# AlkA Protein Is the Third Escherichia coli DNA Repair Protein Excising a Ring Fragmentation Product of Thymine †

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# AlkA Protein Is the Third *Escherichia coli* DNA Repair Protein Excising a Ring Fragmentation Product of Thymine[†]

Cyril V. Privezentzev,[‡] Murat Saparbaev,[‡] Aruna Sambandam,[§] Marc M. Greenberg,*,[§] and Jacques Laval*,[‡]

*Groupe Reparation des lesions Radio- et Chimio-Induites, UMR 8532 CNRS, Institut Gustave Roussy, 94805 Villejuif Cedex, France, and Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523*

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ABSTRACT: Various forms of oxidative stress lead to the formation of damaged bases including *N*-(2-deoxy-$\beta$-D-erythro-pentofuranosyl)-*N*-3-(2R-hydroxyisobutyric acid)-urea or αRT, the fragmentation product of thymine formed from 5R-thymidine C5-hydrate upon hydrolysis. It was shown that αRT is excised by *Escherichia coli* Fpg and Nth proteins. Here we report that when present in DNA, αRT is, in addition, a substrate for the *E. coli* AlkA protein with an apparent $K_m$ value of $\cong$170 nM. αRT positioned opposite T, dG, dC, and dA were efficiently excised by AlkA protein from duplex oligodeoxynucleotides in the following order: dA ≈ T ≫ dC ≈ dG. This is the first example of the excision of a ring opened form of a pyrimidine by AlkA protein and also the first example where the same DNA base lesion is excised by three different DNA glycosylases of the base excision repair pathway. The present results suggest possible structural similarity of the active site between *E. coli* AlkA, Fpg, and Nth proteins.

Oxidative DNA damage, including oxidized purines and pyrimidines, occurs during normal cell metabolism and by exposure to ionizing radiation and certain chemicals (*1*, *2*). Damaged bases are endowed with cytotoxic or mutagenic properties and are believed to be at the origin of aging and cancer. Thymidine glycol blocks replication and, therefore, is assumed to be lethal in vivo (*3−5*). *N*-(2-Deoxy-$\beta$-D-erythro-pentofuranosyl)-*N*-3-(*2R*-hydroxyisobutyric acid)-urea (αRT)[1] is the respective fragmentation product of 5R-thymidine C5-hydrate formed upon hydrolysis. Kinetic measurements indicate that the activity of Klenow (exo⁻) fragment is inhibited by the presence αRT in the DNA template. However, despite the reduction in $V_{max}$, these experiments indicate that αRT base pairs to nucleotide triphosphate and should be thought as an instructive lesion (*6*). Such properties imply that the αRT lesion could have biological significance in vivo.

In mammalian cells, in yeast and in *E. coli*, the elimination of oxidized bases is mediated by base excision repair pathway involving, in the first step a DNA−glycosylase activity (*7*, *8*). There are two classes of DNA glycosylases (i) monofunctional DNA glycosylases and (ii) the DNA glycosylases with associated AP lyase activity that in addition to their DNA glycosylases incise DNA at abasic sites by a $\beta$-elimination (*9*). Most DNA glycosylases have broad substrate specificity. For example, the Fpg protein removes 8-hydroxyguanine, imidazole ring opened purines and αRT (*9−11*). The Nth enzyme repairs a number of oxidized pyrimidine bases (*12*, *13*). We have shown that both the *E. coli* Nth and Fpg proteins remove αRT when present in DNA, the Fpg protein being the most efficient (*11*). Furthermore, it was shown that human cells extracts have enzymatic activity excising with high efficiency αRT from duplex DNA (*11*).

The *E. coli* AlkA protein was primarily described as an enzyme excising alkylated bases (*14*, *15*). It is also involved in the repair of formyluracil (*16*), hypoxanthine (*17*), ethano-(*18*), and ethenobases (*19*), suggesting that the protein has broad substrate specificity. The crystal structure of AlkA protein has been solved (*20*, *21*). It has been suggested that the widening of the active site located in the cleft between two domains of the AlkA protein could explain the broad spectrum of modified bases excised by this protein. The αRT residue has no intact aromatic structure. We expected that if such a modified base is a substrate for the AlkA protein, it will give new insight into the mechanism of recognition of a modified base by this repair enzyme which shows similarity with the 3D structure of the Nth protein. Therefore, we investigated the activity of the AlkA protein toward this ring-fragmented base.

This study shows that the AlkA protein acts as a DNA-glycosylase on the ring fragmentation product of thymine, αRT. In addition, the kinetic constants of the AlkA protein for excision of αRT residue are measured.

## EXPERIMENTAL PROCEDURES

*Materials*. Molecular biology products were purchased from Roche. The 30-mer oligonucleotide substrate containing αRT at position 11 was synthesized as described (*22*). The

* To whom correspondence should be addressed. (J.L.) Phone: (33) 1 42114824. Fax: (33) 1 42114454. E-mail: jlaval@igr.fr. (M.M.G, to whom questions concerning DNA synthesis and characterization should be addressed) Phone: (970) 491-7972. Fax: (970) 491-1801. E-mail: mgreenbe@lamar.colostate.edu.

[‡] Groupe Reparation des lesions Radio- et Chimio-Induites.
[§] Colorado State University.

[1] Abbreviations: AP-sites, apurinic/apyrimidinic sites; αRT, *N*-(2-Deoxy-{3-D-erythro-pentofuranosyl)-*N*-3-(2R-hydroxyisobutyric acid)-urea; AlkA protein, *E. coli* 3-methyladenine-DNA glycosylase II; Fpg protein, *E. coli* formamidopyrimidine-DNA glycosylase; Nfo protein, *E. coli* endonuclease IV; Nth protein, *E. coli* endonuclease III.

sequence used was: 5′−d(TGA CTG CAT AαRTG CAT GTA GAC GAT GTG CAT). Four complementary oligonucleotides, containing dA, dG, dC, or T at the position opposite to αRT were synthesized using standard methods. Single-strand 30-mer was 5′-end labeled by T4 polynucleotide kinase (New England, Biolabs) in the presence of [$\gamma$-$^{32}$P]ATP (4500 Ci/mmol, ICN Pharmaceuticals) and purified as described (*23*). The [$^{32}$P]-labeled oligonucleotide containing αRT residue was hybridized with the respective oligonucleotide containing one of the four naturally occurring deoxynucleotides opposite αRT at a 1:2 molar ratio by heating at 60 °C for 3 min in a buffer containing 75 mM NaCl, 7.5 mM sodium citrate, pH 7.0.

*Enzyme Purification.* AlkA protein was purified to homogeneity from *E. coli* JM 105 harboring plasmid pAlkl0 (*24*). A 6-L culture was grown, at 37 °C, in LB medium supplemented with ampicillin (100 $\mu$g/mL) to OD$_{600nm}$ = 0.8 and then was induced, at the same temperature, with 0.5 mM IPTG for 4 h. Bacteria were collected by centrifugation, washed with 0.3 M Tris, pH 8.0, 5 mM EDTA, resuspended in buffer containing with 0.3 M Tris, pH 8.0, 5 mM EDTA, 0.2 M NaC1, 0.5 mM PMSF, 2 mM $\beta$-mercaptoethanol (buffer A) and frozen at −20 °C. Cells were lysed using three freeze−thaw cycles, 0.1 mg/mL lysozyme was added after first cycle. Resulting suspension was centrifuged in a Beckman 42.1 rotor at 30 krpm for 1 h at 4 °C. The supernatant was passed through column packed with 100 mL QMA anion-exchange (Waters-Accell) preequilibrated in buffer A. The eluate was dialyzed against buffer B (buffer A, but without NaCl), centrifuged at 10000*g* for 20 min and the supernatant was loaded on column packed with 30 mL of Phospho-Ultrogel A6R resin (Biosepra, Paris) equilibrated with buffer B. After column washing proteins were eluted with a linear gradient of NaCl 0 to 1 M in buffer B. The active fractions were pooled and supplemented with ammonium sulfate (0.5 g/mL). The precipitate was collected by centrifugation and disolved in 10 mM HEPES−KOH, pH 7.5, 0.5 mM EDTA, 1 M NaCl, 10 mM $\beta$-mercaptoethanol (final volume 2 mL) that was loaded on a gel filtration column (140 mL of ACA-54 resin, IFB-LKB) and eluted with the same buffer. The active fractions were pooled, supplemented with ammonium sulfate up to 1 M, and loaded on Phenyl Sepharose CL-4B column (10 mL, Pharmacia) preequilibrated with buffer C (20 mM Tris, pH 8.0, 2 mM EDTA, 0.8 M ammonium sulfate, 1 M NaCl, 0.1 mM PMSF, 2.5 mM $\beta$-mercaptoethanol). The column was eluted with a linear gradient of buffer C (ammonium sulfate from 0.8 to 0 M and glycerol from 0% to 5%). Active fractions were pooled, dialyzed against buffer D [20 mM Hepes-KOH pH 7.8, 50 mM KC1, 1 mM EDTA, 5 mM $\beta$-mercaptoethanol, 10% glycerol (w/v)] and loaded on FPLC MONO-S column. The protein were eluted with a linear KCl gradient from 0 to 1 M in the buffer D.

The ANPG40, APDG, MAG, and Tag I proteins were prepared as described (*17, 19, 25*). The Nth protein was prepared as previously described (*26*).

*Enzyme Assays.* The release of αRT residue was measured by the cleavage of the oligonucleotide containing a single lesion at a defined position. The standard assay for AlkA protein activity (50 $\mu$L final volume) contained 5′-[$^{32}$P]-αRT· dA duplex (0.5 pmol) in 70 mM Hepes-KOH, pH 7.8, 1 mM EDTA, 5 mM $\beta$-mercaptoethanol, 100 $\mu$g/mL BSA. After
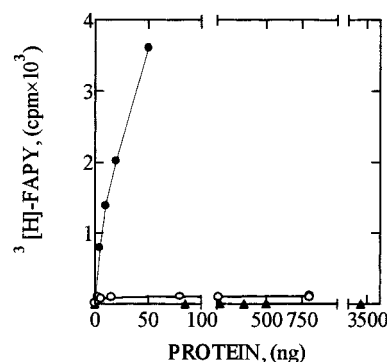


FIGURE 1: Fapy-DNA glycosylase activity of wild-type Fpg and AlkA proteins and mutant AlkA W218K protein. Increasing amount of the various proteins, [Fpg wild-type (●), AlkA wild-type (○), and AlkA W218K (▲)] were incubated in the presence of [$^{3}$H]-Fapy-(poly dG-dC) and the [$^{3}$H]Fapy residues released were quantified. For details see Experimental Procedures.

incubation at 37 °C, for a given time in the presence of the AlkA protein, the abasic sites were cleaved by piperidine treatment (10% piperidine at 37 °C for 30 min) unless otherwise stated. The products of the reaction were analyzed as described (*19*). The gels were exposed to a Storm 840 Phosphor Screen and the amounts of radioactivity in the bands were quantified using the ImageQuaNT Software. The [$^{32}$P]-labeled 10-mer generated by sequential action of the AlkA protein and light piperidine treatment corresponds to the amount of αRT excised by the DNA glycosylase. The Fapy DNA glycosylase activity of the proteins was measured as described (*10*).

*Site-Directed Mutagenesis.* Site-directed mutagenesis for the construction of Trp218 to Lys218 (W218K) AlkA mutant was performed by using QuikChange site-directed mutagenesis kit by Stratagene and the relevant primers. The DNA sequence was verified by using Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer).

## RESULTS

*Ring Fragmentation Product of Thymine, αRT, Is Removed by AlkA Protein.* To investigate the repair of αRT by the AlkA protein we used the oligonucleotide containing αRT described previously (*11*) and highly purified AlkA protein. Special precautions were taken in order to avoid possible traces contamination by Fpg or Nth proteins. Therefore two additional steps were used in addition to the previously described purification (*27*). As shown in Figure 1, this preparation was devoid of detectable activity on a substrate containing [$^{3}$H]Fapy residues. The small amount of radioactivity (about 90 cpm) released by the AlkA protein is independent of the enzyme concentration and represents the excision of the 3-methylguanine residues present in the substrate (*15*) (Figure 1). It should be noted that the W218K AlkA mutant does not recognize 3-methyladenine or 7-methylguanine residues. The AlkA protein preparation was also devoid of activity using substrate containing abasic sites (data not shown), ruling out possible contamination by the Nth protein. These data rules out possible contamination by these two DNA glycosylases that are known to excise αRT residues.

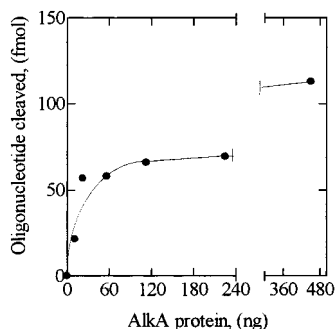The 5′-[$^{32}$P]-labeled oligonucleotide duplex containing αRT was incubated with increasing concentrations of the

FIGURE 2: Concentration-dependent cleavage of the 5′-[³²P]-labeled oligonucleotides containing αRT residue by the AikA protein. Oligonucleotide duplex (500 fmol) was incubated with increasing amounts of enzyme in 50 μL of reaction mixture for 60 min at 37 °C. For details see Experimental Procedures.
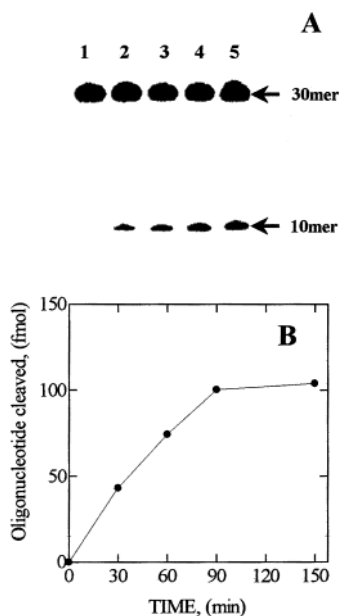


FIGURE 3: Time-dependent cleavage of the 5′-[³²P]-labeled oligo-nucleotides containing αRT residue. The AlkA protein (450 ng) was incubated with αRT/A oligonucleotide duplex for increasing periods of time. The reaction products were analyzed on 20% PAGE 7 M urea gel. (A) Lane 1, 30-mer oligonucleotide containing αRT; lanes 2−5, 30-mer oligonucleotide containing αRT incubated for 30, 60, 90, and 150 min, respectively. (B) The radioactivity of the bands corresponding to native and incised oligonucleotides pre-sented in panel A was quantified. For details see Experimental Procedures.

AlkA protein. Since AlkA protein is a DNA-glycosylase lacking AP-lyase activity, piperidine treatment was used to reveal AP-sites. The AlkA protein excises αRT in a dose-dependent manner (Figure 2) and release of the lesion as a function of time was linear during at least the first 60 min of incubation (Figure 3).

Since the AlkA protein is one of the many enzymes of the 3-methyladenine DNA glycosylase family, we investi-gated whether other members of this family, namely, human ANPG, rat APDG, *E. coli* Tag1, *S. cerevisiae* MAG pure proteins, at high concentrations, could also excise αRT residues from the oligonucleotide containing αRT opposite to Adenine. Since these enzymes are devoid of AP-lyase activity the samples after treatment with the various enzymes were further subjected to piperidine treatment in order to reveal any AP-site generated by the excision of αRT residue.
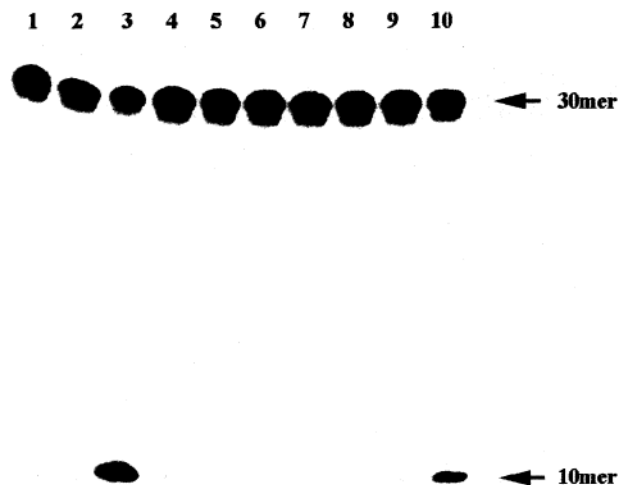


FIGURE 4: Action of various 3-meAde-DNA glycosylases on the 30-mer duplex αRT/A oligonucleotide. The 5′-[³²P]-labeled αRT/A was incubated with pure 3-meAde-DNA glycosylases of different origin at 37 °C for 30 min (unless otherwise stated). Except for the control αRT/A-oligonucleotide and this oligonucleotide treated with Nth, the products of reactions subsequently were subjected to piperidine treatment in order to reveal abasic sites generated by DNA glycosylases devoid of β-lyase activity. Lanes: 1, control αRT/A oligonucleotide; 2, as 1, but treated 1 M piperidine at 37 °C for 30 min; 3, Nth protein, 80 nM (100 ng); 4, TagI, 350 nM (1.0 unit); 5, ANPG40, 50 nM (0.06 unit); 6, ANPG40, 90 nM (0.1 unit); 7, ANPG40, 900 nM (1.0 unit); 8, APDG, 500 nM (0.54 unit); 9, MAG, 70 nM (0.04 unit); 10, AlkA, 250 nM (0.23 unit). The products of the reaction were analyzed by electrophoresis on a denaturing 20% polyacrylamide gel and visualized using the Phosphorimager (model Storm 840). For details see Experimental Procedures.

The results presented in Figure 4 show that the AlkA protein is the only 3-methyladenine DNA glycosylase from the various origin tested able to excise αRT residues. In this experiment the Nth protein was used as a positive control.

*Kinetics of AlkA Protein-Mediated Excision of αRT from Oligonucleotide Duplex.* The excision of αRT by the AlkA protein from oligonucleotide containing αRT exhibits Michae-lis-Menten kinetics. To measure the efficiency of the excision, the apparent $K_m$ and $k_{cat}$ values were extracted from a Lineweaver−Burk plot using varying concentrations of the 30-mer αRT/A duplex oligonucleotide as a substrate (Figure 5). The $K_m$ for the AlkA protein is ∼170 ± 30 nM, $k_{cat} \approx (4.1 \pm 1.1) \times 10^{-3}$ min$^{-1}$ and $k_{cat}/K_m \approx 2.45 \times 10^{-5}$ (nM × min)$^{-1}$ (Table 1). For comparison, respective values for other reported AlkA protein substrates are also provided (Table 1).

*Base Pair Specificity of AlkA Protein.* The specificity of the AlkA protein was investigated when acting on duplex oligonucleotides containing each of the four naturally oc-curring nucleotides opposite αRT. The four duplexes con-taining αRT·dN base pairs are all substrates for AlkA protein. The AlkA protein preferentially excises the αRT residue when it is opposite dA and T, followed by dC and dG (dA ≈ T ≫ dC ≈ dG) (Figure 6).

## DISCUSSION

The data presented above show that the AlkA protein excises the ring fragmentation product of thymine, αRT. The role of AlkA protein in the repair of alkylation damage (*14, 15*), products of oxidation (*16*), hypoxanthine (*17*), ethano-
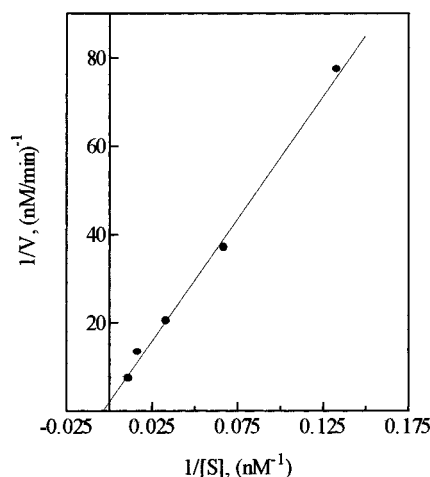
FIGURE 5: Lineweaver-Burk plot for AlkA protein excision of αRT. AlkA protein was incubated with increasing amounts of substrate for various periods of time and the initial velocities determined and plotted against the concentration of αRT.
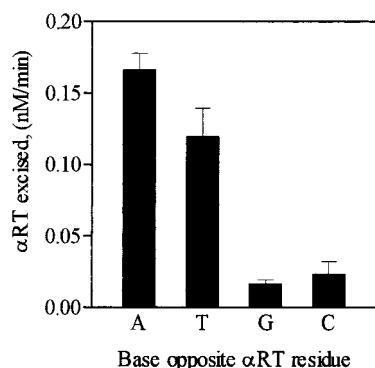


FIGURE 6: Cleavage of oligonucleotide duplexes containing different bases opposite the αRT residue by the AlkA protein. The oligonucleotide containing αRT was annealed with the complementary 30-mer oligonucleotide to generate the following mismatches αRT·dA, αRT·dG, αRT·dC, and αRT·T, which were used as substrates for AlkA protein. The excision of αRT was measured by determining the amount of oligomer migrating at the position of the l0-mer using 375 ng of protein and 10 nM oligonucleotide.

(18) and ethenobases (19), is well documented. The present data add a new substrate that is structurally unique compared to the previously reported oxidized pyrimidines, suggesting an additional function of the AlkA protein in the repair of oxidized pyrimidines. Previously reported modifed bases excised by AlkA protein have an intact aromatic structure. This is a first example when AlkA protein removes fragmented base.

Little is known about the biological implications of αRT residues in DNA. However, previously reported properties

of the lesion (6, 22) and the demonstration that it is repaired by Fpg and Nth proteins in vitro with high efficiency (11) strongly suggest that such a modified base cannot be tolerated in vivo. The fact that αRT is substrate for three different DNA glycosylases involved in base excision repair in vitro, does not necessarily indicate that all of these enzymes play an equal role in the processing of the lesion in vivo. The apparent $K_m$ of Fpg protein for αRT is 7.8 nM, whereas values of Nth protein and AlkA protein for the lesion are in the same order of magnitude, 84 and 170 nM, respectively (11, and this paper). The comparison of the kinetic data for the excision of αRT by these three enzymes suggests that, in vivo, αRT could be preferentially repaired by the Fpg protein. The comparison of the various substrates of the AlkA protein presented in Table 1 includes lesions present in large polynucleotides and short oligonucleotides and could lead to potential artifacts. This possibility was explored using AlkA protein (as well as others 3-methyladenine DNA glycosylase of different origins) and various substrates containing hypoxanthine residues (28). The results show that oligonucleotides containing a unique modified base are the best substrates.

The preferential recognition of a modified residue paired with one of the four natural bases in duplex DNA is an important characteristic of DNA glycosylases. As already shown for the Fpg and Nth proteins (11), the AlkA protein removes αRT from all the mismatches. This suggests that these three DNA repair proteins could recognize αRT per se via the flip-out mechanism. The AlkA protein preferentially excises the αRT residue when it is opposite dA, T, followed by dC, dG (dA ≈ T ≫ dC ≈ dG). This is different from the excision pattern reported previously for the Fpg protein (αRT·dC > αRT·T > αRT·dG > αRT·dA) and for the Nth protein (αRT·dG >αRT·dA > αRT·T > αRT·dC). It has been proposed that the structural alterations of the duplexes vary in the following order: αRT·dA < αRT·dG < αRT·T < αRT·dC. Therefore, one may propose that the recognition of αRT by AlkA protein is similar to that of 7-methylguanine residues which does not depend on damage-induced helix distortions (29).

The fact that αRT is repaired by three different proteins suggests that some structural similarity in the active site could exist in the superfamily of DNA repair glycosylases/lyases and similarity in the mechanisms of damaged base recognition. The crystal structures of AlkA and Nth proteins have been solved (20, 21, 30) but not for the Fpg protein. The three-dimensional structure of AlkA protein reveals similarities with the Nth protein (20, 21). The active site of both enzymes is located in a cleft between two helical domains, carrying catalytically important residues. The active site cleft

Table 1: Kinetic Constants for AlkA Protein for the Excision of ART and Various Other Lesions from DNA

| lesion recognized | $K_m$ (nM) | $K_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (nM × min)$^{-1}$ | ref |
|---|---|---|---|---|
| αRT[a] | 170 ± 30 | $(4.1 ± 1.1) × 10^{-3}$ | $2.45 × 10^{-5}$ | this work |
| 3-methyladenine | 9.2 | 0.5 | 0.1 | 14 |
| 7-methylguanine | 11 | | | 14 |
| hypoxanthine | 420 ± 100 | $0.84 × 10^{-3}$ | $2 × 10^{-6}$ | 17 |
| ethenoadenine | 800 ± 350 | $\sim 10^{-3}$ | $\sim 10^{-6}$ | 19 |
| 5-formyluracil | 4.4 | | | 16 |
| 5-hydroxymethyluracil | 4000 | | | 16 |

[a] The data presented were calculated from four independent experiments.

of the AlkA protein is narrower than that of Nth protein, and unusually rich in electron-donating aromatic residues, whereas the solvent-filled pocket of Nth protein is lined by the side chains of polar amino acids. Although the 3D-structure of Fpg protein has not yet been solved, the results presented above suggest that the active site of the Fpg protein could be organized in a manner similar to that of the AlkA and the Nth proteins.

The conserved location of catalytically essential residues in the active sites of AlkA and Nth proteins is important to explain fundamental similarities in the reaction chemistry of DNA repair glycosylases and glycosylases/lyases (*20*). The catalytically crucial residues were found, by mutational approaches, to be Lys120 and Asp138 for the Nth protein (*30*) and Trp218 and Asp238 for the AlkA protein (*20, 21*). In the case of the Nth protein, Asp138 deprotonates Lys120, which attacks the glycosidic bond to form Schiff base (*31*). In the case of AlkA protein, two different mechanisms were proposed. In the first one, Asp238 deprotonates water attacking the glycosidic bond of the substrate (*20, 21*). The second mechanism involves a $S_N1$ type interaction in which Asp238 stabilizes the reaction intermediate, by either ionic or covalent interactions (*32*). This second mechanism does not require water as a nucleophile as it is the case for UDG (*33*) or the ANPG protein, the human counterpart of AlkA protein (*34*). The role of AlkA protein Trp218 carrying electron-rich aromatic ring is believed to recognize the positively charged alkylated bases via $\pi$-donor-acceptor interactions (*20*). Using site-directed mutagenesis, it was shown that the mutation of Trp218 to Ala in the AlkA protein rendered cells methylmethane sulfonate-sensitive (*21*). In the case of the recognition of αRT by the AlkA protein, we attempted to investigate the role of Trp218 by replacing it by a Lys residue. The mutant protein was overexpressed and purified to homogeneity (data not shown). This mutation resulted in the loss of the DNA glycosylase activity on both αRT residues and alkylated bases. In addition, this mutation did not introduce AP-lyase activity into the AlkA protein (data not shown). This result shows that Trp218 is also involved in the excision of αRT residues. It also strongly suggests that a single active site is responsible for the elimination of both 3-methyladenine and αRT residues. The AlkA wild-type and its mutated W218K counterpart are produced and purified under identical conditions. The observation that the preparation of the mutated protein does not excise αRT residues rules out that the αRT DNA glycosylase activity could be due to an unknown DNA glycosylase contaminant in the AlkA protein preparation.

An other model, based on ability of the AlkA protein to excise unmodified bases, has been proposed (*35*). The authors observed a 8-fold increase in the enzymatic guanine removal by lowering the pH of the reaction from 7.9 to 6.5, showing that protonated bases are the most prone to excision by AlkA protein. This fact suggests that the enzyme reduces the activation energy of base−sugar bonds largely without specificity for a given base structure. As it concerns αRT excision by the AlkA protein, by lowering the pH of the reaction from 7.9 to 6.5, we also observed an increase (2−3-fold) of the initial velocity of excision of this lesion (data not shown). In conclusion, the AlkA protein has, so far, the broadest substrate specificity among the DNA glycosylases studied. The overlapping substrate specificity

and the common structural basis for base excision repair opens the way for the possible construction of a universal DNA glycosylase.

## ACKNOWLEDGMENT

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