The Escherichia coli 3-Methyladenine DNA Glycosylase AlkA Has a Remarkably Versatile Active Site*

Patrick J. O’Brien and Tom Ellenberger†
From the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

3-Methyladenine DNA glycosylase II (AlkA) from Escherichia coli is induced in response to DNA alkylolation, and it protects cells from alkylated nucleobases by catalyzing their excision. In contrast to the highly specific 3-methyladenine DNA glycosylase I (E. coli TAG) that catalyzes the excision of 3-methyl adducts of adenine and guanine from DNA, AlkA catalyzes the excision of a wide variety of alkylated bases including N-3 and N-7 adducts of adenosine and guanosine and O2 adducts of thymidine and cytidine. We have investigated how AlkA can recognize a diverse set of damaged bases by characterizing its discrimination between oligonucleotide substrates in vitro. Similar rate enhancements are observed for the excision of a structurally diverse set of substituted purine bases and of the normal purines adenine and guanine. These results are consistent with a remarkably indiscriminate active site and suggest that the rate of AlkA-catalyzed excision is dictated not by the catalytic recognition of a specific substrate but instead by the reactivity of the N-glycosidic bond of each substrate. Damaged bases with altered base pairing have a modest advantage, as mismatches are processed up to 400-fold faster than stable Watson-Crick base pairs. Nevertheless, AlkA does not effectively exclude undamaged DNA from its active site. The resulting deleterious excision of normal bases is expected to have a substantial cost associated with the expression of AlkA.

The reactivity of nucleobases in DNA renders their spontaneous alkylation by cellular metabolites unavoidable, and exposure to exogenous alkylating agents greatly increases the amount of DNA damage (for review, see Refs. 1 and 2). Alkylated bases block DNA-templated activities such as replication and transcription, and they cause mutations during DNA replication. The efficient repair of alkyl base adducts is complicated by their chemical diversity. For example, purines can be alkylated at positions N-1, N-3, and N-7 of the purine ring and at the exocyclic O6 of guanine, and pyrimidines can be alkylated on O2 of cytosine and thymine or O4 of thymine. An elaborate DNA repair response has evolved to process these diverse lesions, either by the direct reversal of alkylation or more commonly via base excision repair (2).

The base excision repair pathway is initiated by DNA repair glycosylases that locate damaged bases within genomic DNA and catalyze the hydrolysis of the N-glycosidic bond to release the damaged base, resulting in the formation of an abasic site. Completion of the repair pathway requires the subsequent action of an abasic site-specific endonuclease, a deoxyriboendonucleotidase, and a DNA polymerase. The DNA glycosylases that initiate repair expose substrate nucleotides in double-stranded DNA by the process of base flipping (3, 4). Enzymatic specificity could be manifested by preferential binding to damaged DNA, by differential base flipping of damaged nucleotides, or by selective engagement of the active site with flipped-out substrates that are damaged. In Escherichia coli two alklyation-specific DNA glycosylases have been identified that catalyze the excision of cytotoxic 3-methyladenine lesions (5). 3-Methyladenine DNA glycosylase I (TAG),1 the product of the tag gene, is constitutively expressed and has a narrow substrate range. TAG catalyzes the excision of 3-alkyl-substituted adenine or guanine, but it does not recognize other alkylated bases (6). 3-Methyladenine DNA glycosylase II, encoded by the alkA gene, is normally expressed at low levels and up-regulated following exposure to DNA alkylating agents as part of the adaptive response (7–9). AlkA has a very broad substrate range, catalyzing the excision of N-3- and N-7-alkyl purines as well as O2-alkyl pyrimidines (6, 10). In addition to these common alkyl adducts, AlkA has been shown to excise such disparate lesions as the cyclic adducts eA and eC (3,N4-ethenocytosine), deaminated bases such as hypoxanthine and xanthosine, and the oxidative lesions oxanine and 5-formyluracil (11–14). AlkA homologs are found in many prokaryotic and eukaryotic organisms, but in plants and vertebrates this enzyme is replaced with another broadly specific DNA glycosylase AAG.

This broad substrate range of AlkA is remarkable, and it raises the question of whether such a diverse range of DNA lesions can actually be recognized as being different from the vast excess of normal, unmodified bases. The broad substrate range of AlkA differs markedly from other well characterized DNA glycosylases such as those that are specific for uracil, 8-oxoguanine, thymine, and adenine. In each case, specific binding interactions allow the damaged base or bases to be distinguished from the undamaged bases in DNA. The broad specificity of AlkA is at odds with the discrimination against undamaged bases, which are generally smaller than alkylated bases.

1 The abbreviations used are: TAG, 3-methyladenine DNA glycosylase I; AAG, human alkyladenine DNA glycosylase (also known as MPG); AlkA, 3-methyladenine DNA glycosylase II; P, purine; 3mA, 3-methyladenine; 7mG, 7-methylguanine; eA, 1N4-ethenoadenine; Hx, hypoxanthine; Pyr, pyrrolidine; Aza, 1-azaribose.
substrates. AlkA has been shown to have low levels of activity for the excision of each of the normal bases from DNA (15). The deleterious excision of undamaged bases is a likely explanation for the toxicity or increased mutation rate that is associated with the overexpression of AlkA or its yeast homolog Mag1 (15–17).

We have characterized the glycosylase activity of AlkA toward a variety of damaged and undamaged bases in defined oligonucleotides. We find that AlkA prefers to excise bases from nucleotides that are mispaired. Damaged bases that interfere with base pairing in DNA are more readily flipped-out into the active site, providing some selectivity for excision of the damaged base. A comparison of the rate enhancements for excision of structurally disparate bases reveals a remarkably nonspecific active site that can accommodate a broad range of substrate bases (15). Indeed, we find that the preferential excision of alkylated bases can be quantitatively explained by the decreased N-glycosidic bond stability of N-alkylated bases. The poor discrimination between damaged and undamaged bases by AlkA is manifest by the frequent excision of undamaged bases, providing an explanation for why expression of AlkA is tightly repressed under normal growth conditions. However, such a broadly specific enzyme may offer an evolutionary advantage because it is immediately available to process new types of DNA damage before a specific response can evolve.

**EXPERIMENTAL PROCEDURES**

DNA substrates of the sequence 5'-GAGTACATCCCTTCCTCCCT-CCAT annealed to the complementary oligonucleotide 5'-ATGGAGAGAAGGAGGATGCTATCG, in which lesion X is paired with base Y (XY), were prepared as described previously (18). DNA inhibitors of the sequence 5'-GACTACATGATTGGCTCACTT annealed to the complementary oligonucleotide 5'-AAGTAGGCAACCATGTAGTG-AGTC were prepared, in which Z was 1-azaribose (Aza (3, 19)) or pyrrolidine (Pyr (20, 21)). Wild-type and mutant (D238N) AlkA proteins were overexpressed in E. coli and purified as described previously (22).

The protein concentration was determined by absorbance at 280 nm using the calculated extinction coefficient of 6.7 × 10^4 M⁻¹ cm⁻¹.

General Kinetic Methods—Glycosylase activity was measured using a 32P-based assay. Single-stranded oligonucleotides were 5'-labeled with T4 polynucleotide kinase, annealed to a complementary oligonucleotide, and incubated with AlkA. Reactions were quenched by the addition of sodium hydroxide (0.2 M final), and abasic DNA sites were subsequently cleaved by heating (0.2 M sodium hydroxide, 70 °C, 10 min). Samples were analyzed with formamide loading buffer and resolved by denaturing PAGE. Product and substrate bands were quantified by fluorography (Fuji BAS1000), and the fractional extent of reaction was monitored as a function of time. Enzymatic rate constants were obtained from exponential fits to the data (F = 1 - e⁻ᵏᵗ) in which F is the fraction of product, t is time, and k is the observed rate constant. To ensure single-turnover conditions, the concentration of AlkA was kept in excess of the concentration of DNA. For the determination of kₗ, the concentration of enzyme was varied over a range at least 10-fold above the Kₘ for DNA and 10-fold below the observed Kₘ for binding, and the concentration of enzyme was varied over the range from 5-fold below to 5-fold above the Kₘ. Under these conditions the dissociation constants were calculated by fitting the model for a single binding site to the data (Fobs = Kd([DNA] + [E]), in which the [E] refers to the total concentration of protein and Fobs is the fraction of DNA bound). For the tight binding DNA inhibitors these conditions could not be satisfied, and the concentration of DNA was within 3–5-fold of the Kₘ for DNA binding. In these cases the concentration of DNA had to be considered Fobs = Kd([DNA] + [E] + Kₘ)/([DNA][E]+Kₘ+Kcat+Kₘ) where Kcat is the turnover number (data not shown).

**RESULTS**

**AlkA-catalyzed Excision of 7-Methylguanine**—We sought to quantify and dissect the substrate specificity of AlkA to understand the physical basis for how a broad substrate specificity can be achieved. To measure the specificity for damaged DNA we characterized the AlkA-catalyzed base excision of both damaged and undamaged bases. 7-Methylguanosine can be site-specifically incorporated into a defined oligonucleotide, so we first characterized the AlkA-catalyzed reaction toward a 25-mer oligonucleotide duplex containing a single 7-methylguanosine lesion (23). Like 3-methyladenosine, this lesion bears a positive charge (Fig. 1), and it has a greatly destabilized N-glycosidic bond. The activity of AlkA toward 7-methylguanosine and other lesions was compared with the activity toward undamaged oligonucleotides to provide a measure of the specificity for DNA damage.

Many DNA glycosylases are inhibited by their product, an abasic site in DNA, so we measured single-turnover base excision kinetics. The single-turnover rate constant with saturating amounts of AlkA (kₗ) is analogous to the rate constant for multiple-turnover (kcat), but it does not include any of the steps associated with product release (Fig. 2). Single-turnover excision of 7-methylguanine (7mg) by AlkA follows a single exponential with a rate constant of 1.2 min⁻¹ (data not shown). This rate constant is ~2 orders of magnitude greater than the kcat values that have been previously reported for AlkA (0.01–0.07 min⁻¹ (14, 24)), presumably because of the slow rate of product
release that affects the steady-state rates. The single-turnover excision of 7mG catalyzed by AlkA showed a $K_m$ value of $-200$ nM, which is larger than the previously reported $K_m$ values for multiple-turnover excision. Presumably the $K_m$ for multiple turnover includes a contribution from binding with high affinity to the abasic product. This rate constant for single-turnover excision of 7mG is similar to the rate constant for steady-state excision of 3mA ($k_{cat} = 0.5$ min$^{-1}$).

Opposing Base Specificity of AlkA and the Involvement of the Base Flipping Step in Substrate Selection—The relative ease of base flipping can significantly affect the activity of DNA glycosylases (25–27). To test whether the favorable base pairing of 7mG-C hinders AlkA-catalyzed glycosylase activity we compared the ability of AlkA to excise 7mG from different mismatched base pairs. AlkA does exhibit greater activity toward mismatched base pairs, with complete excision of 7mG occurring within a few seconds for 7mG-T and 7mG-A mismatches (data not shown). To more accurately measure these rates, the experiments were repeated at a lower temperature (Table I). AlkA is 25-fold more efficient at removing 7mG from either 7mG-T or 7mG-A mismatches than from a 7mG-C Watson-Crick base pair. The natural 7mG lesion occurs in 7mG-C base pairs and is therefore not a particularly good substrate for AlkA. These opposing base effects are consistent with the expected base pairing stability of 7mG and suggest that stable base pairing provides a barrier to substrate exposure.

1,N$^6$-Ethenoadenosine is a bulky alkylated adduct of adenosine that does not stably pair with thymidine, providing an opportunity to further test the origin of the opposing base effects observed for 7mG. The AlkA-catalyzed excision of 1,N$^6$-ethenoadenosine (eA) from DNA showed little dependence upon the identity of the opposing base. Less than 2-fold differences were observed for excision of eA when it was paired opposite from A, T, or C (Table I), suggesting that AlkA does not have a strong preference for the identity of the opposing base. This is consistent with the absence of specific protein contacts to the opposing base in the crystal structure of AlkA bound to a DNA inhibitor (3). The observed 5-fold decrease in the rate constant for excision of eA from an eA-G pair relative to an eA-A pair (Table I) is consistent with the favorable base pairing interactions of an eA-G base pair observed in the crystal structure of an eA-containing oligonucleotide duplex (25, 28). The absence of strong opposing base effects in the excision of eA and the inverse correlation between base pair stability and excision rate for 7mG and for normal purines (see below) suggest that the base flipping step serves as a barrier to the excision of normal bases present in Watson-Crick base pairs.

AlkA-catalyzed Excision of Normal Bases—AlkA shows low levels of activity toward all four of the normal DNA bases in genomic DNA with the greatest activity toward G and A (15). As 7mG is more efficiently excised from a mismatch (Table I), we surmised that AlkA might excise normal bases more efficiently if they reside in mismatched base pairs. This possibility was tested by measuring the AlkA-catalyzed excision of G and A with different opposing bases. Both G and A are preferentially excised from mismatched base pairs in a defined oligonucleotide sequence (Fig. 3). As much as 400-fold greater glycosylase activity was observed for excision of mismatched purines relative to purines in Watson-Crick base pairs (Table I). Surprisingly, additional sites of AlkA-catalyzed base excision were detected (Fig. 3). Notably purines were excised from an A-T base pair and the neighboring G-C base pair near the end of the oligonucleotide with rate constants significantly larger than those observed for the excision of either G-C or A-T present at the central position of this 25-mer oligonucleotide (Fig. 3 and data not shown). This preferential excision could be due to the different sequence context or to an end-binding effect. AlkA showed preferential excision of normal purines from sites near the 5′ end of other oligonucleotides that were examined but also excises internal purines when they are present in the sequence (data not shown). This suggests the presence of hot spots in the genome for gratuitous repair that could significantly increase mutation rates at these sites.

To confirm that AlkA was responsible for the excision of unmodified bases from mismatched base pairs, we purified an

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**Table I**

| Base | G | A | 7mG$^b$ | eA$^b$ |
|------|---|---|--------|-------|
| Thymine | 0.22 | 0.0023 | 0.98 | 0.68 |
| Cytosine | 0.0040 | 0.091 | 0.02 | 0.54 |
| Guanine | 0.081 | 0.012 | 0.46 | 0.22 |

$^a$ For single-turnover excision ($k_{cat}$). Normalized rates are shown, obtained by dividing the rate constant by the maximal rate constant for that substrate. Measurements are the average of ≥3 independent determinations, and the S.D. is ≤15% of the value. Unless otherwise indicated, the reaction conditions were 50 mM sodium acetate, pH 6.0, 100 mM ionic strength, 0.1 mg/ml bovine serum albumin in 1 mM dithiothreitol, 1 mM EDTA, and 37 °C.

$^b$ 22 °C, sodium HEPES, pH 7.7.
products of 4, 5, and 7 nucleotides correspond to excision of A5,G 6, and A8, respectively. The 12-nucleotide product is the expected product resulting from excision of the central purine, and the additional toward mismatched base pairs (Table I). The identity of the DNA products was determined by comparison with an acid depurination sequencing ladder (data not shown). Control reactions without enzyme (lanes 1 and 14) indicate that the oligonucleotides remain intact in the absence of glycosylase. AlkA-catalyzed excision of G (lanes 2–13) and A (lanes 15–26) show time-dependent glycosylase activity toward normal purines. AlkA shows the greatest activity toward mismatched base pairs (Table I). The identity of the DNA products was determined by comparison with an acid depurination sequencing ladder (data not shown). The 12-nucleotide product is the expected product resulting from excision of the central purine, and the additional products of 4, 5, and 7 nucleotides correspond to excision of A5, G6, and A8, respectively.

FIG. 3. **AlkA preferentially excises normal purines from mismatched base pairs.** The base pair specificity of AlkA was determined by comparing glycosylase activity toward each of the possible base pairs, and an autoradiograph of a representative gel is shown. Oligonucleotide duplexes (25 base pairs) containing the indicated central base pair were incubated with AlkA for 2, 16, and 40 h, then the abasic sites were cleaved with sodium hydroxide, and the samples were analyzed on a 15% polyacrylamide gel under denaturing conditions (see “Experimental Procedures”). Control reactions without enzyme (lanes 1 and 14) indicate that the oligonucleotides remain intact in the absence of glycosylase. AlkA-catalyzed excision of G (lanes 2–13) and A (lanes 15–26) show time-dependent glycosylase activity toward normal purines. AlkA shows the greatest activity toward mismatched base pairs (Table I). The identity of the DNA products was determined by comparison with an acid depurination sequencing ladder (data not shown). The 12-nucleotide product is the expected product resulting from excision of the central purine, and the additional products of 4, 5, and 7 nucleotides correspond to excision of A5, G6, and A8, respectively.

active site mutant (D238N) that has no detectable glycosylase activity toward alkylated bases (Ref. 22 and data not shown) and tested its activity toward purine-containing mismatches. The D238N mutant does not show detectable glycosylase activity toward mismatch-containing oligonucleotide duplexes, either toward the central mismatch or toward the correctly paired bases near the end of the DNA that were excised by wild-type AlkA (data not shown). This confirms that AlkA, and not another contaminating glycosylase, is responsible for the mismatch-specific base excision that we observe.

Undamaged purines are clearly better substrates than undamaged pyrimidines, since excision of normal bases occurs preferentially at purines in a Watson-Crick paired DNA duplex (Fig. 3). However, AlkA can slowly excise normal pyrimidines from PCR-amplified DNA (15), and some alkylated and oxidized pyrimidines are relatively good substrates (29). We did not detect excision of normal pyrimidines from Watson-Crick base pairs using our assay, presumably because the rate constants for excision of normal purines are substantially greater than for excision of normal pyrimidines, and the presence of the resulting apurinic sites inhibits any subsequent binding to and excision of pyrimidine bases. However, AlkA-catalyzed excision was observed for pyrimidine-pyrimidine mismatches (Table II). The single-turnover rate constants for excision of undamaged pyrimidines are only 9% (C-C), 1% (T-C), and 0.1% (U-C) that of the rate constant for excision of G from a G-T mismatch. These results confirm and extend the previous finding that AlkA has significant glycosylase activity toward each of the normal bases in DNA (15).

**DNA Binding by AlkA—**Specificity for damaged bases could derive from tighter binding to damaged nucleotides relative to undamaged DNA or from more efficient catalysis once bound to a lesion. We assayed binding of AlkA to damaged and undamaged DNA either by measuring the concentration dependence for the single-turnover glycosylase reaction or by directly measuring DNA binding with a fluorescence anisotropy-based assay (see “Experimental Procedures”). For substrates in which DNA binding is in rapid equilibrium (i.e. substrate dissociation is much faster than the rate constant for bond cleavage) the $K_{D}$ is simply the $K_{D}$ for substrate binding. For substrates that are very slowly excised by AlkA, we measured DNA binding to the wild-type enzyme before appreciable base excision occurs. For more reactive substrates we used the catalytically inactive mutant D238N to prevent excision during the binding assay.

We first measured the binding of DNA inhibitors containing positively charged abasic site analogs that bind tightly to AlkA (3, 19, 20). Binding constants of ~20 nm were measured for both 1-azadeoxyribose- and pyrrolidine-containing DNA (Table II). The tight binding to these transition state analogs allowed for stoichiometric titration of DNA with protein to determine the binding stoichiometry and the fraction of active protein (see supplemental material). The results are consistent with a 1:1 complex of AlkA bound to DNA with >80% of the AlkA protein competent for binding to DNA. We next measured affinity for oligonucleotides containing either Watson-Crick base pairs or single mismatches, and the apparent binding affinities are reported (Table II). Both modified and unmodified oligonucleotide duplexes bound with similar affinity ($K_{D}$ ~150–450 nM). These results indicate that AlkA binds nonspecifically to DNA, and neither mismatches nor 7mG lesions are specifically recognized in the ground state.

**Structure-Activity Comparison for AlkA-catalyzed Excision of Substituted Purines and Normal Bases from DNA—**To evaluate to what extent AlkA discriminates against normal bases during the hydrolysis of the N-glycosidic bond, we compared the rate enhancements for the excision of a variety of normal and modified purines. As nucleotides with modified nucleobases are known to vary in their spontaneous rates of N-glycosidic bond hydrolysis, a direct comparison of the enzymatic rate constants cannot identify critical features of the substrate that are necessary for catalytic recognition. To evaluate how well different DNA lesions are accommodated in the transition state for N-glycosidic bond cleavage, it is necessary to normalize for the different intrinsic reactivities of their N-glycosidic bonds. The rate enhancement is defined as the ratio of the enzymatic rate constant ($k_{cat}$) divided by the rate constant for the nonenzymatic reaction ($k_{non}$). It provides a measure of the catalysis provided by the enzymatic reaction for each substrate. The interactions that are responsible for substrate recognition in the transition state can be identified by
Experimental Procedures.

Kd error as the acetate, pH 6.0, 1 mM dithiothreitol, 1 mM EDTA, and 0.1 mg/ml bovine serum albumin. The ionic strength was adjusted to 100 mM with NaCl.

The rate constant for base excision was measured with saturating AlkA. Measurements are the average of 3 independent determinations and the S.D. is ±15% of the value in all cases. Unless otherwise stated, enzymatic reaction conditions are 37 °C, 50 mM sodium acetate, pH 6.0, 1 mM dithiothreitol, 1 mM EDTA, and 0.1 mg/ml bovine serum albumin. Theionic strength was adjusted to 100 mM with NaCl.

The rate enhancement is defined as $k_{\text{cat}}/K_{\text{m}}$, and the catalytic proficiency is defined as $(k_{\text{cat}}/K_{\text{m}})w$ in which $k_w$ is the bimolecular nonenzymatic rate constant for $N$-glycosidic bond hydrolysis ($k_w = k_{\text{non}}/55$ M).

The D238N mutant was used to prevent excision of 7mG. The mutant bound to G with a $K_d$ value of 230 nM, which is the same within error as the $K_d$ value for wild-type AlkA binding to this DNA ($K_d = 280$ nM).

The rate constant was estimated by measuring the rate constant at 24 and 21 °C and extrapolating to 37 °C assuming a linear temperature dependence. The temperature dependence for excision of 7mG follows the same temperature dependence between 37 and 4 °C (see supplemental material).

The nonenzymatic data are for 37 °C from Ref. 26 unless otherwise indicated.

Unless otherwise indicated, the binding constants were determined by fluorescence anisotropy for binding to wild-type AlkA as described under “Experimental Procedures.”

We compared activity toward substrates differing in shape, charge, and hydrogen bonding ability (Fig. 1). Unlike 7mG, which bears a positive charge, 1,N6-ethenoadenine (eA) is an alkylated base that is uncharged. Hypoxanthine (Hx) is a small neutral lesion resulting from oxidative deamination of A. Both eA and Hx are good substrates for the human 3-methyladenine DNA glycosylase but have been reported to be relatively poor substrates for AlkA (11, 12). Purine (P) is not known to naturally occur in DNA, but it has no exocyclic substituents and thus serves as a valuable reference point from which to identify interactions with specific substituents. Different pH dependencies for the excision of positively charged and neutral substrates were observed (see supplemental material), so we report the rate constants at the optimum pH for each substrate (Table II). Since the glycosylase activity of AlkA is sensitive to base pairing interactions of the target base (Table I), we compare substrates in their least stable base pairs. In this context, the rate enhancements reflect specific interactions with the extrahelical base in the transition state for $N$-glycosidic bond cleavage.

As expected, the single-turnover rate constant for excision of 7mG is by far the largest of any substrate tested (Table II). The excision rates are $10^5$-fold lower for eA and P, $10^4$-fold lower for Hx, and $10^3$-fold lower for the normal purines A and G. However, after normalization for the vastly different spontaneous rates of depurination, the resulting rate enhancements for excision of these neutral substituted purines are essentially identical to that for excision of 7mG (Fig. 4). These results indicate a remarkable absence of specific interactions with the nucleobase in the transition state for enzymatic cleavage of the $N$-glycosidic bond.

In contrast to purine substrates, the rate enhancements toward pyrimidine bases appear to be significantly smaller (Table II and Fig. 4). The small rate enhancements for pyrimidines relative to purines could reflect catalytic interactions that are specific for purines but might also be attributed to an underestimate of the rate constant for the spontaneous hydrolysis of pyrimidine nucleosides in DNA (Table II). For example, the greater surface area of a purine base could facilitate positioning of the nucleobase leaving group in the active site pocket. A pyrimidine base with a smaller surface area might be more difficult to position in the large active site pocket observed in crystal structures of AlkA (3, 22).

Another comparison that is commonly used to quantify enzymatic catalysis is the catalytic proficiency, which is defined as the apparent second order rate constant for the enzyme-catalyzed reaction divided by the second order nonenzymatic rate constant ($k_{\text{cat}}/K_{\text{m}}w$). Unlike the rate enhancement, the catalytic proficiency of an enzyme accounts for its ability to bind substrates selectively. In general, the catalytic proficiency

| Substrate | $k_{\text{off}}$ | $k_{\text{cat}}/K_{\text{m}}$ | $K_d$ | $k_{\text{cat}}/K_d$ | Rate enhancement | Catalytic proficiency |
|-----------|-----------------|-----------------------------|------|---------------------|------------------|----------------------|
is a useful comparison to evaluate which substrate is the better substrate for an enzyme because it considers both differences in binding and in the rate of the chemical step. However, to address how different substrates are accommodated in the transition state it is pertinent to compare the rate enhancements. The catalytic efficiencies for AlkA-catalyzed excision of substituted purines and pyrimidines closely parallel the rate enhancements for the same substrates (Fig. 4). This is because the $K_d$ values for the different substrates are remarkably similar (Table II). The larger values of the catalytic efficiencies result from the high (non-specific) affinity for DNA. The catalytic efficiencies and rate enhancements both indicate that AlkA provides substantial catalytic assistance for the excision of both normal and modified purines, with little regard for the type of substrate.

**DISCUSSION**

We have quantified the substrate specificity of AlkA to determine how it discriminates between damaged and undamaged bases and to identify the physical basis for this specificity. The specificity for substrates competing at a single active site is given by the ratio of their respective $k_{cat}/K_m$ values. Since there are $\approx 10^7$ bases in the *E. coli* genome, a specificity of $>10^7$ would be required to selectively excise a single lesion from the vast excess of normal genomic DNA. We have measured $k_{cat}/K_m$ values for the excision of the most abundant methyl adduct, 7mG, as well as for the excision of unmodified G and A. The resulting specificity of $2 \times 10^5$ to $4 \times 10^6$ for excision of 7mG and 3mA, relative to G and A, is substantial but insufficient by itself to prevent the excision of significant numbers of undamaged bases (Table III).

The initiation of base excision repair involves first locating a site of damage from among an excess of undamaged DNA. Subsequently, the damaged nucleotide is flipped out of the DNA duplex where it engages the catalytic groups in the active site. Discrimination between damaged and undamaged bases could occur during initial binding to DNA or during the later stages of base flipping and N-glycosidic bond hydrolysis. However, little or no discrimination was observed for binding DNA with a 7mG lesion, a mismatch, or normal Watson-Crick base pairs (Table II). The nonspecific binding of AlkA to DNA suggests that the discrimination between damaged and undamaged bases occurs later in the reaction. We first discuss the effects of base pairing interactions on the base flipping step and then discuss possible mechanisms for discrimination between damaged and undamaged bases in the active site.

**An Unfavorable Equilibrium for Base Flipping**—The graphic pictures of distorted DNA and extrahelical sugar or nucleotide provided by the crystal structures of DNA repair glycosylases in complex with DNA substrates or inhibitors has led to the notion that these enzymes excel at stabilizing an extrahelical base. However, if the extrahelical conformation of a nucleotide were too stable, then an opportunity would be lost to discriminate between damaged and undamaged bases and undesirable substrates might be bound in the flipped out conformation. The inverse correlation between base pair stability and the efficiency of AlkA-catalyzed base excision (Table I) is evidence of an unfavorable equilibrium for base flipping of normal and methylated purines by AlkA ($K_{flip} \leq 1$; Fig. 2). In other words, the ground state complex of AlkA bound to DNA retains the base pairing interactions between the target base and its base pairing partner. Consistent with this notion, mismatched base pairs do not show significantly tighter binding to AlkA in the ground state (Table II). An unfavorable equilibrium for base flipping selectively enhances the reaction with modified bases that form unstable base pairs without requiring specific recognition of the modified base. Because many damaged bases have impaired hydrogen bonding ability they will be preferentially flipped-out by AlkA and hence are more likely to be excised.

The functional homolog of AlkA in human cells, AAG, shows a similar unfavorable equilibrium for base flipping (26). Both enzymes have broad substrate specificities, and thus the extrahelical conformation cannot be fully stabilized by specific contacts. DNA repair glycosylases with narrow substrate specificities such as TAG, UDG, and MutY might be better at capturing their flipped out substrates. An unfavorable equilibrium for base flipping ensures the preferential excision of damaged bases with impaired hydrogen bonding or base stacking ability, and it provides a general mechanism for discriminating against undamaged, Watson-Crick paired DNA.

**A Remarkably Versatile DNA Glycosylase Active Site**—We have measured the catalytic power of AlkA for the excision of different substituted purines from DNA to evaluate whether specific functional groups on these substrates either contribute to or interfere with catalysis. The glycosylase activity is inversely proportional to the stability of the base pair for all bases that were examined (Table II). Therefore, we have compared the rate enhancements for mismatched bases to minimize base pairing effects and instead focus on how purine ring substitutions affect transition state stabilization by AlkA. Interactions that favor the reaction of damaged bases or disfavor...
spontaneous hydrolysis (i.e., the intrinsic reactivity relative to G-alkylated pyrimidines such as O2-methyl-C and 5-formyl-U (6, 10). Damage specificity between two substrates competing for a single active site is given by the ratio of the specificity constants $(k_{\text{cat}}/K_m^a)/k_{\text{cat}}/K_m^b)$. The enzymatic specificities are given relative to excision of G from a G:C base pair.

The specificity for a theoretical enzyme that does not discriminate between substrates was defined as the ratio of the rate constants for hydrolysis of G and A:C were used, because the rate constants for hydrolysis of G:C and A:T are not known. N-Glycosidic bond hydrolysis is expected to be slower for Watson-Crick paired bases, because it is more difficult for water to attack a nucleotide in DNA (e.g., 7mG-C is 6-fold less reactive than 7mG:T, Table II).

The specificity for excision of methylated and unmodified purines in DNA is plotted from Table II. The concentration of repair enzyme, and the damaged DNA prior to DNA replication, will depend upon the abundance of damaged bases (Table II). Nevertheless, AlkA can excise damaged pyrimidines such as O2-methyl-C and 5-formyl-U (6, 10, 13). It remains to be tested whether AlkA shows a larger rate enhancement toward these more bulky and positively charged adducts.

A Threshold Model for Excision of Lesions with Destabilized N-Glycosidic Bonds: The discovery that N-alkylation of purines greatly destabilizes the N-glycosidic bond suggested a compelling general strategy for the selective excision of alkylated bases (15, 30). A nonspecific enzyme could provide uniform transition state stabilization for the hydrolysis of all nucleotide substrates and could still ensure preferential action on damaged nucleotides with destabilized N-glycosidic bonds (Fig. 5; Ref. 15). This simple idea was subsequently complicated by the finding that the broadly specific DNA glycosylases responsible for the repair of alkylation damage can also catalyze the excision of neutral alkyl adducts such as eA and the neutral deaminated bases Hx and xanthosine (11, 12, 14, 18). To resolve this apparent paradox, we have determined the rate enhancements for excision of a positively charged lesion 7mG, various neutral lesions, and the normal undamaged bases in a defined sequence context. The results reveal that the same rate enhancements are provided toward damaged and undamaged purine bases once the effects of base pairing are taken into account (Fig. 4). This provides quantitative evidence in favor of the model that AlkA-catalyzed excision efficiency is dictated by the chemical stability of the N-glycosidic bond and not the shape-selective recognition of damaged bases (15).

Moreover, all of the specificity for excision of methylated purines of AlkA can be attributed to the difference in N-glycosidic bond stability between N-methylated and unmodified bases (Table III). Fig. 5 illustrates how the nonspecific stabilization of depurination by AlkA results in the preferential excision of alkylated purines (see also Ref. 15). Because the spontaneous hydrolysis of 3-methyl and 7-methyl purine adducts is quite rapid, only a modest rate enhancement of $-10^2$ is required for biologically viable rates of excision. Although slow in comparison with many other cellular reactions, the rate constants for excision of 7mG and 3mA are sufficiently fast to ensure repair of methyl adducts prior to replication given the small number of lesions likely to occur during any given cell cycle. In the absence of specific recognition of the alkyl adducts, a larger rate enhancement would result in even more gratuitous repair at undamaged sites in the genome.

**TABLE III**

| Substrate | E. coli AlkA | Human AAG |
|-----------|--------------|-----------|
|           | $k_{\text{cat}}/K_m^{a,b}$ | Intrinsic reactivity, specificity |
|           | $s^{-1}$ | | $s^{-1}$ |
| 7mG-C     | $8.7 \times 10^4$ | $9.0 \times 10^4$ | $3.4 \times 10^5$ |
| 3mAAT     | $1.7 \times 10^5$ | $1.8 \times 10^6$ | $6.7 \times 10^5$ |
| HxT       | $1.4 \times 10^5$ | $1.5 \times 10^6$ | $26$ |
| GC        | $0.94$ | $(1)$ | $1.1$ |
| AT        | $0.48$ | $0.5$ | $0.6$ |

$^a$ $k_{\text{cat}}/K_m$ values for excision at pH 7 (Table II; see supplemental material).

$^b$ Specificity between two substrates competing for a single active site is given by the ratio of the specificity constants $(k_{\text{cat}}/K_m^a)/k_{\text{cat}}/K_m^b$.

**FIG. 5.** A threshold model to explain why AlkA does not require enhanced catalytic specificity toward methylated bases. A shaded horizontal line indicates the biological threshold of adequate DNA repair activity. This level of activity, which is required to repair damaged DNA prior to DNA replication, will depend upon the abundance of damaged bases, the concentration of repair enzyme, and the rate constant for DNA repair. The rate constants for spontaneous (○) and AlkA-catalyzed (●) N-glycosidic bond hydrolysis for methylated and unmodified purines in DNA are plotted from Table II. The arrows indicate the rate enhancements achieved by AlkA. The similar rate enhancements for excision of normal and methylated bases are sufficient to clear the methylated bases from the genome while resulting in some deleterious excision of normal bases.
Broad Substrate Specificity of AlkA

Comparison of Human and Prokaryotic Repair of DNA Alkylation Damage—There appears to be a biological niche for a broadly specific DNA repair glycosylase that can recognize multiple types of alkyl-base lesions, because all cell types from bacteria to humans have such an enzyme. In bacteria this glycosylase is AlkA, and a closely related glycosylase Mag1 is found in yeast (31). The functional homolog in humans and multicellular eukaryotes is AAG. The specificity for alkylated bases and for the deaminated base Hx is summarized in Table III. Although humans have ~100-fold larger genome than E. coli, the specificity of AAG for methylated bases is only ~10-fold larger than that of AlkA. This implies that human cells bear a greater cost of gratuitous repair than prokaryotic cells despite the greater specificity of AAG.

One marked difference is that AAG shows ~10^6-fold specificity toward Hx lesions, whereas AlkA exhibits only 150-fold specificity. The substantial specificity toward Hx lesions is suggestive of a biological role for AAG as a Hx DNA glycosylase in human cells (26, 32, 33). The modest specificity of AlkA toward Hx can be attributed to the less stable base pair of Hx. Another DNA glycosylase, endonuclease V, appears to be responsible for the repair of Hx in E. coli (for review, see Ref. 34).

The rate enhancements for AAG and AlkA-catalyzed excision of substituted purines are compared in Fig. 6. Although both AAG and AlkA have relatively open binding pockets, it is apparent that the AAG active site exhibits much greater discrimination in recognizing its substrates. The substantial discrimination between uncharged damaged bases and normal purines can be attributed to specific contacts that exclude the exocyclic amino groups of G and A from an otherwise forgiving active site pocket (26, 35). In contrast, AlkA bears the hallmark of a truly nonspecific enzyme providing a constant rate enhancement toward structurally dissimilar substrates.

Summary—These results confirm and extend the initial report that AlkA instigates gratuitous repair, excising normal bases from DNA (15). We provide quantitative evidence that AlkA has a remarkably versatile active site that can accommodate a broad range of substituted nucleobases providing a similar amount of catalysis in each case. This nonspecific destabilization of the N-glycosidic bond (i.e. stabilization of the transition state for hydrolysis) provides for the preferential repair of DNA lesions with weakened N-glycosidic bonds. Although N-alkyl purines are destabilized by a factor of ~10^6-fold relative to normal purines, this specificity is insufficient to prevent the deleterious excision of normal bases because there is a vast excess of normal bases in the genome. Apparently this cost is an acceptable means of providing broad coverage for the excision of rare abnormal bases for which a more specialized repair system does not exist. Another DNA repair pathway with broad substrate specificity, nucleotide excision repair, is known to act on undamaged DNA and the biological consequences of this gratuitous repair have been discussed (36, 37).

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Patrick J. O’Brien and Tom Ellenberger

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