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A distinct *Tth*MutY bifunctional glycosylase that hydrolyzes not only adenine but also thymine opposite 8-oxoguanine in the hyperthermophilic bacterium, *Thermus thermophilus*

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ABSTRACT

Oxidative damage represents a major threat to genomic stability because the major product of DNA oxidation, 8-oxoguanine (GO), frequently mispairs with adenine during replication. We were interested in finding out how hyperthermophilic bacteria under goes the process of excising mispaired adenine from A/GO to deal with genomic oxidative damage. Herein we report the properties of an *Escherichia coli* MutY (*Ec*MutY) homolog, *Tth*MutY, derived from a hyperthermophile *Thermus thermophilus*. *Tth*MutY preferentially excises on A/GO and G/GO mispairs and has additional activities on T/GO and A/G mismatches. *Tth*MutY has significant sequence homology to the A/G and T/G mismatch recognition motifs, respectively, of MutY and Mig.*Mth*I. A substitution from Tyr112 to Ser or Ala (Y112S and Y112A) in the putative thymine-binding site of *Tth*MutY showed significant decrease in DNA glycosylase activity. A mutant form of *Tth*MutY, R134K, could form a Schiff base with DNA and fully retained its DNA glycosylase activity against A/GO and A/G mispair. Interestingly, although *Tth*MutY cannot form a trapped complex with substrate in the presence of $NaBH_4$, it expressed AP lyase activity, suggesting Tyr112 in *Tth*MutY may be the key residue for AP lyase activity. These results suggest that *Tth*MutY may be an example of a novel class of bifunctional A/GO mismatch DNA glycosylase that can also remove thymine from T/GO mispair.

1. Introduction

Reactive oxygen species are generated as by-products of oxidative phosphorylation, or from exposure to ionizing radiation to cause extensive base damage that is mainly repaired through base excision repair (BER) pathway [1–3]. Among numerous oxidative damage-related DNA base modifications identified to date, 8-oxo-7,8-dihydro-2′-deoxyguanine (GO) is the most abundant [4]. In *Escherichia coli*, the mutagenic effects of guanine oxidation are countered by the GO system, which consists of three enzymes: (i) MutT, an 8-oxo-dGTPase that prevents the incorporation of 8-oxodGMP into nascent DNA; (ii) MutM (also known as Fpg), a DNA glycosylase/lyase that excises GO and its opened-ring form, formamidopyrimidine (Fapy), from base pairs with C; and (iii) MutY, a monofunctional DNA glycosylase that excises adenines from A/GO mispairs [5].

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In particular, MutY hydrolyzes the N-glycosylic bond of the mispaired adenine base, generating an apurinic/apyrimidinic (AP) site opposite the GO base (AP/GO) [6]. Subsequent repair of the AP site is initiated by another BER enzyme, AP endonuclease [7].

In addition to the four regular bases, 5-methylcytosine (5-meC) is present in the DNA of nearly all organisms [8–10]. In bacteria, the presence of 5-meC within target sequences prevents them from being cleaved by endogenous restriction endonucleases that protects them from pathogens such as viruses. 5-meC forms base pair with guanine and serves various biological functions under a normal condition [11]. However, spontaneous hydrolytic deamination of 5-meC residues results in T/G mismatches, a pre-mutagenic lesion requiring DNA repair. In bacteria, the T/G mispairs are repaired by a very short-patch repair (VSR) endonuclease, nicks the DNA backbone 5′ of a T/G mismatch [12] and a T/G or U/G mismatch-specific glycosylase (MUG). T/G mismatch-specific HhH DNA glycosylases (Migs) from the thermophilic archaeon *Methanobacterium thermoautotrophicum* [8] and the hyperthermophilic archaeon *Pyrobaculum aerophilum* [13] counteract spontaneous mutagenic deamination of 5-meC residues during DNA base excision repair. In mesophilic bacteria such as *E. coli*, DNA mismatch glycosylases (such as MutY) act mainly on A/GO and A/G mismatches, whereas the mismatch DNA glycosylases (such as Mig) of the archaebacteria, *M. thermoautotrophicum* and *P. aerophilum*, display most activity towards T/G and U/G mismatches.

Thermus thermophilus is an aerobic hyperthermophilic bacterium with an optimal growth temperature of ∼85 °C [14]. We identified an open reading frame (ORF, TTC1535) that encodes a putative protein (*Tth*MutY) of 325 amino acids with a molecular weight of 36,575 Da in the complete *T. thermophilus* genomic DNA sequence, which is homologous to the gene encoding *E. coli* MutY protein. From this we produced recombinant TTC1535 protein and found that it could process not only A/GO, A/G, and G/GO substrates but also T/GO. Since hyperthermophilic bacteria are at higher risk of C/G → T/A transition mutations due to greater chance of spontaneous hydrolysis of cytosine and 5-meC at 85 °C than at 37 °C [15], it is reasonable to anticipate a presence of more effective DNA damage repair system in these than in other organisms. We show here that *Tth*MutY is a bifunctional DNA glycosylase that can remove mismatched bases from T/GO as well as from A/GO, A/G and G/GO.

2. Materials and methods

2.1. Enzymes and other materials

The amylose resin and pMal-c2x vector DNA restriction endonuclease, *E. coli* exonuclease III, and T4 DNA ligase were purchased from New England Biolabs (USA). *Taq* DNA polymerase was from Takara (Japan) and *E. coli* MutY was obtained from Trevigen (USA). The FPLC system, columns, and the [γ-^{32}P]ATP were obtained from Amersham Biosciences (Sweden). The genomic DNA of *T. thermophilus* was purchased from the ATCC (USA).

2.2. DNA substrates

The oligonucleotides used in this work were 32-mers (5′-GGATCCTCTAGAG TC[X]ACCTGCAGGCATGCAA-3′) or 39-mers (5′-GGATCCTCTAGAGTC[Y] ACCTGCAGGCATGCAAGCTTGAG-3′), where X denotes uracil (U), 3-methyladenine (3-meA), or 7-methyladenine (7-meG), and Y denotes for 8-oxoguanine (GO) or an apurinic/apyrimidinic (AP) site. These oligonucleotides that contain a single modified base at position 16 were purchased from Bio-Synthesis Inc. (USA). Oligonucleotides with a complementary base (A, T, G, or C) opposite the X (or Y) were used as complementary strands. Double-stranded substrates containing thymine glycol (Tg) residues were prepared by treatment of 32-mer oligonucleotides containing single T residues (5′-AGGAAGAGGAAAGGAG[T]GAAGGGAGAGAGGAG-3′) with OsO_4 before annealing them with the complementary strand, as previously described [16]. The oligonucleotides with complementary bases opposite T were obtained from Cosmo (Korea). The oligonucleotides containing an X, Y, or Tg and their complementary oligonucleotides were 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase at 37 °C. Unincorporated [γ-^{32}P] ATP was removed using a Microspin G-50 column (Sweden). Duplexes were prepared by annealing them using 1.5-fold molar excess of unlabeled complementary strand in buffer (20 mM Tris–Cl, pH 7.4, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 3% glycerol). The annealing mixtures were heated to 75°C for approximately 5 min and slowly cooled to room temperature. The annealed DNA was then ethanol precipitated, dried and resuspended in double-distilled water.

2.3. Construction of genes encoding TTC1535 and derivatives

The complete coding sequence of TTC1535 was amplified by PCR using TTC1535-forward (5′-GCAGGATCCGTGGAGGCCT-GGCAGAAAGCC-3′) and TTC1535-reverse (5′-GTACGCGTCG-ACCTATGCGTCCGGGAGGGGGAC-3′) primers. The underlined sequences indicate *Bam*HI and *Sal*I restriction sites used for direct cloning. PCR was performed in 100 μl reaction mixtures containing 100 ng of genomic DNA, 200 μM dNTPs, 2 units of *Taq* DNA polymerase, 10 μl of 10× reaction buffer, and 1 pmol of each primer. After 5 min denaturation at 94 °C, PCR was carried out (30 cycles) at 94 °C (30 s), 58 °C (30 s) and 72 °C (30 s), followed by extension for 5 min at 72 °C. The amplified DNA fragments were digested with *Bam*HI and *Sal*I, and ligated with the maltose-binding protein (MBP) fusion vector, pMal-c2xthr. The resulting plasmid (pMalthr-TTC1535) was then introduced into *E. coli* TB1. TTC1535 mutants (Y112S, Y112A and R134K) were constructed from pMalthr-TTC1535 using the 'megaprimer' mutagenesis technique [17]. The megaprimer was prepared by PCR starting with 10 ng pMalthr-TTC1535, 100 pmol TTC1535Y112S-reverse (5′-CACCGCCGCCGCGGTG-GAAGGCCCGAGGCCGGG-3′) or TTC1535Y112A-reverse (5′-C-ACCGCCGC-CGCGGTCGCAGGCCCGAGGCCGGG-3′) or TTC-1535R134Kreverse (5′-GAG-GCGGGAGAGGACCCTCTTGACGT-TCCCGTCCACCGC-3′) and 100 pmol TTC1535-forward primer. The underlined sequences indicate the altered codons. The PCR product (megaprimer) was eluted using the agarose gel, and a second PCR was performed using the product and TTC1535-reverse primer. The resulting fragment was digested

with *Bam*HI and *Sal*I and ligated with pMal-c2xthr. A gene encoding the double mutant of TTC1535 (Y112S/R134K) was constructed from the single mutant TTC1535 (Y112S) gene using the same procedure. DNA sequences were confirmed using an ABI 373 automatic DNA sequencer (USA).

2.4. Protein purification

E. coli TB1 harboring pMalthr-TTC1535 (*Tth*MutY) was grown in LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with 100 μg/ml of ampicillin at 37 °C. At an OD of 0.5 at 600 nm the recombinant protein was induced with 0.2 mM IPTG, and incubated for additional 16 h at 18 °C. Cells were harvested and resuspended in buffer A (20 mM Tris–Cl, pH 6.8, 100 mM NaCl and 1 mM DTT) and lysed by ultrasonication. The supernatant from centrifugation at 15,000 rpm for 30 min at 4 °C was loaded onto an amylose resin affinity column, and the MBP-*Tth*MutY fusion protein was eluted with 10 mM maltose in buffer A. The fusion protein was then cleaved with thrombin (10 U/mg of fusion protein) for 16 h at 4 °C and the cleavage mixture was dialyzed in buffer B (20 mM Tris–Cl, pH 6.8, 20 mM NaCl and 1 mM DTT). The resulting mixture was applied to a DEAE anion-exchange FPLC column pre-equilibrated with buffer B. Fractions containing *Tth*MutY were collected using buffer B as an eluent (linear gradient from 20 to 600 mM NaCl). *Tth*MutY, which eluted around 200 mM NaCl, was further purified with a Mono Q anion-exchange FPLC column and concentrated by ultrafiltration using a YM10 membrane (USA). All the procedures for obtaining the mutant TTC1535 proteins were the same as those of wild type TTC1535 (*Tth*MutY). The proteins were stored at −20 °C at concentrations of 5 mg/ml in final buffer.

2.5. Assay of TthMutY activity

DNA cleavage by *Tth*MutY proteins was assayed at 60 °C using 5 pmol of protein and 1 pmol of radiolabeled 39-mer (or 32-mer) oligonucleotide duplexes in a reaction buffer containing 20 mM Tris–Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA and 1 mM DTT. The reactions was quenched using phenol/chloroform and ethanol precipitation, and the resulting oligonucleotides were resuspended in 20 μl of the formamide loading buffer (0.05% bromophenol blue, and 0.05% xylene cyanol), heated for 5 min at 90 °C, and subjected to electrophoresis on a denaturing 20% polyacrylamide gel containing 7 M urea in 1× TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA). The gel was dried, placed on an imaging plate, and DNA cleavage products were quantified using a BAS2000 image analyzer (Japan).

2.6. DNA trapping with $NaBH_4$

*Tth*MutY and its mutants were incubated with 1 pmol of radiolabeled 39-mer oligonucleotide duplexes in the presence of 20 mM Tris–Cl, pH 7.5, 1 mM DTT, 50 mM NaCl, and 50 mM $NaBH_4$ in a total volume of 10 μl. The reaction mixtures were incubated at 60 °C for 30 min and mixed with 2.5 μl of the 5× SDS loading buffer (100 mM Tris–Cl, pH 6.8, 10% SDS, 20% glycerol, 5% 2-mercaptoethanol and 0.2% bromophenol blue). Samples were boiled at 90 °C for 5 min, and electrophoresed on a 10% SDS-polyacrylamide gel. The trapping efficiencies were quantified using a BAS2000 image analyzer.

3. Results

3.1. Purification of proteins

The sequence of the *T. thermophilus* genome has been published [14]. A substantial proportion of the coding genes of *T. thermophilus* are annotated as MutY homologues proteins. The amino acid sequence of TTC1535 showed 39.8% identity with *Ec*MutY, and its 196 N-terminal region showed 30% identity with the 221 amino acid residues of Mig.*Mth*I (Fig. 1). The final preparation of TTC1535 (*Tth*MutY) produced a single band of more than 95% purity (Fig. 2). The molecular mass of the protein was determined to be 36 kDa (*Tth*MutY) on a denaturing gel, corresponding to the theoretically calculated value. The purified *Tth*MutY was incubated for 10 min at different temperatures, and the amount remaining in solution was determined by the Bradford protein assay. About 66% of

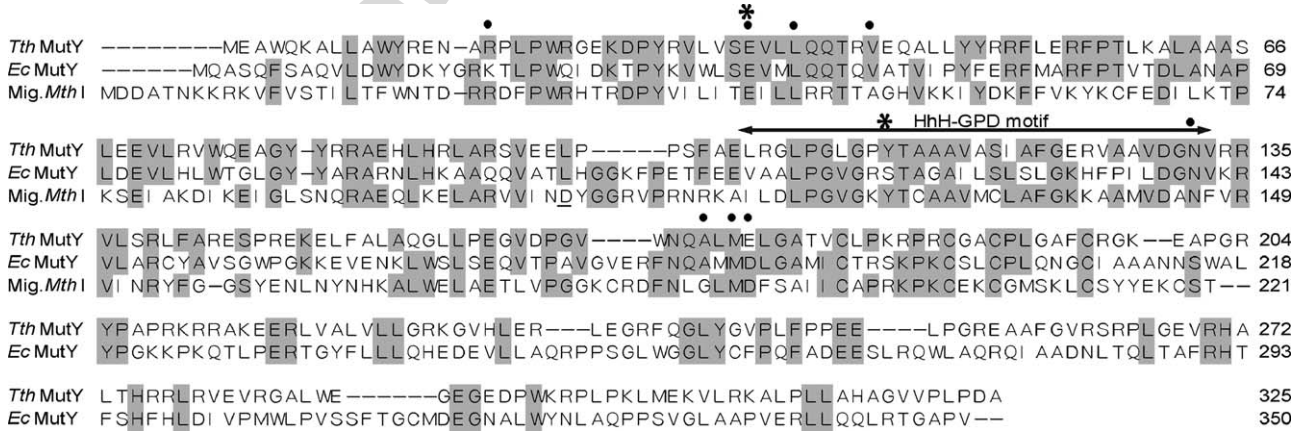


Fig. 1 – Alignment of *Tth*MutY and its homologues. The shadowed boxes represent conserved amino acid residues. The highly conserved HhH-GPD motif is indicated. Filled circles indicate *Ec*MutY amino acid residues involved in adenine binding and asterisks indicate Mig.*Mth*I amino acid residues relate of recognition of thymine in T/G mispair that are conserved in *Tth*MutY. Multiple alignments were carried out using the program CLUSTAL W.

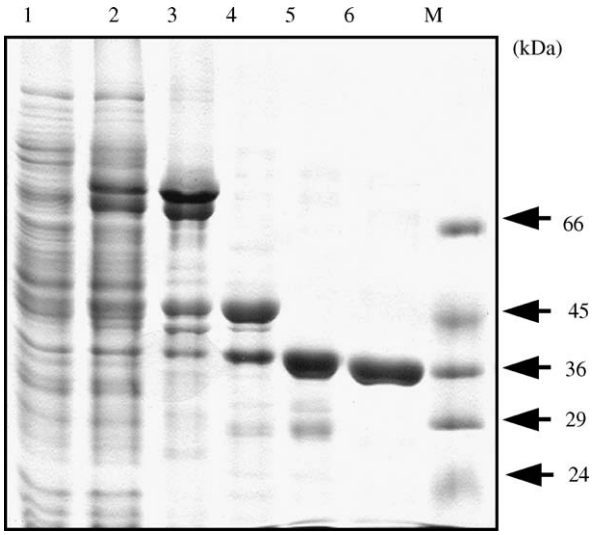


Fig. 2 – Purification of recombinant *Tth*MutY protein. To evaluate the purity and estimate the molecular weight, the product at each step was analyzed on an SDS-12% polyacrylamide gel and stained with Coomassie brilliant blue. Lane 1, control (crude extract of cells harboring vector pMalthr); lane 2, soluble extract of cells harboring pMalthr-*Tth*MutY after IPTG induction; lane 3, fraction eluted from an amylase affinity column; lane 4, proteins after thrombin treatment; lane 5, fraction eluted from a DEAE sepharose anion-exchange column; lane 6, pure *Tth*MutY from a MonoQ anion-exchange column; lane M, protein molecular weight marker.

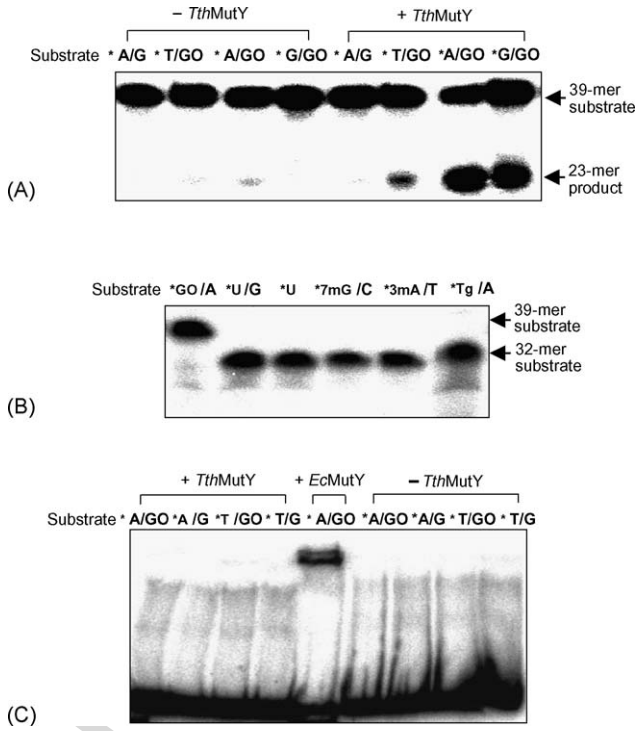


Fig. 3 – Mismatch glycosylase activity of *Tth*MutY. (A) Purified *Tth*MutY (5 pmol) was incubated with 1 pmol of 5′-end labeled adenine (A)- or thymine (T)-containing 39-mer oligonucleotide duplexes with 8-oxoguanine (GO) or guanine (G) in the complementary strand at 60 °C for 30 min. The cleavage products were analyzed using 20% denaturing polyacrylamide gel electrophoresis and a BAS2000 image analyzer. (B) Assay of *Tth*MutY with other unusual bases. *Tth*MutY protein was incubated with 1 pmol of oligonucleotide substrate (containing apurinic/apyrimidinic (AP) site or 8-oxoguanine (GO), 39-mer; oligonucleotides containing uracil (U), thymine-glycol (Tg), 3-methyladenine (3mA), or 7-methylguanine (7mG), 32-mer) under the same reaction conditions. Only the reaction products of *Tth*MutY was treated with 100 mM NaOH and heated. (C) Cross-linking *Tth*MutY to DNA containing A or T opposite GO- or G-containing substrate. The products were analyzed on a 12% SDS-PAGE gel. Five picomoles of *Tth*MutY protein was used in the reactions. *E. coli* MutY (EcMutY) served as a positive control.

the *Tth*MutY remained soluble after 10 min at 80 °C (data not shown). To eliminate endogenous *E. coli* MutY, the finally purified protein sample was heated for 10 min at 70 °C since it is completely denatured by this treatment.

3.2. *Substrate specificity of TthMutY*

E. coli MutY (*Ec*MutY) is an adenine DNA glycosylase active on DNA substrates containing A/G, A/GO, or A/C mismatches; in addition to weak guanine glycosylase activity on G/GO-containing DNA [18]. The *Ec*MutY and yeast MutY excise guanine mispaired with GO, as well as removing adenine from opposite mutagenic GO [19,20]. The DNA glycosylase activity of *Tth*MutY was assayed using defined double-stranded oligonucleotides containing A/G, T/GO, A/GO and G/GO. We found the strand cleavage activity of *Tth*MutY was specific for the A/GO-containing strand. In addition, activity on G/GO- and T/GO-containing oligonucleotide duplex was also observed (Fig. 3A), however, no activity was observed for C/GO-containing oligonucleotide duplex (data not shown). The thymine-binding pocket of Mig.*Mth*I appears to be similar to that of the adenine-binding pocket of MutY such that thymine should be allowed to form specific contacts with Glu42 and Tyr126, as suggested by the mutagenesis results [21]. Furthermore, *Tth*MutY also possesses two amino acid residues (Glu34 and Tyr112) that can also act as key determinants of thymine specificity (Fig. 1). This may account for how it processes T/GO mismatches. *Tth*MutY had no activity on substrates with GO/A, U/G, single U, 7mG, 3mA, and Tg/A (Fig. 3B) as well as on normal base mismatch containing oligonucleotide duplex beside A/G mismatch (data not shown).

*Ec*MutY forms a covalent complex with its substrate but does not catalyze β-elimination [22]. Lys142 in *Ec*MutY was shown to form a cross-link with substrate DNA following reduction with $NaBH_4$ [20]. *Ec*MutY (K142Q) was unable to form a Schiff base that selectively impairs the processing of DNA containing A/G mispairs but not of DNA containing A/GO [23]. Trapping assay revealed *Tth*MutY does not form a complex with A/GO (Fig. 3C), probably due to the presence of Arg in

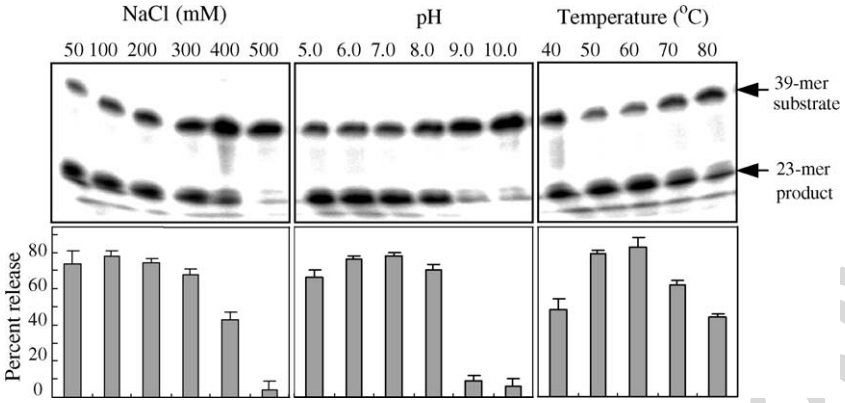


Fig. 4 – The effects of pH, temperature and salt concentration on *Tth*MutY activity. One picomole of A/GO-containing 39-mer oligonucleotide duplex was incubated with *Tth*MutY (5 pmol) for 30 min. Each sample was electrophoresed on a 20% polyacrylamide gel and quantified using a BAS2000 image analyzer. The effect of pH was measured in various buffers at 60 °C. The following buffers were used; MES buffer for 5.0–6.0, Tris–Cl buffer for pH 7.0–8.0, CHES buffer for pH 9.0–10.0. The effect of temperature was measured in Tris–Cl buffer, pH 7.0, and the effect of salt (NaCl) in Tris–Cl buffer, pH 7.0 at 60 °C. The quantitative data in the bottom graph were obtained from at least three independent experiments.

place of the Lys at the corresponding position of *Ec*MutY in the motif (Fig. 1).

We analyzed the DNA glycosylase activity of *Tth*MutY in the pH range 5.0–10.0 on the 39-mer duplex substrate containing A/GO. *Tth*MutY was incubated for 30 min in various buffers at 60 °C. The optimum pH of *Tth*MutY was approximately 6.0 (Fig. 4). When *Tth*MutY was incubated with various concentrations of NaCl, a significantly higher activity was observed in the presence of 50–100 mM NaCl (Fig. 4). Temperature dependence study of *Tth*MutY DNA glycosylase activity showed that it was 2.2-fold higher at 60 °C than at 40 °C (Fig. 4). Above 70 °C, *Tth*MutY activity *Tth*MutY activity diminished, probably due to partial denaturation of the DNA substrate (Fig. 4).

3.3. *Catalytic activity and substrate specificity of the TthMutY mutants*

*Tth*MutY efficiently removed adenine from A/GO mispairs, in addition to activity on T/GO duplexes (Fig. 3). In order to identify the amino acid residues that are involved in catalysis, site-directed mutagenesis was performed. Because alignments with *Ec*MutY and *M. thermoautotrophicum* Mig.*Mth*I suggested that Tyr112 of *Tth*MutY may be involved in thymine recognition of T/G mismatches, as well as catalytic activity (Fig. 1). We mutated Tyr112 of *Tth*MutY to Ser and Ala, respectively. In previous report on the *E. coli* MutY (*Ec*MutY), the activities of wild-type and three mutant proteins (K142A, K142Q and K142R) were compared using A/GO and A/G substrates [23]. Mutant K142A and K142Q displayed reduced activity toward A/G compared to the wild-type *Ec*MutY, as did K142R but to a lesser degree [23]. In *Ec*MutY, Lys142 is involved in discrimination between the *syn* and *anti* conformations of the nucleoside opposite adenine [23]. Arg134 of *Tth*MutY at the position corresponding to *Ec*MutY Lys142 was substituted to lysine. At neutral pH, the A/GO mispair exists as A (*anti*)/GO (*syn*) [24,25], whereas A/G exist as A (*anti*)/G (*anti*) [26,27]. At lower pH, the A/G pair becomes A (*anti*)/G (*syn*) ($pK_a = 6.0$) due to protonation of adenine at N1 position, which enables it to form a hydrogen bond with N7 of guanine [25]. At lower pH, *Ec*MutY showed significantly increased activity on A/G [28].

To evaluate the reaction rate with respect to mismatch substrates, *Tth*MutY and its mutants were compared using DNA duplexes containing A/GO, G/GO, T/GO and A/G. The cleavage activity of *Tth*MutY mutant Y112A was diminished from all substrates (Fig. 5). The reaction rate of mutant Y112S with substrate containing A/GO or G/GO was also significantly reduced (Fig. 5A and B), suggesting the lack of aromatic ring in serine in comparison to tyrosine may play a significant role in reducing the enzyme activity. The mutant R134K had slightly decreased activity on A/GO and G/GO mispair (Fig. 5A and B) while its activities on T/GO and A/G mispair were not noteworthy (Fig. 5C and D). Double mutant Y112S/R134K showed significantly decreased cleavage activity for all substrates (Fig. 5). Fig. 6 showed mutant R134K re-created the Schiff base-forming HhH motif of the AP lyases, but its catalytic activity appeared no different from wild-type *Tth*MutY (Fig. 5). These results suggest that Tyr112 of *Tth*MutY is important for thymine recognition in T/GO mismatch but it is not the key catalytic residue for DNA glycosylase activity. Although mutant R134K can form a trapped complex with A/GO (or A/G) substrate, it may not likely involved in change A (*anti*)/G (*anti*) to A (*anti*)/G (*syn*).

3.4. *TthMutY is a bifunctional DNA glycosylase, and Tyr112 is important for AP lyase activity*

There are two classes of DNA glycosylases with distinct substrate specificities: the monofunctional glycosylase and the glycosylase associated with apurine/apyrimidine (AP) lyase activity [2,3]. Although MutY can form a covalent intermediate with its DNA substrates, its possession of 3′ AP lyase activity is controversial. We tested AP lyase activity of *Tth*MutY toward AP-containing duplexes. We observed that AP site of substrate could be cleaved by *Tth*MutY. The 16-mer DNA prod-

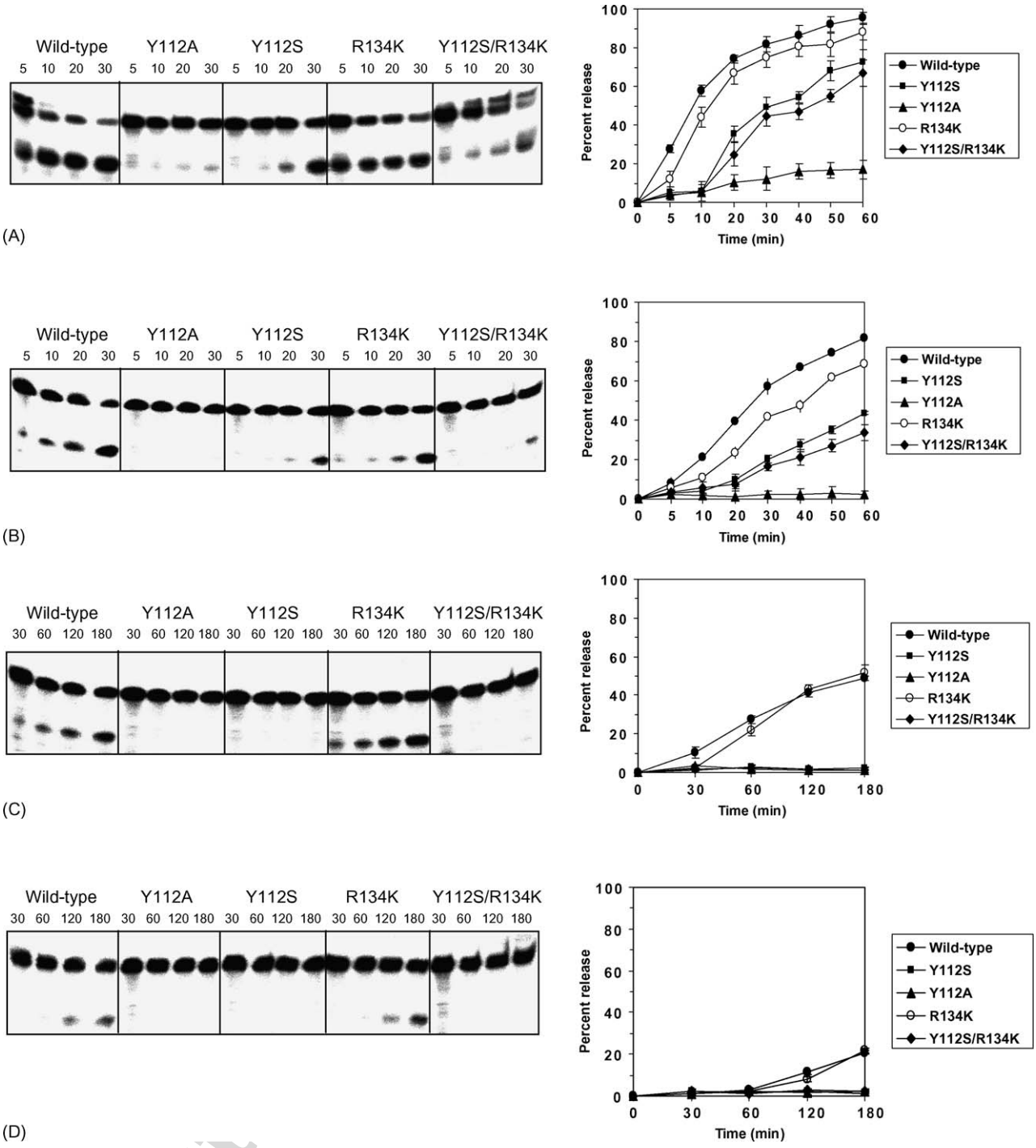


Fig. 5 – Time dependence activity of wild-type T*th*MutY and its mutants. Reactions were carried out at 60 °C with oligonucleotide substrates containing (A) A/GO, (B) G/GO, (C) T/GO and (D) A/G. At the indicated times, samples were treated with 100 mM NaOH at 95 °C for 10 min and cleavage products were analyzed using 20% denaturing polyacrylamide gel electrophoresis and a BAS2000 image analyzer. Quantitative data were obtained from at least three independent experiments.

uct cleaved by T*th*MutY was run slower than the 3′-OH product of *E. coli* exonuclease III (Fig. 7A). The T*th*MutY protein catalyzed a β-elimination reaction at the AP site and thus generated 3′-α,β-unsaturated aldehyde sugar termini at the incision site. To investigate the critical site for AP lyase activity of T*th*MutY, cleavage activities of mutant proteins were assayed using AP site-containing DNA substrate. As shown in Fig. 7B, both wild-type T*th*MutY and mutant R134K could cleave AP/G-containing DNA, whereas two mutants (Y112S and Y112A) were completely abolished AP lyase activity (Fig. 7B). These results suggest that Tyr112 is an important amino acid for AP lyase activity of T*th*MutY.

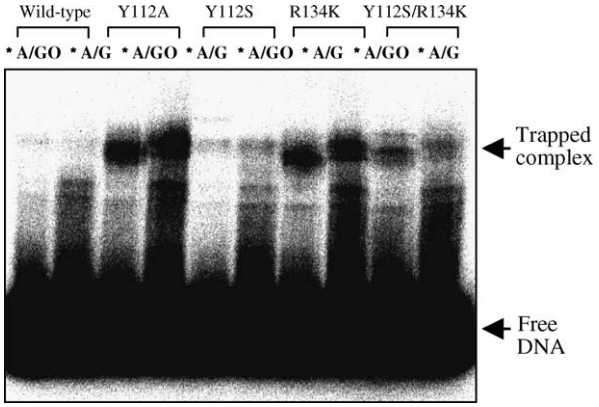
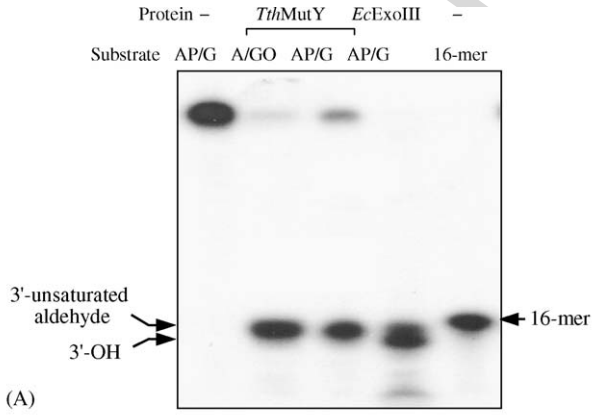


Fig. 6 – Cross-linking of wild-type *Tth*MutY and mutant proteins with A/GO- or A/G-containing substrates. Five picomoles of protein was used in all reactions. The products were analyzed using a 12% SDS-PAGE gel and a BAS2000 image analyzer.

4. Discussion

Oxidative damage represents major threats to genomic integrity. Since the rate of these damages rise with temperature, hyperthermophilic organisms are anticipated to possess highly efficient repair system to remove the oxidatively damaged bases from their DNA. Among various types of oxidized damage in DNA, 8-oxoguanine (GO), the oxidized forms of guanine, can pair not only with cytosine but also with adenine, guanine, or thymine in the template DNA during DNA replication [29]. In *E. coli*, GO is excised from oxidatively damaged DNA by MutM (also known as Fpg) [30]. MutM preferentially cleaves GO paired with G, C, and T, but not A [31], whereas *Afogg* of a hyperthermophilic archaeon, *Archaeoglobus fulgidus*, prefers GO paired with C followed by G > T > A [32]. When C/GO is not repaired by MutM, adenines are frequently incorporated opposite GO bases during DNA replication. MutY enzymes prevent G:C to T:A transitions by excising adenines from A/GO. The T/GO mismatches are derived from deamination of 5-methylcytosine opposite GO [33], as well as by misincorporation of T opposite template GO [34]. The removal of GO from



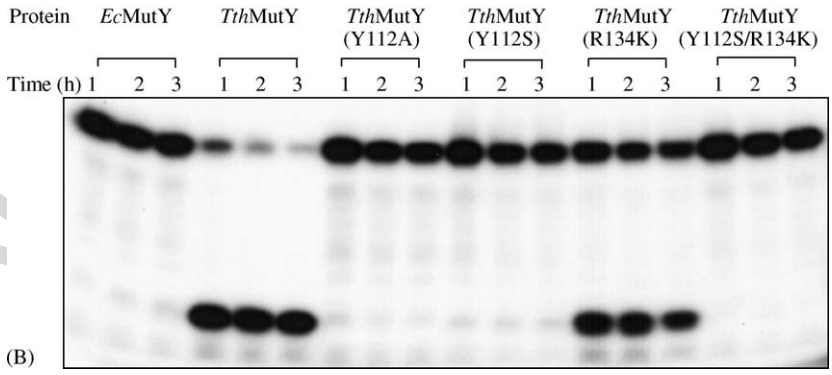


Fig. 7 – Identification of AP lyase activity of *Tth*MutY. (A) *Escherichia coli* exonuclease III (*Ec*ExoIII) (1 pmol) and *Tth*MutY (5 pmol) activities were assayed with apurinic/pyrimidinic (AP) site-containing substrate (AP/G mismatch) for 30 min at 37 and 60 °C, respectively. Samples were treated at 95 °C for 10 min and products were analyzed using 20% denaturing polyacrylamide gel electrophoresis and a BAS 2000 image analyzer. (B) *Ec*MutY activity was assayed at 37 °C and *Tth*MutY and its mutants activities were assayed at 60 °C. Samples were treated with dye at 95 °C for 10 min. Five picomoles of protein was used in all reactions.

T/GO mismatches with GO on the parental strand leads to G:C to A:T transitions [2]. *T. thermophilus* is an aerobic hyperthermophilic eubacterium with an optimal growth temperature of ~85 °C. Formation of T/GO and G/GO mispairs is favored over A/GO in this species [8,35]. We demonstrated *Tth*MutY was able to remove thymine from T/GO (Fig. 3). However, we failed to find a Mig-like ORF in the sequence of *T. thermophilus*. These results suggest that *Tth*MutY may be the only glycosylase in *T. thermophilus* capable of excising thymine from T/GO mismatches. Therefore *Tth*MutY maybe involved with prevention of G:C to A:T mutations as well as G:C to T:A mutations.

We demonstrated recombinant *Tth*MutY has DNA glycosylase activity that excises thymine opposite GO as well as adenine opposite GO. We also demonstrated Tyr112 residue may be critical for recognizing the thymine in T/GO mismatch. *Tth*MutY may be closer to the Mig family rather than clustered with other bacterial MutY proteins since its Tyr112 residue is in the HhH motif. The amino acid sequences of *Ec*MutY involved in adenine recognition of DNA substrates are conserved in Mig.*Mth*I, except two amino acid residues [34]. Interestingly, the substitution of two amino acid residues in the thymine binding motif of Mig.*Mth*I (L187Q and A50V) resulted in altering the substrate discrimination between T/G and A/G, making the recognition rate of T/G 56-fold slower than that of A/G 2.1-fold faster [36]. The amino acid sequences of putative adenine-binding pocket of *Tth*MutY are the same as that of *Ec*MutY adenine-binding pocket (Fig. 1). Also Glu37 and Tyr112 residues of *Tth*MutY correspond to Glu42 and Tyr126 thymine-binding residues of Mig.*Mth*I. When Tyr112 of *Tth*MutY was replaced with alanine or serine, it was found that the resulting two *Tth*MutY mutants (Y112A and Y112S) were unable to cleave T/GO-containing substrate (Fig. 5C). However, mutant Y112S retained activity for excising adenine from A/GO-containing substrate (Fig. 5A). We furthermore showed that two mutant proteins (Y112S and Y112A) had much lower activity on A/GO-containing substrate than the wild-type protein. Y112S has somewhat of catalytic activity on A/GO-containing substrate, whereas Y112A has a very limited activity on the same substrate (Fig. 5A). On the other hand, both Y112A and Y112S mutants had significantly decreased catalytic activity on T/GO mispair. It may be that the tyrosine 112 of *Tth*MutY is important residue involved in thymine recognition of T/GO mismatch. Although tyrosine and serine has different structures, they commonly have a hydroxyl group in the side chain but not alanine, suggesting Y112S has a little influence than Y112A on the protein conformation, following different catalytic activities between both mutants on A/GO-containing substrate.

In Mig.*Mth*I, substitution of serine for Tyr126 essentially inactivated the glycosylase activity of the enzyme and substitution of lysine also resulted in loss of activity even though it retained the Schiff base form [21]. Interestingly, we observed that *Tth*MutY cleaved AP-containing substrate DNA with a sufficient AP lyase activity (Fig. 7A) but that AP lyase activity disappeared in Y112 mutants (Fig. 7B). Human MutY and *E. coli* endonuclease VIII (*Ec*Nei) do not possess the conserved serine residue found in *E. coli* MutY, but instead possesses a tyrosine residue [37,38]. Human MutY is a monofunctional glycosylase but *Ec*Nei is a bifunctional glycosylase that removes thymine glycol, dihydrothymine and urea from double-stranded DNA and cleaves the DNA strand at AP sites [39]. We postulate that Tyr112 of *Tth*MutY has a function similar to that of Tyr170 of *Ec*Nei. In *E. coli* MutY, Asp138 activates a water molecule by abstracting a proton, attacking the C1 carbon of the adenosine and releasing the mispaired adenine base [40]. Tyr112 in *Tth*MutY also has a role similar to that of Tyr126 in Mig.*Mth*I. It recognizes thymine residue in T/GO mispair and is important for AP lyase activity. It may be that the key residue for glycosylase activity is Asp129, like Asp138 in *Ec*MutY because mutant Y112A and Y112S have a glycosylase activity. Actually, the glycosylase activity was completely abolished in mutant D138N of *Ec*MutY [41]. Presumably Tyr112 of *Tth*MutY is of evolutionary importance since life at high temperature is under a high level of DNA oxidation and that an efficient repair is required for maintaining the fidelity of the genomic DNA.

Although the mutant R134K could form a trapped complex with A/GO and A/G substrate, its catalytic activity on the A/G-containing substrate was not compromised (Fig. 5), suggesting Lys134 of mutant R134K is unable to modulate A (*anti*)/G (*anti*) mispair conversion into A (*anti*)/G (*syn*). The *syn* conformation is thermodynamically favored over *anti* conformation for an isolated GO nucleoside; however both rotamers are found in GO-containing duplex DNA, with *syn* being preferred for GO opposite adenine or guanine [24,25,33] and *anti* being preferred for GO opposite cytosine [33]. In the MutM active site, the GO nucleoside adopts a *syn* glycosidic torsion angle [42]; conversely, the same lesion is recognized by human Ogg1 in the *anti* glycosidic conformation [43]. The mono-functional glycosylases such as Mig.*Mth*I and MutY bind to their target nucleotides in the *syn* conformation normally seen in dsDNA [19]. Similarly, *Tth*MutY prefers the *syn* conformation found in normal monofunctional glycosylases. *Tth*MutY mutant Y112A could also form trapped complex with DNA substrate like mutant R134K (Fig. 6). We postulate that Tyr → Ala substitution would make conformational change in this protein, so another lysine might contribute forming a trapped complex with DNA substrate. In the case of *E. coli* MutY (*Ec*MutY), the lysine residue (Lys142) would be available for initiating the lyase reaction through the formation of a Schiff base intermediate [44]. However, K142A mutant of *Ec*MutY could be covalent trapped protein–DNA complexes by chemical reduction in mild reducing agent ($NaCNBH_3$) [45], suggesting lysine residues other than Lys142 could also participate in formation of protein–DNA complex and the flexibility of amino acids including lysine residues such as Lys157 and Lys158 is important factor in formation of the Schiff base intermediate. Interestingly, *Tth*MutY has a lysine residue (Lys149) corresponding to that of *Ec*MutY (Lys157) (Fig. 1).

Even though *Tth*MutY shares common features such as the recognition motifs for adenine in A/G mispairs and for thymine in T/G mispairs with the MutY and Mig families, our site-directed mutagenesis experiments strongly suggest that *Tth*MutY does not fall into any known group of mismatch DNA glycosylases of the HhH superfamily. *Tth*MutY is functionally similar to MutY family but differs from Mig family by lack of activity against U/G mispair. Taken together, our observations suggest that *Tth*MutY may be a novel type of mismatch DNA glycosylase.

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