in 25mer duplex DNA at 37 °C, pH 6.0; from Ref. 23.

^eFor release of eA opposite T in 25mer duplex DNA at 37 °C, pH 6.0; from Ref. 42.