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Characterization of a Noncanonical Purine dNTP Pyrophosphatase from *Archaeoglobus fulgidus*

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Abstract DNA can oxidatively be deaminated by ROS, which converts DNA base amino groups to keto groups and can trigger abnormal mutations, resulting in mutagenesis in organisms. In this study, a noncanonical purine dNTP pyrophosphatase (*Af*PPase) from a hyperthermophilic archaeon *Archaeoglobus fulgidus*, which hydrolyzes aberrant nucleoside triphosphates, was overexpressed in *E. coli*, purified, and characterized. The purified *Af*PPase showed remarkably high activity for XTP and dITP, suggesting that the 6-keto group of these nucleotides is critical for the reactivity. Under optimal reaction conditions, the reaction rate for these substrates was about 120 times that with dGTP. Therefore, *Af*PPase may play a significant role in DNA repair by hydrolysis of noncanonical nucleotides before they are misincorporated into DNA.

Key words: *Archaeoglobus fulgidus*, hyperthermophile, mutation, noncanonical, dNTP pyrophosphatase

Cellular DNA is at risk for damage by UV light, ionizing radiation, reactive oxygen species (ROS), or chemical mutagens. Of these damage risks, oxidative damage of DNA is an important factor in mutagenesis in all organisms. Several types of oxidative DNA lesions are formed; for example, strand breaks, base-less sugars, and a range of different oxidized DNA bases [2, 7, 9, 13, 15, 21]. The oxidative deamination of DNA is caused by transitions, which converts DNA base amino groups to keto groups and can trigger abnormal mutations [25], and this DNA damage occurs not only in duplex DNA strands [12, 16], but also in the free nucleotides [3]. Thus, guanine is converted to xanthine, adenine to hypoxanthine, and cytosine to uracil in this process [20, 24, 25]. These bases can increase the mutation rate of DNA, and specifically, hypoxanthine is biologically important because transitions (A:T to G:C) are induced at the site damaged by the oxidative deamination [12, 16].

Most organisms have a repair system that is specific for these deaminated bases in DNA. DNA glycosylase, endonuclease V, which attacks DNA containing hypoxanthine or xanthine, has been previously isolated from *E. coli* [10, 28], and two DNA repair enzymes, 3-methyladenine DNA glycosylase and methylpurine DNA *N*-glycosylase, which are capable of removing hypoxanthine from hypoxanthine-mismatch duplex DNA, have also been identified from eukaryotes and prokaryotes [22]. However, the oxidative deamination of bases, such as dITP and XTP, occurs not only in DNA strands, but also in the free nucleotide pool [3]. Recently, a hypothetical protein (termed *Ec*O197) has been identified from *E. coli* as a dITP- and XTP-hydrolyzing protein, suggesting that this protein plays an important role in preventing misincorporation of these aberrant nucleotides into DNA [6, 28]. This protein hydrolyzes damaged purine nucleotides that contain the 6-keto group; however, it has little activity toward canonical nucleotides.

In order to understand the noncanonical purine-hydrolyzing protein from archaea and to compare its biochemical properties with eubacterial protein, we searched for *Ec*O197 homologs in the *Archaeoglobus fulgidus* VC-16 genome. *A. fulgidus* is a type of *Archaeoglobales* strain, which grows optimally at 83°C. Although the entire genome sequence has been determined [17], the noncanonical purine-hydrolyzing protein from *A. fulgidus* has not yet been identified. Hyperthermophilic organisms living in a high temperature environment are at especially high risk of DNA damage by base deamination, because high temperature can significantly promote base deamination [19]. Thus, it

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is highly possible that these organisms may have more effective DNA damage repair systems than other organisms. In this study, we identified and characterized a noncanonical purine dNTP pyrophosphatase from *A. fulgidus* and named it as the *Af*PPase.

Identification and Recombinant Protein Purification of *Af*2237

The entire genomic sequence of a hyperthermophilic archaeon, *A. fulgidus*, has been published [17]; however, the function of a significant fraction of the coding genes and their products remains to be elucidated. Among these genes, the ORF *Af*2237 (Swiss-Prot No. O28046) has until now been annotated as a putative HAM1 protein with unknown function in the genome database (TIGR Microbial Database: http://www.tigr.org/tdb/mdb/mdbcomplete.html). Nevertheless, using a local similarity of the BLAST program at NCBI [1], 35.4% of the overall amino acid sequence of *Af*2237 was found to be identical (55.2% similarity) to a dITP- and XTP-hydrolyzing protein (*Ec*O197) from *E. coli* [6]. The *Af*2237 protein is composed of 181 amino acids with a predicted molecular mass of about 21 kDa and pI 6.3. Using the ClustalX program [11], the homology between *Af*2237 and the *Ec*O197 protein was found to appear throughout the entire amino acid sequence (Fig. 1). An analysis of the sequence alignment revealed several highly conserved regions.

The protein encoding *Af*2237 was expressed in *E. coli* BL21 (DE3) as a hexa-histidine-tagged recombinant protein. *A. fulgidus* (DSM, Germany) was cultivated under the condition described previously [26], and genomic DNA was prepared using a Genomic DNA Midi Kit (Qiagen, Germany). The *Af*2237 gene was amplified from a genomic DNA by PCR using two oligonucleotide primers (forward, 5'-CGTTCACATATGTACTTCATAACG-3'; reverse, 5'-CATGTCGGATTCTTAAATTTTATTCTCCTT-3') at the amino and carboxy terminal ends of the ORF, and the PCR product was cloned into an expression vector, pET28a vector (Novagen, U.S.A.), which places a hexa-histidine tag at the 5'-end of the gene. The recombinant plasmid pET28a-*Af*2237 was then introduced into *E. coli* BL21 (DE3) for protein expression. *E. coli* BL21 (DE3) cells harboring the recombinant plasmid were induced to express proteins by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the growth medium. The high level of the recombinant protein was expressed in the soluble fraction and was heated at 80°C for 30 min; a fraction of the *E. coli* proteins became denatured and were removed by centrifugation, whereas recombinant protein was recovered as a soluble protein in the supernatant. The histidine-tagged *Af*2237 protein was eluted with imidazole from the Ni-NTA affinity column (Qiagen, Germany), while the other proteins flowed through. The final product had a single band at the 21 kDa region on 15% SDS-PAGE, which is compatible with the predicted molecular mass of *Af*2237 protein, with more than 95% purity (Fig. 2). The recombinant protein was stored at 1 mg/ml concentration at −80°C. Protein concentration was determined by the Bradford method [4], using bovine serum albumin as a standard. A soluble protein of *E. coli Ec*O197, which is homologous with *Af*2237, had previously also been purified [6]. Finally, we investigated various properties of the *Af*2237 protein to elucidate its biochemical function and whether the recombinant *Af*2237 protein had an enzyme activity similar to the *Ec*O197 protein.

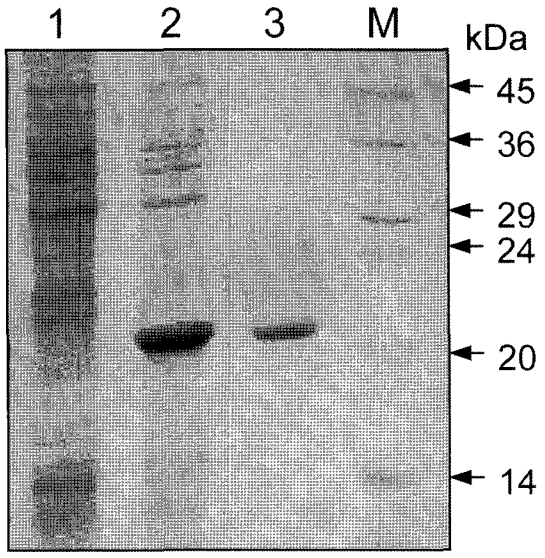

Fig. 2. Purification of recombinant protein.
Preparation at each step was analyzed by staining the 15% SDS-polyacrylamide gel with Coomassie brilliant blue. Lane 1, control (crude extract of cells harboring vector pET28a); lane 2, soluble extract of cells harboring pET28a-*Af*2237 after 4 h of induction with IPTG; lane 3, protein fraction eluted from Ni-NTA affinity column after heat denaturation; lane M, protein molecular weight markers.

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Af2237  - - - MYF I TSNEGKFREVREMASKYGIE EWLK- - - MEYIEPQGSSLEEIARLSAEMLAEKVEGEFVI  61
EcO197  MQKVVLATGNVGKVRELASLLSDFGLDIVAQTDLGVDSAEETGLTFIENAILKARHAAKVTALPAIA  67

Af2237  EDSGLFVEALKGFPGPYSS- - - - - YVFKTIGNEGILKLMEGVEN- - RKAYFMAVVAYFDGKEVRT- -  119
EcO197  DDSGLAVDVLGGAPGIYSARYSGEDATDQKNLQKLLETMKDVPDDQRQARFHCVLVYLRHAEDPTPL  134

Af2237  - FTGKVEGEISREMRGTQGFGYDPIFLYGN- - KTFAEMATEEKNQVSHRRKAFEEFFRWLKENKI  181
EcO197  VCHGSWPGVITREPAGTGGFGYDPIFFVPSEGKTAAELTREEKSAISHRGQALKLLLDALRNG- -  197
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Fig. 1. Sequence alignment of *Af*2237 and a homolog from *E. coli* (*Ec*O197).
The shadowed boxes represent conserved amino acid residues. Alignment was performed with the program ClustalX.

Substrate Specificity

Hydrolysis of some noncanonical nucleoside triphosphates, including dITP and XTP, by the *Ec*O197 protein has previously been examined [6]. In the present study, we determined whether the *Af*2237 protein functioned as a nucleotide-hydrolyzing enzyme. Therefore, the reaction was carried out in a 40-μl reaction mixture containing 50 mM CAPS buffer (pH 10.0), 2 mM $MgCl_2$, 100 μM substrate, and *Af*2237 protein (1–4 μg), at 80°C for 10 min, and was stopped by the addition of trichloroacetic acid (final concentration 0.1%). The reaction mixture was then analyzed on a Hypersil SAX 5 μ HPLC column (ThermoHypersil, U.K.) equipped with a recording integrator [14]. The nucleotides were bound to the column in water and eluted with a linear gradient formed from 5 mM ammonium phosphate (pH 2.8) to 750 mM ammonium phosphate (pH 3.7) at a flow rate of 1 ml/min. First, the *Af*2237 protein was tested for its ability to hydrolyze XTP, which is a typical substrate of *Ec*O197. As shown in Fig. 3A, the *Af*2237 protein efficiently hydrolyzed XTP to XMP. The *Af*2237 protein was more active at 80°C than at 37°C, whereas the activity of *Ec*O197 from *E. coli* was greatly reduced at high temperature (Fig. 3B). This observation indicates that the activity of *Af*2237 was not due to contamination of *Ec*O197 during protein purification.

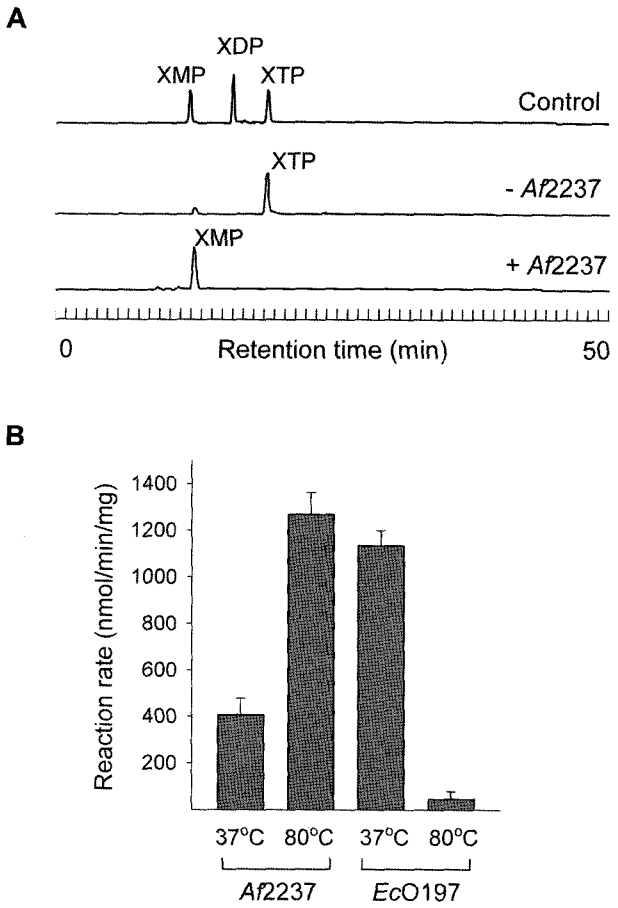

Fig. 3. XTP-hydrolyzing activity of *Af*2237 protein.

A. HPLC chromatograms showing XTP-hydrolyzing activity. Reaction was performed in 40 μl of a reaction mixture, containing 50 mM CAPS buffer, pH 10.0, 2 mM $MgCl_2$, and 4 μg of *Af*2237 protein, at 80°C for 10 min. The reaction product was analyzed using a Hypersil SAX 5 μ HPLC column. Top, XMP, XDP, and XTP as a control; middle, the reaction product of XTP in the absence of *Af*2237; bottom, the reaction product of XTP incubated with *Af*2237. **B.** Comparison of XTP-hydrolyzing activity of *Af*2237 and *Ec*O197 at different temperatures. Reaction was performed in 40 μl of a reaction mixture, containing 50 mM CAPS buffer, pH 10.0, 2 mM $MgCl_2$, and 1 μg of protein, at 80°C or 37°C for 10 min. Results are shown as the mean of three separate experiments.

Table 1. Substrate specificity of *Af*2237.

Substrates	Reaction rate (nmol/min/mg)
XTP	1,268±101
dITP	1,022±133
ITP	1,048±121
dGTP	10.5±2.1
dATP	0.8±0.4
dCTP	0.5±0.3
dTTP	0.9±0.4
dUTP	5.1±1.1
GTP	12.3±3.9
ATP	0.7±0.3
CTP	0.4±0.2
UTP	3.3±0.8
8-oxo-dGTP	0.7±0.4
8-Br-dGTP	0.8±0.5

Reaction was carried out in 40 μl of a reaction mixture, containing 50 mM CAPS buffer, pH 10.0, 2 mM $MgCl_2$, and 1 μg of *Af*2237 protein, at 80°C for 10 min, and the reaction mixtures were analyzed using a Hypersil SAX 5 μ HPLC column. Rates of hydrolysis of nucleoside triphosphates were determined with three independent experiments.

Next, the specificity of the *Af*2237 protein for nucleotide hydrolysis was examined using various nucleoside triphosphates under the same condition (Table 1). Of all the nucleotides tested, only dITP (ITP) and XTP were hydrolyzed efficiently. In particular, the reaction rate of XTP was more than 120 times higher than that of dGTP. The *Af*2237 protein hardly hydrolyzed pyrimidines, dATP, dCTP, and dTTP, whereas purine dGTP was hydrolyzed with modest efficiency, among the common nucleotides (Table 1). Noncanonical purine dNTPs (dITP, ITP, and XTP) were preferred substrates of *Af*2237; therefore, we named *Af*2237 as non-canonical purine dNTP pyrophosphatase (*Af*PPase).

Base analogs, such as hypoxanthine (HX, base of dITP) and xanthine (X, base of XTP), can arise from adenine and guanine, respectively, by oxidative deamination of DNA [20]. HX residues are mutagenic, since they give rise to A:T→G:C transitions [16]. Therefore, using duplex substrates (Fig. 4) containing an HX:T pair, whether *Af*PPase has endonuclease activity was investigated. The HX-containing oligonucleotide was labeled on the 5'-end with [γ-^{32}P]ATP

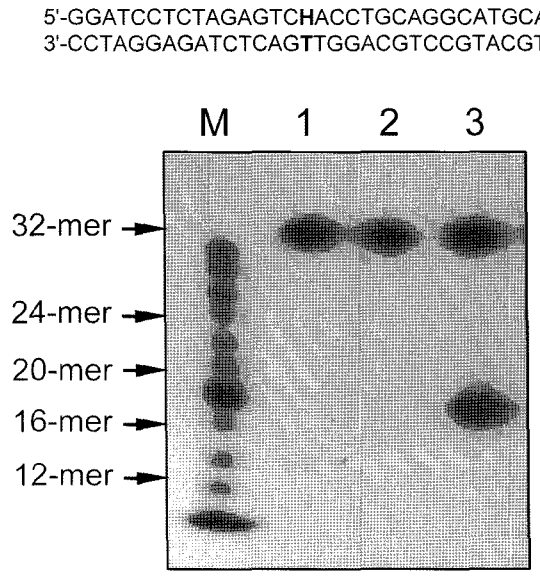

Fig. 4. Endonuclease activity assay.
*Af*2237 protein was incubated with 100 pmol of 5'-labeled HX-containing duplex (top sequence) for 30 min. The products were analyzed by denaturing PAGE and BAS2000 image analyzer. Lane M, oligonucleotide size marker; lane 1, HX:T only; lane 2, HX:T incubated with *Af*2237; lane 3, HX:T incubated with *E. coli* endonuclease V.

(Amersham Biosciences, Sweden), using T4 polynucleotide kinase (Takara, Japan) at 37°C. Unincorporated [γ-^{32}P]ATP was removed by purification of the oligonucleotide using the Microspin G-50 column (Amersham Biosciences, Sweden). The duplexes were prepared by annealing with an unlabeled complementary strand at a 1.5-fold molar excess in a 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 mixture 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. The reactions were performed in a 40-μl reaction mixture containing 4 μg of *Af*PPase and 100 pmol of radiolabeled 32-mer oligonucleotide duplexes. After termination of the reaction with phenol/chloroform and ethanol precipitation, the oligonucleotides were resuspended in 20 μl of formamide loading buffer containing 0.05% bromophenol blue and 0.05% xylene cyanol. The samples were then heated for 5 min at 90°C and subjected to electrophoresis through a denaturing 15% polyacrylamide gel containing 7 M urea. After the gel was dried and placed on an imaging plate, intensities of bands in the autoradiograms were then measured, using a BAS2000 image analyzer (Fuji, Japan). *E. coli* endonuclease V (Trevigen, U.S.A.) was used as a control for this experiment. As seen in lane 2 of Fig. 4, the strand cleavage activity by *Af*PPase was not observed for any of the HX-containing strands; however, *E. coli* endonuclease V showed HX-containing strand cleavage (Fig. 4, lane 3). These residues have been found to be removed by some DNA glycosylases [10, 22, 23]. *Af*PPase can hydrolyze only nucleoside triphosphate to its monophosphate form, and pyrophosphate and dITP (ITP) and XTP are the specific substrates for the *Af*PPase. This sanitizing reaction has also been seen with dUTPase [8, 18] and 8-oxo-dGTPase [5], removing aberrant nucleotides from the nucleotide pool. Since *Af*PPase efficiently hydrolyzed noncanonical purine nucleotides, this protein also seems to play an important role in the DNA repair system in *A. fulgidus*.

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