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# Repair activities of 8-oxoguanine DNA glycosylase from *Archaeoglobus fulgidus*, a hyperthermophilic archaeon

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**Abstract**

Oxidative DNA damage is caused by reactive oxygen species formed in cells as by products of aerobic metabolism or of oxidative stress. The 8-oxoguanine (8-oxoG) DNA glycosylase from *Archaeoglobus fulgidus* (*Af*ogg), which excises an oxidatively-damaged form of guanine, was overproduced in *Escherichia coli*, purified and characterized. *A. fulgidus* is a sulfate-reducing archaeon, which grows at between 60 and 95°C, with an optimum growth at 83°C. The *Af*ogg enzyme has both DNA glycosylase and apurinic/apyrimidinic (AP) lyase activities, with the latter proceeding through a Schiff base intermediate. As expected for a protein from a hyperthermophilic organism, the enzyme activity is optimal near pH 8.5 and 60°C, denaturing at 80°C, and is thermally stable at high levels of salt (500 mM). The *Af*ogg protein efficiently cleaves oligomers containing 8-oxoG:C and 8-oxoG:G base pairs, and is less effective on oligomers containing 8-oxoG:T and 8-oxoG:A mispairs. While the catalytic action mechanism of *Af*ogg protein is likely similar to the human Ogg1 (*hOgg*1), the DNA recognition mechanism and the basis for 8-oxoG substrate specificity of *Af*ogg differ from that of *hOgg*. 

*Keywords:* DNA repair; 8-Oxoguanine DNA glycosylase; *Archaeoglobus fulgidus*; Apurinic/apyrimidinic (AP) lyase

## 1. Introduction

Oxidative damage to DNA is an important factor in mutagenesis in all aerobic organisms and has been postulated to contribute to a wide range of diseases, including atherosclerosis, strokes and autoimmune syndromes [1,2]. Several types of oxidative DNA lesions are formed, for example, strand breaks, baseless sugars and a range of different oxidized DNA bases [3].

The most well-characterized oxygen free radical-induced alteration in DNA is 8-oxoguanine (8-oxoG) [4,5]. The presence of 8-oxoG residues in the template during replication induces G:C → T:A transversions, whereas A:T → C:G transversions can arise from the incorporation of 8-oxoG containing deoxyribonucleotides formed in the nucleotide precursor pool. In response, cells have evolved multiple mechanisms to repair this oxidative damage. Studies with *Escherichia coli* have shown that 8-oxoG is excised from oxidatively-damaged DNA by a DNA repair enzyme known variously as MutM [6] or formamidopyrimidine DNA glycosylase [7,8], and 8-oxoG DNA glycosylase (Ogg) [9].

Two 8-oxoG-specific glycosylase/lyase enzymes, termed Ogg1 and Ogg2, have been isolated from *Saccharomyces cerevisiae* [10]. Yeast Ogg1 has both 8-oxoG:C-specific base-excision DNA repair activity,

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and also an intrinsic β-lyase activity, which proceeds through a Schiff base intermediate. In contrast, Ogg2 preferentially acts on 8-oxoG:G. Based on sequence homology with yeast Ogg1, the cDNA of human Ogg, including several splice variants, has been cloned and expressed in *E. coli* by several groups [11–13]. The mammalian Oggs are 8-oxoG DNA glycosylase/AP lyases like *E. coli* Fpg and yeast Ogg1.

Human cell extracts possess two antigenically distinct Ogg activities with an identical reaction mechanism [14]. The *hOgg*1, identical to the cloned enzyme, cleaves 8-oxoG when paired with C, T and G but not with adenine in DNA. However, the newly discovered 36 kDa *hOgg*2 preferentially acts on 8-oxoG paired with G and A.

In order to understand prokaryotic base excision repair and compare the properties with eukaryotic enzymes, we searched for prokaryotic thermostable DNA glycosylase superfamily homologs. Recently, a thermostable 8-oxoG DNA glycosylase from *Methanococcus jannaschii* (*Mj*ogg) has been expressed in *E. coli* and characterized [15]. We have found that *Archaeoglobus fulgidus* open reading frame *Af*0371 shares 47% sequence identity with *Mj*ogg (*Mj*0724). *A. fulgidus* is the first hyperthermophilic and sulphur-metabolizing organism to have its genome sequence determined [16]. Growth occurs at between 60 and 95°C, with an optimum growth at 83°C. In the present work, a thermostable 8-oxoG DNA glycosylase from *Archaeoglobus fulgidus* (*Af*ogg) has been expressed in *E. coli*, purified and characterized.

## 2. Materials and methods

### 2.1. Enzymes and other materials

Oligonucleotides for the polymerase chain reaction (PCR) primer and substrate were purchased from Bio-Synthesis Inc. Amylose resin and the pMal-c2x vector were purchased from New England Biolabs. Restriction endonuclease and T4 DNA ligase were obtained from Promega. *Taq* DNA polymerase was a product of Takara. All the instruments and columns for FPLC were supplied by Amersham Pharmacia Biotech. Oligonucleotide sizing markers and [$\gamma$-$^{32}$P] ATP were also purchased from Amersham Pharmacia Biotech. *E. coli* Fpg protein was provided by Dr. Hiroaki Terato (Hiroshima University, Japan). All other reagents used in this study were of the analytical grade commercially available.

### 2.2. Genomic DNA preparation from A. fulgidus

The hyperthermophilic archaeon *A. fulgidus* (DSM no. 4304) was purchased from Deutsche Sammlung von Mikroorganism und Zellkultren GmbH (DSM, Germany) and grown at 85°C. *A. fulgidus* cells were cultivated under conditions as reported previously [17], and genomic DNA was prepared using a Genomic DNA Midi Kit (Qiagen GmbH, Hilden, Germany).

### 2.3. Protein expression and purification

The complete coding sequence of *Af*ogg was amplified by PCR, with the pair of DNA primers: 5′-GCCTCACGTGGATCCATTGAGAAAGCAATTT CAAGG-3′ (where the underlining indicates the *Bam*HI site), and 5′-GCTAGGCATGTCGACTTACTT CAAGACCTTACCCGT-3′, (where the underlining indicates the *Sal*I site). The PCR was performed in 100 μl reaction mixtures containing 500 ng of genomic DNA as a template, 200 μM dNTPs, 2 units of *Taq* polymerase, 10 μl of 10 × reaction buffer and 1 pmol of each primer. After 5 min denaturation at 94°C, the PCR was performed followed by 25 cycles of amplification at 94°C (1 min), 52°C (40 sec) and 72°C (50 sec), and the reaction was completed by extension for 7 min at 72°C. The *Af*ogg gene was cloned in the maltose binding protein (MBP) fusion vector, pMal-c2x. The amplified DNA fragment was digested with *Bam*HI and *Sal*I and ligated with the pMal-c2x expression vector. The recombinant plasmid, pMal-*Af*ogg DNA, was introduced into *E. coli* TB1. The sequence of the gene in the expression vector was confirmed with an ABI 373 DNA automated sequencer.

*E. coli* TB1 harboring pMal-*Af*ogg was grown in rich broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.2% glucose) containing 100 μg/ml of ampicillin at 37°C to a density of $A_{600}$ of 0.5. The recombinant protein was induced with 0.3 mM isopropyl-D-thiogalactopyranoside (IPTG) at 24°C for 6 h. The cell pellets, obtained by centrifugation at 4000 rpm for 20 min, were resuspended in buffer

A (20 mM Tris–Cl, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 1 mM DTT). The cells were disrupted by ultrasonication and the lysate was centrifuged at 15,000 rpm for 30 min. The supernatant was loaded onto amylose resin affinity column. The MBP-*Af*ogg fusion protein was eluted with 10 mM maltose in buffer A. The fusion protein was cleaved by factor Xa protease (10 U/mg of fusion protein) digestion for 12 h at room temperature. The cleavage mixture of fusion protein was dialyzed in buffer B (20 mM Tris–Cl, pH 7.4, 20 mM NaCl, 1 mM EDTA, and 1 mM DTT) and then applied to a MonoS cation-exchange FPLC column pre-equilibrated with buffer B. The fractions containing the homogeneous *Af*ogg were collected with a linear gradient of 20 mM–1 M NaCl and concentrated by ultrafiltration using an Amicon YM10 membrane (Amicon Co.). The *Af*ogg protein was stored at −20°C at a concentration of 1 mg/ml.

### 2.4. Mass spectrometry

MALDI-TOF MS analysis was performed on a HP G2025A (Hewlett Packard, Palo Alto, CA, USA) linear type time-of-flight mass spectrometer operating in the positive ion mode of detection. The instrument equipped with an oscilloscope (LeCroy 9350AM, USA) was used to increase peak intensity and sensitivity by multiplying the rate of signal sampling. Typically, the spectra from 60–120 laser shots were summed to obtain the final spectrum. The spectra obtained were reconstructed by Igor pro 3.15 software. The MALDI-TOF MS matrix solution was prepared by mixing cyano-4-hydroxycinnamic acid (CHCA) with ethanol-ammonium bicarbonate (1:1, v/v) solution or ethanol-ammonium citrate (1:1, v/v) solution. After vortexing 2 μl of the matrix and 1 μl of the sample in an Eppendorf tube, 1 μl of mixture solution was loaded onto the target. For the molecular mass determination, the acceleration voltage was set to 25 kV and spectra were calibrated with cytochrome *c*, myoglobin and bovine serum albumin as external standards.

### 2.5. Preparation of oligonucleotide duplex substrates

The 39-mer oligonucleotide containing a single 8-oxoG residue at position 16 was purchased from Bio-Synthesis, Inc. (USA). The oligonucleotide sequence used in this work is the following: 5′-GGATCCTCTAGAGTC[8-oxoG]ACCTGCAGGC ATGCAAGCTTGAG-3′ The complementary sequences with A, T, G or C opposite 8-oxoG were also obtained from Bio-Synthesis Inc. The 8-oxoG-containing oligonucleotide was labeled on the 5′-end with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (Takara) at 37°C. Unincorporated [$\gamma$-$^{32}$P]ATP was removed following purification of the oligonucleotide using the Microspin G-50 column (Amersham Pharmacia Biotech). The duplexes were prepared by annealing with an unlabeled complementary strand at a 1.5-fold molar excess in buffer (20 mM Tris–Cl, pH 7.4, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 3% glycerol). To generate duplex molecules, the annealing reaction was heated to 75°C for approximately 5 min and slowly cooled to room temperature. The annealed DNA was eluted by ethanol precipitation, dried and resuspended in double-distilled water.

### 2.6. DNA cleavage assay

DNA cleavage reactions of *Af*ogg protein were performed in 40 μl reaction mixtures with 20 ng of *Af*ogg protein at 50°C. Reactions were carried out with 1 pmol of radiolabeled 39-mer oligonucleotide duplexes in the reaction buffer containing 20 mM Tris–Cl, pH 8.5, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA. After termination of the reaction with phenol/chloroform and ethanol precipitation, the oligonucleotides were resuspended in 20 μl of the formamide loading buffer (containing 0.05% bromophenol blue and 0.05% xylene cyanol). The samples were heated for 5 min at 90°C, and subjected to electrophoresis through a denaturing 15% polyacrylamide gel containing 7 M urea in 1 × TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). The gel was dried and placed on an imaging plate. The amount of DNA cleavage products was quantified using a BAS2000 image analyzer (Fuji, Japan).

### 2.7. DNA trapping assay with $NaBH_4$

*Af*ogg protein was incubated with 1 pmol radiolabeled 39-mer oligonucleotide duplexes in the presence of 20 mM Tris–Cl, pH 8.5, 1 mM EDTA, 1 mM DTT, and 50 mM $NaBH_4$ in a total volume of 10 μl. The reaction mixture was incubated at 50°C for 30 min and mixed with 2.5 μl of the 5 × SDS loading buffer

(100 mM Tris–Cl, pH 6.8, 10% SDS, 20% glycerol, 5% 2-mercaptoethanol, and 0.2% bromophenol blue). Samples were boiled at 90°C for 5 min, and electrophoresed on a 10% SDS-polyacrylamide gel. The trapping efficiencies were quantified using a BAS2000 image analyzer.

### 2.8. Thermal denaturation and thermostability

Thermal denaturation of *Af*ogg protein was measured by far-UV circular dichroism (CD) at 222 nm. We used a J-715 spectropolarimeter (Jasco, Japan) equipped with the temperature-controlled liquid system. Measurements were performed in a solution at pH 7.5 containing 20 mM Tris–Cl and 0.2 mg/ml of *Af*ogg protein. Thermostability of *Af*ogg protein was also determined. *Af*ogg protein was incubated at 80°C in 20 mM Tris–Cl buffer, pH 7.5 with each salt concentration. Aliquots were withdrawn at periodic intervals and kept on ice. The residual activity of aliquots was measured under DNA cleavage assay conditions as described above after all samples had been collected at various times. The relative activity of the enzymes was defined as the residual activity of the enzyme compared to the activity of the unheated enzyme.

## 3. Results

### 3.1. Purification of Afogg protein

The sequence of the entire genome of *A. fulgidus* was published [16]. A significant fraction of the coding genes from *A. fulgidus* are annotated as hypothetical proteins with unknown function. By searching the *A. fulgidus* genome for ORFs encoding hypothetical proteins, we identified the ORF *Af*0371 (hitherto hypothetical protein) as a presumed 8-oxoG DNA glycosylase (*Af*ogg) showing sequence homology to the endonuclease III family of DNA repair enzymes.

For searching the presumed DNA glycosylase in *A. fulgidus* genome, sequence search of the ORFs of unknown function (hypothetical proteins) was undertaken using a local similarity of BLAST at NCBI (http://www.ncbi.nlm.nih.gov) [18]. The amino acid sequences of hypothetical genes that their amino acid residue number is below 400 were compared with the known proteins in the database BLAST. Some members of hypothetical genes including *Af*0371 showed relatively high hit score for each of the DNA binding or endonuclease family (data not shown). Two ORFs (*Af*1692 and *Af*0383) of those have been already annotated as endonuclease III or endonuclease III-like protein. The selected candidates were scrutinized in order to determine if these proteins had a helix-hairpin-helix (*HhH*) motif. A DNA binding function of the *HhH* motif has been proposed in endonuclease III family [19,20]. The *Af*0371 showing a *HhH* motif homology was selected (Fig. 1).

The amino acid sequence of *Af*0371 exhibited 20–23% identity with *E. coli* endonuclease III, *Aquifex aeolicus* endonuclease III, and *Pyrobaculum aerophilum* putative DNA glycosylase (data not shown). The amino acid sequence of *Af*0371 (*Af*ogg) also aligns with that of human Ogg1 (*hOgg*1) in Fig. 1, showing 20% of sequence identity, but high conservation of amino acid residues critical for the catalytic reaction and helix-hairpin-helix motif (*HhH* motif) [11]. We investigated the function of this protein in order to determine if *Af*0371 protein has indeed 8-oxoG DNA glycosylase activity.

The *Af*0371 gene (594 bp) was amplified by PCR using two oligonucleotide primers (Section 2) at the amino and carboxy terminal ends of the ORF. To obtain the soluble fraction of *Af*ogg protein, we cloned the *Af*ogg gene into the pMal-c2x MBP-fusion vector. The recombinant plasmid (pMal-*Af*ogg) was introduced into *E. coli* TB1 for protein expression. *E. coli* TB1 cells harboring the pMal-*Af*ogg plasmid were induced to express the MBP-*Af*ogg fusion protein by addition of IPTG to the growth medium. MBP-*Af*ogg fusion protein was purified through amylose resin affinity column chromatography. As the molecular weight of the MBP and *Af*ogg is 43 kDa and 22 kDa, the full-length of fusion protein was confirmed to be about 65 kDa by SDS-PAGE (Fig. 2). The MBP-*Af*ogg fusion protein was cleaved with factor Xa and *Af*ogg protein was further purified by monoS cation-exchange chromatography. The resultant purified *Af*ogg protein has additional six amino acids (ISEFGS) at its N-terminus after factor Xa cleavage. The final preparation has produced a single band of *Af*ogg protein by SDS-PAGE with >95% purity (Fig. 2). The yield of purified *Af*ogg protein was about 10 mg per 1 l of culture.

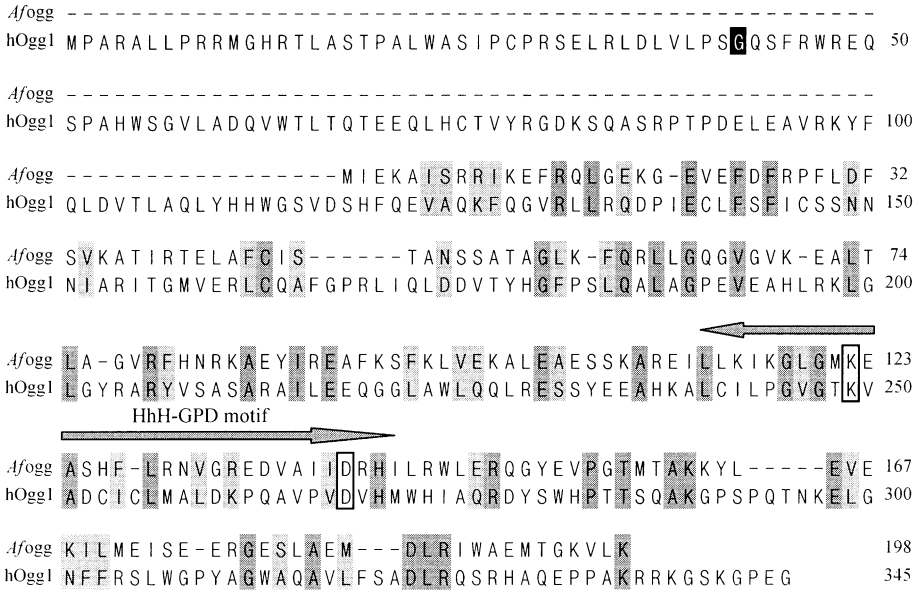


Fig. 1. Sequence alignment of *Af*ogg and *hOgg*1. The catalytic sites (*Lys*122 and *Asp*140) are in open boxes. The shadow boxes represent conserved amino acid residues and the glycine in the black box is the specificity-determined residue of *hOgg*1. The arrow represents highly conserved *HhH*-GPD motif.

The molecular mass of the purified recombinant protein was determined by mass spectrophotometry (Maldi-TOF). In the acid condition (pH 1.5), the mass spectrum of *Af*ogg protein showed molecular ions $(M + H)^+$ at 23,124 Da, which appeared to be a monomer (Fig. 3). The molecular size of *Af*ogg was also estimated by a gel filtration chromatography using a Superdex 200 FPLC column and this gave a single symmetrical peak yielding the same result as from the mass spectrophotometer (data not shown). These results indicate that *Af*ogg exists as a monomer.

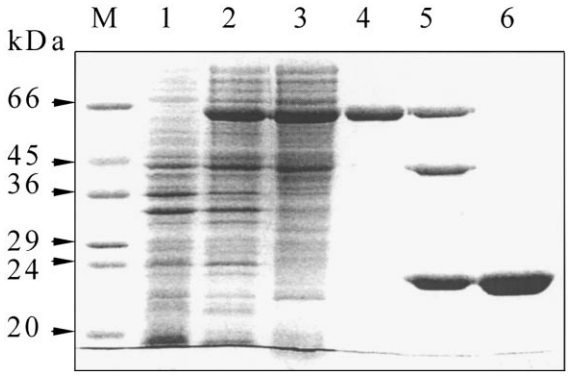


Fig. 2. Purification steps of recombinant *Af*ogg protein. Each step was analyzed by staining the 15% SDS-polyacrylamide gel with coomassie brilliant blue. Lane M: protein molecular weight makers; lane 1: control (crude extract of cells harboring vector pMal-c2x); lane 2: crude extract of cells harboring pMal-*Af*ogg; lane 3: soluble fraction after cell disruption; lane 4: fraction eluted from an amylose affinity column chromatography; lane 5: proteins after factor Xa treatment; lane 6: purified *Af*ogg protein from MonoS cation-exchange column chromatography.

### *3.2. 8-oxoG DNA glycosylase activity of Afogg protein*

The DNA glycosylase/lyase activity of *Af*ogg was examined using a 39-mer oligonucleotide duplex containing a single 8-oxoG:C pair. *Af*ogg specifically incised oligonucleotide duplexes containing 8-oxoG:C (Fig. 4A). Since the oligonucleotide containing 8-oxoG was labeled on the 5′-end with [$\gamma$-$^{32}$P]ATP, we could observe that the strand cleavage activity of *Af*ogg was specific for the 8-oxoG-containing strand of the oligonucleotide duplex on the gel. Strand-specific cleavage of the 8-oxoG:C-containing oligonucleotide was observed after incubating oligonucleotide

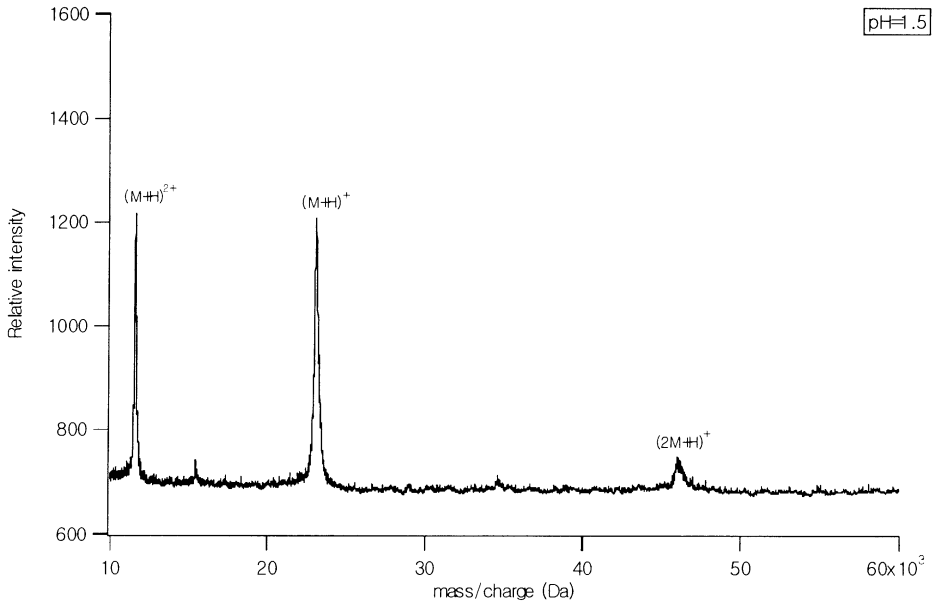


Fig. 3. MALDI-MS spectrum of *Af*ogg protein in pH 1.5 (2-propanol:water:formic acid solution = 2:3:1 v/v) with CHCA matrix.

duplexes with *Af*ogg, versus no incision of G:C-containing oligonucleotides as a control (Fig. 4A).

As all DNA glycosylases that can initiate 8-oxoguanine excision (DNA glycosylase activity) are classified with one of two types of lyase activity at the AP site created by initial glycosidic bond scission [21], we examined the cleavage mechanism of *Af*ogg. The different reaction mechanisms of DNA glycosylase/AP lyase enzymes, $\beta$-elimination or $\delta$-elimination, can be identified by the distinct electrophoretic mobilities of the oligonucleotide products. *E. coli* Fpg forms 5′ and 3′ phosphate termini via $\delta$-elimination, whereas eukaryotic Ogg enzymes (human, yeast Ogg1) produce 5′-phosphates and 3′-$\alpha$,$\beta$-unsaturated aldehyde, 4-hydroxy-2-pentenal termini, resulting from a $\beta$-elimination reaction. The *Af*ogg protein catalyzed a $\beta$-elimination reaction at the AP site produced by excision of the 8-oxoG and thus generated 3′-$\alpha$,$\beta$-unsaturated aldehyde sugar termini at the incision site. The gel mobilities of the cleavage products with *Af*ogg protein treatment were thus slower than that of *E. coli* Fpg (Fig. 4A).

We confirmed this $\beta$-elimination reaction mechanism of DNA cleavage by *Af*ogg under piperidine treatment [21]. After the enzymatic reaction with *Af*ogg, the samples were treated with 10% piperidine at 90°C. In the piperidine treatment according to time course, reaction products of *Af*ogg protein were converted to the $\delta$-elimination products migrating slightly faster than $\beta$-elimination products (Fig. 4B). Comparative experiments with *E. coli* Fpg on the same substrate showed a formation of $\delta$-elimination products. These results suggest that *Af*ogg protein has the same reaction mechanism as the eukaryotic 8-oxoG DNA glycosylase.

Besides the duplex substrate containing 8-oxoG, several oligonucleotide duplexes containing thymine glycol (TG) and alkylating bases, 3-methyladenine or 7-methylguanine, were examined as substrates for *Af*ogg protein, and they were not hydrolyzed (Fig. 4C). The 32-mer duplex substrates containing hypoxanthine (HX:T), uracil (U:A) or A:G mismatch were also tested and no activities were detected (data not shown). The reaction conditions, for example, concentration of substrate and protein, reaction time, and buffer conditions, were varied as well as complementary bases (A, T, G or C) opposite to damaged base, but the activity of *Af*ogg protein was not observed for these substrates of damaged base in any reaction conditions.

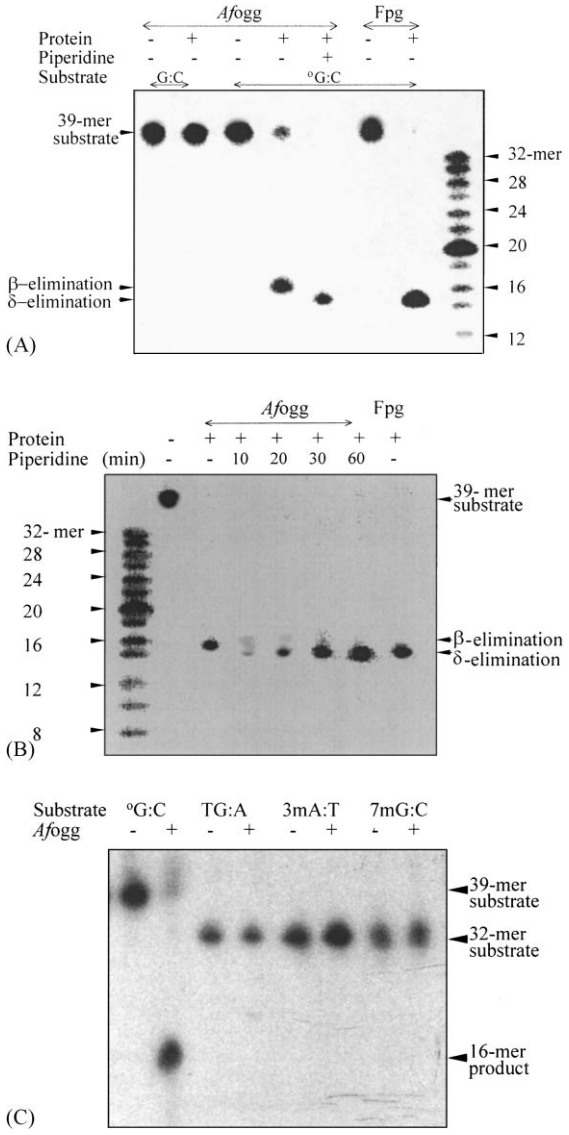


Fig. 4. The 8-oxoG DNA glycosylase activity and AP lyase reaction mechanism of *Af*ogg. (A) The 8-oxoG specific strand cleavage by *Af*ogg. Purified *Af*ogg protein (20 ng) was incubated with 1 pmol of 5′-end labeled G- or 8-oxoG-containing 39-mer oligonucleotide duplex with C in the complementary strand at 50°C for 30 min. *E. coli* Fpg was reacted at 37°C. The cleavage products were analyzed by 15% denaturing polyacrylamide gel electrophoresis and BAS2000 image analyzer. (B) $\beta$-elimination activity by *Af*ogg. After enzymatic reaction with *Af*ogg protein, the samples were treated with 10% piperidine at 90°C according to the indicated time course (0–60 min). (C) DNA cleavage activity of *Af*ogg protein against duplex substrates containing other damaged bases (TG: thymine glycol; 3 mA: 3-methyladenine; 7 mG: 7-methylguanine). Each of damaged bases in the 32-mer oligonucleotide sequence is at position 16.

### 3.3. Substrate specificity and optimal conditions for DNA glycosylase activity

The specificity of DNA glycosylase activity of *Af*ogg was compared using 39-mer DNA duplexes containing an 8-oxoG residue opposite each of the four natural DNA bases (A, T, G and C). DNA cleavage reactions by *Af*ogg with 1 pmol of substrate were performed at 50°C. *Af*ogg recognized 8-oxoG paired with C most efficiently and then G > T > A (Fig. 5). Human Ogg1 strongly preferred substrate

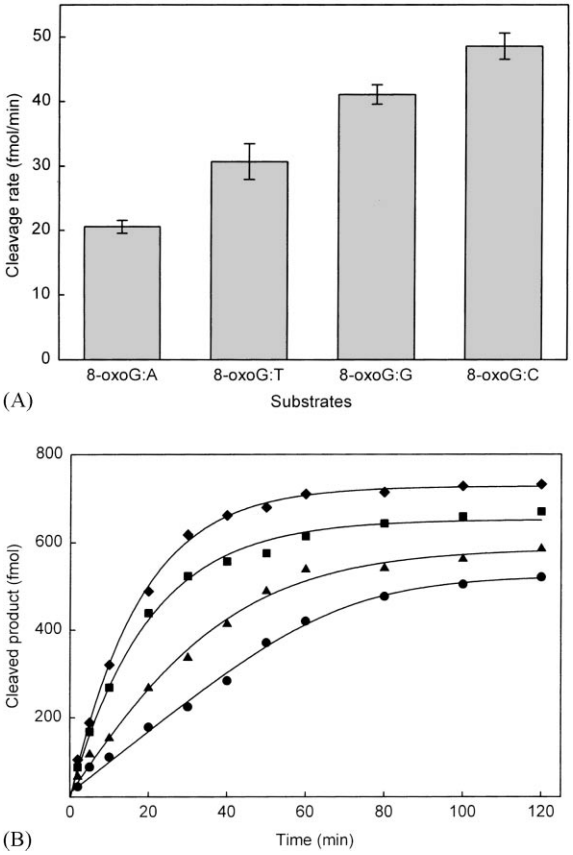


Fig. 5. Substrate specificity of *Af*ogg: (A) DNA cleavage reactions for substrate specificity were performed in 40 ul reaction mixtures containing 20 mM Tris–Cl buffer, pH 8.5 with 20 ng of *Af*ogg protein and 1 pmol substrate at 50°C. Reaction samples were electrophoresed on a 15% polyacrylamide gel. Cleavage products were quantified using a BAS2000 image analyzer. Initial reaction rates were obtained by fitting to a Michaelis–Menten equation; (B) time course of cleavage activity for the four different 8-oxoG-containing substrates (8-oxoG:A: (●); 8-oxoG:T: (▲); 8-oxoG:G: (■); 8-oxoG:C: (◆)).

DNA duplexes containing an 8-oxoG:C pair, followed by 8-oxoG:T and 8-oxoG:G, but had the poorest activity on 8-oxoG:A [14]. The 8-oxoG paired with T, G, or C was a good substrate for *E. coli* Fpg, whereas 8-oxoG:A was poorly recognized [8].

DNA glycosylase activity of *Af*ogg was analyzed in the pH range of 6.0–10.5 with the 39-mer duplex substrate containing 8-oxoG:C. *Af*ogg protein was incubated for 30 min in various buffers at 50°C. The optimum pH of *Af*ogg protein was approximately 8.5 (Fig. 6A). Dependence of DNA glycosylase activity by *Af*ogg on temperature was also investigated. *Af*ogg was 4.2-fold more active at 60°C than at 30°C (Fig. 6B). Since $T_m$ of the DNA substrates (39-mer) of *Af*ogg was 74°C, the activity above 60°C is not convincible. However, the actual optimum temperature of *Af*ogg protein might be higher than 60°C. The effects of salt (NaCl) on the enzyme activities were also investigated. Enzymes were incubated with various concentrations of NaCl. The *Af*ogg protein exhibited significantly higher activities in the presence of 100 mM NaCl (Fig. 6C).

### 3.4. *Afogg-substrate complex*

To verify the mechanism of *Af*ogg protein, we examined the DNA trapping assay with $NaBH_4$ [10].

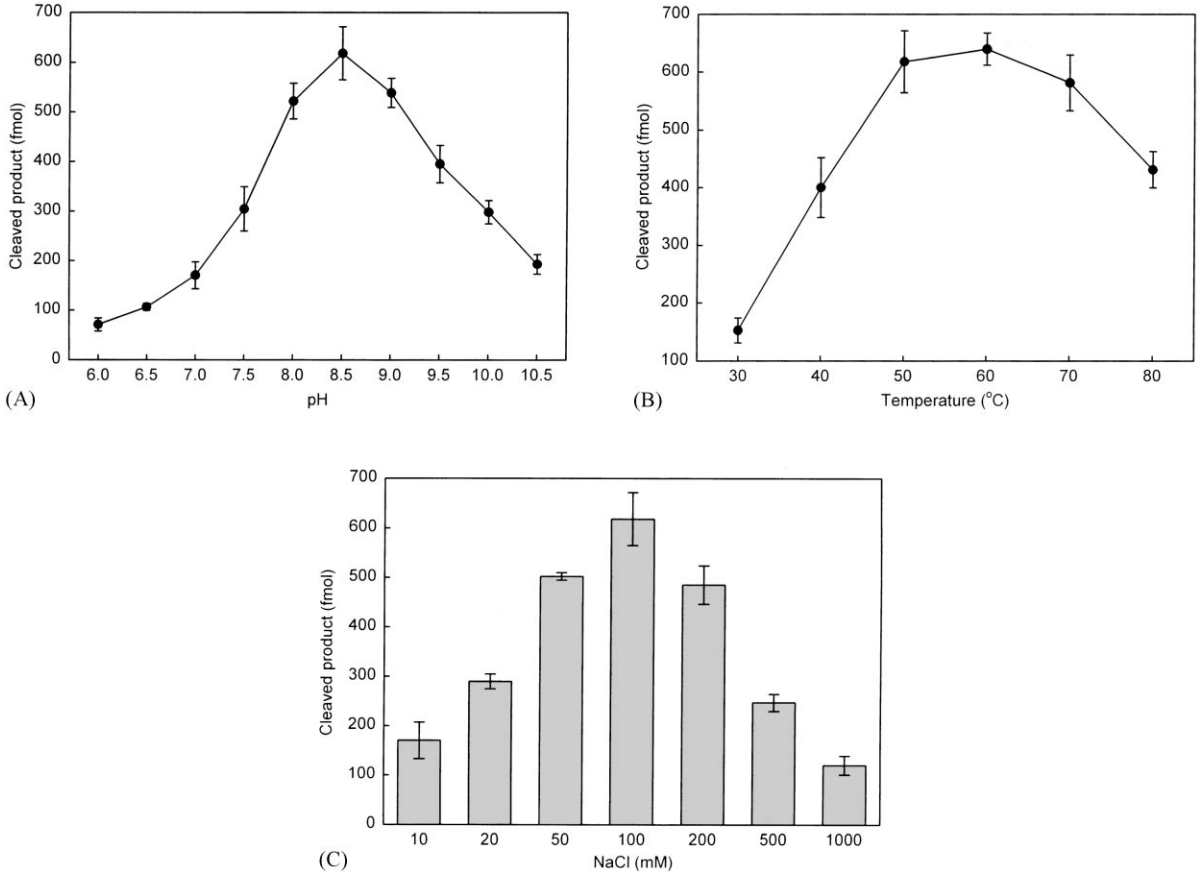


Fig. 6. The effects of pH, temperature and salt concentration on *Af*ogg activity. The reaction mixture containing 1 pmol of 8-oxoG:C-containing 39-mer oligonucleotide duplex was incubated for 30 min with *Af*ogg. Each sample was electrophoresed on a 15% polyacrylamide gel and quantified using a BAS2000 image analyzer. (A) The activity assays of pH effect were performed in various buffers at 50°C. The following buffers were used; MES buffer for pH 6.0–6.5, HEPES buffer for pH 7.0–7.5, Tris–Cl buffer for pH 8.0–8.5, CHES buffer for pH 9.0–9.5, and CAPS buffer for pH 10.0–10.5. (B) Assays of temperature effect were performed at various temperatures using Tris–Cl buffer, pH 8.5. (C) The effects of salt (NaCl) concentration were assayed in Tris–Cl buffer, pH 8.5 at 50°C.

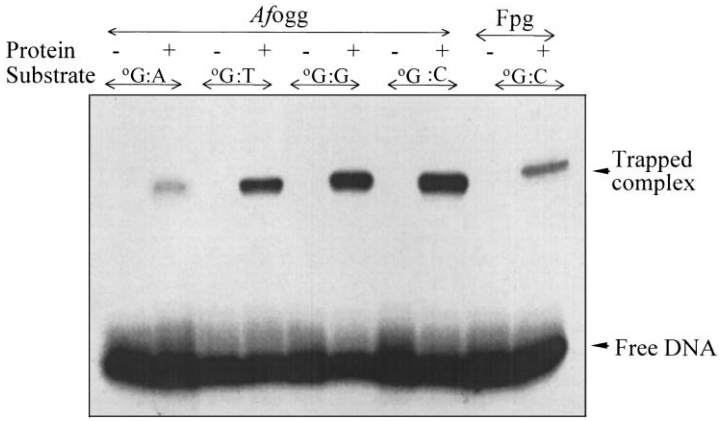


Fig. 7. DNA trapping assay of *Af*ogg protein. *Af*ogg was incubated with 1 pmol of 39-mer oligonucleotide duplexes containing 8-oxoG:A, 8-oxoG:T, 8-oxoG:G or 8-oxoG:C in the presence 50 mM $NaBH_4$. The reaction mixture was incubated at 50°C for 30 min. Protein-DNA complexes were analyzed by 12% SDS-PAGE and BAS2000 image analyzer.

DNA glycosylases with associated AP lyase activity, including *hOgg*1, yOgg1 and *E. coli* Fpg, form a transient Schiff base (imine intermediates) in the reaction with substrates containing 8-oxoG that can be trapped by $NaBH_4$ to generate a covalent protein-DNA complex. Such complexes can be detected as a stable protein-DNA complex on SDS-polyacrylamide gel electrophoresis. DNA substrates containing 8-oxoG were incubated with *Af*ogg protein in the presence of $NaBH_4$ and the complexes were analyzed by SDS-PAGE. The DNA trapping assays with *Af*ogg protein showed a distinct preference for C opposite 8-oxoG (C > G > T > A) (Fig. 7). These results confirmed the data from the DNA cleavage assay. Because of the different molecular mass of Fpg (∼ 30 kDa) and *Af*ogg (∼ 22 kDa), the trapped complex with *Af*ogg had a slightly higher mobility than that of Fpg.

### *3.5. Thermal denaturation and thermostability of Afogg*

Thermal denaturation of *Af*ogg was examined using a far-UV CD spectropolarimeter. The thermal melting curve was measured at 1°C intervals with an average time of 60 s at each temperature, and the change in ellipticity monitored at 222 nm. *Af*ogg was stable at high temperatures, but was denatured irreversibly above 80°C (Fig. 8A). As this temperature is below the optimum growth temperature for *A. fulgidus*, we examined whether ionic pressure could further stabilize the protein.

The thermostability of *Af*ogg protein was investigated by incubation at 80°C in buffer containing salt (20 mM or 500 mM NaCl). Aliquots were taken at periodic intervals and the residual activity of each aliquot was measured. As shown in Fig. 8B, *Af*ogg is relatively stable against heat inactivation in the buffer containing a high concentration of NaCl. In the presence of a high concentration of the salt, *Af*ogg retains about 50% activity after heating for 50 min. The optimum salt concentration for the thermostability of *Af*ogg was examined between 0.02 and 1 M NaCl. The thermostability of *Af*ogg protein was the highest near 0.5 M NaCl (data not shown). The thermostability of *Af*ogg protein was maintained under the high salt concentration, while its catalytic activity was inhibited in buffer more than 100 mM NaCl. Although, the enzymes from thermophiles show an intrinsic thermostability, several enzymes are not intrinsically stable [22,23]. The extrinsic factors, such as, salts may play a role in the stabilization of the proteins. Some hyperthermophilic archaea indeed contain high level of salts with various compatible solutes [23–27]. *A. fulgidus* was grown on a completely mineral salts medium and their concentration up to 350 mM of mostly NaCl [17,28].

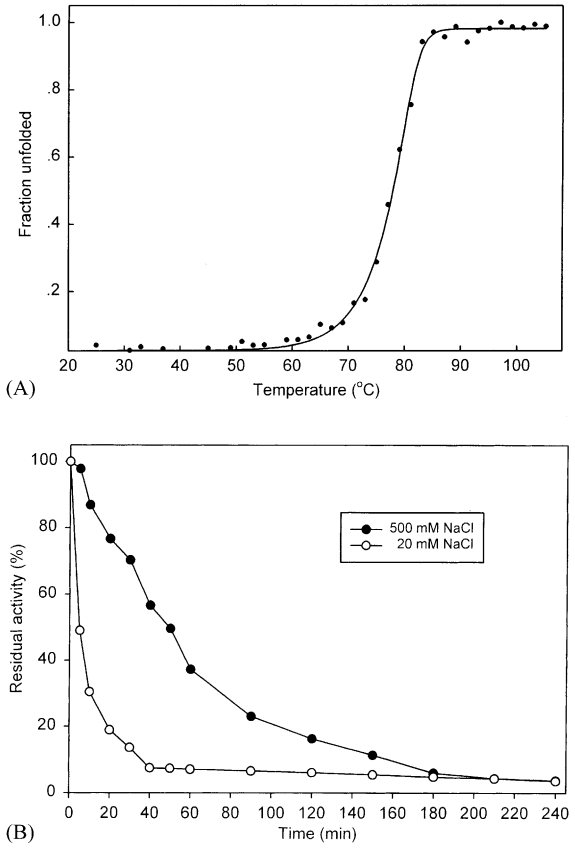


Fig. 8. Thermal denaturation and thermostability of *Af*ogg protein. (A) Thermal denaturation measured by CD at 222 nm. Measurements were performed in a solution at pH 7.5 containing 20 mM Tris–Cl and 0.2 mg/ml of *Af*ogg protein. Thermal melting curves were measured at 1°C intervals with an averaging time of 60 s at each temperature, and the change in ellipticity was monitored. The raw data of the residue molar ellipticity value were transformed into fraction of protein unfolded. (B) *Af*ogg was incubated at 80°C with each salt concentration (20 or 500 mM NaCl). Aliquots were withdrawn at periodic intervals and kept on ice, and then DNA cleavage reactions were carried out with 1 pmol of radiolabeled substrate in 20 mM Tris–Cl, pH 8.5. Cleavage products were quantified using a BAS2000 image analyzer. The relative activity of the enzymes was presented as the residual activity of the enzyme compared to the activity of the unheated enzyme control.

## 4. Discussion

We obtained the soluble recombinant protein of ORF *Af*0371 from the hyperthermophilic archaeon, *A. fulgidus*. The putative 8-oxoG DNA glycosylase/AP lyase activity of *Af*0371 was determined using an oligonucleotide duplex containing 8-oxoG:C, and *Af*0371 showed activity against this substrate. The purified recombinant protein has a molecular mass of 22.6 kDa, as determined by mass spectrophotometry and confirmed by gel filtration chromatography. Recently, thermostable proteins from *Thermus thermophilus* and *Methanococcus jannaschii*, were also characterized to have 8-oxoG DNA glycosylase activities [15,29]. MutM from *T. thermophilus* is 42% identical, and has the same cleavage mechanism, as *E. coli* Fpg protein. MutM retains activity at high temperature (60°C) and its secondary structure up to 75°C. The factors contributing to the thermostability of *T. thermophilus* MutM protein have been suggested from comparison of amino acid composition with that of *E. coli* Fpg protein. *T. thermophilus* MutM has a small number of amino acids (Cys, Asn, Gln, Met) known to be unstable at high temperature compared to those of the *E. coli* protein, and also has more Pro and Arg residues than Fpg. These amino acids in *T. thermophilus* MutM are concerned with peptide folding and hydrogen bonds in protein structure formation [29].

The amino acid sequence of *Af*ogg protein was compared with several 8-oxoG DNA glycosylases (Table 1). The frequency of the charged amino acid residues, Asp, Glu, Lys, and Arg, in *Af*ogg protein is rather high (33%) compared to that of *hOgg*1 (21%) and *E. coli* Fpg (24%). *Mj*ogg also shows an abundance (37%) of charged amino acid residues. While $\alpha$D (Phe120 $\sim$ Asp136) and $\alpha$N (Glu298 $\sim$ Trp313) region, surface area of *h*Ogg1 protein, have five and two charged amino acids, respectively, *Af*ogg protein has nine and eight charged amino acids in the region (Glu3 $\sim$ Lys19 and Glu165 $\sim$ Glu179) corresponding to $\alpha$D and $\alpha$N of *h*Ogg1. It has been shown that the frequency of charged amino acids in each protein correlates with the optimum growth temperature of the organism [30], suggesting that the more ion pairs a protein has, the more stable it is, provided that the number of charged amino acids is proportional to the number of ion pairs. This significant effect of ion pairs on the thermostability of protein has been documented [31], and is likely one of several factors contributing to the thermal stability of *Af*ogg.

Several 8-oxoG DNA glycosylases, characterized from human (hOgg1), yeast (yOgg1) and *E. coli* (Fpg), have distinct mechanisms for lyase activity

Table 1
Amino acid composition of *Af*ogg protein compared to those of *Methanococcus jannaschii* Ogg (*Mj*ogg), *Thermus thermophilus* MutM (*Tt*MutM), human Ogg1 (*hOgg*1) and *E. coli* Fpg[a]

| Amino acids | *Af*ogg | *Mj*ogg | *Tt*MutM | *hOgg*1 | Fpg |
|---|---|---|---|---|---|
| Ala | 18 (9.09) | 7 (3.38) | 24 (8.99) | 34 (9.86) | 17 (6.32) |
| Cys | 1 (0.51) | 2 (0.97) | 4 (1.50) | 8 (2.32) | 6(2.23) |
| *Asp* | *5 (2.53)* | *10 (4.83)* | *8 (3.00)* | *14 (4.06)* | *10 (3.72)* |
| *Glu* | *25 (12.63)* | *27 (13.04)* | *23 (8.61)* | *19 (5.51)* | *21 (7.81)* |
| Phe | 11 (5.56) | 11 (5.31) | 14 (5.24) | 11 (3.19) | 6 (2.23) |
| Gly | 14 (7.07) | 10 (4.83) | 29 (10.86) | 23 (6.67) | 21 (7.81) |
| His | 3 (1.52) | 3 (1.45) | 6 (2.25) | 13 (3.77) | 10 (3.72) |
| Ile | 14 (7.07) | 24 (11.59) | 3 (1.12) | 10 (2.9) | 13 (4.83) |
| *Lys* | *19 (9.60)* | *23 (11.11)* | *3 (1.12)* | *12 (3.48)* | *16 (5.95)* |
| Leu | 21 (10.61) | 23 (11.11) | 38 (14.23) | 38 (11.01) | 35 (13.01) |
| Met | 6 (3.03) | 2 (0.97) | 2 (0.75) | 5 (1.45) | 4 (1.49) |
| Asn | 3 (1.52) | 13 (6.28) | 2 (0.75) | 5 (1.45) | 6 (2.23) |
| Pro | 2 (1.01) | 2 (0.97) | 21 (7.87) | 23 (6.67) | 17 (6.32) |
| *Arg* | *17 (8.59)* | *17 (8.21)* | *40 (14.98)* | *27 (7.83)* | *19 (7.06)* |
| Gln | 4 (2.02) | 2 (0.97) | 7 (2.62) | 25 (7.25) | 10 (3.72) |
| Ser | 11 (5.56) | 7 (3.38) | 7 (2.62) | 26 (7.54) | 15 (5.58) |
| Thr | 8 (4.04) | 5 (2.42) | 13 (4.87) | 14 (4.06) | 11 (4.09) |
| Val | 11 (5.56) | 8 (3.86) | 16 (5.99) | 19 (5.51) | 19 (7.06) |
| Trp | 2 (1.01) | 2 (0.97) | 0 (0.00) | 10 (2.90) | 5 (1.86) |
| Tyr | 3 (1.52) | 9 (4.35) | 7 (2.62) | 0 (0.00) | 8 (2.97) |
| Total | 198 (100%) | 207 (100%) | 267 (100%) | 345 (100%) | 269 (100%) |

[a] Percent frequency is also shown in parentheses. The charged amino acid residues are italicized.

[8,10,14]. The mechanism of phosphodiester bond cleavage after glycosylase activity is either through $\beta$-elimination or $\delta$-elimination reaction. Both *h*Ogg1 and yOgg1 have glycosylase/AP lyase activity, which removes the 8-oxoG base lesion from DNA and cleaves the DNA strand at the resulting AP site via $\beta$-elimination. These reactions result in strand breaks 3′ to AP sites, leaving 3′-terminal unsaturated sugar derivatives. In contrast, the *E. coli* Fpg protein undergoes an additional $\delta$-elimination reaction, yielding a single nucleotide gap flanked by 3′ and 5′ phosphate termini. Purified *Af*ogg protein catalyzed only $\beta$-elimination reaction after cleavage of the 8-oxoG glycosidic bond. The different termini were identified by distinct electrophoretic mobilities of the repaired product compared to that of *E. coli* Fpg.

The primary amino acid sequence of *Af*ogg is quite different from the *E. coli* Fpg protein, and, moreover, *Af*ogg lacks the N-terminal proline residue that is responsible for the glycosylase/AP lyase activity of Fpg [32]. Thus, as we have shown, *Af*ogg acts differently than Fpg. *Af*ogg also exhibits low identity (20%) with *hOgg*1, but the important catalytic residues *Lys*122 and *Asp*140 are identical to the active site residues of *hOgg*1 (*Lys*249 and *Asp*268) (Fig. 1), suggesting that *Af*ogg and *hOgg*1 use the same catalytic mechanism. The crystal structure *hOgg*1 bound to an 8-oxoG:C-containing duplex, show that specificity for 8-oxoG derives from a single hydrogen bond to the 8-oxoG N7 position from a glycine backbone carbonyl in the *hOgg*1 N-terminal domain [33]. Interestingly, *Af*ogg has a unique N-terminal sequence that is not similar to *hOgg*1 and does not contain the specificity-determining glycine residue. Thus, the basis for 8-oxoG specificity in *Af*ogg is likely to differ from that of *hOgg*1. The crystal structure determination of *Af*ogg is underway to identify the structural basis for 8-oxoG recognition.

To characterize the biochemical activity of *Af*ogg, we examined the cleavage of oligonucleotide duplexes containing 8-oxoG paired with four different bases (A, T, G or C). Initial rates (fmol/min) were estimated on the basis of the time course of substrate cleavage (Fig. 5), and show that *Af*ogg prefers 8-oxoG paired with C followed by G > T > A. DNA cleavage by the eukaryotic Oggs and Fpg show different

substrate specificities, with Fpg preferentially cleaving 8-oxoG paired with G, C, and T, but not A [8], while *hOgg*1 efficiently cleaves duplexes containing 8-oxoG:C, with 8-oxoG:T and 8-oxoG:G poorer substrates, and 8-oxoG:A practically uncleaved. The Ogg1 enzymes from mouse and yeast show similar substrate specificities as *hOgg*1 [10,21]. The specificity of *Af*ogg for the 8-oxoG:A containing duplex also was the lowest. However, the cleavage rate of this substrate is about 42% relative to that of 8-oxoG:C-containing duplex. The cleavage rate of *Af*ogg on the 8-oxoG:A-containing duplex is relatively high, as shown in *Mj*ogg, but not in other Oggs.

Further characterization of the activity of *Af*ogg was the formation of covalent enzyme-substrate complexes. Recent analysis of 8-oxoG DNA glycosylases has revealed that these proteins form a Schiff base protein-DNA substrate intermediate [10]. Trapped complexes of *Af*ogg with DNA substrates confirmed the results obtained from as cleavage assays. The results suggest that while the chemical reaction mechanism of *Af*ogg is likely similar to the eukaryotic Ogg1 enzymes, the DNA recognition mechanism and the basis for 8-oxoG substrate specificity of *Af*ogg differ from that of eukaryotic 8-oxoG DNA glycosylases. The structure determination of *Af*ogg with an oligonucleotide duplex containing 8-oxoG should help to clarify the unique *Af*ogg recognition mechanism.

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