Methylpurine DNA Glycosylase of the Hyperthermophilic Archaeon *Archaeoglobus fulgidus*†

Nils-Kåre Birkeland,[‡,§] Hilde Ånensen,[||] Ingeborg Knævelsrud,[‡,||] Wenche Kristoffersen,[||] Magnar Bjørås,[⊥] Frank T. Robb,[§] Arne Klungland,[⊥] and Svein Bjelland*[,||]

Department of Microbiology, University of Bergen, Jahnebakken 5, P.O. Box 7800, N-5020 Bergen, Norway, Center of Marine Biotechnology, University of Maryland Biotechnology Institute, East Pratt Street, Baltimore, Maryland 21202, School of Science and Technology, Stavanger University College, P.O. Box 8002, N-4068 Stavanger, Norway, and Centre of Molecular Biology and Neuroscience and Institute of Medical Microbiology, University of Oslo, National Hospital, N-0027 Oslo, Norway

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ABSTRACT: Base excision repair of DNA alkylation damage is initiated by a methylpurine DNA glycosylase (MPG) function. Such enzymes have previously been characterized from bacteria and eukarya, but not from archaea. We identified activity for the release of methylated bases from DNA in cell-free extracts of *Archaeoglobus fulgidus*, an archaeon growing optimally at 83 °C. An open reading frame homologous to the *alkA* gene of *Escherichia coli* was overexpressed and identified as a gene encoding an MPG enzyme (M_r = 34 251), hereafter designated *afalkA*. The purified AfalkA protein differs from *E. coli* AlkA by excising alkylated bases only, from DNA, in the following order of efficiency: 3-methyladenine (m^3A) ≫ 3-methylguanine ∼ 7-methyladenine ≫ 7-methylguanine. Although the rate of enzymatic release of m^3A is highest in the temperature range of 65−75 °C, it is only reduced by 50% at 45 °C, a temperature that does not support growth of *A. fulgidus*. At temperatures above 75 °C, nonenzymatic release of methylpurines predominates. The results suggest that the biological function of AfalkA is to excise m^3A from DNA at suboptimal and maybe even mesophilic temperatures. This hypothesis is further supported by the observation that the *afalkA* gene function suppresses the alkylation sensitivity of the *E. coli tag alkA* double mutant. The amino acid sequence similarity and evolutionary relationship of AfalkA with other MPG enzymes from the three domains of life are described and discussed.

DNA alkylation is an important lethal and mutagenic event in all cells, arising from extrinsic agents or from side reactions of normal metabolic functions, such as methylation (*1*). All the cells that were studied use *S*-adenosylmethionine as a cofactor in enzymatic transmethylation reactions: by bacteria in the methylation of adenine and cytosine to 6-methyladenine and 5-methylcytosine, respectively, and by mammals in the methylation of cytosine to 5-methylcytosine (*2*). However, erroneous nonenzymatic methylation of DNA (and other macromolecules) by *S*-adenosylmethionine as well as by other cofactors, e.g., N^5-methyltetrahydrofolic acid, also occurs at a slow rate (*3*, *4*).

The primary targets for alkylation of DNA are the N and O positions of the DNA bases, where the biological effect is dependent upon the position being affected (*5*−*7*). O^6-Alkylguanine and O^4-alkylthymine have been established as the most mutagenic lesions due to their ability to base pair with thymine and guanine, respectively (*8*, *9*). The abundantly formed 3-methyladenine (m^3A)[1] is the most important cytotoxic damage. This and certain minor products such as 3-methylguanine (m^3G) and O^2-alkylpyrimidines are excised from DNA in vivo by methylpurine DNA glycosylase (MPG) enzymes, leaving behind an abasic or apurinic/apyrimidinic (AP) site (*10*, *11*). This reaction initiates the so-called base excision repair (BER) pathway, which is predominantly completed by reinsertion of only one single nucleotide by the activities of 5′-acting AP endonuclease, DNA deoxyribophosphodiesterase, DNA polymerase, and DNA ligase (*10*−*13*).

The cytotoxicity of 3-methylpurines has been suggested to be caused by protrusion of the N^3-methyl group into the minor groove of the DNA double helix, thereby blocking DNA replication at the site of the lesion (*14*, *15*). *Escherichia coli* possesses two MPG enzymes that excise m^3A from DNA with high efficiency, i.e., the products of the *tag* and *alkA* genes. Tag, or m^3A DNA glycosylase I (M_r = 21 104; *16*, *17*), is constitutively produced and typifies the class I glycosylases, where corresponding genes have been found in bacteria and plants (*18*). AlkA, or m^3A DNA glycosylase

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* To whom correspondence should be addressed: Stavanger University College, P.O. Box 8002, N-4068 Stavanger, Norway. Telephone: 47-51831884. Fax: 47-51831750.

‡ University of Bergen.

§ University of Maryland Biotechnology Institute.

|| Stavanger University College.

⊥ University of Oslo.

[1] Abbreviations: AP, apurinic/apyrimidinic; BER, base excision repair; BSA, bovine serum albumin; HhH, helix−hairpin−helix; HPLC, high-performance liquid chromatography; m^3A, 3-methyladenine; m^7A, 7-methyladenine; m^3G, 3-methylguanine; m^7G, 7-methylguanine; MMS, methyl methanesulfonate; MNU, methyl-*N*-nitrosourea; MPG, methylpurine DNA glycosylase; ORF, open reading frame; 8-oxoG, 7,8-dihydro-8-oxoguanine; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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II (M_r = 31 402; *19*, *20*), is induced in cells exposed to sublethal doses of alkylating agents (*21*, *22*) and typifies the extensively characterized class II glycosylases (*18*), which are found in bacteria and lower eukarya (*10*). A three-dimensional crystal structure of AlkA was determined a few years ago (*23*, *24*). Class III glycosylases are typified by mammalian MPGs and are present in bacteria and higher eukarya (*18*). Recently, two different bacterial MPGs unrelated to classes I−III have been found in *Bacillus cereus* (*18*). In addition, two other enzymes belonging to the endonuclease III (Nth) superfamily of DNA glycosylases (Nth is involved in oxidation repair in *E. coli*) have been characterized: MpgII of *Thermotoga maritima* (*25*) and MagIII of *Helicobacter pylori* (*26*). Whether the latter four enzymes (here designated "class IV") represent three or four new classes of MPGs is being debated.

Despite its possible presence in most or all organisms, no MPG enzymes have yet been characterized from archaea, the third domain of life. However, open reading frames (ORFs) that are significantly homologous to class II or class IV MPGs have been detected in the genomes of several archaeons, including the sulfate-reducing and hyperthermophilic anaerobe *Archaeoglobus fulgidus* (*27*). In the report presented here, we describe the detection of base excision activity in *A. fulgidus* cell-free extracts, and the cloning and overexpression of a class II glycosylase gene from this organism. Enzymatic characterization of the purified protein demonstrated that the enzyme specifically releases methylated purines from DNA.

EXPERIMENTAL PROCEDURES

Methylated Bases. 7-Methyladenine (m^7A) was a gift from T. Lindahl. m^3G and 7-methylguanine (m^7G) were obtained from Fluka (product nos. 67072 and 67073, respectively), and m^3A was obtained from Sigma (product no. M-9281).

Cultivation of A. fulgidus and Preparation of Archaeon Cell Extracts. *A. fulgidus* type strain VC16 (DSMZ 4303; *28*) was grown anaerobically at 83 °C in 20 L carboys under an Ar headspace. Cell-free extracts were prepared as described elsewhere (*29*).

Enzymatic Assays for DNA Glycosylase Activities. Calf thymus DNA (Sigma) was treated with [^{3}H]methyl-*N*-nitrosourea (MNU; 1.5 Ci/mmol, Amersham Pharmacia), resulting in a specific radioactivity of 15 000 dpm/μg. [^{3}H]-Dimethyl sulfate/alkaline-treated poly(dG-dC) containing labeled 2,6-diamino-4-hydroxy-5*N*-methylformamidopyrimidine residues was prepared as described previously (*30*). *E. coli* [*methyl*-^{3}H]thymine-labeled DNA was obtained from New England Nuclear (NET-561). [^{3}H]Uracil-containing DNA (with a specific activity of 1110 dpm/pmol) was a gift from H. Krokan and B. Kavli.

Substrate DNA was incubated with enzyme in 50 μL of 140 mM Mops [3-(*N*-morpholino)propanesulfonic acid], 2 mM EDTA, 2 mM dithiothreitol (pH 7.5), 100 mM KCl, and 10% (v/v) glycerol (reaction buffer) at 70 °C for 10 min, unless otherwise stated, followed by precipitation with ethanol as in the m^3A DNA glycosylase assay (*31*).

Reverse phase high-performance liquid chromatography (HPLC) was performed according to the method of Hofer and Möller (*32*) with some modifications, using isocratic elution (0.5 mL/min, 0.5 min per fraction) of two successively coupled Inertsil 5 ODS-2 columns (150 mm × 4.6 mm, catalog no. 29326, Chromsep, Chrompack) with 50 mM sodium acetate (pH 5.3) and 10% (v/v) methanol (system 1) or 50 mM sodium acetate (pH 4) and 5% (v/v) methanol (system 2). System 2 resulted in broader peaks but provided complete separation of m^3G from m^3A and m^7A from m^7G.

Enzymatic Cleavage of DNA Fragments Containing a Base Lesion Inserted at a Specific Position. A DNA oligomer with 5-formyluracil inserted at a specific position is described elsewhere (*29*). A double-stranded DNA substrate with a 7,8-dihydro-8-oxoguanine (8-oxoG) residue inserted at a certain position was prepared by 5′-^{32}P labeling the oligonucleotide 5′-GGCGGCATGACCC[8-oxoG]GAGGCCCATC-3′ using T4 polynucleotide kinase (MBI Fermentas) and [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia), followed by annealing to complementary strands with A, C, G, or T opposite 8-oxoG. Double-stranded DNA substrates with hypoxanthine (I) and 1,N^6-ethenoadenine (EthA) site specifically inserted opposite T were prepared from 5′-GCTCATGCGCAG[I]-CAGCCGTACTCG-3′ and 5′-CGAGTACGGCGG[EthA]-GGGCGCATGAGC-3′, respectively, as described above. Enzymatic cleavage reactions and analysis of the products were essentially performed as described previously (*33*).

Cloning of the A. fulgidus AF2117 ORF. General cloning techniques were performed as described by Sambrook et al. (*34*). Amplification of the gene was performed using the oligonucleotide primers 5′-TACATATGTGGAGAATTGAG-CTGAAGC-3′ (forward) and 5′-TAGGATCCGGGGAA-CATCAAACTAATTCTG-3′ (reverse), containing *Nde*I or *Bam*HI restriction sites (underlined), respectively, for directional cloning into an expression vector. Polymerase chain reactions (PCRs) were set up with 1.25 units of *Pfu* DNA polymerase (Stratagene), 200 μM dNTP, each primer at 1 μM, ~100 ng of *A. fulgidus* DNA as a template, and buffer supplied by Stratagene, in a total volume of 50 μL. The reaction mixture was incubated at 95 °C for 5 min and then subjected to 15 cycles each with denaturation for 30 s at 95 °C, annealing for 30 s at 51 °C, and elongation for 6 min at 72 °C, followed by 15 cycles where the annealing temperature was changed to 60 °C.

The PCR product was first cloned into a blunt end cloning vector and subsequently transferred into expression vector pET-11a (Novagen). Prior to ligation into the pCR-Blunt vector using the Zero Blunt PCR cloning kit (Invitrogen), the PCR product was purified using the QIAquick PCR purification kit (Qiagen). A pCR-Blunt plasmid carrying the DNA insert was digested with *Nde*I and *Bam*HI. The excised fragment, as well as pET-11a digested with *Nde*I and *Bam*HI, were purified by extraction from an agarose gel using a QIAquick gel extraction kit (Qiagen). Following ligation overnight at 16 °C, the ligation mixture was used to transform competent *E. coli* DH5α cells (GibcoBRL). A pET-11a plasmid (pET-11a/af2117) harboring the cloned gene was then transferred into the expression host *E. coli* BL21(DE3). The nucleotide sequence of the insert was verified by sequencing both DNA strands. Prior to expression, the BL21-(DE3) strain was transformed by pSJS1240 (*35*), containing the genes encoding the rare *E. coli* tRNAs for arginine (AGA) and isoleucine (ATA), which are frequently used by hyperthermophiles.

Expression of the AF2117 ORF and Purification of AfalkA Protein. BL21(DE3)/pSJS1240 cells harboring pET-11a/

af2117 were grown at 37 °C in LB medium containing 100 μg/mL ampicillin and 30 μg/mL spectinomycin to an OD_{600} of 0.6. The cells were induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h and harvested at 4 °C by centrifugation at 7000*g* for 10 min. The cell pellet was washed once with ice-cold 50 mM Tris-HCl, 50 mM NaCl buffer (pH 7.6) and resuspended in the same buffer. The protein extract (fraction I) was prepared from the cell suspension by treating the sample twice in a French press. The cell lysate was incubated at 70 °C for 20 min, and denatured proteins were removed by centrifugation at 10000*g* for 15 min. The supernatant (fraction II) was applied to a HiTrap SP Sepharose column (1 mL, Amersham Pharmacia) equilibrated with 50 mM Mes [2-(*N*-morpholino)ethanesulfonic acid], 1 mM EDTA, 1 mM dithiothreitol (pH 6), and 5% (v/v) glycerol, where the proteins were eluted with a stepwise NaCl gradient (from 0.1 to 1 M). Fractions (2 mL each) were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE; Bio-Rad 12% Tris-HCl gel; Bio-Rad BroadRange 161-0318 protein standards).

Alkylation Survival Measurements. The two bacterial strains that were employed, AB1157 (wild type) and BK2118 (*tag alkA*), are derivatives of *E. coli* K-12 (*36*). Methyl methanesulfonate (MMS) was supplied by Aldrich (product no. 12,992-5). Exponentially growing cells were exposed to 5 or 50 mM MMS for the time periods indicated in M9 buffer at 37 °C, followed by washing in the same buffer and seeding of appropriate dilutions on LB plates, where bacterial colonies were counted the next day. The pET-11a/af2117 expression vector containing *afalkA* was transformed into AB1157 and BK2118 by electroporation.

General Procedures. Protein concentrations were determined by the method of Bradford (*37*) using bovine serum albumin (BSA) as a standard.

RESULTS

Enzymatic Release of Methylated Bases from DNA by Archaeon Cell-Free Extracts. This investigation was initiated by an interest in testing whether the archaeon *A. fulgidus* might contain an enzyme that releases methylated bases from DNA, since the growth conditions of this hyperthermophile suggest a sufficiently high rate of spontaneous removal of such bases by H_2O-mediated hydrolysis. However, incubation of [^{3}H]methylated calf thymus DNA with *A. fulgidus* cell-free extracts at 70 °C resulted in a protein-dependent release of ethanol-soluble radioactivity (Figure 1). In contrast, no release of radioactive material from aged [*methyl*-^{3}H]-thymine-labeled DNA was observed, indicating a lack or a low level of DNA glycosylase functions directed against some oxidized thymines, e.g., 5-(hydroxymethyl)uracil and 5-formyluracil in *A. fulgidus* (*29*). The latter observation also excludes a possible contribution by unspecific nuclease activities to the release of radioactive material in the DNA glycosylase assay system.

Identification of a Gene Encoding MPG in A. fulgidus. A recent determination of the complete genome sequence of *A. fulgidus* revealed an ORF (AF2117) homologous to the *E. coli* gene (*alkA*) encoding the m^{3}A DNA glycosylase II (*27*), which was considered a good candidate for the gene encoding the putative MPG enzyme detected in *A. fulgidus*

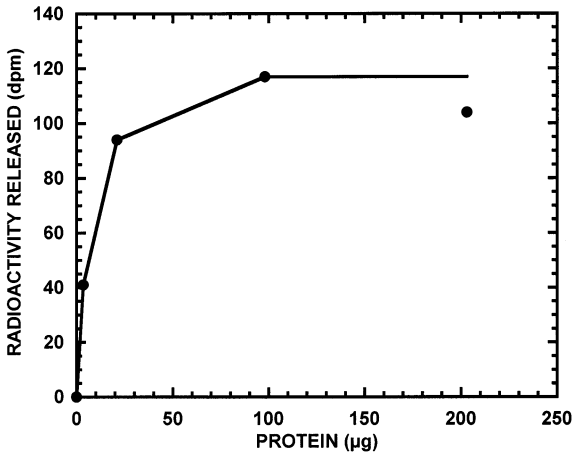

FIGURE 1: Protein dependence for the excision of methylated bases from [^{3}H]MNU-treated DNA by cell-free extract prepared from *A. fulgidus* cells. Protein extract was incubated with alkylated calf thymus DNA (2000 dpm, 0.6 pmol of methylated DNA bases) in 50 μL of 70 mM Mops, 1 mM EDTA, 1 mM dithiothreitol (pH 7.5), 100 mM KCl, and 5% (v/v) glycerol at 70 °C for 10 min. Each value represents the average of three independent measurements (deviation from the mean, ≤4.8%). The background value obtained from incubations without enzyme (195 dpm) was subtracted.

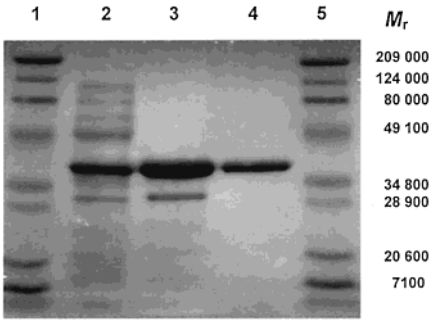

FIGURE 2: SDS−PAGE of different fractions obtained during purification of the overproduced AF2117 gene product (AfalkA). Proteins were separated on a 12% (w/v) polyacrylamide gel and stained with Coomassie blue: lanes 1 and 5, molecular weight markers [from top to bottom, myosin (M_r = 209 000), β-galactosidase (M_r = 124 000), BSA (M_r = 80 000), ovalbumin (M_r = 49 100), carbonic anhydrase (M_r = 34 800), soybean trypsin inhibitor (M_r = 28 900), lysozyme (M_r = 20 600), and aprotinin (M_r = 7100)]; lane 2, fraction I (crude extract, 20 μg); lane 3, fraction II (crude extract heat-treated at 70 °C for 20 min and centrifuged, 11 μg); and lane 4, fraction III (2.9 μg).

cell-free extracts. The AF2117 ORF was cloned and overexpressed in *E. coli*. SDS−PAGE of crude protein extracts prepared from *E. coli* cells expressing AF2117 indicated an M_r of 40 000 for the overproduced protein (Figure 2), which is significantly higher than the M_r of 34 251 estimated from the amino acid sequence. The thermophilic nature of the protein was confirmed by heating the extract at 70 °C for 20 min, which demonstrated that virtually only the "AF2117" protein resisted denaturation. Apart from one band at an M_r of ~30 000, the overproduced protein was electrophoretically pure after the heat treatment (Figure 2). When this partially purified fraction was incubated with [^{3}H]methylated DNA at 70 °C, radioactivity was released in a protein-dependent manner (data not shown). This indicates that the AF2117 ORF, which hereafter will be designated *afalkA*, encodes an MPG enzyme.

Table 1: Purification of AfalkA

fraction	volume (mL)	protein (mg)	specific activity (pmol of m^3A released/ mg of protein)	purification (-fold)
protein extract (I)	5.5	80	7.8	1
heat treatment (II)	5	41	17	2.2
SP Sepharose (III)	4	6.7	1100	140

Purification, Substrate Preference, and Kinetics of the AfalkA Enzyme. Because AfalkA resisted inactivation by heating at 70 °C for 20 min, a treatment which denatures most *E. coli* proteins (Figure 2), crude *E. coli* extracts with overproduced protein (fraction I) were routinely subjected to such treatment as the first step in the purification of AfalkA, as previously used in the purification of uracil DNA glycosylase of *A. fulgidus* (Afung; *29*). Following centrifugation, the supernatant (fraction II) was applied to a strong cation exchange column (SP Sepharose) at pH 6, where the AfalkA protein eluted from the gel at 0.4 and 0.5 M NaCl and appeared to be electrophoretically pure (Figure 2, fraction III). The specific activity of the enzyme at the various steps of the purification is indicated in Table 1.

Analysis of the protein dependence for the excision of methylated purines from [^{3}H]MNU-treated DNA by purified AfalkA (fraction III) showed that 3.2% of the methyl label in the substrate was excised by the smallest amount of enzyme that was employed (0.035 pmol). Increasing the enzyme concentration 100-fold resulted in a release of 5.9% (Figure 3) and 1000-fold of only 8.2% of the radioactivity (data not shown), corresponding to 8−9% of the methyl label as m^3A in MNU-treated DNA (*6*). The results thus indicate that m^3A is efficiently excised from alkylated DNA by AfalkA. AfalkA-mediated release of larger amounts of radioactivity (up to 33%) following incubations with large amounts of enzyme can only be explained by slow excision of m^7G (data not shown), which is present in 65−70% of the methyl label in MNU-treated DNA (*6*).

As well as providing evidence that AfalkA is a true DNA glycosylase, HPLC analysis of radioactive material released following incubations at 50 and 65 °C confirmed the efficient release of m^3A and the slow release of m^7G by the enzyme (Figure 4A,B); m^3A was released by similar rates at both temperatures, whereas m^7G was released slowly at 65 °C and extremely slowly at 50 °C (Figure 4B). In addition, HPLC analysis demonstrated the ability of AfalkA to excise the minor alkylation products m^3G and m^7A from [^{3}H]MNU-treated DNA at both temperatures (Figure 4C), although with significantly lower efficiency than m^3A. Like m^3A, and contrary to m^7G, however, both of these lesions were released quantitatively from DNA at 65 °C by the highest concentrations of enzyme that were employed. Thus, 1.1 and 0.94% of the radioactivity were recovered as m^3G and m^7A, respectively, following HPLC (Figure 4C), which corresponds to the previously determined contents of 0.6−1.9% of the methyl label as m^3G and 0.8−2% as m^7A in MNU-treated DNA (*6*). To summarize, the analysis of the protein-dependent release of the different methylated bases at 65 °C by AfalkA indicated the following order for the excision efficiency: $m^3A \gg m^3G \sim m^7A \gg m^7G$. This contrasts with *E. coli* AlkA, which excises m^3A and m^3G equally efficient from DNA (*38*).

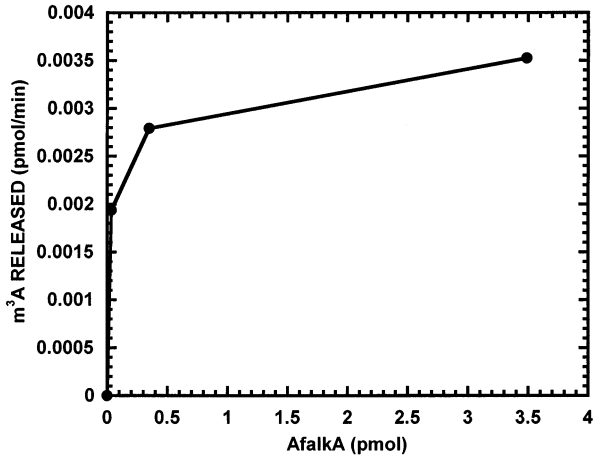

FIGURE 3: Protein dependence for the excision of methylated bases from [^{3}H]MNU-treated DNA by AfalkA. An increasing amount of AfalkA protein (0.035−700 pmol) was incubated with alkylated calf thymus DNA (2000 dpm, 0.6 pmol of methylated DNA bases) in 70 mM Mops, 1 mM EDTA, 1 mM dithiothreitol (pH 7.5), 100 mM KCl, and 5% (v/v) glycerol at 70 °C for 10 min, where material released by the smallest quantities of enzyme, which corresponds to the excision of m^3A, is presented. Each value represents the average of two independent measurements (deviation from the mean, ≤1.9%). The background value obtained from incubations without enzyme (198 dpm) was subtracted.

To specifically examine the efficiency of excision of m^3A from [^{3}H]methylated DNA by AfalkA, initial velocities of methylated base removal were measured as a function of substrate concentration under conditions where virtually only m^3A is enzymatically released, i.e., using 0.633 pmol of AfalkA over a substrate range of 1000−5000 dpm of [^{3}H]-MNU-treated DNA (0.6−3 nM m^3A) at 70 °C (see Figure 3). Analysis of the results with a Lineweaver−Burk plot indicated an apparent K_m of 2.2 nM (Figure 5), which is 5 times lower than the value of 10.8 nM measured for *E. coli* AlkA (*39*). The V_{max} for the AfalkA-catalyzed excision of m^3A from DNA was determined to be 0.0077 pmol/min, resulting in a k_{cat} of 0.012 min^{-1} and a k_{cat}/K_m of 0.0055 nM^{-1} min^{-1}; these values are on the same order of magnitude as the respective values of 0.03 min^{-1} and 0.0028 nM^{-1} min^{-1} determined for AlkA (*39*).

Partially purified AfalkA protein (fraction II) contained no uracil DNA glycosylase activity as analyzed by possible release of uracil from [^{3}H]uracil-containing DNA at 60 and 95 °C (data not shown). In addition, no activity toward imidazole-damaged m^7Gs was detected (data not shown), demonstrating that AfalkA is devoid of formamidopyrimidine DNA glycosylase activity. Experiments with defined DNA oligomers containing 5-formyluracil paired with the four common normal bases were also performed. However, no excision of 5-formyluracil by AfalkA was observed (data not shown). This contrasts with *E. coli* AlkA, which has been shown to remove the lesion from DNA when paired with A or G (*39*, *40*). Incubations of the purified enzyme (fraction III, 2.9 pmol; 50 °C for 10 min) with defined DNA oligomers containing 8-oxoG opposite the four common normal bases showed no AfalkA-mediated excision of this oxidized base (data not shown). Similar results were obtained using defined DNA oligomers with hypoxanthine and 1,N^6-ethenoadenine placed opposite thymine (data not shown), which contrast with *E. coli* AlkA that exhibits activity toward these latter lesions (*41*, *42*).

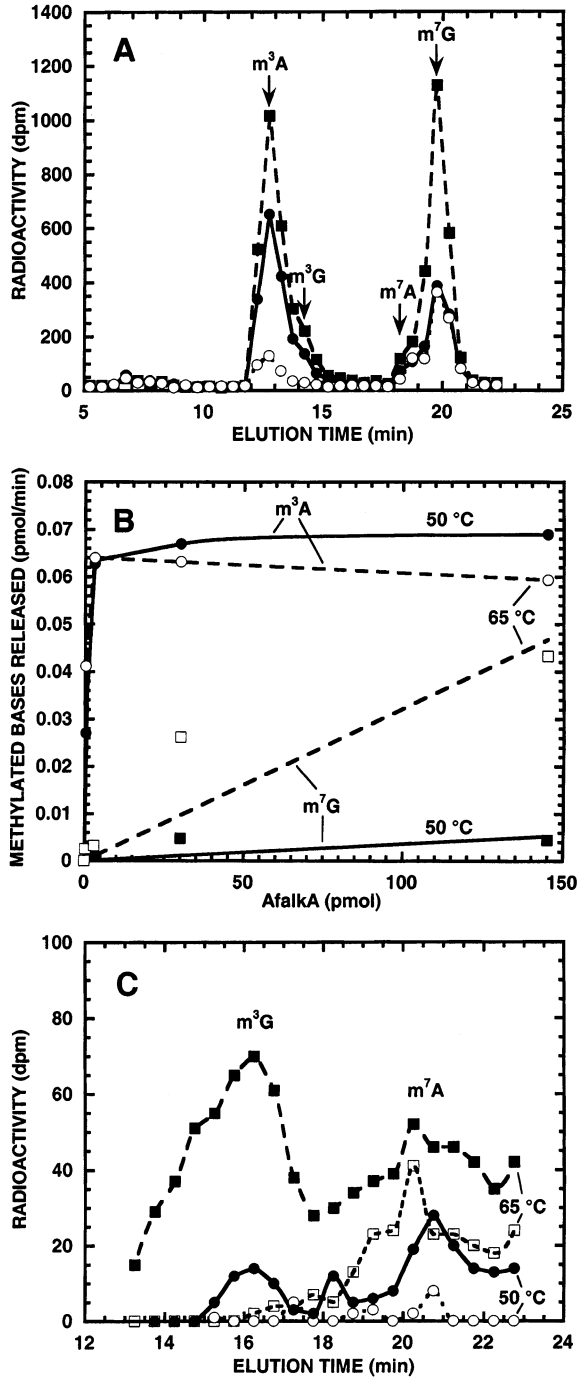

FIGURE 4: Reverse phase HPLC of methylated bases released from [^{3}H]MNU-treated DNA by AfalkA. Enzyme (0, 0.3, 3, 30, and 145 pmol) was incubated with alkylated calf thymus DNA (40 000 dpm, 12 pmol of methylated DNA bases) in reaction buffer at 50 or 65 °C for 10 min, followed by analysis of the ethanol-soluble material by HPLC. (A) Elution profiles (system 1) of material released by an increasing amount of AfalkA at 65 °C: (○) 0 (control), (●) 0.3, and (■) 145 pmol. (B) Protein dependence curves for enzymatic excision of the major alkylation products m^{7}G [(■) 50 and (□) 65 °C] and m^{3}A [(●) 50 and (○) 65 °C]. (C) Elution profiles (system 2) of material released as m^{3}G and m^{7}A at 50 °C [(○) 0 (control) and (●) 145 pmol] and 65 °C [(□) 0 (control) and (■) 145 pmol] by AfalkA.

Inhibition of AfalkA Activity. The m^{3}A-releasing activity of AfalkA was unaffected (i.e., showed no product inhibition) by the addition of m^{3}A (5 mM) to the incubation mixture (Table 2), as previously reported for *E. coli* AlkA (*38*).

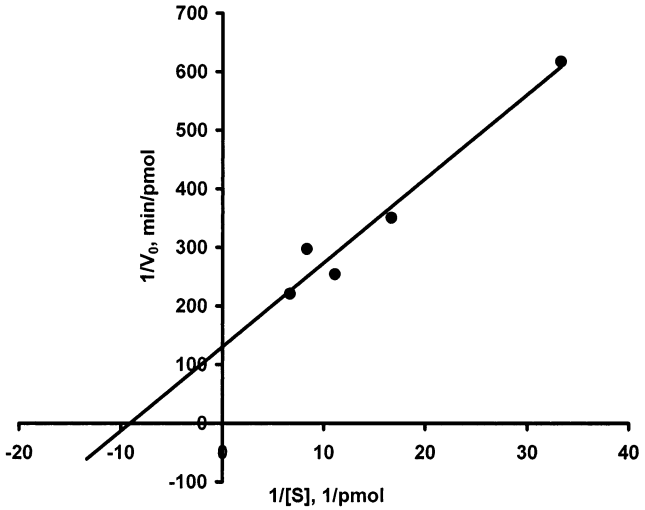

FIGURE 5: Lineweaver−Burk plot for the excision of m^{3}A from [^{3}H]MNU-treated DNA by AfalkA. Enzyme (0.633 pmol) was incubated with an increasing amount of alkylated calf thymus DNA (1000−5000 dpm, 0.03−0.15 pmol m^{3}A) in reaction buffer at 70 °C for 10 min. Each value represents the average of two independent measurements (deviation from the mean, ≤4.8%). The background value obtained from incubations without enzyme (155 dpm) was subtracted.

Table 2: Effect of Metal Ions, Sulfhydryl-Blocking Agent, and Methylated Bases on AfalkA Activity[a]

addition	concentration (mM)	enzyme activity (%)
none		100
$MgSO_4$	2	112 ± 4
$CaCl_2$	2	103 ± 6
$MnCl_2$	2	102 ± 3
$FeCl_3$	2	17 ± 4
$ZnSO_4$	0.1	114 ± 1
	2	23 ± 11
p-hydroxymercuribenzoate	1	29 ± 7
m^{3}A	5	110 ± 5
m^{3}G	5	120 ± 7

[a] Enzyme (0.117 pmol) was incubated with [^{3}H]MNU-treated calf thymus DNA (2000 dpm, 0.06 pmol of m^{3}A) in reaction buffer for 10 min. Each value represents the average ± the standard deviation of three independent measurements.

Similarly, no inhibition of AfalkA was observed using the same concentration of m^{3}G as well as 2 mM of $MgSO_4$, $CaCl_2$, or $MnCl_2$. In contrast, *p*-hydroxymercuribenzoate (1 mM) caused a 70% decline in AfalkA activity, indicating an essential thiol as the denominator for enzyme activity. AfalkA activity was similarly inhibited by 2 mM $FeCl_3$ or $ZnSO_4$ (Table 2).

Temperature and pH Dependence of AfalkA Activity. AfalkA activity was detectable from 20 to 80 °C, but varied significantly, when incubated with [^{3}H]methylated DNA (Figure 6A). Thus, a 50 times larger amount of enzyme (5.75 pmol) was needed to demonstrate significant activity at 20 °C (data not shown). AfalkA exhibited a broad optimum of activity around 65−75 °C (Figure 6A), which is ∼10 °C lower than the optimal growth temperature of *A. fulgidus*. In addition, the thermostability of the protein was demonstrated by the fact that 60% of enzyme activity was left following incubation for 1 h at 90 °C compared to incubation of the enzyme at 60 °C (data not shown). AfalkA is consequently more heat-stable than the two other character-

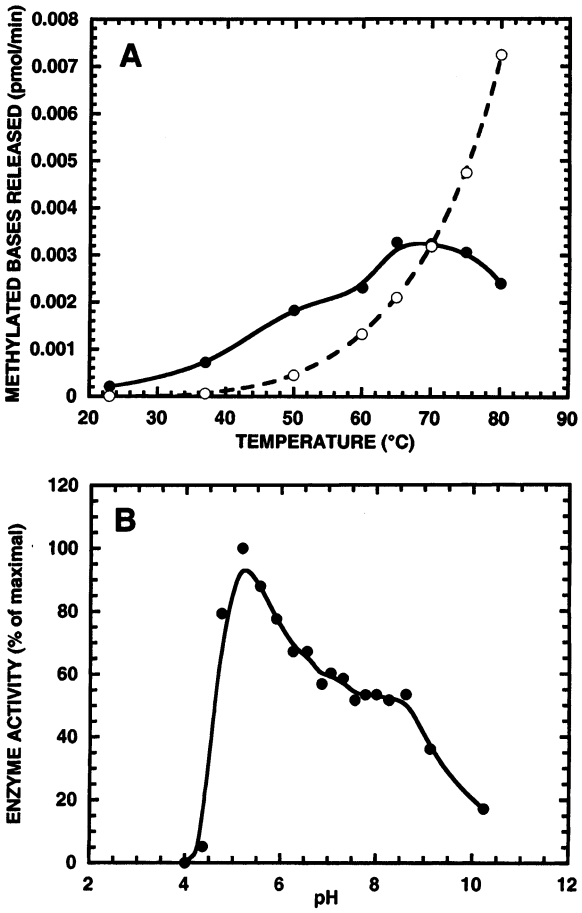

FIGURE 6: Temperature (A) and pH dependence (B) for the excision of m^3A from [^{3}H]MNU-treated DNA by AfalkA. Enzyme (0.117 pmol) was incubated with alkylated calf thymus DNA (2000 dpm, 0.6 pmol of methylated DNA bases/0.06 pmol of m^3A) for 10 min in (A) reaction buffer at different temperatures [(●) AfalkA-excised material and (○) nonenzymatically released material] or (B) universal buffer (*44*) containing 1 mM EDTA, 1 mM dithiothreitol, 100 mM KCl, and 5% (v/v) glycerol at different pH values and 70 °C (100% enzyme activity = 0.002 pmol/min). Each value represents the average of two or three independent measurements [deviation from the mean, (A) ≤7.7 and (B) ≤4.8%]. The background value obtained from incubations without enzyme [(A) ≤37 °C, 33 dpm; >37 °C, 33 dpm + the value indicated (○); (B) 45 dpm, except at pH 4 (82 dpm) and pH 4.4 (62 dpm)] was subtracted.

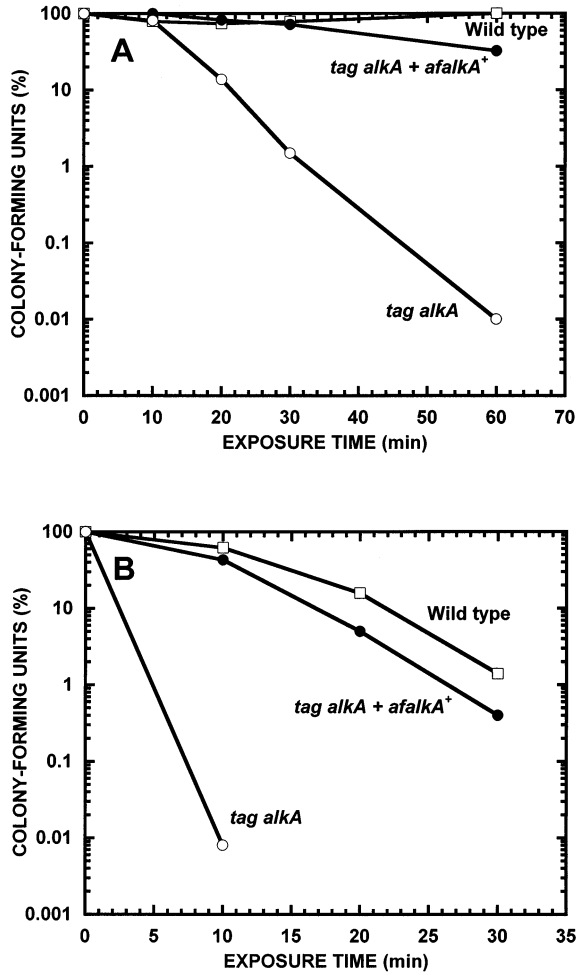

FIGURE 7: Suppression of the MMS sensitivity of the *E. coli tag alkA* double mutant by *afalkA*. The colony-forming ability of cells exposed to 5 (A) or 50 mM MMS (B) for the indicated period of time was determined for the wild type, the wild type transformed with the pET-11a/af2117 expression vector containing the *afalkA* gene, the *tag alkA* double mutant, and the *tag alkA* double mutant transformed with pET-11a/af2117. The wild type transformed with pET-11a/af2117 exhibited a response to the MMS treatment identical to that of the wild type without this vector (data not shown).

ized DNA glycosylases of *A. fulgidus*, the 8-oxoG DNA glycosylase (Afogg; *43*) and Afung (*29*).

To analyze the pH dependence of enzyme activity, AfalkA was incubated with [^{3}H]methylated DNA at 70 °C in a modified universal buffer at different pH values (*31*, *44*), showing that the enzyme exhibited a distinct peak of optimal activity at pH 5.2. This is similar to the optimal pH of 4.8 determined for Afung (*29*), but significantly different from the optimal pH of ~8.5 reported for Afogg (*43*). AfalkA displayed more than 50% of maximal activity from pH 4.8 to 8.6 (Figure 6B).

Suppression of the Alkylation Sensitivity of the E. coli tag alkA Double Mutant by afalkA. Following transformation of the expression vector containing *afalkA* into the very alkylation sensitive *tag alkA* double mutant (BK2118; *36*), significantly more cells survived the exposure, approaching the resistance of the wild type (AB1157), at both 5 and 50 mM MMS. At the longest times of exposure, survival measured as colony-forming units increased several thousand fold (Figure 7A,B). These in vivo results confirm the in vitro demonstration of significant enzyme activity of AfalkA at 37 °C (Figure 6A).

Sequence Comparisons and Phylogeny. Careful alignment of the amino acid sequence of AfalkA with those of *E. coli* AlkA and *Saccharomyces cerevisiae* Mag demonstrates an extensive homology among MPGs from the three domains of life (Figure 8A). Determination of the crystal structure together with site-directed mutagenesis has identified certain amino acid residues as being critical for enzyme activity of *E. coli* AlkA (*23*, *24*, *46*). For instance, Asp238 (in AlkA) is conserved in all members of the AlkA family and probably participates in the catalytic mechanism as a nucleophile to attack C1′ of the deoxyribose residue, promoting release of the alkylated base (*46*). With regard to the amino acid residues lining the suggested active site pocket of AlkA, two of six are identical and four of six are similar to those of AfalkA (Table 3), thus explaining the similarity between the two enzymes in methylated base removal. However, the remaining two putative active site residues are not conserved

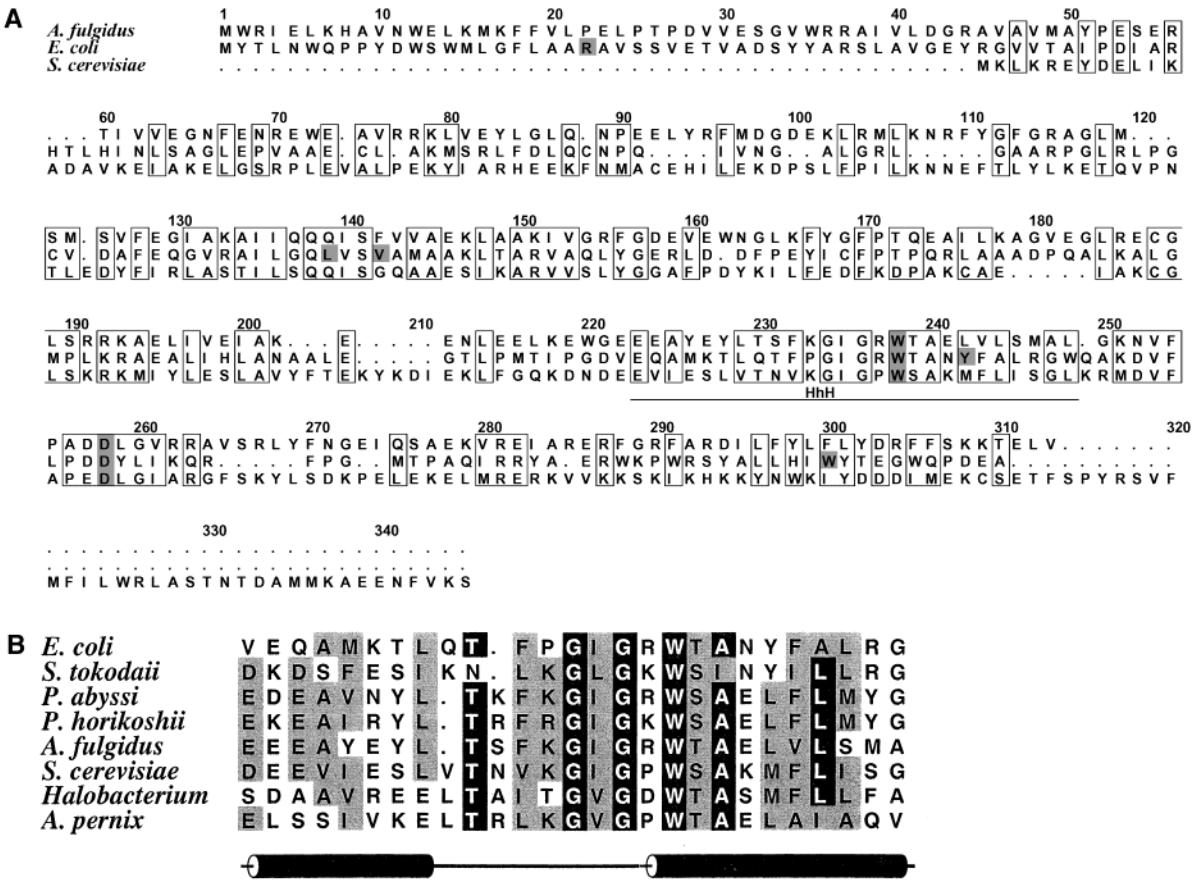

FIGURE 8: Comparison of the sequence of AfalkA to those of other proteins. (A) Alignment of the amino acid sequence of AfalkA with the homologous sequences of AlkA (*E. coli*) and Mag (*S. cerevisiae*). Positions forming the suggested active site in AlkA of *E. coli*, including the catalytic aspartate (D238, *E. coli* numbering; D258 in the alignment), are shaded. The DNA-binding helix−hairpin−helix (HhH) motif is underlined. The sequences were aligned using ClustalW (*45*; Genetics Computer Group). (B) Evolutionary conservation of the HhH motif in archaea. Shown are the partial amino acid sequence alignments of AlkA homologues identified in archaeal genome sequences and the HhH motif in *E. coli* AlkA and *S. cerevisiae* Mag. Highly conserved residues are boxed in black, whereas less well-conserved residues are highlighted in gray. The helices in the *E. coli* HhH motif are indicated below the alignment. Abbreviations (SWISS-PROT/TrEMBL accession numbers in parentheses): *A. fulgidus* (O28163); *A. pernix*, *Aeropyrum pernix* (Q9YFG9); *E. coli* (P04395); *Halobacterium*, *Halobacterium* sp., strain NRC-1 (Q9HQ62); *P. abyssi*, *Pyrococcus abyssi* (Q9UZ73); *P. horikoshii*, *Pyrococcus horikoshii* (O58476); *S. cerevisiae* (P22134); *S. tokodaii*, *Sulfolobus tokodaii* (Q972N8).

Table 3: Amino Acid Residues Forming the Suggested Active Site Pocket in AlkA of *E. coli* and the Proposed Corresponding Residues in AfalkA and Mag of *S. cerevisiae*[a]

alignment no. (see Figure 8A)	amino acid residue			proposed function
	AlkA	AfalkA	Mag	
22	$Arg^{b}22$	$Pro^{h}22$		
142	$Val^{h}128$	$Phe^{h}133$	$Gly^{b}98$	van der Waals surface
238	$Trp^{h}218$	$Trp^{h}221$	$Trp^{h}189$	van der Waals surface
242	$Tyr^{p}222$	$Leu^{h}225$	$Met^{h}193$	van der Waals surface
258	**$Asp^{a}238$**	**$Asp^{a}240$**	**$Asp^{a}209$**	nucleophile
300	$Trp^{h}272$	$Phe^{h}282$	$Ile^{h}251$	π-cation interaction

[a] The AlkA residues are selected according to the report of Hollis et al. (*46*). The residue believed to participate directly in the catalytic reaction is indicated in bold. The superscript letters denote the following: a, ionizable (acidic); b, ionizable (basic); h, hydrophobic; and p, polar (nonionizable).

between AlkA and AfalkA; Arg22 and Tyr222 of AlkA have been replaced with Pro and Leu in AfalkA, respectively (Figure 8A and Table 3). It is tempting to speculate whether the presence of the basic and/or polar residue(s) in AlkA in place of the hydrophobic residues present in AfalkA might explain the different activity exhibited toward the polar base damage 5-formyluracil (ref *39* and data not shown). It is also noteworthy that the proposed active site residues seem to be more conserved between AfalkA and the Mag glycosylase of *S. cerevisiae* (*47*, *48*) than between AfalkA and AlkA (Table 3), which is in accord with the much more similar substrate specificity observed between the former than the latter (*49*).

The AlkA DNA glycosylase family (or class II glycosylases) belongs to the endonuclease III or HhH-GPD superfamily of base excision repair proteins, which also includes MutY and 8-oxoG DNA glycosylases. These enzymes all share a common helix−hairpin−helix (HhH) motif, which is proposed to act as a DNA-binding domain (*12*). Alignment of this motif from putative archaeal AlkA homologues with those from *E. coli* AlkA and *S. cerevisiae* Mag shows that it is highly conserved also in the archaeal domain of life (Figure 8B). A phylogenetic tree based on MPG sequences from archaea, yeast, and bacteria (Figure 9) shows that MPGs from hyperthermophilic archaea form a separate cluster, which in addition includes AlkA from *Bacillus subtilis*. This indicates a lateral gene transfer event from archaea to *Bacillus*. AlkA homologues from Gram-negative bacteria appear to be monophyletic, while the yeast enzymes may have two different origins. However, low bootstrap values in the central region of the tree indicate that the branching

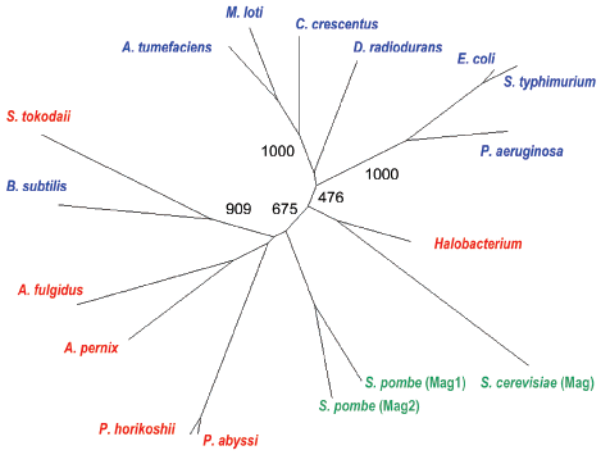

FIGURE 9: Unrooted phylogenetic tree of AlkA and its homologues. The sequences were aligned using ClustalW (*45*), and a distance analysis was performed using Protdist (Phylip, version 3.573c). The phylogenetic tree was constructed using the neighbor-joining algorithm and displayed by the Drawtree program (Genetics Computer Group). Bootstrap values obtained for 1000 iterations are indicated at major nodes. Color codes are as follows: red for archaea, blue for bacteria, and green for eukarya. Abbreviations in addition to those explained in Figure 8 (SWISS-PROT/TrEMBL accession numbers in parentheses): *A. tumefaciens*, *Agrobacterium tumefaciens* (Q8U9Y7); *B. subtilis* (P37878); *C. crescentus*, *Caulobacter crescentus* (Q9A693); *D. radiodurans*, *Deinococcus radiodurans* (Q9RRB0); *M. loti*, *Mesorhizobium loti* (Q98G59); *P. aeruginosa*, *Pseudomonas aeruginosa* (Q9I339); *S. pombe*, *Schizosaccharomyces pombe* (Q92383 and O94468); *S. typhimurium*, *Salmonella typhimurium* (Q8ZNQ5).

order is uncertain. The lack of AlkA homologues in higher eukaryotes implies a prokaryotic origin of the Mag enzymes in yeast, and the bifurcated shape of the tree further implies that *alkA* genes are ancient, and were probably present before the divergence of the two prokaryotic domains. However, in several archaeal and bacterial species, AlkA homologues cannot be detected, indicating that this type of gene has frequently been lost during the process of speciation. No AlkA homologues could be found in other completely sequenced archaeal genomes such as that of *Thermoplasma* spp., *Sulfolobus solfataricus*, and methanogens.

DISCUSSION

Purine residues are hydrolytically released from DNA relatively frequently due to the instability of the *N*-glycosyl bond, forming AP sites as the most abundant DNA lesion in all cells (*1*). For instance, it has been estimated that ~2000−10000 purines are lost every day from the DNA of a human cell by thermal disruption. At higher temperatures, the frequency of such reactions is significantly increased (*50*). Because alkylation at most positions, including m^3A and m^7G, causes a further decrease in the stability of the *N*-glycosyl bond (*14*) resulting in a short half-life of such residues at high temperatures, it was somewhat surprising when we detected MPG activity in cell-free extracts prepared from the hyperthermophilic *A. fulgidus* (Figure 1). The complete genome sequencing of this organism revealed, however, the presence of an ORF (AF2117) homologous to the *alkA* gene of *E. coli* (*27*). Here we describe cloning and overexpression in *E. coli* of this putative glycosylase gene of *A. fulgidus* (Figure 2), followed by enzymatic characterization of the purified protein. The results show that the encoded protein releases m^3A efficiently and m^3G, m^7A, and m^7G less efficiently from DNA (Figure 4A−C). The AF2117 ORF was thus designated *afalkA*. To our knowledge, this is the first report describing an archaeal DNA glycosylase enzyme removing alkylated bases from DNA. The presence of putative genes for O^6-methylguanine DNA methyltransferase (Ogt) and restriction modification enzymes [the latter probably requiring *S*-adenosylmethionine as a cofactor (*2*)] in the *A. fulgidus* genome (*27*) provides further support for the notion that the DNA in this organism is suffering from alkylation damage that needs to be repaired. Recently, two other glycosylases of *A. fulgidus* were overproduced, purified, and characterized: uracil DNA glycosylase (Afung; *29*, *51*) and 8-oxoG DNA glycosylase (Afogg; *43*).

As indicated above, the extensive nonenzymatic release of methylated purines at growth temperatures of hyperthermophiles (≥80 °C) asks for an explanation for why an MPG enzyme is present at all in such organisms. For instance, these results indicate a half-life of ~45 min at 80 °C for the spontaneous removal of methylpurines from DNA (Figure 6A), which means that >90% is spontaneously released after <3 h. The nonenzymatic hydrolysis also acts specifically by releasing the cytotoxic m^3A from DNA at a much higher rate than the innocuous m^7G (*14*). However, AfalkA clearly enhances this effect (Figure 6A).

In contrast to Afung, exhibiting a temperature optimum (~80 °C) for uracil release around the optimal growth temperature of *A. fulgidus* (*29*), AfalkA excises m^3A optimally at 65−75 °C and shows significant activity even at 37 °C (Figure 6A). On the basis of these facts, we suggest that the biological function of AfalkA is to remove m^3A from DNA at suboptimal growth temperatures (64−80 °C) and at temperatures not supporting growth. At growth temperatures above 80 °C (i.e., ≤92 °C), the initial step in the BER pathway may be predominantly nonenzymatic. Efficient removal of m^3A lesions during resting or slow growth at low temperatures, thus preparing the cells for replication of its DNA when growth commences or increases at higher temperatures, may be particularly beneficial to organisms such as *A. fulgidus* VC-16, which may experience quite fluctuating temperatures in its natural habitats. The ability of *afalkA* to complement the alkylation sensitivity of the *E. coli tag alkA* double mutant (Figure 7A,B) strengthens this hypothesis.

An alternative explanation for the presence of an AlkA-like enzyme in *A. fulgidus* could be indicated by the fact that m^3G, which despite its lower abundance may be equally important for cell toxicity as m^3A (*22*), is much more stable to nonenzymatic removal from DNA than m^3A (*14*). However, the low efficiency for the removal of m^3G from DNA displayed by the enzyme argues against this as a significant function of AfalkA.

Each *A. fulgidus* cell contains ~0.033 pg of soluble protein (*29*). Taking this into account and suggesting that AfalkA is the only MPG enzyme present, we can calculate that *A. fulgidus* contains ~125 AfalkA molecules per cell. This is surprisingly similar to the previously determined content of ~200 Tag and ~50 AlkA molecules in an uninduced *E. coli* cell (*31*, *39*), considering the 50% smaller genome of *A. fulgidus* compared to that of *E. coli*. It implies that the MPG function is of similar importance to the two organisms.

One other report describing an MPG of a hyperthermophilic organism recently appeared in the literature. The anaerobic bacterium *T. maritima*, which is capable of growth to 90 °C (optimum at 80 °C), contains a gene for an enzyme (MpgII) that is able to excise both m^3A and m^7G from DNA, although no data were provided to differentiate their abilities as substrates (*25*). MpgII was also reported to be active at both 37 and 65 °C (although without quantitative comparison) and the corresponding gene shown to complement the alkylation sensitivity of the *E. coli tag alkA* double mutant. These results seem to support our conclusion by indicating a similar biological function for MpgII and AfalkA, i.e., to excise m^3A from cellular DNA at suboptimal growth and nongrowth temperatures. That this may be a general adaptive strategy for hyperthermophiles is further supported by the fact that a homologue of the *alkA* gene occurs in the genome of the hyperthermophile *Pyrococcus horikoshii*, which grows optimally at 98 °C (PH0784; *52*).

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