The *Methanothermobacter thermautotrophicus* ExoIII homologue Mth212 is a DNA uridine endonuclease

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Received May 9, 2006; Revised July 13, 2006; Accepted August 3, 2006

ABSTRACT

The genome of *Methanothermobacter thermautotrophicus*, as a hitherto unique case, is apparently devoid of genes coding for general uracil DNA glycosylases, the universal mediators of base excision repair following hydrolytic deamination of DNA cytosine residues. We have now identified protein Mth212, a member of the ExoIII family of nucleases, as a possible initiator of DNA uracil repair in this organism. This enzyme, in addition to bearing all the enzymological hallmarks of an ExoIII homologue, is a DNA uridine endonuclease (U-endo) that nicks double-stranded DNA at the 5'-side of a 2'-d-uridine residue, irrespective of the nature of the opposing nucleotide. This type of activity has not been described before; it is absent from the ExoIII homologues of *Escherichia coli*, *Homo sapiens* and *Methanosarcina mazei*, all of which are equipped with uracil DNA repair glycosylases. The U-endo activity of Mth212 is served by the same catalytic center as its AP-endo activity.

INTRODUCTION

The archaeal domain comprises a large number of thermophilic, extremely thermophilic and hyperthermophilic organisms, which suggests a correspondingly pronounced importance, among archaea, of genome protection against the eroding effect of spontaneous hydrolytic DNA damage. For example, the pre-mutagenic conversion of DNA cytosine to DNA uracil residues will occur at an increased rate compared to that in mesophilic organisms. In contrast to this expectation, no archaeal members of the otherwise almost ubiquitous DNA repair enzymes of the Ung family (1) have been identified to date. Instead, three other families of DNA glycosylases were found to remove uracil residues from DNA in archaea; these are (i) Mig (Mig.*Mth*, *Pa*-Mig) (2,3), (ii) ‘Mj/UDG’ (as exemplified by *mj1434* of *Methanocaldococcus jannaschii*) (4) and (iii) tUDG with its sub-families tUDGA (5) and tUDGB (6,7). The first two families belong to the HhH superfamily, the third to the UDG superfamily.

None of these, however, qualifies as a general archaeal line of defense against DNA cytosine deamination. Mig enzymes have clearly evolved to counteract the deamination of DNA 5-methylcytosine residues (i.e. processing of T/G mismatches) with the lack of discrimination against U/G oppositions as substrates probably owed to its selective neutrality. The *Methanothermobacter thermautotrophicus* genome (8) also contains a reading frame whose sequence is closely related to the *mj1434* gene (*mth746*; 38% identity, 54% similarity at the protein level); its gene product, however, is devoid of DNA uracil glycosylase activity (L.S. and H.-J.F., unpublished data). Lastly, enzymes of the tUDGA and tUDGB families have been identified in both the bacterial (5,7) and the archaeal domain (6,9,10). While tUDGA homologues are present in many archaeal genomes sequenced to date and tUDGB homologues in several, no consistent pattern of occurrence has emerged yet and, significantly, both tUDGA...
and tUDGB are lacking in a number of methanogenic archaea, including M.thermautotrophicus.

Hence, the question as to the DNA uracil repair function specifically relevant to M.thermautotrophicus and the general one (if any) of the entire archaeal kingdom, is still open. Having exhausted all bioinformatics options, we decided to approach the problem by fractionating total cell extract of M.thermautotrophicus while monitoring relevant biochemical activities. Here we report the discovery of a DNA uridine endonuclease (U-endo) activity that is not sequence- and mismatch-specific, carried by the product of gene mth212, a protein that was, until now, only known as a homologue of Escherichia coli ExoIII (11). This kind of activity has not been described earlier; it is clearly competent to specifically incise DNA next to uracil residues and may thus provide an initiation point for repair.

**MATERIALS AND METHODS**

**Strains**

E. coli DH5α (Invitrogen, Carlsbad, CA): F-, o80lacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17 (rK-, mK-), phoA, supE44, thi-1, λ-, gyrA96 (Nalr), relA1, E.coli BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA): E.coli b, F+, ompT, hsdS (rB-, mK-), dcm-, Tet-, gal λ(DE3), endA, Hfr [argU, ileY, leuW, CamR] E.coli XL1-Blue (12): recA1, endA1, gyrA96 (Nalr), thi-1, hsdR17 (rK-, mK-), supE44, relA1, λ-, lac+, [F', proAB lacIΔZΔM15, Tn 10 (TetR)].

M.thermautotrophicus Delta H (DSM 1053) was cultured as described earlier (13), Methanosarcina mazei (DSM 7222) was purchased from DSMZ (Braunschweig, Germany).

**Plasmids**

pCR-Blunt II-TOPO vector for cloning of blunt-ended PCR-products was purchased from Invitrogen (Carlsbad, CA) as part of the ‘Zero Blunt® TOPO’ kit.

pET_B_001 is a derivative of vector pET-21d (Novagen, San Diego, CA), in which the multiple cloning site between NcoI and XhoI is replaced by the following 14 nt section: dTCTGGCAGGCACACA. The chromosomal ung gene of the expression strain BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA) was replaced by a kanamycin resistance gene according to the method of Datsenko and Wanner (16). First, a PCR copy of the kan gene of pKD4, flanked by sequence of the ung locus was prepared using the following primers: UNGEX_UP1: dTAGAAGAAGACGATTGTTAGCGAGATGATTAGGCTTGTTAGGGTCTCTGTCTTTGC and UNGEX_LO1: dTGATAAAATCGACGGGTTGGAACACTTGCATCCCTCCGCCCCAAA-TCATATGAAATATCCCTCTTGA (pKD4 sequence in italics). Successful exchange was confirmed by PCR with primer pair KAN_SEN: dCGGTGCCCCATGAACTGC and Ung_AUS_ANT: dGCTGAAATATCTCTGCGCAA and primer pair KAN_ANT: dCGGCCACAGTCGATGAATCC and Ung_AUS SEN: dCATCAACTTATGCGGGTGTG, where the first primer of each pair primed within the kanamycin resistance gene and the second primer within the flanking region of the ung gene. Absence of Ung activity in cleared cell lysates of the resulting strain BL21_UX was

**Enzyme substrates**

The following oligonucleotides were purchased from Purimex (Grebenstein, Germany) in HPLC-purified and, if necessary, in preparative polyacrylamide gel electrophoresis purified grade.

40-U (40mer) 5′-(F)dGGGTACTTGGCTTACCTGCCCTGUGCAACGTGGCGCCGCAG-3′

40-U_pr (40mer) 5′-(F)dGGGTACTTGGCTTACCTGCCCTGUGCAACGTGGCGCCGCAG-3′

40-Ψ (40mer) 5′-(F)dGGGTACTTGGCTTACCTGCCCTGUGCAACGTGGCGCCGCAG-3′

40-T (40mer) 5′-(F)dGGGTACTTGGCTTACCTGCCCTGUGCAACGTGGCGCCGCAG-3′

40-AP (40mer) 5′-(F)dGGGTACTTGGCTTACCTGCCCTGUGCAACGTGGCGCCGCAG-3′

**Construction of Δung strain**

The chromosomal ung gene of the expression strain BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA) was replaced by a kanamycin resistance gene according to the method of Datsenko and Wanner (16). First, a PCR copy of the kan gene of pKD4, flanked by sequence of the ung locus was prepared using the following primers: UNGEX_UP1: dTAGAAGAAGACGATTGTTAGCGAGATGATTAGGCTTGTTAGGGTCTCTGTCTTTGC and UNGEX_LO1: dTGATAAAATCGACGGGTTGGAACACTTGCATCCCTCCGCCCCAAA-TCATATGAAATATCCCTCTTGA (pKD4 sequence in italics). Successful exchange was confirmed by PCR with primer pair KAN_SEN: dCGGTGCCCCATGAACTGC and Ung_AUS_ANT: dGCTGAAATATCTCTGCGCAA and primer pair KAN_ANT: dCGGCCACAGTCGATGAATCC and Ung_AUS SEN: dCATCAACTTATGCGGGTGTG, where the first primer of each pair primed within the kanamycin resistance gene and the second primer within the flanking region of the ung gene. Absence of Ung activity in cleared cell lysates of the resulting strain BL21_UX was
biochemically confirmed using the uracil DNA glycosylase assay described below.

Isolation of structural genes by PCR and insertion into expression vector

For isolation of genomic DNA from *E. coli* DH5α and *M.thermautotrophicus* 100 mg wet cell mass were suspended in 2 ml SCE buffer (1M sorbitol, 100 mM trisodiumcitrate, 60 mM EDTA) and sonicated on ice for 3 min with a Branson Sonifier 250 (microtrop, output level 5, duty cycle 50%). DNA was isolated by extraction with phenol/chloroform (vol:vol, 1:1) and ethanol precipitation. Genomic DNA from *M. mazei* was isolated similarly starting from a 2 ml liquid culture purchased from DSMZ.

Gene *mth212* was amplified by PCR with primer pair dAT-TATTTAGGTGTTACCATGCGCTGC and dTTATCGAGACGATTTGCTTATTT (NcoI and XhoI sites are underlined). Ten pmoles of each primer and 10 ng *M.thermautotrophicus* genomic DNA were mixed in a total volume of 49 µl 1× Pfu buffer without MgSO₄ (as supplied by the vendor), 0.2 mM dNTPs and 2 mM MgSO₄. After an initial denaturation step at 98°C for 2 min, 1 µl *Pfu* DNA polymerase (2.5 U/µl) was added at 85°C. PCR was run with the following cycling conditions: 5 cycles of 30 s at 94°C, 60 s 50°C, 60 s 72°C and 25 cycles of 30 s at 94°C, 60 s 60.5°C, 60 s 72°C followed by a final elongation step at 72°C for 10 min.

* M. mazei MM.3148 gene was PCR amplified with primers dAAAAGGGGTAAACATTTTATTTTCTCC and dGTTAGCTCGAGTAACATTTTATTTTCTCC (NcoI and XhoI sites are underlined). Amplification conditions were as for *mth212* except that the annealing temperatures for the 5 cycles was raised from 50 to 53°C and for the 25 cycles from 60.5 to 61.5°C.

* E. coli ung gene was PCR amplified with primers dGaTTTACCATGCGCTGC and dGTTACGAGACGATTTGCTTATTT (NcoI and XhoI sites are underlined). Amplification conditions were as described above. The PCR program was: 95°C, 3 min; 85°C, 2 min, addition of polymerase; 30 cycles of 95°C, 1 min; 62°C, 1 min; 72°C, 1 min; final elongation 72°C, 10 min.

* Bacillus* phage PBS-1 *ugi* gene was PCR-amplified from plasmid pEF/ugi (Michael Neuberger, Cambridge) with primers dAAATTCACCATGCGCTGC and dCTGAGCTCGAGTAACATTTTATTTTCTCC (NcoI and XhoI sites underlined). The PCR program was the same as for *E. coli ung* gene, except for a lower annealing temperature (60°C).

PCR products were directly cloned into pCR-Blunt II-TOPO as described by the supplier. NcoI/XhoI and NcoI/SalI fragments were recovered from the respective recombinant TOPO plasmids and inserted into pET_B_001 using standard procedures. The resulting constructs were confirmed by DNA sequence analysis.

Construction of mutant *mth212* coding for *Mth212/D151N*

Site-directed mutagenesis of the *mth212* gene was carried out by a modified QuikChange protocol (Stratagene, La Jolla, CA). Briefly, 70 ng pET_B_001-Mth212 as template were subjected to amplification with *Pfu* DNA polymerase and two mutagenic primers Mth212-D151N_FOR: dGATTATATTGCGGAACTCCAACACAGC, Mth212-D151N_REV: dGCGTGTGTTGAATCCACACACAAAACTCCACATATAATC (nucleotide exchanges marked in bold face). After an initial denaturation step at 95°C for 1 min, the reaction was subjected to 18 cycles of 50 s 95°C, 50 s 60°C and 10 min 72°C followed by a final elongation step at 72°C for 15 min. Reaction products were digested with DpnI at 37°C for 3 h and purified with the NucleoTrap kit (Macherey-Nagel, Düren, Germany) according to the instructions of the supplier. The eluted DNA was used to transform *E. coli* XL-1-Blue (12). Mutants were identified and confirmed by DNA sequence analysis.

Identification of candidate proteins from *M.thermautotrophicus*

Preparation of cell lysates. Up to 5 g of *M.thermautotrophicus* wet cell mass were suspended in 20 ml 20 mM HEPES-KOH, pH 7.6 and sonicated for 2 × 3 min on ice with a Branson Sonifier 250 (Danbury, CT; output level 5, duty cycle 50%). Cells were then passed through a Constant Systems Ltd (Daventay, England) cell disrupter at 180 MPa. 5 µl of benzonase were added and the lysate was incubated at 37°C for 30 min. After heating to 65°C for 30 min to deactivate benzonase, the mixture was centrifuged at 15 000 r.p.m., 4°C for 60 min in an SS-34 rotor (Sorvall, Thermo Electron Corp., Waltham, MA) and the supernatant was saved.

Chromatography steps. The supernatant was immediately fractionated using a heparin column (for details of heparin chromatography see section ‘Overexpression of cloned genes and protein purification’). Fractions with uracil processing activity (see below) were pooled, diluted ~1:25 with 20 mM HEPES-KOH, pH 7.6 and again fractionated by heparin chromatography with a more shallow gradient (0–1.5 M NaCl, eluent volume was increased from 117.8 to 196.3 ml). Fractions with activity were pooled, concentrated to ~1 ml final volume with Vivaspin 20 columns (Vivascience AG, Hanover, Germany) and applied to a HiLoad 16/60 Superdex 75 prep grade gel filtration column (GE Healthcare/Amersham Biosciences, Fairfield, CT, USA). The column was run with 25 mM HEPES-KOH, pH 7.6, 0.5 M NaCl at a flow rate of 1 ml/min. Elution was monitored by measuring the absorption at 280 nm.

Activity screening and protein identification by mass spectrometry. A total of 10 µl aliquots of fractions from corresponding chromatography steps were tested for uracil glycosylase and endonuclease activity (see below) with the following substrates: 40-U/35-G, 40-U/35-A, 40-T/35-G and 40-T/35-C for 30 min on ice.

Lanes from the silver-stained gel were cut into slices and proteins were digested in-gel with trypsin according to Shevchenko (19). Before the resulting peptides were extracted for LC-coupled ESI MS/MS (see below), 0.5 µl of the supernatant from the digest was analyzed by MALDI-ToF MS on a Bruker Reflex IV (Bruker Daltonics, Germany).
Bremen, Germany). The samples were prepared on a stainless steel target by using the thin-layer method with α-cyano-4-hydroxycinnamic acid (CHCA) as matrix as described by Vorm et al. (20). Samples were analyzed under standard conditions by MALDI-ToF mass spectrometry. The main protein component in the sample was identified via peptide mass fingerprint by searching the NCBI nr database with Mascot as a search engine. For extensive peptide sequence analysis, the extracted peptides were analyzed in a LC-coupled ESI Q-ToF mass spectrometer (Q-ToF Ultima, Waters) under standard conditions. Proteins were identified by searching fragment spectra of sequenced peptides against the NCBI nr database using Mascot as search engine.

Overexpression of cloned genes and protein purification

Overexpression and preparation of lysates. A 50 ml overnight culture of BL21_XU cells grown in selective medium, freshly transformed with the respective pET-derivative, was used to inoculate 1 liter dYT medium (16 g tryptone, 10 g Yeast extract, 5 g NaCl in 1 liter H2O) containing 50 μg/ml ampicillin, 50 μg/ml kanamycin and 25 μg/ml chloramphenicol in a 3 liter baffled flask. The culture was grown at 37°C with shaking to an OD600 of 0.6. Gene expression was induced by addition of IPTG to a final concentration of 1 mM. Incubation (as above) was continued for another 3 h. Cells were collected by centrifugation in a Roto Silenta/RP (Hettich, Tuttingen, Germany) at 4000 r.p.m., 4°C, 30 min, resuspended in 25 ml lysis buffer (25 mM

Figure 1. Identification of a DNA U-endo in total protein extract of *M.thermautotrophicus*. (A) Heparin affinity chromatography of a cleared cell lysate from *M.thermautotrophicus*. Abscissa: elution volume, left ordinate: absorbance at 280 nm, right ordinate: NaCl concentration. The bar indicates fractions containing DNA uridine processing activity illustrated in (C) (for details refer to Materials and Methods). (B) Schematic representation of substrates used for assays summarized in (C) (X: U; T; Y: G, A). (C) Right column: Gel electrophoretic analysis of DNA uridine incision assays with pooled fractions of heparin affinity chromatography (A). Left column: Same reactions, carried out with purified *E.coli* Ung. NaOH: Post-reaction treatment with NaOH. EDTA: Presence of 10 mM EDTA in assay buffer. For details refer to Materials and Methods. Electrophoretic position of 23mer is marked separately for each group of products. A second mark, set 30 min after the 23mer, indicates the approximate position of the starting 40mer. Designation of substrates is shown in (B); ssU: single-stranded 40mer with X = U.
HEPES–KOH pH 7.6, 0.5 M NaCl) and recentrifuged at 9000 r.p.m., 4°C, 30 min in an SS-34 Rotor (Sorvall, Thermo Electron Corp., Waltham, MA). The cell pellet was frozen at −80°C until required. The frozen pellet was resuspended in 20 ml lysis buffer and sonicated for 2 × 3 min on ice with a Branson Sonifier 250 (Danbury, CT; output level 5, duty cycle 50%). Cells were disrupted by passage through a Constant Systems Ltd (Daventry, England) cell disrupter at 180 MPa. The cell lysate was centrifuged at 15 000 r.p.m. at 4°C for 60 min in an SS 34 rotor (Sorvall, Thermo Electron Corp.) The supernatant was subjected immediately to immobilized metal ion affinity chromatography (IMAC).

**Immoblized metal ion affinity chromatography.** A column of chelating sepharose Fast Flow (Amersham Biosciences) was prepared from 5 ml slurry in a 10 ml plastic syringe fitted with a plastic frit, charged with Ni²⁺ ions and equilibrated with binding buffer (25 mM HEPES–KOH, pH 7.6, 0.5 M NaCl). Cleared cell lysates containing His-tagged proteins were passed through the Ni-sepharose column by gravity flow. Subsequently, the matrix was washed twice with 10 ml binding buffer before the His-tagged proteins were eluted with a stepwise imidazole gradient of 30, 60, 70, 80, 90, 100, 300 and 500 mM imidazole in binding buffer (10 ml per fraction). Elution was monitored by SDS–polyacrylamide gel electrophoresis. Normally, His6-tagged proteins eluted between 70 and 300 mM imidazole. Corresponding fractions were pooled, desalted by using Vivaspin 20 columns (Vivascience AG, Hanover, Germany) and with exception of Bacillus phage protein Ugi applied to a heparin column (see below).

**Heparin column chromatography.** Heparin affinity matrix (POROS 20 perfusion chromatography resin) was used in a column of 10 mm diameter and 100 mm length (bed volume 7.85 ml) operated with a Vision workstation (Applied Biosystems, Foster City, CA), flow rate 4 ml/min. Basal buffer was 20 mM HEPES–KOH pH 7.6 to which a gradient from 0 to 1.5 M NaCl in a total eluent volume of 117.8 ml was added. Fractions of 1.5 ml were collected. Elution was monitored by measuring the absorption at 280 nm. Peak fractions were analyzed by SDS–polyacrylamide gel electrophoresis. Fractions containing the pure protein were pooled.

**Enzyme activity assays**

**Glycosylase assay.** 0.12 pmol of double- or single-stranded oligonucleotide substrate were preincubated in a total volume of 50 μl 10 mM HEPES–KOH, pH 7.6, 100 mM KCl for 10 min at 37 and 65°C for thermostable enzymes respectively. After addition of 2.4 pmol E.coli Ung or 10 μl of *M.thermautotrophicus* protein mixture incubation was for 1 h at 37 and 65°C respectively. 5 μl of 1 M NaOH were added and the reaction mixture was incubated at 95°C for 10 min. 25 μl loading dye (95% formamide, 20 mM EDTA pH 8.0, 50 mg/ml Dextran-Blue 2000000) were added and samples were kept on ice until gel electrophoretic analysis.

**Endonuclease assay.** 0.12 pmol of double- or single-stranded oligonucleotide substrate were preincubated at 65°C for 10 min in a total volume of 50 μl 20 mM potassium phosphate buffer pH 6.2, 50 mM KCl, 1 mM MgCl₂ and 0.1 mg/ml BSA. 0.12 pmol purified Mth212 protein or 10 μl of *M.thermautotrophicus* protein mixture were added and the reaction incubated for 1 h at 65°C. (Where deviating reaction conditions [e.g. different enzyme/substrate ratio, different reaction times] were applied, they are described in legends to corresponding figures.) 25 μl loading dye (see above) were added and samples were kept on ice until gel electrophoretic analysis.

**Gel electrophoretic analysis.** 7 μl of the reactions resulting from glycosylase or endonuclease assays were loaded onto a 11% polyacrylamide gel (30 cm length, acrylamide:bisacryl- amide = 30:1; gel buffer 106.8 mM Tris-base, 106.8 mM boric acid, 3 mM EDTA, 7 M urea). Gels were run at 52°C in a Pharmacia A.L.F. DNA sequencer at a constant power of 40 W and 2 mW laser power with 89 mM Tris-base,

![Image](https://example.com/image.png)
89 mM boric acid, 2.5 mM EDTA as running buffer. The fluorescent species with the lowest electrophoretic mobility corresponding to the unmodified fluorescently labeled 40mer oligonucleotide was registered after ca. 140 min. Peak areas were quantitated with the Pharmacia Fragment Manager program.

Electrophoretic mobility shift assays (EMSA)

Mth212/D151N (310 ng, 10 pmole) was preincubated for 5 min at 65°C with no or increasing amounts of competitor DNA (pET_B_001, 26–1300 ng) in 9 μl 20 mM phosphate buffer pH 6.2, 50 mM KCl, 1 mM MgCl₂ and 0.1 mg/ml BSA, 10% glycerol. Double-stranded substrate (40-AP-D/35-G or 40-C-D/35-G) was added (1 μl containing 26 ng, 1 pmole). Samples were incubated at 65°C for 5 min and applied to 10% TBE-polyacrylamide gels. Gels were run at room temperature for 50 min at 10 V/cm in 1·TBE and stained with SYBR®Gold (Invitrogen, Carlsbad, CA) according to the supplier's manual. DNA was visualized by UV-Transilluminator at 302 nm.

RESULTS

A 2'-d-uridine-specific DNA nicking activity present in whole-cell extracts of M.thermautotrophicus

Total protein extract of M.thermautotrophicus was fractionated by chromatography using a heparin affinity column; the elution profile is illustrated in Figure 1A. Substrates of the type shown in Figure 1B were synthesized and used in assaying individual fractions for 2'-d-uridine-directed strand nicking, either initiated by a uracil DNA glycosylase or brought about directly by an endonuclease activity. Under nuclease assay conditions (see Materials and Methods), the pool of fractions indicated by the bar (Figure 1A) gave the results illustrated in Figure 1C, right column. U/G substrate is used up in favor of a major reaction product having the same electrophoretic mobility as the marker 23mer carrying a free 3'-OH terminus (Figure 1C, right column). The reaction is Mg²⁺-dependent and treatment of the reaction products with sodium hydroxide has no influence on their electrophoretic mobility. All of these observations are in stark contrast to reactions of the same substrate catalyzed by E.coli Ung uracil

![Figure 3](http://nar.oxfordjournals.org.at/DNAT545.png)

**Figure 3.** Purification of heterologously produced Mth212. SDS–PAGE (15%) of aliquots taken from different stages of protein purification as indicated. M: Molecular weight markers as in Figure 2. Cr: cleared cell lysate (crude extract) of induced BL21/XU cells carrying pET_B_001-mth212 (refer to Materials and Methods). 60–100: elution from Ni-IMAC column (imidazole concentrations given as mM); bracket indicates fractions pooled for subsequent heparin affinity chromatography. H: fraction eluted from heparin column at ca. 650 mM NaCl (for details refer to Materials and Methods).

![Figure 4](http://nar.oxfordjournals.org.at/DNAT546.png)

**Figure 4.** Activity of purified Mth212 protein. Assay as described in Materials and Methods. Substrates (X/Y) are schematically shown in Figure 1B. Single strand substrates (ssX) consist only of the respective 40mers. For ‘NaOH’, ‘EDTA’ and electrophoretic position marks see legend to Figure 1C. ‘AP’ stands for the chemically stable analogue of a base-free DNA site as shown in Figure 5.
DNA glycosylase. In the latter case, the reaction is unaffected by EDTA but treatment with NaOH is required to liberate a 23mer carrying a 3'-terminal phosphate group (compare corresponding tracks of left column). The most straightforward interpretation of this behavior of the cell extract fractions is direct endonucleolytic cleavage of the internucleotidic phosphodiester bond that links the mismatched 2'-d-uridine residue to the 3' oxygen atom of its immediate 5' neighbour. The activity, in addition, was unaffected by the addition of Ugi (data not shown), the polypeptidic inhibitor of uracil DNA glycosylase Ung and related enzymes (21). At this stage, the series of bands moving faster than the 23mer was tentatively attributed to exonucleolytic processing of the primary reaction product.

In order to obtain a first impression of its substrate spectrum, the activity was tested with oligonucleotide duplexes containing U/A oppositions, T/G mismatches and with a single-stranded oligonucleotide containing a single 2'-d-uridine residue (ssU). Again, results are displayed in the right column of Figure 1C, and compared, in each case, with the outcome of a corresponding uracil DNA glycosylase assay employing E.coli Ung enzyme (left column). With the U/A substrate, the 23 mer product is clearly detected, together with its characteristic series of shorter secondary products. After the limited incubation time applied, however, a substantial amount of substrate is left uncleaved, accompanied by its own series of faster moving material. Apparently, U/A is a less efficient substrate for U-directed strand nicking and exonucleolytic degradation acts on substrate and product alike. No trace of the characteristic 25mer is seen with the T/G substrate, but it is subject to exonucleolytic degradation.

Identification of mth212 as a candidate gene for the novel activity

A combination of column chromatography, gel electrophoresis, mass spectrometry and gene cloning was used to
reveal the identity of the protein that carries the nicking activity described in the preceding paragraph. First, the fractionation illustrated in Figure 1A was repeated. Re-chromatography on heparin affinity resin using a more shallow gradient (data not shown) was followed by gel filtration through Superdex 75 (Figure 2A). From the latter column, the activity eluted in two separate peaks which were pooled as indicated. Analysis of the two pools by SDS polyacrylamide gel electrophoresis yielded the pattern of bands shown in Figure 2B. Both lanes of the gel were cut in narrow strips and proteins contained in them digested with trypsin in situ. Fragment peptides were eluted from gel slices and subjected to mass spectrometric analysis as described in Materials and Methods. The fragment patterns were matched against the NCBI database of expected fragments. More than 10 candidate proteins were identified for each of the two lanes. The presence of activity in both pools can be explained either by two different enzymes sharing the same properties or by one enzyme eluting at two different volumes. If the second assumption holds, one would have to expect at least one protein to be present in both pools. By this criterion, the product of mth212 was chosen as the top candidate. A BLAST-search puts it into the category of ExoIII-like AP-endonucleases, a function that was recently verified experimentally (11).

Cloned Mth212 protein exhibits the expected catalytic properties

Gene mth212 was isolated by PCR and inserted into a pET-type expression vector. The protein was produced by heterologous gene expression in E.coli and purified by Ni²⁺ IMAC, followed by chromatography on heparin perfusion resin. (Figure 3, for details refer to Materials and Methods). Purified Mth212 protein was tested for the newly discovered DNA U-endo function and also for activities known to be commonly associated with homologues of E.coli ExoIII (AP-endonuclease, double-strand specific 3'→5' exonuclease). Assay conditions were essentially as previously applied to M.thermautotrophicus cell extract. Results are displayed in Figure 4 (also compare Figure 1C). With respect to U/G, U/A, T/G and ssU substrates, the pattern of activities displayed by the heterologously produced protein (Figure 4, second to fifth set of lanes from top) qualitatively matches that of the pool of proteins obtained upon fractionating total cell extract (Figure 1). Not surprisingly, the enzyme proves to be an AP-endonuclease; in this function it accepts both double- and single-stranded substrates (Figure 4, bottom two groups of lanes). Comparison of reactions of the AP/G and the ssAP substrates also serves to illustrate the double-strand specific exonuclease activity.
expected for an ExoIII homologue (note the absence of products with chain length smaller than 23 in the ssAP reaction). Knowing this also explains the exonuclease activity observed with the fractionated cell extract (Figure 1) as residing, at least in part, in the same protein as the DNA U-endo activity. Surprisingly for a DNase, Mth212 processes the AP/G substrate to the 23mer (but not further) in the presence of EDTA, as has been noted in a similar fashion with the human homologue Ape1 (22). Experiments with prolonged pre-incubation of enzyme with EDTA suggest this may be due to slow release of metal ion (data not shown).

Both AP- and U-endonuclease activities show turnover; complete conversion of substrate to product was demonstrated for substrate/enzyme ratios of up to 50 for the former and 10 for the latter substrate (data not shown).

Again, the ssU substrate is shortened in an apparently endonucleolytic fashion with no correlation of the cleavage point and the position of the single 2′-d-uridine residue (also compare Figure 1C, right column, bottom group of lanes). Possible folding states of the ssU 40mer at 65°C as predicted by a modified Zuker algorithm (23,24) suggest that the endonucleolytic cut may be directed by secondary structure. This interesting property of Mth212 is presently being investigated separately.

Enzymological exploration of Mth212
Since Mth212, like all known ExoIII homologues, is an AP-endonuclease (Figure 3, lanes 1–8 from bottom), the endonucleolytic cleavage product observed could arise from a two-stage reaction: removal of uracil by a uracil DNA glycosylase (activity residing in the same or an accompanying protein), followed by an endonucleolytic cut at the resulting AP-site.

As shown in Figure 5, a 2′-deoxy-pseudouridine-containing substrate is cleaved, which excludes the possibility of a glycosylase activity participating in the reaction. The alternative mechanism of a direct endonucleolytic incision is corroborated by inhibition of the reaction by an internucleotidic phosphorothioate linkage located to the 5′-side of the 2′-d-uridine residue (Figure 5, lane 6). As expected, Ung responds to the modifications by pseudouridine and, alternatively, phosphorothioate, in an exactly complementary fashion (corresponding lanes of left column).

An endonucleolytic cut next to the DNA uridine residue is observed with all four U/X oppositions as substrates (X = A, C, G, T), whereas no incision is seen if U is replaced by T (data not shown). A limited set of different sequence contexts was investigated, none of which was refractory to endonucleolytic scission. There is, however, significant variation of cleavage rate (data not shown).

Figure 7. Enzymological characterization of Mth212/D151N. Products of incubation of U/G and AP/G substrates with Mth212 (left column) and, respectively, Mth212/D151N (right column). Molar ratios of substrate:enzyme are indicated in the gray bars. Assay conditions: Standard substrate concentration and buffer (compare Figure 4), 65°C, 15 min.
In good accord with the optimal and maximal growth temperatures of *M. thermautotrophicus* (65–70°C for optimal, 75°C for maximal growth temperature) (25), AP-endo, U-endo and 3’→5’-exo activities all reach a maximum at 70°C, beyond which they drop sharply (data not shown). U- and AP-endonuclease activities of Mth212 respond differently to varying buffer conditions, as shown in Table 1. Similar observations have been made for AP-endo and damaged-nucleotide incision activities of Ape1 (26). Obviously, Mth212 nucleolytically processes a variety of different DNA substrates with conceivably rather different structural requirements to be met by the protein catalyst. This raises the question as to the number of distinct active sites of Mth212 involved in the various functions. For clarification of this issue, mutant (Mth212/D151N) was constructed in analogy to the previously studied mutant of the human homologue Ape1/D210N which is deficient in AP-endonuclease activity while still binding to substrate (27). Residue 151 was chosen on the basis of the multiple sequence alignment shown in Figure 6. As shown in Figure 7, Mth212/D151N is completely devoid of both DNA U-endo and 3’→5’ exonuclease activities with only a trace of AP endonuclease activity detectable. At the same time, specific binding to an AP/G substrate (Figure 8) proves the mutant protein to be properly folded. Clearly, these data are in accord with the assumption of a single active site serving all of the nucleolytic functions enumerated above.

**Possible DNA U-endo activity of other ExoIII homologues**

The new finding of a general, double-strand-specific DNA U-endo provided incentive to investigate how general this activity may be within the ExoIII family of enzymes. Possible DNA U-endo activity was tested, with negative outcome, for ExoIII homologues from *E. coli*, *Homo sapiens* and *M. mazei* (data not shown), all sharing high sequence similarity with Mth212 (compare Figure 6). AP-endo and 3’→5’ exo activities of the archaeal *M. mazei* homologue (product of *mm3148*) (28), which has not previously been characterized biochemically, were confirmed (data not shown).
DISCUSSION

The product of gene mth212, while displaying all features of a classical ExoIII homologue, in addition cleaves endo-
ucleolytically on the 5’-side of a DNA uridine residue, irrespective of the nature of the opposing nucleotide. At the
same time, the enzyme strictly discriminates against DNA cytidine and thymidine residues. With these properties, Mth212 can plausibly substitute as an initiator of DNA uracil repair for any of the general DNA uracil glycosylases known (Mj1434, Ung, UDGa, tUDGB) (1, 4–7, 10). Projecting the Mth212 amino acid sequence onto known 3D structures of ExoIII-homologues devoid of DNA U-end activity (compare above) did not yield any compelling lead as to the structural roots of the additional function. The subtle discrimination of DNA uracil residues from thymine on one hand and cytosine on the other, a property Mth212 shares with the structurally unrelated UDG family of glyco-
sylases, is especially remarkable since members of the ExoIII family of enzymes typically catalyze a number of dif-
ferent phosphoester hydrolysis reactions on a broad spectrum of substrates. For herpes simplex and human UDG (Ung family), the structural basis of this discrimination has been elucidated by X-ray crystallography (29, 30). It will be inter-
esting to see if and to what extent Mth212, along its own independent evolutionary path, found a different structural
solution to this problem.

ACKNOWLEDGEMENTS

We thank Michael Neuberger for gift of ugi gene. Work at the University of York was supported by a Biotechnology and Biological Sciences Research Council David Phillips Research Fellowship awarded to J.P.J.C. Valuable technical assistance was contributed by Christiane Preiß. Funding to pay the Open Access publication charges for this article were defrayed by regular funds made available to the department of the corresponding author by Georg-August-University, Göttingen.

Conflict of interest statement. None declared.

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