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Excision of uracil from DNA by the hyperthermophilic Afung protein is dependent on the opposite base and stimulated by heat-induced transition to a more open structure

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Abstract

Hydrolytic deamination of DNA-cytosines into uracils is a major source of spontaneously induced mutations, and at elevated temperatures the rate of cytosine deamination is increased. Uracil lesions are repaired by the base excision repair pathway, which is initiated by a specific uracil DNA glycosylase enzyme (UDG). The hyperthermophilic archaeon *Archaeoglobus fulgidus* contains a recently characterized novel type of UDG (Afung), and in this paper we describe the over-expression of the *afung* gene and characterization of the encoded protein. Fluorescence and activity measurements following incubation at different temperatures may suggest the following model describing structure-activity relationships: At temperatures from 20 to 50 °C Afung exists as a compact protein exhibiting low enzyme activity, whereas at temperatures above 50 °C, the Afung conformation opens up, which is associated with the acquisition of high enzyme activity. The enzyme exhibits opposite base-dependent excision of uracil in the following order: U > U:T > U:C ≫ U:G ≫ U:A. Afung is product-inhibited by uracil and shows a pronounced inhibition by *p*-hydroxymercuribenzoate, indicating a cysteine residue essential for enzyme function. The Afung protein was estimated to be present in *A. fulgidus* at a concentration of ∼1000 molecules per cell. Kinetic parameters determined for Afung suggest a significantly lower level of enzymatic uracil release in *A. fulgidus* as compared to the mesophilic *Escherichia coli*.

Keywords: DNA repair; Uracil DNA glycosylase; *Archaeoglobus fulgidus*

1. Introduction

Hydrolytic deamination of cytosine to uracil is next to depurination the most frequent damaging event to DNA [1]. Since persistent uracil residues result in G:C to A:T transition mutations, all cells contain specific uracil DNA glycosylase enzymes (UDG; EC 3.2.2.3) to remove such lesions from DNA [2]. The resulting abasic (AP) site can subsequently be repaired by the sequential action of the following enzymes: 5′-acting AP endonuclease, DNA deoxyribophosphodiesterase, DNA polymerase and DNA ligase. This so-called base

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excision repair pathway, initiated by one of about ten DNA glycosylases with different substrate specificities, is the quantitatively most important repair mechanism for the removal of spontaneously generated base modifications (for review, see [3–5]). A variety of eubacterial and eukaryotic UDGs exhibiting significant selectivity for uracil have been cloned and sequenced, demonstrating a high degree of conservation between distant species [6]. This family of UDGs, typified by the *Escherichia coli* Ung enzyme [2,7], has a very distinct three-dimensional structure, which together with mutational studies reveals the catalytic mechanism [4,8–10].

UDG activity has been detected in a number of different hyperthermophiles, and the inhibition by the *Bacillus subtilis* bacteriophage PBS1 UDG inhibitor protein suggests a conserved tertiary structure [11]. It was rather surprising when none Ung homologues could be identified by sequence analysis of the genomes of several hyperthermophiles (five archaeons and two eubacteria) ([12–17]; http://www.genoscope.cns.fr/cgi-bin/Pab.cgi), suggesting a hitherto unrecognized gene function being responsible for UDG activity in these organisms. Recently, Sandigursky and Franklin [18] cloned and over-expressed an open reading frame (ORF) from the genome of the hyperthermophilic prokaryote *Thermotoga maritima*, exhibiting a low level of homology to the *E. coli* G:T/U mismatch-specific DNA glycosylase (Mug). The purified protein was demonstrated to be a novel type of UDG enzyme able to remove uracil from U:G and U:A base pairs as well as from single-stranded DNA. By means of homology searches they found ORFs homologous to the *T. maritima* UDG gene present in several prokaryotes including the hyperthermophilic archaeon *Archaeoglobus fulgidus*, a strict anaerobe growing optimally at 83 °C [19,20]. Subsequently, they cloned and over-expressed this ORF in *E. coli*. As expected, the purified His-tagged Afung protein exhibited UDG activity [21].

We here provide physicochemical characterization of the "unmodified" Afung protein, showing that the enzyme excises uracil from DNA with a much lower efficiency than the mesophilic Ung of *E. coli*. We also demonstrate that the rapid increase in activity of Afung at thermophilic temperatures is accompanied by significant changes in protein structure.

2. Materials and methods

2.1. Materials

E. coli [methyl-^{3}H]thymine-labeled DNA was obtained from New England Nuclear (NET-561). Radioactivity released from the DNA during storage was removed by ethanol precipitation and three washes (of the pellet) with 70% ethanol. [^{3}H]uracil-containing DNA (with a specific activity of 1110 dpm/pmol) was a gift from Drs. H. Krokan and B. Kavli. DNA and poly(dG)/poly(dC) were alkylated with [^{3}H]methyl-*N*-nitrosourea (21 Ci/mmol); alkylated poly(dG)/poly(dC) was thereafter incubated for 2 days at pH 11 to convert 7-methylguanine residues into 2,6-diamino-4-hydroxy-5*N*-methylformamidopyrimidine residues [22]. The 23- and 36-mer oligonucleotides 5′-GGCGGCATGACCCXGAGGCCATC-3′ and 5′-TTGACATTGCCCTGGAGAXCTCCTAGACGAATTCCC-3′, where X is the dNMP of 7,8-dihydro-8-oxoguanine and 5-formyluracil, respectively, were annealed to a complementary oligonucleotide where one of the four normal bases was placed opposite the lesion. The 10-mer 5-formyluracil-containing oligonucleotide 5′-GGAGAXCTCC-3′ used to construct the latter was a gift from Dr. A. Matsuda.

2.2. Cultivation of A. fulgidus

A. fulgidus type strain VC16 (DSMZ 4303) [19,20] was grown anaerobically at 83 °C in 20 l carboys under Ar in a medium containing (per l): 18 g NaCl, 7.4 g $MgSO_4 \cdot 7H_2O$, 2.75 g $MgCl_2 \cdot 7H_2O$, 0.32 g KCl, 0.25 g NH_4Cl, 0.14 g $CaCl_2 \cdot 2H_2O$, 0.14 g $K_2HPO_4 \cdot 3H_2O$, 2 mg $(NH_4)Fe(SO_4)_2 \cdot 6H_2O$, 0.03 g yeast extract and 10 ml trace element solution [23]. Lactate (final concentration, 15 mM) was added as a substrate. Sodium dithionite (0.1 g) and 0.5 M Na_2S (0.5 ml) were added to the medium (per l) as reducing agents. The pH was adjusted with KOH to 6.5–6.7. Cells were harvested in the stationary phase by tangential cassette filtration (Millipore pellicon 0.22 Micron GUPP, Lot P30M7301, cassette 33) followed by centrifugation at 7000 × *g*. The cells were stored at −20 °C.

2.3. Preparation of archaeon cell-free extracts

The cells were thawed and re-suspended by vortex mixing in 350 mM Mops, pH 7.5, 5 mM EDTA,

5 mM dithiothreitol, 25% (v/v) glycerol. The cells were lyzed by freezing in liquid nitrogen followed by gentle thawing in a water bath at room temperature and vortex mixing, repeated three times. Cell debris was removed by high-speed centrifugation in a micro-centrifuge. Cell extracts were stored at −70 °C. The protein extracts contained 5–20 mg protein/ml.

2.4. Enzymatic assays for DNA glycosylase activities

Substrate DNA was incubated with protein extract/enzyme in 50 μl 70 mM Mops, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 100 mM KCl, 5% (v/v) glycerol (reaction buffer) for 10 min, unless otherwise stated, followed by precipitation with ethanol and determination of the amount of radioactivity in the supernatant as in the 3-methyladenine DNA glycosylase assay [24]. Control values from incubations without enzyme were subtracted. Addition of bovine serum albumin (BSA) to a final concentration of 20 μg/ml caused a 100% increase in Afung (fraction III; see Table 1) activity as measured at 95 °C for 10 min. Thus, this concentration of BSA was added as a stabilizer prior to incubation with purified enzyme. However, addition of more BSA (up to and including 150 μg/ml) did not cause a further increase in enzyme activity. One unit of UDG is defined as the amount of enzyme that catalyzes the release of 1 pmol of uracil per min under standard conditions at 80 °C. All incubations were performed on a water bath in closed 1 ml Eppendorf tubes.

2.5. Assays for enzymatic cleavage of uracil-containing DNA fragments

Single-stranded 25 or 60-mer oligonucleotides, with a uracil residue inserted at a certain position, were prepared on a commercial DNA synthesizer, purified on 20% denaturing polyacrylamide gels and 5′-[^{32}P]-labeled using T4 polynucleotide kinase and [^{32}P]ATP (Amersham Pharmacia Biotech Inc.). The corresponding double-stranded oligonucleotide substrates were prepared by annealing each [^{32}P]-labeled single-stranded oligomer to a complementary strand with an A, C, G or T residue inserted opposite U. The repair reactions with purified protein or cell-free extracts were performed as described in Section 3.

2.6. Cloning and expression of the *A. fulgidus* AF2277 ORF

Amplification of the *A. fulgidus* AF2277 ORF, which codes for Afung [21], was performed using the oligonucleotide primers 5′-ATGGAGTCTCTGGACGACATAGTCC-3′ (forward) and 5′-TCTATAGGTAATCAAAGAGCGTGGGC-3′ (reverse), where polymerase chain reactions (PCR) were set up with 1 unit *Taq* DNA polymerase (Stratagene), 500 μM dNTPs, 1 μM of each primer, about 200 ng *A. fulgidus* DNA as template and buffer supplied by Stratagene, in a total volume of 50 μl. The reaction mixture was incubated at 94 °C for 2 min, then subjected to 35 cycles (each of 30 s) denaturation at 94 °C, 30 s annealing at 53 °C and 1 min elongation at 68 °C, followed by 10 min with an annealing temperature of 68 °C. The PCR product was cloned into an *E. coli* expression vector (pCR®T7/CT-TOPO TA Cloning® Kit, Invitrogen) followed by transformation into TOP10F′ *E. coli* cells (TOP10F′ One Shot® Competent Cell Kit, Invitrogen) as described by the manufacturer, where 10 ampicillin resistant clones were isolated. The cells (10 × 3 ml) were grown overnight at 37 °C in LB medium containing ampicillin (100 μg/ml), followed by cell harvesting by centrifugation. Plasmids were purified using the StrataPrep™ Plasmid Miniprep Kit (Stratagene) followed by analysis for possible inserts using agarose [1% (w/v)] gel electrophoresis. Clones with inserts detected were digested with *Hin*dIII and

Table 1
Purification of Afung

Fraction	Volume (ml)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Protein extract (I)	10	200	140	1	100
Heat treatment (II)	8	12.8	3900	27	175
HiTrap SP Sepharose (III)	3	1.35	8900	62	42

the direction of the inserts was determined as analyzed by agarose [2% (w/v)] gel electrophoresis. One clone with the insert in the correct, and one clone with the insert in the opposite direction, was obtained, where the correct DNA sequence of the insert in the former clone was verified by sequencing. This plasmid was used to transform competent *E. coli ung*$^-$ cells (BW310; λ^- *ung-1 relA1 spoT1 thi-1*; *E. coli* Genetic Stock Center, Yale University) [25] that had already been transformed with pGP1-2 [26]. The cells were grown at 30 °C in LB medium containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml) to an OD_{600} of 0.68. The T7 RNA polymerase was induced by heat shock treatment when the cells were grown at 42 °C for 3 h, causing expression of the cloned gene. The cells were harvested and the pellet was washed once with 10 ml ice-cold 50 mM Tris–HCl, pH 7.6, 50 mM NaCl and stored at −20 °C. Protein extract was prepared by treating the cells twice in 50 mM Tris–HCl, pH 7.6, 50 mM NaCl in a French Press at 55 MPa.

2.7. Purification of Afung protein

Protein extract (fraction I) was heated at 70 °C for 20 min followed by removal of cell debris by centrifugation. The supernatant (fraction II) was applied to a HiTrap SP Sepharose column (1 ml; Amersham) equilibrated with 50 mM Mes, pH 6, 1 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol, where the proteins were eluted with a step-wise NaCl gradient (0.1–0.5 M). Fractions (1.5 ml each) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and assayed for UDG activity.

2.8. Protein fluorescence measurements

Afung emission spectra were recorded at an excitation wavelength of 280 nm and at a protein concentration of 1 μM. To minimize the effect of fluctuations in the sample, each emission spectrum Φ was provided as the mean of 25 repetitive scans (CAT-mode of fluorimeter) with a scan speed of 1200 nm/min. All measurements were made on a 50 μl sample in a quartz micro-cell using a Hitachi F-4500 spectrofluorimeter.

For heat treatment, protein solution was pipetted into the micro-cell, which was placed in a thermostatted water bath at the desired temperature T (±1 °C). After the treatment, the sample was rapidly transferred from the water bath to the spectrometer (which contained a thermostatted cell holder) and spectra were recorded immediately.

The decomposition of Φ into a linear combination of three-Gaussian functions describing the contributions of tyrosine (f_1) and tryptophan (f_2, f_3), $\Phi = f_1 + f_2 + f_3$ with $f_i(\lambda) = a_i \exp[-\sigma_i(\lambda - \lambda_{i,max})^2]$, was performed as described previously [27]. The extent of conformational changes (protein unfolding) is expressed as the redshift of the tryptophan f_2 contribution [27]. The definition of the f_2 redshift is given in Section 3 (see Fig. 8A and B). The redshift arises due to an increased interaction between the dominating fluorophore (tryptophan) and solvent water [28].

The increase of the protein f_2 redshift as a function of temperature and time was fitted to first-order kinetics. The numerical determination of the rate constants was done on a Macintosh PowerPC using KaleidaGraph™ (data analysis/graphing application for Macintosh and Windows operating systems, Synergy Software, 2457 Perkiomen Avenue, Reading, PA, USA).

2.9. General procedures

Protein concentration was determined by the method of Bradford [29] using BSA as standard. Recombinant DNA techniques were performed according to Sambrook et al. [30].

3. Results

3.1. Enzymatic release of uracil from DNA by archaeal cell-free extracts

This investigation was initiated by an interest in testing the ability of the archaeon *A. fulgidus* to release uracil from DNA. The growth conditions of this hyperthermophile suggest a high rate of spontaneous hydrolytic deamination of DNA-cytosines into uracils [31]. As expected, incubation of [^{3}H]uracil-labeled DNA (where uracil is inserted opposite adenine) with *A. fulgidus* cell-free extracts around optimal growth temperature and at neutral pH resulted in a protein and time-dependent release of uracil. At the lowest protein concentrations, the reaction followed a

strictly linear course (Fig. 1A). A maximum release of 16.3 pmol/min/mg protein was observed following the first 5 min of incubation (data not shown). By contrast, no release of radioactive material was observed from aged [methyl-^{3}H]thymine-labeled DNA (data not shown), indicating a lack or a low level of DNA glycosylase activity directed against oxidized thymines in *A. fulgidus*. Such DNA contains substantial amounts of 5-hydroxymethyluracil and 5-formyluracil as well as various ring-contracted and fragmented forms of thymine [32]. The lack of enzymatic activity directed towards this substrate also excludes a possible contribution by unspecific nuclease activities to the release of radioactive material in the glycosylase assay systems for uracil and other damaged bases. When a defined DNA sequence (60-mer) with uracil inserted at a certain position opposite guanine was used as substrate for 0.1, 1 and 10 μg protein extract, protein and temperature-dependent excision of uracil was observed in the temperature range 37–77 °C (Fig. 1B).

3.2. Physicochemical and enzymatic characteristics of Afung protein over-produced in E. coli

Crude protein extract prepared from UDG-deficient (*ung*$^-$) *E. coli* cells expressing *afung* contained high UDG activity at 80 °C (data not shown). The thermophilic nature of the enzyme was confirmed when crude extract was heated at 70 °C for 20 min. SDS-PAGE showed that over-expressed Afung resisted denaturation and appeared as the major band on the gel with a M_r of 27,000 (see Fig. 2A, fraction II), which is significantly higher than the M_r of 22,718 estimated from the amino acid sequence. In agreement with the results already described for *A. fulgidus* cell-free extracts, the heat-treated Afung-enriched extract contained no detectable glycosylase activity

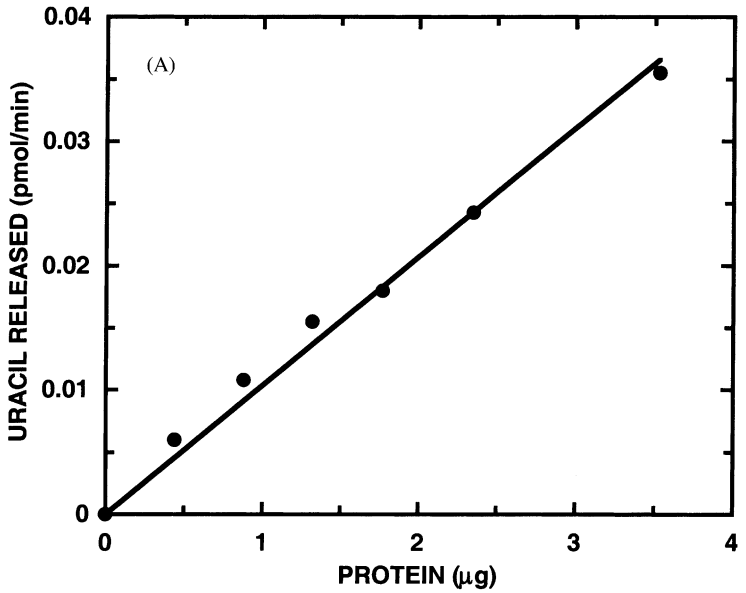

Fig. 1. Excision of uracil from DNA by cell-free extracts prepared from stationary *A. fulgidus* cells. (A) Protein extracts (0.44–141 μg) incubated with [^{3}H]uracil-containing DNA (2400 dpm; 2.2 pmol DNA-uracils) in reaction buffer at 80 °C for 10 min, where only the strictly linear portion of the protein dependence curve is presented; increasing the amount of protein above 30 μg caused no significant increase in uracil excision, i.e. no more than 0.12–0.13 pmol uracil was released per min. Each value represents the mean of two independent measurements. (B) Excision of uracil from a double-stranded DNA oligomer containing a U:G base pair. Protein extract (0–10 μg) was incubated with 60-mer oligonucleotide (5′-TAGACATTGCCCTCGAGGTAUCATGGATCCGATTTCGACCTCAAACCTAGACGAATTCCG-3′ with complementary strand; 100 fmol) in 10 μl reaction buffer for 30 min at the temperatures indicated. As a result of uracil excision and base-catalyzed phosphodiester bond cleavage, each [^{32}P]end-labeled 60-mer substrate is converted into one 39-mer and one 21-mer product where the 21-mer is [^{32}P]-labeled and thus, appears on the picture.

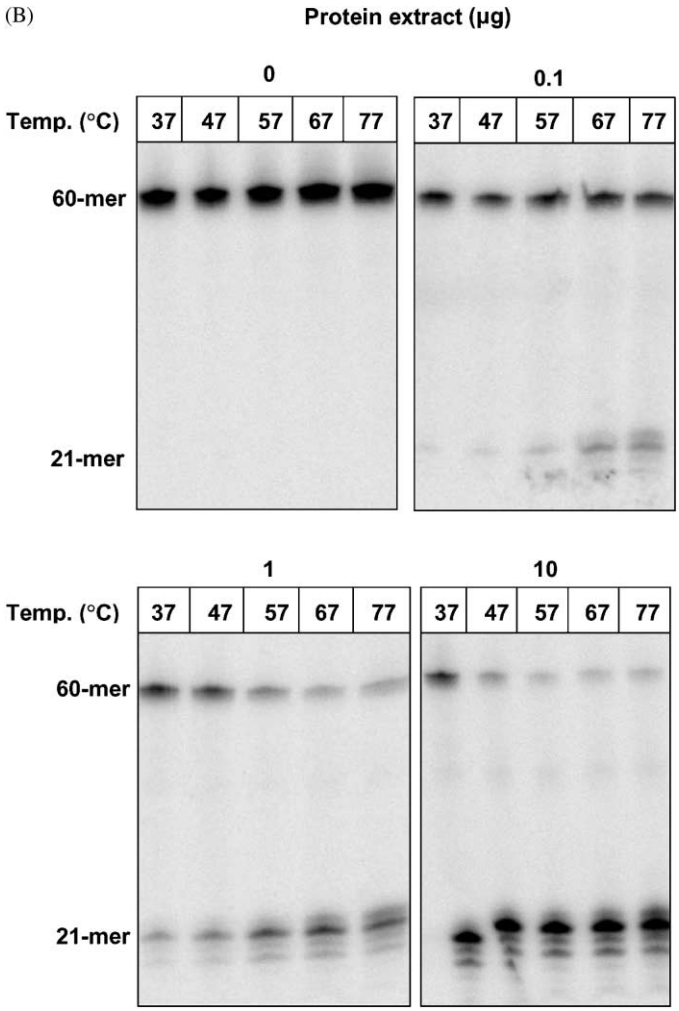

Fig. 1 (*Continued*).

directed towards oxidized thymines (data not shown). In addition, no activity towards methylated purines in [^{3}H]methyl-*N*-nitrosourea-treated DNA or towards imidazole-damaged 7-methylguanines was detected (data not shown), demonstrating that Afung is devoid of methyl purine and formamidopyrimidine DNA glycosylase activity, respectively.

Because Afung resisted inactivation by heating at 70 °C for 20 min, which denature most *E. coli* proteins (Fig. 2A), crude *E. coli* extracts with over-expressed protein (fraction I) were routinely subjected to such treatment as the first step in the purification of Afung. Following centrifugation the resultant supernatant (fraction II) was applied to a strong cation exchange column (SP Sepharose) at pH 6, where Afung protein eluted from the gel at 0.3 M NaCl and appeared electrophoretically pure after this step (Fig. 2A; fraction III). Isoelectric focusing of Afung on polyacrylamide gels resulted in only one protein band (Fig. 2B) corresponding to a pI of 5.67 ± 0.05 (three independent measurements), confirming the electrophoretic purity of the protein as determined by SDS-PAGE. This value is one unit lower than the pI of 6.75 calculated from the amino acid content [21]. Analysis of protein dependence for the excision of uracil from [^{3}H]uracil-labeled DNA by purified Afung showed

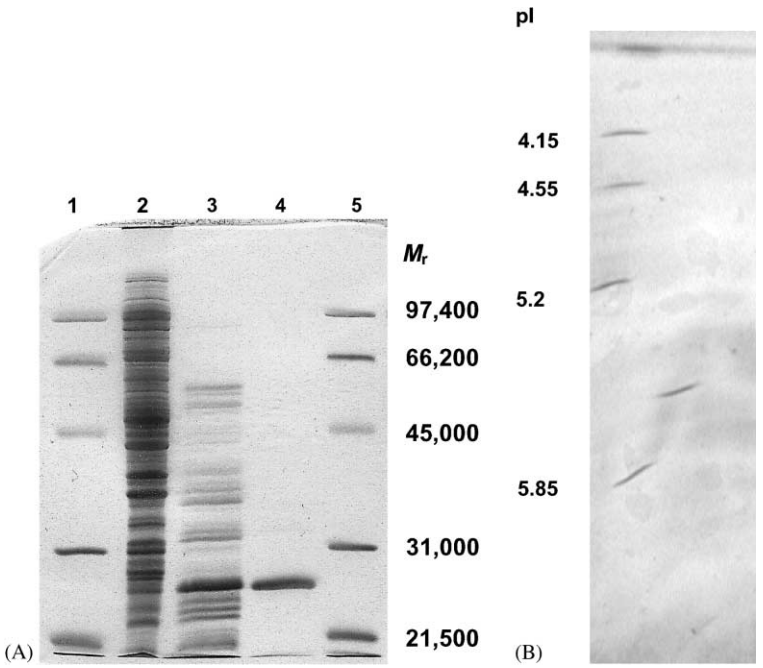

Fig. 2. SDS-PAGE (A) and isoelectric focusing (B) of different fractions obtained during purification of Afung. (A) Proteins were separated on a 12% (w/v) polyacrylamide gel and stained with Coomassie Blue. Lanes 1 and 5, molecular weight markers [from the top: phosphorylase b (M_r 97,400), BSA (M_r 66,200), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000) and soybean trypsin inhibitor (M_r 21,500)]; 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); lane 4, fraction III (2.9 μg). (B) Proteins were separated on an Ampholine PAGplate pH 4.0–6.5 precast gel (Code no. 80-1124-81, Amersham) and stained with Coomassie Blue. Lane 1, pI markers [from the top: glucose oxidase (pI 4.15), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.2), and bovine carbonic anhydrase B (pI 5.85)]; lane 2, Afung (fraction III) (4.4 μg).

that ∼70% of the uracil in the substrate (2.2 pmol) was excised by ∼2 pmol Afung after 10 min of incubation (data not shown). The specific activity and recovery of the enzyme at the various steps of purification are shown in Table 1.

3.3. Kinetics of excision of uracil by Afung

To specifically examine the efficiency of uracil excision from [^{3}H]uracil-labeled DNA by Afung, initial velocities were measured as a function of substrate concentration. Enzymatic release of uracil was determined over a substrate range of 4.7–170 nM using 4.14 pmol of Afung in incubations at 70 and 95 °C, to obtain results for both double- and single-stranded DNA under non-optimal conditions, respectively. In addition, incubations were also performed at 80 °C, i.e. close to the optimal growth temperature of *A. fulgidus*. Analysis of the results by Lineweaver–Burk plots (Fig. 3) indicated an apparent K_m of 1.34 μM at 70 °C, of 1.51 μM at 80 °C and of 0.536 μM at 95 °C (Table 2). These K_m values obtained for uracil are

Table 2
Kinetic parameters of Afung

Enzyme (°C)	K_m (μM)	V_{max} (pmol/min)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1}\,min^{-1}$)
70 (dsDNA)	1.34	4.34	1.05	0.782
80	1.51	4.92	1.19	0.786
95 (ssDNA)	0.536	2.06	0.498	0.929

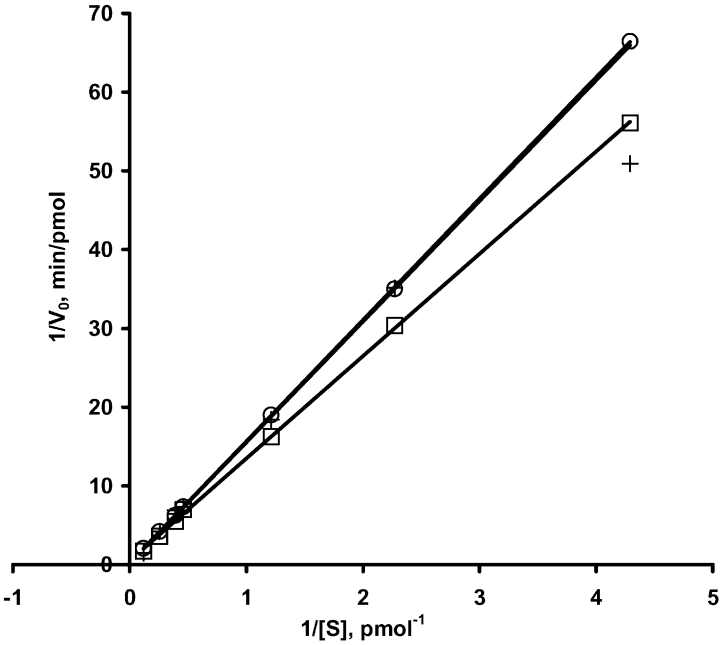

Fig. 3. Lineweaver–Burk plots for excision of uracil from DNA by Afung at different temperatures. Enzyme (4.14 pmol) was incubated with an increasing amount of [^{3}H]uracil-containing DNA (0.233–8.49 pmol DNA-uracils) in reaction buffer at pH 7.5 for 10 min at 70 (dsDNA; (○)), 80 (+) or 95 °C (ssDNA; (□)). Each value represents the median of 2–4 independent measurements.

quite close to the value of 0.5 μM measured at 70 and 95 °C by Sandigursky and Franklin [21], who used about five times higher substrate concentrations. However, the turnover number (k_{cat}) of about one uracil released per min as determined in the present investigation (Table 2) is 1–2 orders of magnitude lower than the turnover number determined by Sandigursky and Franklin [21] possibly caused by non-identical (i.e. His-tagged versus non-His-tagged) enzyme preparations. The different kinetic parameters (K_m, V_{max}, k_{cat}, k_{cat}/K_m) calculated from the Lineweaver–Burk plots are shown in Table 2.

3.4. Opposite base-dependent excision of uracil by Afung

To analyze the efficiency of uracil excision from the U:A match and the different mismatches of the uracil base, including when present in single-stranded DNA, a certain amount of a defined DNA sequence (25-mer) with uracil inserted at a specific position was used as substrate for an increasing amount of Afung. After incubation at 50 °C—to keep the temperature well below the melting point of these short oligomers—for 10 min, the following order for the efficiency of uracil excision was observed: U > U:T > U:C ≫ U:G ≫ U:A (Fig. 4). By contrast, no excision of thymine was observed in any context of base pairing using an identical DNA sequence (data not shown). The opposite base-dependent excision of uracil by Afung accords with the widely accepted flipping-out mechanism for DNA glycosylase action. The enzyme has to disrupt the base pairing and stacking interactions of uracil in double-stranded DNA to be accommodated in the active site pocket [5], and the rate is determined by the forces of the base pairing or base stacking interactions. Sandigursky and Franklin [21] have previously reported Afung-mediated excision of uracil from U:G and U:A base pairs present in a defined 30-mer DNA sequence, however, without providing data to differentiate their abilities as substrates.

Similar experiments, using defined DNA oligomers with all possible matches and mismatches of 7,8-dihydro-8-oxoguanine and 5-formyluracil with the

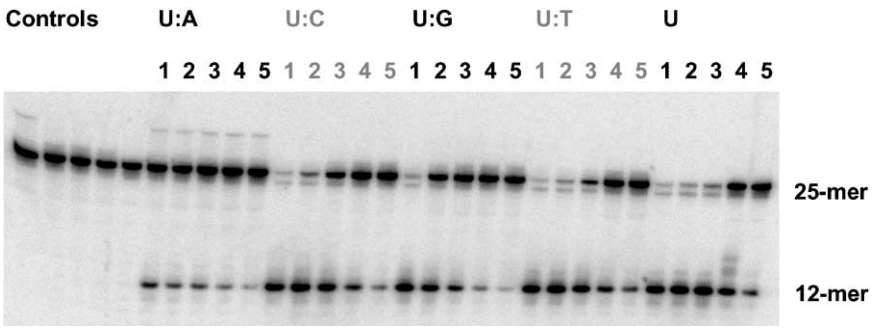

Fig. 4. Opposite base-dependent excision of uracil from a defined DNA sequence by Afung. Enzyme (7.35×10^{-4}–7.35 pmol) was incubated with 25-mer oligonucleotides (5′-GCTCATGCGCAGUCAGCCGTACTCG-3′ with/without complementary strand; 100 fmol), only differing by containing a different base opposite to uracil as indicated, in 20 μl reaction buffer at 50 °C for 10 min. Lanes labeled U represent experiments with the uracil-containing oligonucleotide without complementary strand. For each type of substrate, the following amount of enzyme was used: Lane 1, 7.35 pmol; lane 2, 0.735 pmol; lane 3, 7.35×10^{-2} pmol; lane 4, 7.35×10^{-3} pmol; lane 5, 7.35×10^{-4} pmol. The controls represent incubations without enzyme where each lane (from left to right) represents U:A, U:C, U:G, U:T and U. As a result of uracil excision and base-catalyzed phosphodiester bond cleavage, each [^{32}P]end-labeled 25-mer substrate is converted into two 12-mer products where one of them is [^{32}P]-labeled and thus, appears on the picture.

four common normal bases, were performed. However, no excision of these oxidized bases by Afung was observed (data not shown).

The capacity of Afung to recognize and remove uracil irrespective of base context or DNA conformation makes it similar to the Ung family of UDGs rather than the Mug family of G:T/U mismatch-specific DNA glycosylases [33]. Members of the Ung family have a high affinity for uracil in both single- and double-stranded DNA. Mug-like enzymes require double-stranded DNA and only excises uracil from the G:U mismatch.

3.5. Stimulation and inhibition of Afung activity

The uracil-releasing activity of Afung was unaffected by the addition of uridine, deoxyuridine, thymidine and thymine to a concentration of 5 mM (Table 3). However, addition of uracil resulted in 50% inhibition of both the uracil-releasing activity of Afung and cell-free extracts, in agreement with Sandigursky and Franklin [21] who demonstrated product inhibition of Afung by uracil. Interestingly, *p*-hydroxymercuribenzoate (1 mM) caused a virtually complete inhibition of Afung and the UDG activity

Table 3
Inhibition of UDG activity of Afung and *A. fulgidus* cell-free extracts[a]

Addition	Concentration (mM)	Enzyme activity % (±S.D.)	
		Afung	Extract
None	0	100	100
Uracil	5	49 ± 17 (10)	51 ± 8 (6)
Uridine	5	94 ± 17 (6)	114 ± 10 (4)
Deoxyuridine	5	88 ± 18 (6)	119 ± 25 (4)
Thymine	5	79 ± 17 (10)	97 ± 19 (6)
Thymidine	5	94 ± 9 (6)	102 ± 20 (4)
p-Hydroxymercuribenzoate	1	6.3 ± 6.7 (4)	0 (4)
	4	0 (4)	0 (4)

[a] Purified Afung (0.2 pmol) or *A. fulgidus* protein extract (1.77 μg) was incubated with [^{3}H]uracil-containing DNA (2400 dpm; 2.2 pmol DNA-uracils) in reaction buffer with 20 μg/ml BSA at 80 °C for 10 min. Numbers of independent experiments are indicated in parenthesis.

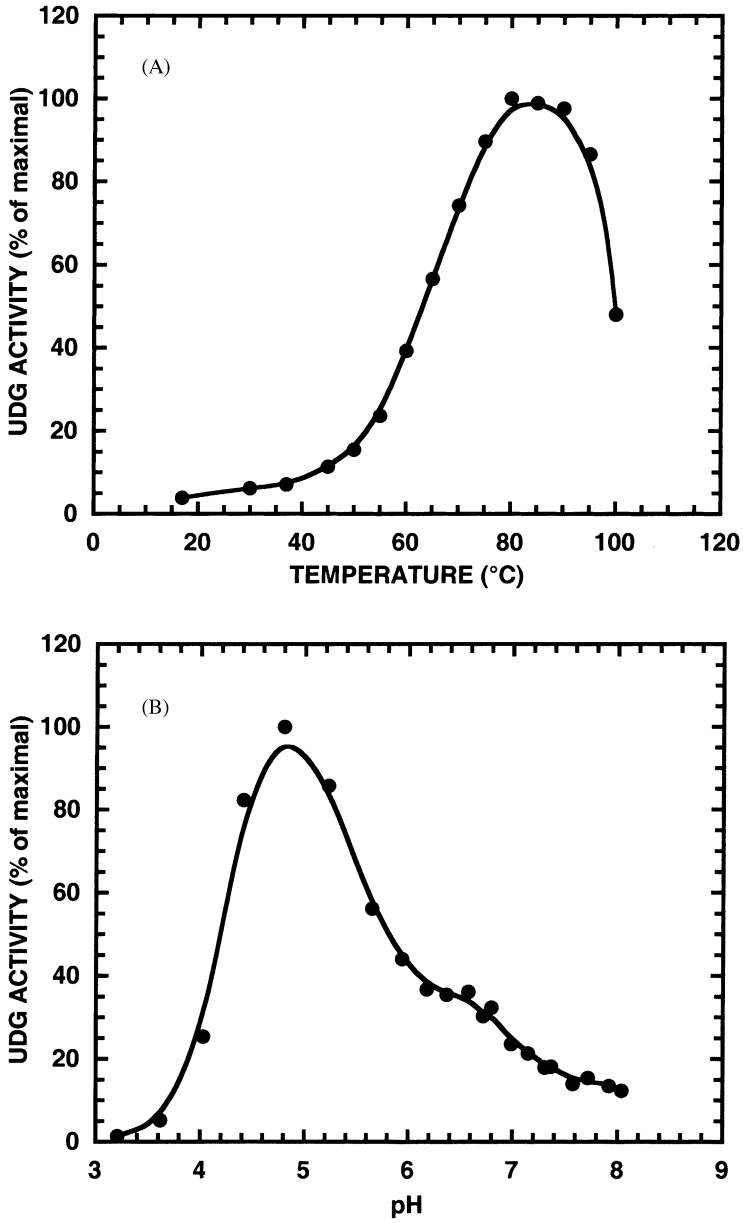

Fig. 5. Temperature (A) and pH dependence (B) for the excision of uracil from DNA by Afung. Enzyme [0.9 pmol (A); 0.2 pmol (B)] was incubated with [^{3}H]uracil-containing DNA (2000 dpm; 2 pmol DNA-uracils) for 10 min in reaction buffer at different temperatures (A) or in universal buffer [34] containing 1 mM EDTA/1 mM dithiothreitol/100 mM KCl/5% (v/v) glycerol at different pH values, at 80 °C (B). Each value represents the median of four (A) or three (B) independent measurements.

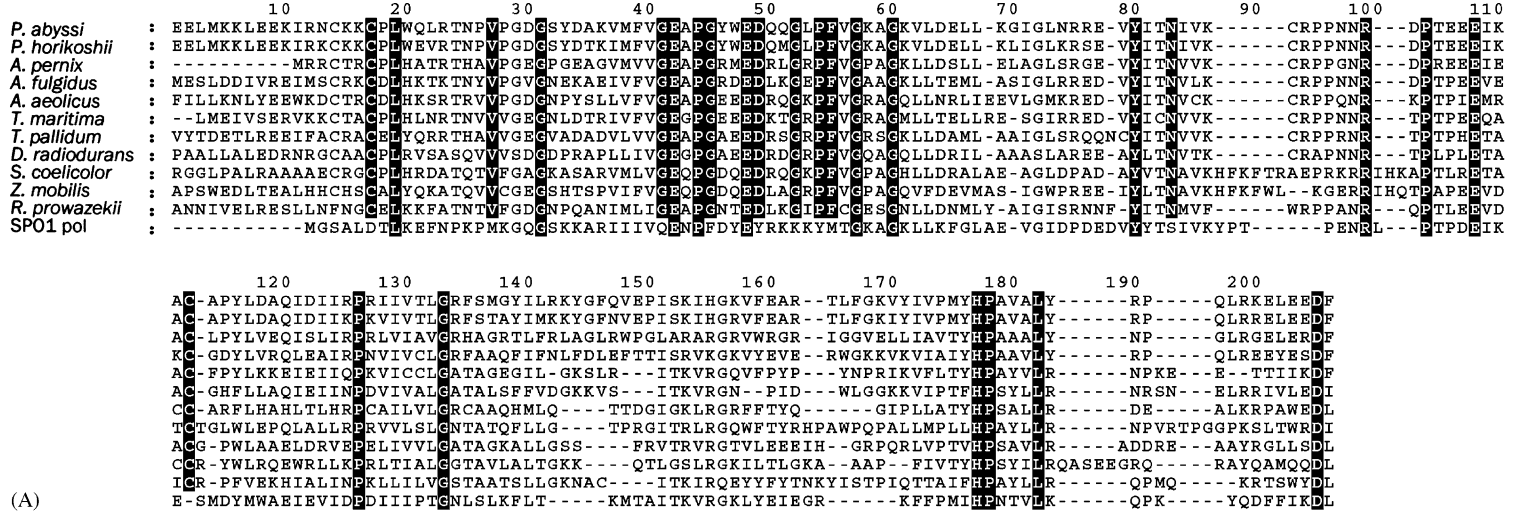

Fig. 6. Amino acid alignment (A) and phylogenetic relationships (B) of Afung homologues. (A) The sequences were identified using BLAST software [35] at NCBI (url: http://www.ncbi.nlm.nih.gov/) and aligned using CLUSTAL W [36]. Conserved residues are shaded. Only the most conserved region, corresponding to amino acids 1–181 of the *A. fulgidus* protein, was included in the alignment. (B) The tree was rooted using the sequence of the N-terminus of the bacteriophage SPO1 DNA polymerase as outgroup. The tree was constructed using CLUSTAL W. Positions with gaps were excluded. The numbers at the nodes are bootstrap values and show the frequency of the topology shown to the right of each node calculated per 1000 trees tested. Only bootstrap values larger than 500 are indicated.

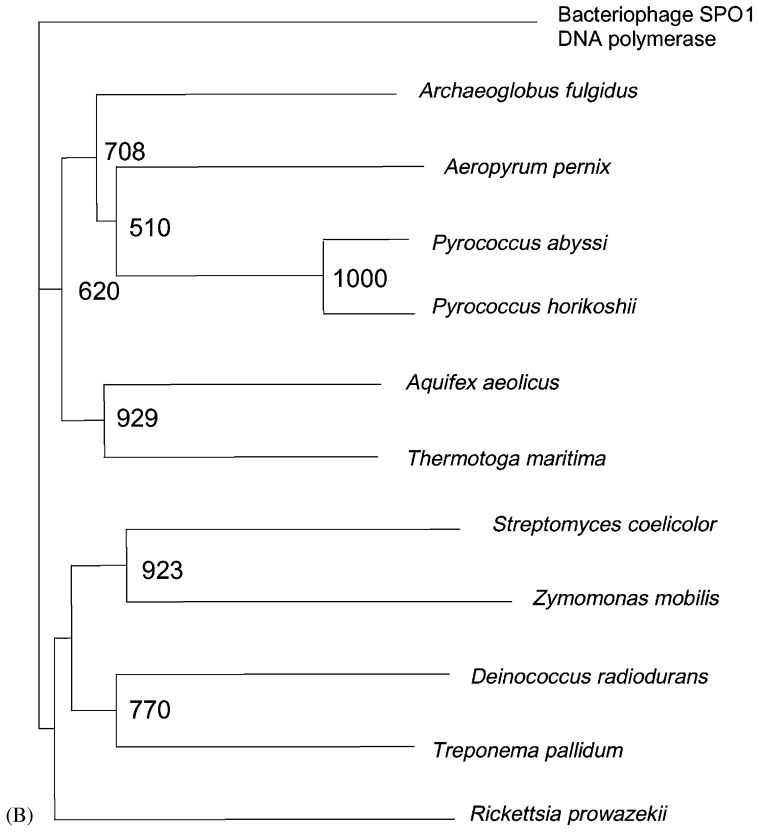

Fig. 6 (*Continued*).

present in *A. fulgidus* extracts, indicating an essential thiol as denominator for enzyme activity (Table 3).

3.6. Temperature and pH dependence of Afung activity

To determine the temperature dependence for uracil release by Afung, enzyme was incubated with [^{3}H]uracil-labeled DNA at 15 different temperatures in the range 15–100 °C. The results show that UDG activity was detected at all temperatures but varied significantly in the temperature range investigated. Afung exhibited a broad optimum of activity around 80–90 °C close to the optimal growth temperature of 83 °C for *A. fulgidus* (Fig. 5A).

Afung was also incubated with substrate at 80 °C in a modified universal buffer of different pH values [24,34], to analyze the pH dependence of enzymatic uracil release. The results show that optimum of activity for Afung is at pH 4.8 (Fig. 5B).

3.7. Sequence comparison and phylogeny

Database searches have previously identified homologues of Afung both in bacteria and Archaea [18,21]. We have aligned 11 similarly sized bacterial and archaeal homologues of Afung giving *E*-values of less than 7e-21 in a BLASTP search using the amino acid sequence of Afung (Fig. 6A). Sequence identity with Afung ranges from 35 to 53%, and the alignment revealed 27 invariable amino acid residues including two conserved cysteines (Cys 47 and 141). The 210 amino acid N-terminus of the 928 amino acid bacteriophage SPO1 DNA polymerase also shares homology with these proteins, giving an *E*-value of 9e-20 in BLASTP searches. SPO1 DNA contains 5-hydroxymethyl-

uracil instead of thymine [37], and its DNA polymerase might share a domain with Afung more or less specific for binding of 5-hydroxymethyluracil and uracil excluding the more hydrophobic thymine. Alignment of the N-terminus of SPO1 DNA polymerase with Afung revealed that it lacks 10 of the 27 invariable amino acid residues shaded in Fig. 6A, including the conserved cysteines, which consequently are possible constituents of the glycosylase active site in Afung. The conserved amino acids of Afung and the SPO1 DNA polymerase might be crucial for determining recognition sites for 5-hydroxymethyluracil/uracil-containing DNA.

A phylogenetic tree for the 11 Afung homologues was constructed, and rooted using the N-terminus of the bacteriophage SPO1 DNA polymerase as an outgroup (Fig. 6B). The tree reveals a clustering of the sequences into a hyperthermophilic group comprising Archaea and the hyperthermophilic bacteria, and a mesophilic group comprising a great variety of bacterial lineages.

3.8. Functional stability and conformational changes of Afung as a function of temperature

When Afung protein was incubated in reaction or potassium phosphate buffer at different elevated temperatures for an increased period of time, the catalytic function declined. Afung was most stable in phosphate compared to reaction buffer, where in the former buffer the enzyme function declined rather slowly with time at 60 °C, but rapidly at 80–100 °C (Fig. 7 and data not shown).

We addressed the question as to whether the loss in Afung catalytic function caused by high temperatures is a result of significant conformational changes of protein structure, and decided to investigate this by fluorescence spectroscopy. Following excitation at 280 nm, the emission spectrum (Φ) of Afung was decomposed with high precision into the tyrosine contribution f_1 and the tryptophan contributions f_2 and f_3. Phenylalanine does not contribute to protein fluorescence under these conditions [27]. Fig. 8(A and B) shows the change of the emission spectrum after a 40 min storage of Afung at 80 °C. The f_2 redshift is defined by $\lambda_{2,\max}(T) - \lambda_{2,\max}(20\,^{\circ}\mathrm{C})$ and considered to reflect the putative unfolding/denaturation of the protein. In fact, the kinetics of the f_2 redshift increase

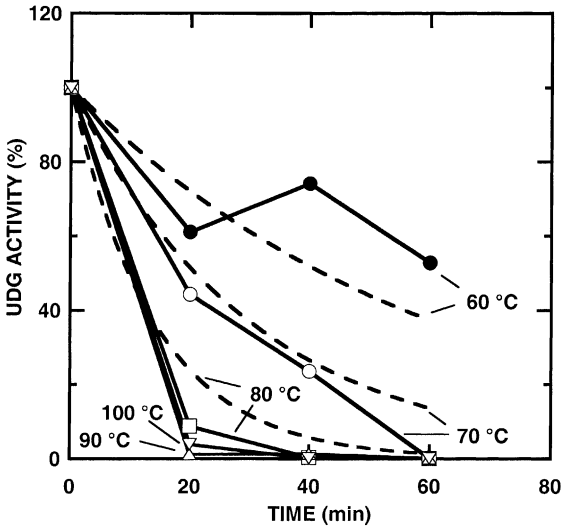

Fig. 7. Functional stability of Afung at different temperatures. Afung (0.083 μM) was incubated in 50 mM potassium phosphate buffer, pH 7.5 at the temperatures and for the time periods indicated. Following heat treatment, enzyme (4.14 pmol) was incubated with [^{3}H]uracil-containing DNA (2400 dpm; 2.2 pmol DNA-uracils) in reaction buffer at 80 °C for 10 min. Dashed lines show, for comparison, the first-order decreases (%) in Afung structural integrity at 60, 70 and 80 °C as determined from the kinetic fluorescence denaturation experiments presented in Fig. 8C.

at 60, 70 and 80 °C (Fig. 8C) is almost identical with the corresponding kinetic data demonstrating loss of enzyme activity at these temperatures (Fig. 7). Following 1 h heat treatments the f_2 redshift of Afung was recorded in the range 50–80 °C. No significant change in redshift from 20 °C to a couple of degrees below 50 °C could be detected, indicating the presence of a compact protein structure (Fig. 8D) with low catalytic activity (Fig. 5A). The f_2 redshift increased linearly from 50 up to 70 °C demonstrating protein unfolding (Fig. 8D). This unfolding of Afung starting at 50 °C is associated with a significant increase in, and is probably necessary for efficient induction of, enzyme activity (Fig. 5A). However, the loss of Afung activity at high temperatures, which correlated with the f_2 redshift kinetics (Figs. 7 and 8C), indicates that certain stabilizing agents (probably acting as chaperones) are necessary to keep Afung stable at the high growth temperatures of *A. fulgidus.* From the obtained rate constants at 60, 70 and 80 °C, we can use the Arrhenius equation ($k = A\,\mathrm{e}^{-E_a/RT}$) to determine the activation energy E_a for the unfolding process. When ln k is plot-

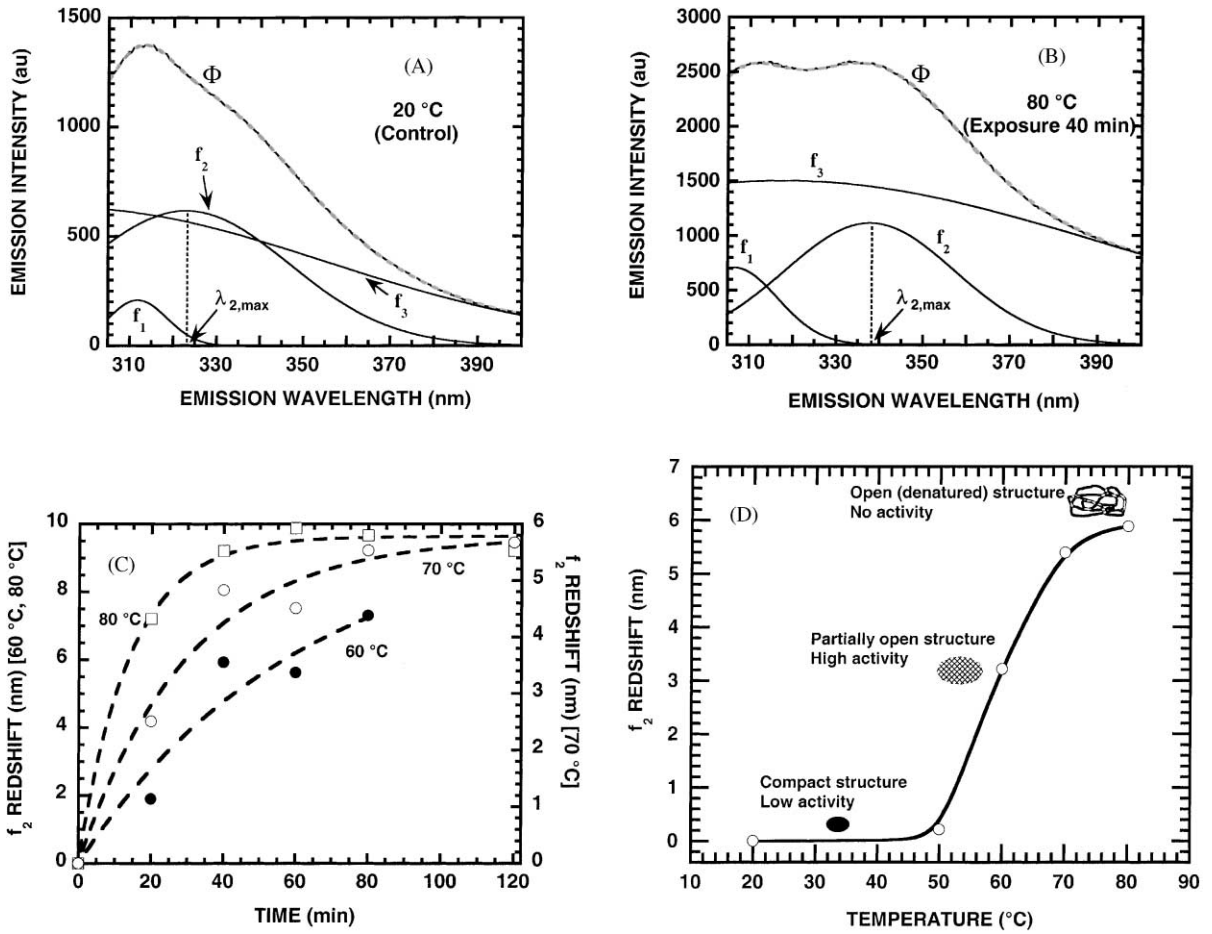

Fig. 8. Fluorescence (f_2 redshift) of the single tryptophan (Trp 195) in Afung following excitation at 280 nm at different temperatures. (A and B) Emission fluorescence spectra Φ (hidden black line) of Afung (1 μM) following incubation in 0.05 M potassium phosphate buffer, pH 7.5 at 20 °C (A) and 80 °C for 40 min (B). The dashed gray line shows the fit of the linear combination $f_1 + f_2 + f_3$ to the experimental spectrum. Correlation coefficients (r) of the fitted three-Gaussian functions are 0.99998 (A) and 0.99992 (B). Dotted lines show the individual f_i contributions to Φ. The wavelength indicated ($\lambda_{2,max}$) corresponds to the f_2 maximum. Note the increase of $\lambda_{2,max}$. The f_2 redshift is defined as $\lambda_{2,max}(80\,^\circ C) - \lambda_{2,max}(20\,^\circ C)$. (C) First-order kinetic increase of the f_2 redshift at 60, 70 and 80 °C. The experimental data were fitted to the expression $A = (f_2\,\text{redshift}) = A\bullet(1-\exp(-kt))$, where $A\bullet$ is the equilibrium f_2 redshift, k is the rate constant for the putative unfolding process, and t is time in min. The half-life of protein unfolding is calculated as $\ln 2/k$. Rate constants k determined are: 60 °C, 0.0164 min^{-1} ($r = 0.9669$); 70 °C, 0.0332 min^{-1} ($r = 0.98579$); 80 °C, 0.0713 min^{-1} ($r = 0.99754$). (D) The f_2 redshift plotted as a function of temperature. Prior to fluorescence spectrophotometric analysis, Afung (1 μM) was incubated in 50 mM potassium phosphate buffer, pH 7.5 for 1 h at the temperatures indicated.

ted as a function of $1/T$ (T is the temperature in Kelvin) the data points approximate a straight line, where $-E_a/R$ is the slope (R is the gas constant). From the plot, it was calculated that the activation energy for the unfolding process is 72 kJ/mol (data not shown). Deactivation energies in the range 51–654 kJ/mol have been calculated for other enzymes from *A. fulgidus* [38–40].

4. Discussion

A significant level of UDG activity was detected in crude extracts prepared from *A. fulgidus* stationary phase cells (Fig. 1A and B). The activity appeared to be of about the same order of magnitude as reported for several other hyperthermophilic archaeons and

bacteria as well as for the mesophilic *E. coli* and for different human tissues [11,41]. Thus, it was surprising that the complete genome sequence of *A. fulgidus* failed to indicate any putative UDG gene [13]. However, Sandigursky and Franklin [18] recently identified an ORF from *A. fulgidus* sharing significant homology with the UDG gene from the hyperthermophilic eubacterium *T. maritima*, an enzyme distantly related to the *E. coli* G:T/U mismatch-specific DNA glycosylase (Mug). The His-tagged version of the over-produced protein exhibited UDG activity and the gene was designated *afung* [21].

Is Afung the major DNA glycosylase enzyme for uracil removal in *A. fulgidus*, or alternatively, does it represent a less abundant UDG enzyme? An alternative candidate for removal of uracil from DNA was the putative gene product of the AF1692 ORF, a homologue of the *E. coli* Nth enzyme (endonuclease III). Nth has been shown to excise a variety of oxidatively damaged bases including 5-hydroxyuracil from DNA [42]. Indeed, a Nth/MutY homologue from the hyperthermophilic archaeon *Pyrobaculum aerophilum* has recently been shown to excise uracil from U:G mismatches [43]. Another possible UDG enzyme in *A. fulgidus* was the putative gene product of the AF2117 ORF sharing significant homology with the *E. coli* AlkA enzyme (3-methyladenine DNA glycosylase II). The AlkA enzyme excises the oxidized bases 5-formyluracil and 5-hydroxymethyluracil from DNA [32]. We over-expressed the enzymes coded by AF1692 and AF2117 ORFs in *E. coli*. Extensively purified samples of both proteins were analyzed for the presence of UDG activity. However, no DNA glycosylase activity for uracil was found to be present in neither the AF1692 nor AF2117 protein (Birkeland, et al., unpublished results). Since these two putative glycosylase genes could be ruled out as possible UDG genes, the hypothesis that Afung is the principal UDG of *A. fulgidus* was strengthened. The present study provides further evidence for this by showing a similar pattern of inhibition of Afung and of UDG activity in cell-free extracts when different agents were added to the incubation mixture (Table 3). This conclusion is also supported by the observation that the uracil-releasing activity of Afung and of *A. fulgidus* cell-free extracts have similar requirement for the opposite base (Fig. 4; Knævelsrud, et al., unpublished results).

The fluorescence signal (λ_{max}) from the single tryptophan in Afung protein (Fig. 6A) was followed by spectrometric measurements to analyze conformational changes in Afung as a function of temperature. Comparing these conformational changes with UDG activity measured at different temperatures (Fig. 5A), the following conclusions may be drawn. Below 50 °C, Afung has a relatively compact structure exhibiting low enzymatic activity. From 50 °C and above, extensive conformational changes take place (Fig. 8D) causing a large increase in enzyme activity (see Fig. 5A). Thus, active enzyme corresponds to a certain kind of open structure(s).

Each *A. fulgidus* cell contains about 0.033 pg soluble protein (data not shown). Taking this into account and suggesting that Afung is the only UDG enzyme present, it can be calculated that *A. fulgidus* contains ~1000 Afung molecules per cell, i.e. $\sim 9.5 \times 10^{-4}$ Afung molecules per G:C base pair in cellular DNA. It has previously been determined that *E. coli* contains ~300 Ung molecules per cell [2,44] which results in $\sim 1.3 \times 10^{-4}$ molecules per G:C base pair. However, the turnover number for Ung in *E. coli* has been determined to be ~800 uracils released per min [2] as compared to ~1 uracil released per min by Afung (Table 2). Consequently, *E. coli* has a much more efficient UDG enzyme than *A. fulgidus*, especially when taking into account the lower level of cytosine deamination taking place at moderate compared to high temperatures [31]. The deamination rate in ssDNA at 80 °C is $4.2 \times 10^{-8}\,s^{-1}$ [45]. From this number and the cytosine content in *A. fulgidus* DNA, it can be calculated that maximal 1.33 uracil residues are formed per min, a sufficiently low number to being handled by Afung. The deamination rate in dsDNA at 37 °C is $\sim 7 \times 10^{-13}\,s^{-1}$ [45], which should result in a four order of magnitude lower level of uracil formation in *E. coli*. It is a puzzle why *E. coli* has such a huge over-capacity for uracil excision.

The phylogenetic tree shows a grouping of the Afung homologues from the hyperthermophilic eubacteria *Aquifex aeolicus* and *T. maritima* with those from Archaea. These two species represent the most deeply branching bacterial lineages and are the only hyperthermophiles in the bacterial domain. It has furthermore been shown that lateral gene transfer between *T. maritima* and Archaea has been extensive [17]. The Afung homologues from mesophilic

bacteria, however, form a monophyletic clade, indicating evolution from a single origin.

As mentioned previously, none of the hyperthermophiles carry homologues of the classical UDG family as typified by the *E. coli* Ung enzyme. However, the *ung* gene is widespread in mesophilic bacteria, where *Treponema pallidum* and *Rickettsia prowazekii* are the only mesophiles of the 23 bacterial species so far characterized by complete genome sequencing that lack an *ung* homologue. Thus, in these species the gene responsible for excision of uracil might have been replaced by an Afung homologue [46,47]. It is interesting to note that *Deinococcus radiodurans*, which is an extremely radiation resistant microbe with a high DNA repair capacity, contains both UDG homologues. It should also be recognized that none homologues of either the *afung* or *ung* gene can be detected using BLAST searches against the genome sequences of the methanogens *Methanococcus jannashii* and *Methanobacterium thermoautotrophicum*, indicating that still another uracil-removing enzyme must be present in Archaea.

Recent studies using sequence profile searches, multiple alignment analysis and protein structure comparisons propose that all known UDG enzymes form a single protein super-family with a distinct structural fold and a common evolutionary origin [46,47]. The presently characterized UDGs were grouped into four families sharing very limited sequence similarity. The ancestral UDG was suggested to most closely resemble members of the "Afung" family. It calls for further inquiries for this group of enzymes.

In addition to Afung, an 8-oxoguanine DNA glycosylase of *A. fulgidus* (Afogg) has very recently been extensively purified and characterized [48]. It is interesting to note that even strictly anaerobic organisms may need enzymes specifically involved in the excision of certain oxidized base damages.

Acknowledgements

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