In Vivo, in Vitro, and X-ray Crystallographic Analyses Suggest the Involvement of an Uncharacterized Triose-phosphate Isomerase (TIM) Barrel Protein in Protection against Oxidative Stress

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Background: Characterization of conserved hypothetical proteins is essential for finding novel biological phenomena. Results: A hypothetical protein, TTHB071, has a phosphatase-like trinuclear zinc center and bound reductants in a zinc-mediated manner. Disruption of tthb071 increased the sensitivity to oxidative stress. Conclusion: TTHB071 would protect cells from oxidative stress through controlling the metabolism of reductants. Significance: Our findings may explain how cells avoid oxidative damage.

Accumulating genome sequences have revealed the existence of a large number of conserved hypothetical proteins. Characterization of these proteins is considered essential in the elucidation of intracellular biological pathways. Our previous transcriptomic analysis suggested that, in Thermus thermophilus HB8, loss of an oxidized DNA-repairing activity leads to the up-regulation of a function-unknown gene, tthb071, which is conserved in a wide range of bacteria. Interestingly, the tthb071 gene product, TTHB071, showed a significant primary structure similarity to apurinic/apyrimidinic (AP) endonucleases, which are required for the repair of oxidized DNA. In the present study, we observed that disruption of tthb071 increases the H2O2 sensitivity in T. thermophilus HB8, suggesting the involvement of tthb071 in a protection mechanism against oxidative stress. However, purified TTHB071 exhibited no AP endonuclease or DNA-binding activities, indicating that TTHB071 plays no major role in repairing oxidative DNA damage. Then we determined the three-dimensional structure of TTHB071 complexed with zinc ions by x-ray crystallography. In addition to the overall structural similarity, the zinc-binding fashion was almost identical to that of the phosphatase active site of an AP endonuclease, implying that TTHB071 possesses a phosphatase activity. Based on the structural information around the zinc-binding site, we investigated the binding of TTHB071 to 14 different compounds. As a result, TTHB071 favorably bound FMN and pyridoxal phosphate in a zinc ion-mediated manner. Our results suggest that TTHB071 protects the cell from oxidative stress, through controlling the metabolism of reductants.

A large number of genomes have been sequenced over the past 3 decades, showing that there are numerous hypothetical proteins whose amino acid sequence is conserved in a large part of organisms. Characterization of these conserved hypothetical proteins is considered essential in the search for a novel biological phenomenon and also in the elucidation of partially understood biological pathways. The extremely thermophilic eubacterium, Thermus thermophilus HB8, is a promising model organism for such studies, as evidenced by the ongoing Whole Cell Project on T. thermophilus HB8 (1). T. thermophilus HB8 has a relatively small genome size (2.2 Mbp), and its intracellular biological systems are thought to consist of minimum essential components. Low redundancy of the homologous components in this bacterium is expected to enable the straightforward characterization of conserved hypothetical proteins. Furthermore, proteins from T. thermophilus HB8 are extremely thermo- and suitable for physicochemical characterization, including x-ray crystallographic analysis (2). In the Whole Cell Project on T. thermophilus HB8, researchers have been cooperating in an attempt to characterize all of the proteins from this bacterium (see the Structural-Biological Whole Cell Web site), using a range of methodologies, including structure (2), transcriptome (3–6), interactome (7), proteome, and metabolome (8) analyses. As a part of this project, we have attempted to elucidate how cells avoid oxidative stress in T. thermophilus HB8 (9–12). Here, we describe the characterization of a hypothetical protein that is suggested to participate in a DNA-repair pathway or a protection mechanism against oxidative stress.
Characterization of a Function-unknown TIM Barrel Protein

In the cell, reactive oxygen species (ROS) arise as a consequence of extracellular oxidative stress and intracellular aerobic metabolism. These ROS oxidize a wide range of biomolecules, including proteins, lipids, carbohydrates, and DNA. The ROS-induced oxidation denatures proteins, lipids, and carbohydrates, which results in severe dysfunction of the cellular activities. The radical attacks on DNA generate oxidized bases, such as thymine glycol, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 7,8-dihydro-8-oxoguanine (8-oxoguanine), and 5-formyluracil. These modifications stall the RNA and DNA polymerases and form stable hydrogen bonds with multiple base partners, making them toxic and mutagenic, respectively. Although oxidative stress-induced mutagenesis can serve as a driving force for evolution to survive in an unconventional environment, cells need to avoid frequent alteration of their genomes during a short period. Therefore, cells are equipped with mechanisms for detoxifying ROS and repairing oxidative DNA damage.

Cells are known to possess a variety of ROS-detoxifying systems. Enzymes, such as catalases, superoxide dismutases, peroxidases, and NAD(P)H/FMN-dependent oxidoreductases, reduce superoxide or hydrogen peroxide. Small oxidases, and NAD(P)H/FMN-dependent oxidoreductases, detoxifying ROS and repairing oxidative DNA damage. To avoid frequent alteration of their genomes during a short period, cells need to prevent oxidative DNA damage. The ROS-induced oxidation denatures proteins, lipids, and carbohydrates, which results in severe dysfunction of the cellular and molecular functions of the thhb071 gene product, TTHB071.

EXPERIMENTAL PROCEDURES

Reverse Transcription-PCR—Reverse transcription-PCR was performed by a previously described procedure (26) using a PrimeScript RT-PCR kit (Takara, Shiga, Japan). The forward and reverse primers used were 5′-CTTCCGGGCGGCAAG-3′ and 5′-CTCCAGGGGCTCCCTCC-3′, respectively (BEX Co., Tokyo, Japan).

Culture Conditions for T. thermophilus HB8—T. thermophilus HB8 was grown at 70 °C in TR medium: 0.4% (w/v) tryptone (Difco), 0.2% (w/v) yeast extract (Oriental Yeast, Tokyo, Japan), and 0.1% (w/v) NaCl (pH 7.5) (adjusted with NaOH). To prepare plates, 1.5% (w/v) gellan gum (Wako, Osaka, Japan), 1.5 mm CaCl2, and 1.5 mm MgCl2 were added to the TR medium.

Disruptions of thhb071, thhb070, and uvrC—The thhb071, thhb070, and uvrC null mutants of T. thermophilus HB8 (∆thhb071, ∆thhb070, and ∆uvrC) were generated by substituting the target gene with the thermostable kanamycin resistance gene, Htk (27), through homologous recombination as described previously (28, 29). The plasmids for gene disruptions were derivatives of the pGEM-T Easy vector (Promega Co., Madison, WI), constructed by inserting Htk, flanked by ~500-bp upstream and downstream sequences of each gene. The 500-bp DNA fragments from upstream of each gene were amplified by PCR using primer sets 5′-GCTCCCAAAAAGGAGGCCGGCACT-3′ and 5′-CGCGGTCAACGTCTAGAGGAGGAGGCCAGCCTG-3′, 5′-GGGCGTGCTTCCACTCGG-3′ and 5′-CGCCGTCACGTCTAGTGGTGTGGAC-3′, and 5′-CGAGGTCCGGGTTCGGCG-3′ and 5′-CGCCGGTCACGGGTGCTTTGGGAC-3′ for thhb071, thhb070, and uvrC, respectively. The 500-bp DNA fragments from downstream of each gene were amplified by PCR using primer sets 5′-TGTGTTGTCATACGCTTTACATGGGAGACG-3′ and 5′-ACGTTCACGGAGGGCGGGC-3′, 5′-TGTGTTGTCATACGCTTTACATGGGAGACG-3′ and 5′-ACGTTCACGGAGGGCGGGC-3′, respectively.

The thhb071 gene was disrupted as described previously (28). Gene disruptions were confirmed by PCR amplification using the isolated genomic DNAs as templates (supplemental Fig. S2). A T. thermophilus HB8 strain lacking the mutM gene (∆mutM) was generated as described previously (12).

Examination of sensitivity to H2O2—The sensitivity of wild-type, ∆thhb071, ∆thhb070, and ∆mutM strains of T. thermophi-

5 The abbreviations used are: ROS, reactive oxygen species; AP, apurinic/apyrimidinic; EndoIV, endonuclease IV; PLP, pyridoxal phosphate; TPP, thiamine pyrophosphate; ICP-ES, inductively coupled plasma emission spectrometry; TIM, triose-phosphate isomerase.
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*lus* HB8 to H$_2$O$_2$ was examined by a procedure described previously (12). Briefly, the precultured cells were suspended in 3 ml of TR medium and cultured to 1 × 10$^8$ cells/ml. Forty-five microliters of each culture was mixed with 5 μl of 0, 100, 200, 300, 400, or 500 mM H$_2$O$_2$. After incubation at 25 °C for 2 min, 2 μl of each mixture was spotted onto a TR plate, followed by incubation at 70 °C for 8 h.

The sensitivity to H$_2$O$_2$ was also examined by monitoring the growth curve of *T. thermophilus* HB8 in H$_2$O$_2$-containing medium. The wild-type, Δsthb071, and ΔmutM strains of *T. thermophilus* HB8 were grown in 3 ml of TR medium for 16 h. The precultured cells were suspended in 3 ml of TR medium to A$_{600}$ = 0.15. After incubation at 70 °C for 2 h, 30 μl of 0 or 250 mM H$_2$O$_2$ was added. The cell cultures were incubated at 70 °C for 6 h. The growth of the cells was monitored by measuring the A$_{600}$.

**Examination of Sensitivity to Low Temperature**—The precultured wild-type and Δsthb071 strains of *T. thermophilus* HB8 were suspended in 3 ml of TR medium and cultured to 1 × 10$^8$ cells/ml. The 1 × 10$^3$, 5 × 10$^3$, 1 × 10$^4$, and 5 × 10$^4$ cells of each strain were spotted onto a TR plate and incubated at 55 or 45 °C for 48 or 144 h, respectively.

**Examination of Sensitivity to UV Light**—The precultured wild-type, Δsthb071, and ΔuvrC strains of *T. thermophilus* HB8 were suspended in 3 ml of TR medium and cultured to 1 × 10$^8$ cells/ml. Two microliters of each culture was spotted onto a TR plate and irradiated with 254- or 312-nm UV light at a dose rate of 1.9 J m$^{-2}$ s$^{-1}$ for various periods.

**Estimation of the Mutation Frequency under Oxidative Stress**—The mutation frequency of *T. thermophilus* HB8 was estimated based on the frequency of streptomycin-resistant mutants measured by means of the modified Luria-Delbrück fluctuation test (30), as described previously (12). A single amino acid substitution in the streptomycin-binding site of the ribosomal protein S12, or a point mutation in 16 S rRNA, leads to the acquisition of streptomycin resistance (31). The precultured wild-type, Δsthb071, and ΔmutM strains of *T. thermophilus* HB8 were diluted 1:60 with 3 ml of TR medium and then shaken at 70 °C for 2 h. After the addition of 30 μl of 0 or 250 mM H$_2$O$_2$, cells were cultured at 70 °C for 5 h. Then 1 ml of each culture was spread on a plate containing 50 μg/ml streptomycin. The same cultures were diluted 1:10$^5$ with TR medium, and 100 μl of each diluted culture was spread on a drug-free plate. The plates were incubated at 70 °C for 20 h. The frequency of streptomycin-resistant mutants per 10$^8$ cells was calculated from the numbers of colonies formed on the streptomycin-containing and drug-free plates.

**Preparation of Proteins**—The TTHB071 coding sequence was amplified by PCR using *T. thermophilus* HB8 genomic DNA. Amplification was carried out according to standard protocols, and the amplified gene fragments were ligated into a pT7Blue vector (Merck) at the EcoRV site, which was confirmed by sequencing. The fragment with the target gene from pT7Blue-Δsthb071 was ligated into pET-11a (Merck) at the NdeI and BamHI sites. *Escherichia coli* Rosetta2(DE3)pLysS (Merck) cells were transformed with the resulting plasmid and cultured at 37 °C to 4 × 10$^8$ cells/ml in 1.5 liters of LB medium containing 50 μg/ml ampicillin. The cells were then incubated for 6 h in the presence of isopropyl-β-D-thiogalactopyranoside, harvested by centrifugation, and stored at −20 °C.

The following procedures were carried out at room temperature unless stated otherwise. Frozen cells (16 g) were thawed, suspended in 160 ml of buffer I (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, and 1 mM EDTA), and disrupted by sonication on ice. The lysate was incubated at 70 °C for 10 min and centrifuged (38,000 × g) for 60 min at 4 °C. Ammonium sulfate was added to the resultant supernatant to a final concentration of 1.5 m, and the supernatant was loaded onto a TOYOPEARL Ether-650 M (Tosoh, Tokyo, Japan) column (bed volume, 15 ml) that was equilibrated with buffer II (50 mM Tris-HCl (pH 7.5) and 1 mM EDTA) containing 1.5 m ammonium sulfate. Proteins were eluted with a linear gradient of 1.5–0 m ammonium sulfate (total volume, 150 ml). The resultant supernatant was dialyzed against buffer II and loaded onto a TOYOPEARL SuperQ-650 M (Tosoh) column (bed volume, 20 ml) that was equilibrated with buffer II. Proteins were eluted with a linear gradient of 0–1 m NaCl (total volume, 200 ml). Fractions containing TTHB071 were collected and concentrated using a Vivaspin concentrator (molecular weight cut-off, 5,000). The concentrated solution was applied to a Superdex 75 HR 10/30 column (GE Healthcare) that was equilibrated with buffer III (20 mM Tris-HCl (pH 7.5) and 100 mM KCl) and eluted with the same buffer using an AKTA explorer system (GE Healthcare). The fractions containing TTHB071 were concentrated and stored at 4 °C. At each step, the fractions were analyzed by SDS-PAGE. The concentration of the purified protein was determined using the molar absorption coefficient at 278 nm ($ε_{278} = 27,500$ m$^{-1}$ cm$^{-1}$), calculated according to the formula of Kuramitsu et al. (32). Approximately 45 mg of TTHB071 was obtained from 16 g of cells. *T. thermophilus* HB8 EndoIV was prepared as described previously (25).

**Examination of AP Endonuclease and AP Site Binding Activities**—The synthesized oligonucleotide containing an AP site analog (BEX Co.) (′-GGGTGGTGTGGCTTGAATGGCTCAT-3′, where X represents tetrahydrofuran) was 5′-labeled using T4 polynucleotide kinase and [γ-$^{32}$P]ATP and annealed with a complementary strand (′-ATGACAAACTAAGGCAACTACC-3′). The resultant 10 nm double-stranded DNA (dsDNA) was reacted with 1 μM TTHB071 or *T. thermophilus* HB8 EndoIV in 50 mM Tris-HCl, 20 or 100 mM KCl, and metal ions (pH 7.5) at 37 °C for 30 min. The reaction was discontinued by the addition of 2X denaturing dye (50 mM EDTA, 20 mM NaOH, 80% (w/v) deionized formamide, 0.1% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanol). The mixture was then analyzed by 20% (w/v) denaturing PAGE (8 M urea) and autoradiography. For the electrophoretic mobility shift assay, 0.5 or 10 μM TTHB071 or *T. thermophilus* HB8 EndoIV was incubated in 50 mM Tris-HCl, 100 mM KCl, and 10 nm 5′-labeled dsDNA containing an AP site analog (pH 7.5) at 37 °C for 30 min. The reaction was stopped by the addition of 5X native dye (5 mM EDTA, 50% (w/v) glycerol, and 0.05% (w/v) bromophenol blue). The mixture was then analyzed by 8% (w/v) native PAGE and autoradiography.
Crystallization, Data Collection, and Structure Determination—Crystallization of TTHB071 was performed by the hanging drop vapor diffusion method. Drops (1 μl) of 10 mg/ml protein solution were mixed with 1 μl of 0.1 M HEPES (pH 7.5), 0.05 M magnesium chloride hexahydrate, and 30% (v/v) polyethylene glycol monomethyl ether 550 and equilibrated against 0.2 ml of the reservoir solution at 20 °C. For cryoprotection, the crystals were transferred to a crystallization solution containing 22.5% (v/v) ethylene glycol and then flash-frozen in a liquid nitrogen stream (−196 °C). For ZnCl2-soaked crystals, a cryosolution containing 10 mM ZnCl2 was used. Diffraction data were collected on beamline BL26B2 at SPring-8 (Hyogo, Japan). The data were processed by HKL2000 program package (33). The phase was determined by zinc single-wavelength anomalous dispersion using the PHENIX program (34), and structural refinement was carried out using the programs CCP4 suite, Coot, and Refmac5 (35–37). The stereochemistry of the structure was checked using the program PROCHECK (38). Data collection and refinement statistics are shown in Table 1.

Surface Plasmon Resonance (SPR) Analysis—SPR analysis was performed at 25 °C using a Biacore 3000 system (GE Healthcare). TTHB071 was diluted with 10 mM acetate (pH 4.5) to 0.1 mg/ml and immobilized on a CM4 sensor chip by amine coupling, followed by blocking with ethanolamine hydrochloride. More than 10,000 resonance units of TTHB071 were immobilized. Control cells were also blocked by ethanolamine hydrochloride in a similar manner to TTHB071 cells to reduce nonspecific electrostatic binding. The running buffer was composed of 10 mM HEPES-NaOH, 100 mM KCl, and 1 mM EDTA or 0.1 mM ZnCl2 (pH 7.5) at 25 °C and was filtered and degassed. The concentrations of FMN, pyridoxal phosphate (PLP), and thiamine pyrophosphate (TPP) were determined by the molar extinction coefficients of ε268 = 3.14 × 104 (in water), ε295 = 6.7 × 103 (in 0.1 N HCl), and ε323 = 11.2 × 103 M−1 cm−1 (pH 7.35), respectively. Each compound diluted with running buffer was injected over TTHB071 at a flow rate of 30 μl/min. After binding for 6 min, the complexes were dissociated using running buffer without compounds. The dissociation constant (Kd) was calculated by fitting the average resonance unit of the steady state (Req) to the following equation using Igor 4.03 (Wavemetrics),

\[
R_{eq} = R_{max}[S]/([S] + K_d) \tag{Eq. 1}
\]

where \( R_{eq} \) and \( R_{max} \) correspond to [ES] and [E]p, respectively, and [S] is free concentration of the compounds. \( R_{eq} \) value of each binding curve was determined by fitting the curve to the following equation using Igor 4.03,

\[
R_t = R_{eq} - A(e^{-kt}) \tag{Eq. 2}
\]

where \( R_t \), \( A \), and \( k \) are response units at each time point, an amplitude constant, and the binding rate constant of the first-order reaction, respectively.

Inductively coupled plasma emission spectrometry (ICP-ES)—The sample buffer and 4 μM TTHB071 were analyzed by the CIROS CCD ICP-ES instrument (Rigaku, Tokyo, Japan) in a buffer containing 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl. Solutions of Zn(NO₃)₂ (Wako) (0, 5, 10, 25, 100, and 500 μg/liter) in 0.1 M HCO₃ and iron atomic absorption standard solution (Sigma-Aldrich) (0, 5, 10, 25, 100, and 500 μg/liter in 1% (v/v) HCl) were used to construct the calibration curves for estimating the zinc and ferric ion concentrations in the sample solutions.

Examination of Xylose Isomerase Activity—The substrate 10 mM D-xylose (Wako) was reacted with 0 or 1 mg/ml TTHB071 in 50 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl in the presence of 5 mM CoCl₂, MgCl₂, MnCl₂, or 1 mM ZnCl₂ at 37 °C for 16 h or 55 °C for 6 h. As a positive control, 10 mM D-xylose was also incubated with 0 or 1 mg/ml xylose isomerase A (Thermostable Enzyme Laboratory Co., Kobe, Japan) in 50 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl and 5 mM CoCl₂ at 70 °C for 15 min. The generated D-xylulose was detected by the cysteine/carbazole/sulfuric acid method (39).

Docking Simulation—The binding energies were calculated by the AutoDock Vina program (40). The hydrogen-containing model structure file of TTHB071 chain D and the PDBQT molecular structure file of the ligands were prepared by AutoDock tools. The area used for calculation of docking is as follows: center_x = 50, center_y = 2.5, center_z = 77, size_x = 30, size_y = 50, and size_z = 30 Å, respectively, for chain D of TTHB071 (Protein Data Bank code 3AYV).

RESULTS

Up-regulation of tthb071 in the mutS2-lacking Strain (ΔmutS2) of T. thermophilus HB8—Our previous transcriptional study suggested that the expression level of tthb071 in ΔmutS2 was up-regulated 2.6-fold compared with the wild type (12). In order to confirm the up-regulation of tthb071 in ΔmutS2, we performed reverse transcription-PCR. As shown in Fig. 1A, we confirmed a significant increase in the mRNA level of tthb071 in ΔmutS2. Previous studies demonstrated the involvement of mutS2 in the repair of oxidative DNA damage (12, 23). In addition, the amino acid sequence of TTHB071 shows a significant similarity to those of AP endonucleases that are generally required for the repair of oxidatively damaged DNA. Therefore, the mutS2 disruption-dependent up-regulation of tthb071 led us to hypothesize that tthb071 is involved in the protection mechanisms against oxidative stress.

Disruption of tthb071 Caused an Increase in H₂O₂ Sensitivity—To investigate whether tthb071 is associated with the mechanisms for protection against oxidative stress, we generated a tthb071-lacking strain of T. thermophilus HB8 (Δtthb071) (supplemental Fig. S2) and examined its sensitivity to H₂O₂-induced oxidative stress. The wild type and Δtthb071 were incubated with various concentrations of H₂O₂ and spotted onto a plate. As a positive control, a strain lacking mutM (ΔmutM), which encodes a base excision repair glycosylase, was also subjected to the same experiment. Fig. 1B shows the significantly increased H₂O₂ sensitivity of Δtthb071 compared with the wild type. Although the wild type was viable even when mixed with 30 mM H₂O₂, Δtthb071 hardly grew under the condition of 10 mM H₂O₂. The increased H₂O₂ sensitivity of Δtthb071 was further confirmed by monitoring its growth curve under H₂O₂-induced stress. As shown in Fig. 1C, the addition of 2.5 mM H₂O₂ resulted in a decrease in the growth

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It was previously reported that expression of tthb071 was dramatically up-regulated when wild-type *T. thermophilus* HB8 was subjected to a rapid temperature downshift (from 70 to 45 °C) (41). In our oxidative stress experiments, we could not exclude the possibility that cells were exposed to such a cold shock during experimental manipulations. Therefore, we examined whether disruption of *tthb071* has any effect on growth under low temperature conditions. Our results showed that disruption of *tthb071* caused no growth delay of *T. thermophilus* HB8 at 45 and 55 °C (supplemental Fig. S3). Thus, our oxidative stress experiments would accurately address the effect of *tthb071* disruption on the sensitivity of *T. thermophilus* HB8 to *H₂O₂*-induced oxidative stress.

On the megaplasmid pTT27 of *T. thermophilus* HB8, *tthb071* is located directly upstream of *ttb070*, which encodes SurE protein, a 5'-nucleotidase (42). To some extent, therefore, the disruption of *tthb071* might influence the expression of *ttb070*. In order to rule out the possibility that the observed effect of *tthb071* disruption on *H₂O₂* sensitivity was caused by the perturbation of expression of *ttb070*, we examined the *H₂O₂* sensitivity of Δ*tthb070*. The results clearly showed that disruption of *ttb070* had no effect on the tolerance of *T. thermophilus* HB8 to *H₂O₂* (supplemental Fig. S4). These findings strongly indicate that the loss of the function of *tthb071* increases the *H₂O₂* sensitivity of *T. thermophilus* HB8.

We also tested the tolerance of Δ*tthb071* to a different type of DNA-damaging stress (i.e. UV light irradiation). Wild type, Δ*tthb071*, and Δ*uvc* were spotted onto a plate and irradiated with 254- or 312-nm UV light for various periods. The *uvc* gene product is known to function as a nucleotide excision repair endonuclease, which is essential for the repair of UV light-induced DNA damage. As shown in Fig. 1D, Δ*uvc* showed remarkable sensitivity to 254- and 312-nm UV light irradiation, indicating that a significant amount of DNA damage was generated during the UV light irradiation. However, the tolerance of Δ*tthb071* to 254- and 312-nm UV light irradiation was quite similar to that of the wild type (Fig. 1D), suggesting that *tthb071* does not play a major role in the repair of UV light-induced DNA damage.

The results of our in vivo experiments demonstrated that Δ*tthb071* shows sensitivity not to a broad range of stresses but specifically to oxidative stress. Considering the sequence similarity of TTHB071 to other AP endonucleases, we anticipated that TTHB071 possesses an AP endonuclease activity, which is utilized for the repair of oxidative stress-induced DNA damage.

**FIGURE 1. Effect of tthb071 disruption on the tolerance of *T. thermophilus* HB8 to *H₂O₂*.** A, reverse transcription-PCR confirmed the up-regulation of *tthb071* in ΔmutS2. DNA fragments in the *tthb071* gene were amplified using total RNA as templates and then subjected to agarose gel electrophoresis. *M*, wild type, and ΔS2, 100-bp ladder DNA size marker, wild type, and ΔmutS2, respectively. B, wild type and Δ*tthb071* were incubated in medium containing the indicated concentrations of *H₂O₂*, and then cells were spotted onto plates and incubated at 70 °C for 8 h. C, growth curves of wild type (red) and Δ*tthb071* (blue). Precultured cells were inoculated to 3 ml of medium to an *A₅₆₀* value of 0.15. After incubation at 70 °C for 2 h, *H₂O₂* was added to a final concentration of 0 (circles) or 2.5 mM (triangles). Experiments were performed in triplicate. Error bars, S.D. D, UV light tolerance. Wild type, Δ*uvc*, and Δ*tthb071* were spotted onto plates and irradiated with 312-nm (top) or 254-nm (bottom) UV light for the indicated periods. After irradiation, plates were incubated at 70 °C for 8 h.
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In order further to test whether TTHB071 participates in DNA repair, we examined the effect of tthb071 disruption on the frequency of H$_2$O$_2$-induced mutagenesis. The mutation frequency was estimated based on the frequency of streptomycin-resistant mutants. It is known that streptomycin resistance-based measurement of mutation frequency detects the frequency of single-base substitutions, including AT-CG transversion and AT-GC transition mutations (43). These can be generated by certain types of oxidative DNA damage, such as 8-oxoguanine and 5-formyluracil. As a result, $\Delta tthb071$ showed no significant increase in mutation frequency under the oxidative stress caused by 2.5 mM H$_2$O$_2$, whereas $\Delta$mutM exhibited a remarkably increased mutation frequency under the same condition (Fig. 2C). Thus, our in vitro and in vivo experiments suggested that tthb071 does not participate in DNA repair, at least for mutagenic oxidative DNA damage.

**TTHB071 Exhibited No Xylose Isomerase Activity**—Because the amino acid sequence of TTHB071 can be classified into the superfamily that includes xylose isomerases (supplemental Fig. S1B), we tested the xylose isomerase activity of TTHB071. The substrate D-xylose was incubated with TTHB071 or a positive control enzyme (xylose isomerase A), and then, the product, D-xylulose, was detected by cysteine/carbazole/sulfuric acid method. Xylose isomerases generally require a divalent cation to exert their effective activities; therefore, the reactions were performed in the presence of a range of divalent cations. When D-xylose was reacted with xylose isomerase A, the absorption spectrum with a maximum at 540 nm was observed, which represents the production of D-xylulose (supplemental Fig. S5). On the other hand, no spectral change was observed when D-xylose was reacted with TTHB071 (supplemental Fig. S5), indicating the lack of xylose isomerase activity in TTHB071.

**Presence of a Phosphatase-like Trinuclear Zinc Center in the Structure of TTHB071**—As indicated above, our primary structure-based prediction failed to identify the molecular function of TTHB071. To obtain more precise clues to the molecular function of TTHB071, we determined the three-dimensional structure of TTHB071 at 1.85 Å by x-ray crystallographic analysis (Table 1 and Fig. 3). Because the molecular function of a protein is fundamentally based on its tertiary structure, determination of the three-dimensional structure often contributes to prediction of the molecular function of a hypothetical protein (44, 45). The structure analysis in the Whole Cell Project on *T. thermophilus* HB8 has revealed that tertiary structure determinations have a potential to give us clues to characterize ~60% of the function-unknown proteins (see the Structural-Biological Whole Cell Web site).

As expected from the primary sequence analysis, TTHB071 has an eight-stranded $\alpha/\beta$-barrel (TIM barrel) structure, without any additional domain. The overall structure of TTHB071 was highly homologous to that of EndoIV (Fig. 3A). Importantly, TTHB071 contains a trinuclear zinc center with 9 coordinated residues in the possible active site (Fig. 3, B and C, and supplemental Fig. S6). These zinc ions are also found in the native crystal (Table 1). Moreover, ICP-ES revealed that purified TTHB071 contained zinc ions (Table 2). These results suggested that the zinc ions observed in the crystal are derived from the expression host, *E. coli*. It has been reported that the trinuclear metal center forms the active sites for the phosphomonoesterase and phosphodiesterase activities of EndoIV, UV damage endonuclease, PHP domains of DNA polymerases, nuclease P1, and phospholipase C (46–50). The zinc-binding manner of TTHB071 is particularly similar to that of EndoIV, which coordinates three zinc ions by using 5 histidine, 2 glutamate/asparagine, and 2 aspartate residues (Fig. 3, C and D, and supplemental Fig. S1A). This finding indicates that TTHB071 also possesses a phosphoesterase activity. However, the arginine residue (Arg-37 in *E. coli* EndoIV), which is important for...
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TABLE 1
Data collection and refinement statistics

| Parameter                        | Native        | Zn\(^{2+}\)-soaked | Zn\(^{2+}\)-soaked (SAD)* |
|----------------------------------|---------------|---------------------|---------------------------|
| **Data collection**              |               |                     |                           |
| Space group                      | P2\(_1\)      | P2\(_1\)            | P2\(_1\)                   |
| Cell dimensions                  |               |                     |                           |
| a, b, c (Å)                      | 66.1, 92.6, 85.8 | 67.1, 93.4, 87.4  | 66.6, 93.3, 87.4           |
| α, β, γ (degrees)                | 90.0, 96.5, 90.0 | 90.0, 95.9, 90.0   | 90.0, 95.8, 90.0           |
| Wavelength                       | 1.00          | 1.282               |                           |
| Resolution (Å)                   | 50.1-1.95 (1.98-1.95) | 50-1.85 (1.88-1.85) | 50-3.15 (3.15-3.10)       |
| Rmerge                          | 0.046 (0.320) | 0.055 (0.253)      | 0.096 (0.271)              |
| I/σ(I)                           | 26.5 (3.7)    | 49.4 (11.8)         | 21.9 (7.4)                 |
| Completeness (%)                 | 99.1 (96.8)   | 99.6 (98.9)         | 99.7 (99.9)                |
| Redundancy                       | 3.7 (3.4)     | 14.8 (14.1)         | 7.8 (7.8)                  |
| **Refinement**                   |               |                     |                           |
| Resolution (Å)                   | 50.0-1.95     | 50.0-1.85           |                           |
| No. of reflections               | 74,034        | 86,357              |                           |
| Rwork/Rfree                      | 0.204/0.234   | 0.172/0.205         |                           |
| No. of atoms                     |               |                     |                           |
| Protein                          | 7,553         | 7,617               |                           |
| Ligand/Ion                       | 12            | 12                  |                           |
| Water                            | 425           | 779                 |                           |
| B-Factors                        |               |                     |                           |
| Protein                          | 26.7          | 19.4                |                           |
| Ligand/Ion                       | 79.6          | 65.4                |                           |
| Water                            | 31.0          | 29.5                |                           |
| r.m.s. deviations\(^\dagger\)   |               |                     |                           |
| Bond lengths (Å)                 | 0.007         | 0.013               |                           |
| Bond angles (degrees)            | 1.05          | 1.37                |                           |
| Ramachandran plot                |               |                     |                           |
| Most favored (%)                 | 90.6          | 91.1                |                           |
| Generously allowed (%)           | 0.1           | 0.1                 |                           |
| Additional allowed (%)           | 0             | 0                   |                           |
| Protein Data Bank code           | 3AYT          | 3AYV                |                           |

\(^a\) Single-wavelength anomalous dispersion.
\(^b\) One crystal was used for each data set. Values in parentheses are for the highest resolution shell.
\(^c\) Root mean square deviations.

AP site recognition in EndoIV (51), is not found in TTHB071 (Fig. 3B). Instead, in TTHB071, several aromatic residues (including Tyr-29, Phe-58, and Trp-59) are located over the trinuclear zinc center. These residues form a hydrophobic protrusion, which is not found in E. coli EndoIV (Fig. 3, E and F, and supplemental Fig. S1A). These aromatic residues may be stacked with a ring structure of the substrate or have other roles. The structural comparison between TTHB071 and E. coli EndoIV suggested that the substrate for TTHB071 is not an AP site but rather another compound carrying a phosphate and a ring structure.

**Screening of the Substrate for TTHB071**—Based on the results of our structural analysis, we used SPR to measure the binding activity of TTHB071 for a range of compounds containing a phosphate and a ring structure. We tested ADP, ATP, NTP, dNTP, NAD\(^+\), NADH, NADP\(^+\), NADPH, PLP, co-enzyme A (CoA), FMN, FAD, TPP, and riboflavin in the running buffer with 1 mM EDTA or 0.1 mM ZnCl\(_2\). We detected the binding responses for TPP, FMN, CoA, PLP, NAD, NADP, and ATP with 1 mM EDTA, and for TPP, FMN, FAD, CoA, PLP, NAD, and NADP with 0.1 mM ZnCl\(_2\). Among them, binding responses of TTHB071 for FMN, TPP, and PLP were significant. On the other hand, the binding responses for the remaining compounds exhibited obscure concentration dependence and could not be fitted by a theoretical curve, indicating that they did not reflect physiologically meaningful binding. Then we determined the dissociation constants for FMN and TPP in the presence of 1 mM EDTA and for FMN and PLP in the presence of 0.1 mM ZnCl\(_2\) (Fig. 4 and Table 3). The maximum tested concentration of FMN in the presence of 1 mM EDTA was 40 mM because of sensorgram disorders. In the presence of 0.1 mM ZnCl\(_2\), concentrations of PLP higher than 1 mM increased the binding response once the plateau had been reached. This increased response was thought to represent nonspecific binding of PLP to basic residues of a protein; therefore, responses detected below 1 mM PLP were used to determine the \(K_d\) value. The relatively high \(K_d\) value for FMN in the presence of EDTA was dramatically decreased in the presence of ZnCl\(_2\). The same tendency was also observed for the binding of PLP.

Such zinc ion-mediated binding of FMN and PLP is consistent with the structural features of the possible active site in TTHB071, where the terminal phosphate of FMN or PLP could be trapped by the trinuclear zinc center. This notion is supported by a docking simulation performed by AutoDock Vina (40) (Fig. 5). In the generated model structure of FMN-TTHB071 and PLP-TTHB071 complexes, FMN and PLP are located at the possible active site pocket, and the terminal phosphates are trapped by the three zinc ions (Fig. 5, A and B). However, the phosphates of TPP were not trapped by zinc ions in the obtained docking model (Fig. 5C).

We subsequently examined the phosphoesterase activity of TTHB071 by HPLC, using a MonoQ 5/50 GL (GE Healthcare) and KCl gradient. This form of anion exchange chromatography can be used to separate compounds according to the numbers of phosphate groups. We observed that TTHB071 exhibited no hydrolase activity for FMN, TPP, PLP, NAD, NADP, AMP, dAMP, GMP, and dGMP in the presence of magnesium, manganese, or zinc ions (data not shown). The lack of phospha-
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Superposition was performed by the DaliLite program (59). The posed on chain D of TTHB071 (Protein Data Bank code 3AYV). The structure mutant complexed with DNA containing an AP site analog (51) was super-

Mean square deviation, and sequence identity were 19.6, 2.8 Å, and 18%, respectively.

FIGURE 3. Structural comparison between TTHB071 and E. coli EndoIV (Protein Data Bank code 2NQJ). A, the comparison of overall structures of TTHB071 (orange) and E. coli EndoIV (dark blue). The E. coli EndoIV E261Q mutant complexed with DNA containing an AP site analog (51) was superposed on chain D of TTHB071 (Protein Data Bank code 3AYV). The structure superposition was performed by the DaliLite program (59). The Z-score, root mean square deviation, and sequence identity were 19.6, 2.8 Å, and 18%, respectively. Orange and dark blue spheres represent zinc ions from TTHB071 and E. coli EndoIV, respectively. B, active site comparison of TTHB071 and E. coli EndoIV. Three zinc ions of the two structures were superposed using the PyMOL Pair Fitting program. The conserved 9 residues coordinating three zinc ions in TTHB071 (Fig. 3C) are indicated by orange letters. Arg-37 and Tyr-72 required for AP site binding and the endonuclease activity of E. coli EndoIV (51) are indicated by dark blue letters. C, 9 residues coordinating three zinc ions in TTHB071. The 2Fα − Fc electron density map around active site residues is shown as a gray mesh contoured at 2 σ. D, residues coordinating three zinc ions in E. coli EndoIV. Note that Glu-261 was mutated to Gln-261. E, electrostatic potential surface of TTHB071. PyMOL plugin APBS (60) was used for the calculation of electrostatic potential. The positions of aromatic hydrophobic residues in B are indicated by a white dashed circle. The gray spheres represent zinc ions. F, electrostatic potential surface model of E. coli EndoIV. The DNA containing an AP site analog is shown as a semitransparency model. The Arg-37 and Tyr-72 are labeled as in B.

tase activity against FMN and PLP, despite the fact that these were bound by TTHB071 in a zinc ion-mediated manner, was surprising.

We identified a characteristic, negatively charged, long narrow cleft around the zinc-binding site of TTHB071 (Fig. 3E). If this cleft was the substrate-binding pocket, a relatively long compound would be preferably caught. In agreement with this, docking simulation by AutoDock predicted plausible binding of diadenosine tetraphosphate (Ap₄A) to this site (Fig. 3D). Nonetheless, no hydrolyzing activity of TTHB071 against Ap₄A or its analogous compounds (Ap₃A, p₄A, Ap₅A, Gp₃G, Gp₄G, and Gp₅G) was observed (data not shown).

DISCUSSION

The major purpose of this study was to determine the cellular and molecular functions of a conserved uncharacterized protein, TTHB071. In vivo experiments indicated that the cellular function of TTHB071 is related to a mechanism to protect cells from oxidative stress. In order to determine the molecular function of TTHB071, we performed a variety of in vitro experiments and also x-ray crystallographic analysis. ICP-ES revealed that purified TTHB071 retains zinc ions, although they were purified using the buffer containing 1 mM EDTA (Table 2), indicating that TTHB071 tightly binds zinc ions in the cell. X-ray crystallographic analysis revealed that the zinc ion-binding manner of TTHB071 is almost identical to that of DNA repair endonucleases, such as EndoIV. EndoIV utilizes three zinc ions to hydrolyze a phosphodiester bond at AP sites. The similarity in zinc-binding fashion implies that TTHB071 also possesses a phosphoesterase activity (Fig. 3, C and D). We clarified the zinc ion-mediated binding ability of TTHB071 to FMN and PLP (Fig. 4 and Table 3). The determined Kₐ values of TTHB071 for FMN and PLP are comparable with the intracellular concentration of FMN (∼90 μM) (52) and the Kₐ value of PLP phosphatases (53, 54), implying that TTHB071 can bind these in the cell. On the basis of these results, we discuss the possible molecular functions of TTHB071.

The first possibility is that TTHB071 is an enzyme that requires FMN, PLP, or an analogous compound as a cofactor. For example, a variety of FMN-dependent oxidoreductases with a TIM barrel fold, such as yeast old yellow enzyme (55, 56), are involved in reduction of oxidized molecules. However, this may be unlikely for the following reason. When FMN and PLP are involved in reduction of oxidized molecules. It is well known that FMN and FAD are essential for various types of oxidoreductases, such as glutathi-

| Metal       | Ratio⁷ |
|-------------|--------|
| Zinc        | 0.043 ± 0.0063 |
| Iron        | 0.028 ± 0.0075 |
| Magnesium   | ND⁺ |
| Nickel      | ND |
| Cobalt      | ND |
| Manganese   | ND |

⁷ The molar ratio of metal per protein is shown. The values represent means of three independently performed experiments ± S.D. 

ND, not determined.
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one reductases and thioredoxin reductases, and that PLP plays a central role in scavenging singlet oxygen. Perturbation of FMN, FAD, or PLP production would result in the dysfunction of cellular responses to oxidative stress. In addition, a phosphatase activity is known to be required for riboflavin and PLP biosynthesis.

The third possibility is that TTHB071 degrades excess reductants to prevent Fenton reaction-mediated generation of ROS. In mammalian mitochondria, FMN phosphatase and FAD pyrophosphatase activities are thought to be involved in this function (22). This seems consistent with our findings that the structure of TTHB071 is similar to that of zinc-ion-dependent phosphatase and that the detected binding activity to FMN and PLP was also zinc ion-mediated. TTHB071 may protect the cell from oxidative stress by controlling the intracellular amounts of FMN, PLP, or analogous reductants. Nonetheless, we were unable to detect the phosphatase activity against FMN and PLP. There may be a regulatory mechanism for modulating the phosphatase activity of TTHB071 to prevent unrestrained degradation in the cell. The tthb070 locus is located directly downstream of tthb071, and several transcriptomic analyses have indicated that the two loci are transcribed polycistrionically (12,

![Figure 4: SPR analysis of the binding affinity of TTHB071 to some compounds.](image)

**FIGURE 4. SPR analysis of the binding affinity of TTHB071 to some compounds.** SPR sensorgrams of the binding of TTHB071 to FMN (A and C), TPP (B), and PLP (D) are shown. Running buffer containing 1 mM EDTA (A and B) or 0.1 mM ZnCl₂ (C and D) was used. The determined equilibrium points of resonance unit (RU) were plotted against the concentrations of the compounds and fitted using Equation 1. The theoretical curve fitted with each binding curve using Equation 2 is given in supplemental Fig. S7.

**TABLE 3**
The dissociation constants ($K_d$) of TTHB071 and compounds

| Compound | $K_d$ (1 mM EDTA) | $K_d$ (0.1 mM ZnCl₂) |
|----------|------------------|----------------------|
| FMN      | 37 ± 0.11        | 0.18 ± 0.022         |
| PLP      | ND*             | 0.10 ± 0.025         |
| TPP      | 5.4 ± 0.64       | ND*                  |

*ND, not determined. Exhibited obscure concentration dependence and could not be fitted by a theoretical curve.

![Figure 5: Docking simulation of phosphate-containing molecules and TTHB071.](image)

**FIGURE 5. Docking simulation of phosphate-containing molecules and TTHB071.** The binding energies of TTHB071 were calculated with FMN (A), PLP (B), TPP (C), and Ap₄A (D) by the AutoDock Vina program (40). The program suggested nine candidate docking models for each compound. The models in which phosphate of the substrate was bound by zinc ions are shown, except for that of TTHB071-TPP. The calculated binding energies are as follows: FMN (eighth candidate), −7.4 kcal/mol; PLP (second candidate), −9.8 kcal/mol; TPP (first candidate), −7.5 kcal/mol; and Ap₄A (first candidate), −9.8 kcal/mol.
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It may be possible that TTHB070 has a promoting effect on the phosphatase activity of TTHB071.

Finally, it is also possible that TTHB071 is involved in the metabolism pathway for a compound other than FMN and PLP. The local structural feature around the trinuclear zinc-binding site suggested that phosphate compounds containing aromatic ring structures are candidate substrates for TTHB071. Although we tested a wide range of major intracellular compounds containing a ring structure and a phosphate, there will still be the candidates that can be found in the cell. As mentioned earlier, the existence of a narrow cleft around the zinc-binding site implies the binding of a long, phosphate-containing compound as the substrate. The docking simulation predicted a favorable binding of Ap4A to the cleft near the zinc-binding site (Fig. 5D). In this case, the protruding hydrophobic residues over the zinc-binding site seem to cover the active site and phosphates of the substrate. These residues may retain the hydrophobic environment of the active site like Tyr-29 of E. coli EndoV (51).Because the lack of a phosphatase activity against Ap4A causes an increase in H2O2 sensitivity in E. coli (58), Ap4A or its analogous compounds may be a good candidate substrate for TTHB071. Although we observed no hydrolyzing activity of TTHB071 against Ap4A or its analogous compounds (data not shown), we cannot exclude the possibility that the activity of TTHB071 is enhanced when it is required in the cell.

In summary, on the basis of the structural analysis, we proposed that TTHB071 possesses a phosphoesterase activity that is dependent on the trinuclear zinc center. Although SPR experiments indicated that FMN, PLP, or an analogous compound present the possible substrate for TTHB071, the long cleft around the zinc-binding site raises the possibility that another compound, which is a relatively long or stretched molecule, is the true substrate. Given that Δthtb071 showed a high sensitivity to oxidative stress, substrate identification of TTHB071 is crucial to understanding how cells avoid oxidative damage.

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