MazG proteins form a widely conserved family among bacteria, but their cellular function is still unknown. Here we report that Thermotoga maritima MazG protein (Tm-MazG), the product of the TM0913 gene, has both nucleoside triphosphate pyrophosphohydrolase (NTPase) and pyrophosphatase activities. Tm-MazG catalyzes the hydrolysis of all eight canonical ribo- and deoxyribonucleoside triphosphates to their corresponding nucleoside monophosphates and PP\(_i\) and subsequently hydrolyzes the resultant PP\(_i\) to P\(_i\). The NTPase activity with deoxyribonucleoside triphosphates as substrate is higher than corresponding ribonucleoside triphosphates. dGTP is the best substrate among the deoxyribonucleoside triphosphates, and GTP is the best among the ribonucleoside triphosphates. Both NTPase and pyrophosphatase activities were enhanced at higher temperatures and blocked by the α,β-methyleneadenosine triphosphate, which cannot be hydrolyzed by Tm-MazG. Furthermore, PP\(_i\) is an inhibitor for the Tm-MazG NTPase activity. Significant decreases in the NTPase activity and concomitant increases in the pyrophosphatase activity were observed when mutations were introduced at the highly conserved amino acid residues in Tm-MazG N-terminal region (E41Q/E42Q, E45Q, E61Q, R97A/R98A, and K118A). These results demonstrated that Tm-MazG has dual enzymatic functions, NTPase and pyrophosphatase, and that these two enzymatic activities are coordinated.

The members of the MazG protein family are categorized by the homology to Escherichia coli MazG protein. MazG proteins are highly conserved among bacteria and considered to be typical prokaryotic proteins. Although the cellular function of this protein family is still unknown, we have demonstrated that the carboxyl-terminal region of MazG interacts with Era (E. coli Ras-like protein), an essential GTPase in E. coli, and identified E. coli MazG as a nucleoside triphosphate pyrophosphohydrolase (NTPase),\(^1\) which can convert (d)NTP to (d)NMP and PP\(_i\).\(^1\)

There are a few NTPases known in bacteria. The genomic DNA of Thermotoga maritima has been sequenced (\(^2\)) and its analysis using BLAST search reveals that the TM0913 gene encodes the MazG homolog in T. maritima. In this paper, we demonstrated that the MazG protein from T. maritima (Tm-MazG), unlike E. coli MazG, has not only the NTPase activity but also the pyrophosphatase activity, converting (d)NTP to (d)NMP and PP\(_i\), and subsequently hydrolyzing the resultant PP\(_i\) to P\(_i\). By the site-directed mutagenesis, the amino acid residues involved in both enzymatic activities were identified. We demonstrated that Tm-MazG has dual enzymatic functions, NTPase and pyrophosphatase, and that these activities are coordinated probably by having partially overlapping active sites.

**EXPERIMENTAL PROCEDURES**

**Reagents and Enzyme**—Restriction enzymes and DNA-modifying enzymes used for cloning were from New England Biolabs. F\(_7\)DNA polymerase was from Stratagene. Radioactive nucleotides were from Amer sham Biosciences. Nucleoside triphosphates and AMPCPP were purchased from Sigma. Pyrophosphate was provided from the PiPer

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\(^{1}\) The abbreviations used are: NTPase, nucleoside triphosphate pyrophosphohydrolase; Tm-MazG, T. maritima MazG protein; AMPCPP, α,β-methyleneadenosine triphosphate; DTT, dithiothreitol.

\(^{2}\) Lack of the mutT gene increases the spontaneous mutation frequencies from 100- to 10,000-fold over the wild-type level (3–5). An oxidized form of dGTP, 8-oxo-dGTP, is a potent mutagenic substrate for DNA synthesis to induce AT to C-G transversion. 8-Oxo-dGTP can be hydrolyzed and eliminated from the nucleotide pool by MutT protein to prevent misincorporation of 8-oxo-dGTP into DNA (6). Genes for MutT homolog proteins have been identified in Proteus vulgaris and Streptococcus pneumoniae (7, 8). Enzymatic activity similar to that of MutT protein has also been detected in mammalian tissues (9), and the genes for 8-oxo-dGTPase have been identified in humans, mice, and rats by cDNA cloning (10–12). Among the MutT homologs, there is a small conserved region that is involved in the NTPase activity as well as the antimitator activity (13, 14), known as the MutT signature (15). There are other proteins with the MutT signature, such as the protein encoded by E. coli orf17 gene, which has a preference for dATP and is not involved in antimitogenic activity (16). The deletion of the mazG gene did not result in a mutator phenotype in E. coli, suggesting that MazG is not associated with antimitogenic activity (1). Mj0226 from Methanococcus jannaschii efficiently hydrolyzes xanthosine 5′-triposphate to xanthosine 5′-monophosphate and ITP but not the canonical standard nucleotides (17, 18). The inosine triphosphate pyrophosphohydrolase activity has also been identified in human erythrocyes (19). The human inosine triphosphate pyrophosphohydrolase gene has been cloned and named as hITPase, which encodes a protein homologous to the M. jannaschii Mj0226 protein (20). The function of this protein family has been proposed to eliminate minor potentially mutagenic purine nucleoside triphosphates from cell.

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pyrophosphate assay kit purchased from Molecular Probes, Inc. (Eugene, OR).

Strains and Plasmids—The genomic DNA of _T. maritima_ was used as template for PCR to amplify the TM0591 gene with primer 5'-GAATTCATTAGCAAGGCCAGGAACATCTCCCTC-3' (an NdeI site is underlined) and primer 5'-CCCAAGCTTTATGTTCACTCCTCCTC-3' (a HindIII site is underlined). The PCR product was digested with NdeI and HindIII and cloned into the NdeI-HindIII site of pET17b. This plasmid was designated as pET17b-Tm-MazG. pET17b-Tm-MazG was introduced into _E. coli_ BL21(DE3) strain for protein expression and purification.

Protein Expression and Purification—The _E. coli_ BL21(DE3) cells harboring the pET17b-Tm-MazG were grown to midexponential phase in M9 medium supplemented with 0.2% casamino acid and 50 μg of ampicillin/ml, and then the expression of Tm-MazG was induced in the presence of 1 mM isopropyl-β-thiogalactopyranoside for 4 h. The cells were harvested by centrifugation and resuspended in buffer A (100 mM potassium phosphate buffer, pH 6.0, 10 mM β-mercaptoethanol) and then lysed through a French press followed by centrifugation at 8,000 × _g_ for 10 min to remove cell debris and unbroken cells and by ultracentrifugation at 100,000 × _g_ for 1 h to remove membrane and insoluble fractions. The supernatant was treated at 80 °C for 15 min and then centrifuged at 120,000 × _g_ for 15 min to remove denatured _E. coli_ proteins. The resulting supernatant was loaded on a Q-Sepharose column and eluted with buffer A using a gradient of 0.1–1 M potassium phosphate. Fractions containing Tm-MazG were pooled together and dialyzed with buffer B (10 mM potassium phosphate buffer, pH 6.0, and 10 mM β-mercaptoethanol). The protein sample was then loaded on to a hydroxyapatite column (Bio-Rad), which had been equilibrated with buffer B. Tm-MazG was eluted with buffer B using a gradient of 0.01–1 M potassium phosphate. The fractions containing purified Tm-MazG were pooled together and concentrated. Protein concentrations were measured with the Bio-Rad protein assay dye reagent. The Tm-MazG mutants indicated in Fig. 7 were constructed by site-directed mutagenesis. All of the Tm-MazG mutant proteins were purified with the same protocol as mentioned above.

Enzyme Assay—With α-[^32]P-labeled nucleoside triphosphates as substrates, the NTPase activity of Tm-MazG was assayed by measuring the hydrolyzed products from the α-[^32]P-labeled nucleoside triphosphates by TLC. The assay was carried out in 20 μl of reaction buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, and 0.5 μg of Tm-MazG protein at 70°C for 10 min. Kinetic constants were determined with eight different concentrations of each substrate in the range from 0.1 to 2.0 mM. The figures are the mean values from three independent experiments.

### TABLE I

**Comparison of the nucleotide substrate specificity of Tm-MazG**

The NTPase assays were performed in a 20-μl reaction mixture, containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, and 0.5 μg of Tm-MazG protein at 70°C for 10 min. Kinetic constants were determined with eight different concentrations of each substrate in the range from 0.1 to 2.0 mM. The figures are the mean values from three independent experiments.

| Substrate | _K_<sub>m</sub> (μM) | _V_<sub>max</sub> (nmol/min/μg) | _k_<sub>cat</sub> (min<sup>-1</sup>) | _k_<sub>cat</sub>/_K_<sub>m</sub> (min<sup>-1</sup> μM<sup>-1</sup>) | Relative _k_<sub>cat</sub>/_K_<sub>m</sub> |
|-----------|-------------------|-------------------------------|-------------------------------|---------------------------------|------------------|
| dGTP      | 1.0 ± 0.3         | 1.90 ± 0.12                   | 56.6 ± 3.5                    | 56.0                            | 100              |
| dCTP      | 1.7 ± 0.1         | 2.00 ± 0.74                   | 59.6 ± 2.1                    | 34.3                            | 61               |
| dTTP      | 1.5 ± 0.4         | 1.30 ± 0.56                   | 38.7 ± 6.7                    | 26.5                            | 47               |
| dATP      | 3.2 ± 0.8         | 2.25 ± 0.96                   | 67.2 ± 8.0                    | 20.9                            | 37               |
| GTP       | 1.0 ± 0.1         | 1.39 ± 0.11                   | 41.4 ± 3.2                    | 40.6                            | 75               |
| CTP       | 0.4 ± 0.1         | 0.22 ± 0.03                   | 6.4 ± 1.0                     | 17.9                            | 32               |
| ATP       | 1.0 ± 0.3         | 0.39 ± 0.16                   | 11.82 ± 4.7                   | 11.9                            | 21               |
| UTP       | 1.1 ± 0.2         | 0.36 ± 0.05                   | 10.6 ± 1.6                    | 9.8                             | 18               |

FIG. 1. **Nucleoside triphosphate pyrophosphohydrolase activity of Tm-MazG.** A, conversion of ATP, dATP, GTP, and dGTP to their corresponding monophosphates by Tm-MazG. Reactions were performed as described under “Experimental Procedures” with [α-[^32]P]ATP (lane 4), [α-[^32]P]GTP (lane 6), or [α-[^32]P]dGTP (lane 8) as substrate. Lanes 1, 3, 5, and 7 are the controls of each reaction without Tm-MazG. The hydrolysis products were assayed by polyethyleneimine-cellulose thin layer chromatography. B, the GTP hydrolysis activity of Tm-MazG in the presence of nucleotide competitors. The GTP hydrolysis assays were carried out in a 20-μl reaction mixture with 10 μM GTP, 10 μCi of [α-[^32]P]GTP, and 0.5 μg of Tm-MazG at 70°C for 10 min in the presence of 4 mM nucleotide competitor. The hydrolysis products were assayed by polyethyleneimine-cellulose thin layer chromatography. The amounts of [α-[^32]P]GMP produced were estimated with a PhosphorImager. The hydrolysis activity with each nucleotide competitor indicated was depicted relative to the activity without any nucleotide competitor, which was taken as 100%. Each value is the mean from three independent experiments.

Experimental Procedures

_ α-[^32]P-labeled nucleoside triphosphates were visualized by UV shadowing._

TABLE I

**Comparison of the nucleotide substrate specificity of Tm-MazG**

The NTPase assays were performed in a 20-μl reaction mixture, containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, and 0.5 μg of Tm-MazG protein at 70°C for 10 min. Kinetic constants were determined with eight different concentrations of each substrate in the range from 0.1 to 2.0 mM. The figures are the mean values from three independent experiments.
FIG. 2. Product analysis of Tm-MazG-catalyzed GTP hydrolysis. A, production of Pi, in the Tm-MazG-catalyzed GTP hydrolysis. The reaction was performed in a 20-μl reaction mixture with 100 μM GTP and 10 μCi of [γ-32P]GTP. Samples were analyzed by paper chromatography as described under “Experimental Procedures.” Lane 1, control without protein; lane 2, with 1 μg of E. coli MazG at 37°C for 10 min; lane 3, the sample in lane 2 was treated with 0.5 units of yeast inorganic pyrophosphatase at 37°C for 10 min. Lane 4, control without protein; lane 5, with 1 μg of Tm-MazG at 37°C for 5 min; lane 6, with 1 μg of Tm-MazG at 70°C for 10 min. Lane 7, control without protein; lane 8, with 1 μg of Tm-MazG at 70°C for 5 min; lane 9, with 1 μg of Tm-MazG at 70°C for 10 min. B, production of pyrophosphate in the Tm-MazG-catalyzed GTP hydrolysis reaction as an intermediate product. The reaction was carried out at 70°C for 5 min. Lane 1, control without Tm-MazG; lane 2, with 1 μg of Tm-MazG; lanes 3–6, with 1 μg of Tm-MazG at various concentrations of pyrophosphate as indicated.

FIG. 3. Inhibition of the Tm-MazG-catalyzed GTP hydrolysis by pyrophosphate. GTP hydrolysis reactions were carried out at 70°C for 10 min in 20 μl of reaction mixture with 100 μM GTP, 10 μCi of [γ-32P]GTP, and 1 μg of Tm-MazG at various concentrations of pyrophosphate. Lane 1, without pyrophosphate; lanes 2 to 4, with various concentrations of pyrophosphate as indicated. The hydrolysis products were assayed by polyethyleneimine-cellulose thin layer chromatography.

The pyrophosphatase activity was detected with pyrophosphate as substrate. The reaction mixture (20 μl) contained 1 mM pyrophosphate, 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 1 μg of Tm-MazG protein. The reaction was carried out at 70°C for 10 min and terminated by the addition of 5 μl of the stop solution. The amount of P_i released was measured by the standard colorimetric assay method as described by Ames and Dubin (22).

Substrate Specificity of Tm-MazG Protein—The specificity of nucleotide hydrolysis by Tm-MazG protein was examined using various nucleoside triphosphates. The reactions were performed at 70°C for 10 min in 20 μl of reaction mixture containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 0.5 μg of Tm-MazG protein. Rates of hydrolysis of nucleoside triphosphates were determined with six different concentrations of the substrates from 0.1 to 2 mM. Under these conditions, the velocity of the reaction was linear with time. Amounts of (d)NMP product were measured, and the kinetic parameters were calculated from the average values of three independent experiments.

FIG. 4. Effects of AMPCPP on the NTPase and pyrophosphatase activities of Tm-MazG. A, Tm-MazG can hydrolyze ATP and PP, but not AMPCPP. The reaction was performed at 70°C for 10 min in the presence of 1 μg of Tm-MazG in a 20-μl reaction mixture with 1 mM ATP, 1 mM AMPCPP, or 1 mM pyrophosphate as substrate. Amounts of P_i released were measured by the colorimetric assay. B, inhibition of the pyrophosphatase activity by AMPCPP. The reaction mixtures were incubated at 70°C for 2 min in the presence of 1 μg of Tm-MazG at various concentrations of AMPCPP as indicated. Then pyrophosphate was added to a final concentration of 1 mM. The reaction was continued for another 10 min at 70°C. Amounts of P_i, released were measured by the colorimetric assay with the reaction mixture without Tm-MazG as blank control. C, inhibition of Tm-MazG-catalyzed GTP hydrolysis by AMPCPP. Tm-MazG-catalyzed GTP hydrolysis reaction was carried out at 70°C for 10 min in a 20-μl reaction mixture with 100 μM GTP, 10 μCi of [γ-32P]GTP, and 1 μg of Tm-MazG at various concentrations of AMPCPP as indicated. The hydrolysis products were assayed by polyethyleneimine-cellulose thin layer chromatography.

Detection of Pyrophosphate—Nucleoside triphosphate hydrolysis assays were performed as mentioned above with [γ-32P]GTP as substrate. The reaction mixture (5 μl) was mixed with 5 μl of the stop solution to terminate the reaction. The samples (1 μl) were spotted onto a Whatman 3MM paper, which was then developed in a solution containing n-butyl alcohol, n-propyl alcohol, acetone, 80% formic acid, and 30% trichloroacetic acid at a ratio of 40:20:25:25:15 (v/v/v/v/v) in the presence of 0.5 mg/ml EDTA. Then the Whatman 3MM paper was autoradiographed to identify products from [γ-32P]GTP. The unlabeled monophosphate and pyrophosphate were analyzed alongside and visualized as described by Schwemmle and Staeheli (23).

RESULTS

Cloning, Expression, and Purification—MazG family proteins are highly conserved among bacteria. The TM0913 gene,
encoding the MazG homolog in *T. maritima*, was cloned as described under “Experimental Procedures.” Its original GTG start codon was changed to ATG. Expression of the gene cloned in pET-Tm-MazG plasmid transformed in *E. coli* BL21(DE3) resulted in a major band on an SDS-PAGE gel, corresponding to about 20% of total cellular protein. The cell lysate was incubated at 80 °C for 15 min and centrifuged to remove denatured *E. coli* proteins. At this stage, MazG was substantially purified. Tm-MazG was subsequently purified by column chromatography with Q-Sepharose and hydroxyapatite columns, resulting in a highly homogenous protein band in SDS-PAGE. The molecular mass of this product was 29,728 Da as determined by mass spectrometry, which agrees well with the predicted mass of 29,674 Da for recombinant Tm-MazG without the N-terminal Met residue. Thermal denaturation of Tm-MazG protein was examined using a far-UV CD spectropolarimeter. Tm-MazG was very stable at high temperature, and no change was detected up to 85 °C. The purified Tm-MazG showed a CD spectrum of a typical protein containing both α-helices and β-sheets (data not shown).

**Tm-MazG Converts (d)NTP to (d)NMP—**The purified Tm-MazG was incubated with [α-32P]ATP, [α-32P]dATP, [α-32P]GTP, or [α-32P]dGTP at 70 °C for 10 min, and then the nucleotide analysis was performed by polyethyleneimine-cellulose thin layer chromatography. It was found that Tm-MazG could convert ATP/dATP to AMP/dAMP and GTP/dGTP to GMP/dGMP with no detectable nucleoside diphosphate products (Fig. 1A). Further studies revealed that GDP and ADP could not be hydrolyzed by Tm-MazG (data not shown).

With [α-32P]GTP as substrate for Tm-MazG, the GTP hydrolysis activity was tested with unlabeled nucleoside triphosphates as competitors. In the presence of 4 mM competitors (400-fold excess), GTP hydrolysis was effectively blocked by all of the eight canonical nucleoside triphosphates, among which CTP was the strongest competitor. The ribonucleoside triphosphates were stronger competitors than the corresponding deoxyribonucleoside triphosphates (Fig. 1B).

The general kinetic parameters for hydrolysis of the eight canonical nucleoside triphosphates by Tm-MazG were measured (Table I). Among the various nucleoside triphosphates used, the hydrolytic efficiency (kcat/Km) of deoxyribonucleoside triphosphates was higher than that of the corresponding ribonucleoside triphosphates. dGTP was the most preferred substrate for Tm-MazG among deoxyribonucleoside triphosphates, and GTP was the most preferred among ribonucleoside triphosphates. The Km values for the ribonucleoside triphosphates were lower than those for the corresponding deoxyribonucleoside triphosphates except that for GTP, which was similar to that for dGTP. Consistent with the competition experiment results described above, the ribonucleoside triphosphates were stronger competitors, and GTP with the lowest Km value is the strongest inhibitor (Fig. 1B, column 5).

**Production of P i with Pyrophosphate as an Intermediate**—As shown above, Tm-MazG converts (d)NTP to (d)NMP without any detectable production of (d)NDP. Therefore, pyrophosphate should be another product of the Tm-MazG-catalyzed nucleoside triphosphate hydrolysis. To detect the pyrophosphate product in the reaction mixture, the hydrolysis reaction was performed with [γ-32P]GTP as substrate for Tm-MazG (1 μg), and the reaction products were analyzed by paper chromatography and visualized by autoradiography as described under “Experimental Procedures.” A radioactive spot was observed at the position of Pi, but not at the position of pyrophosphate, whether the reaction was performed at 37 °C (Fig. 2A, lanes 5 and 6) or 70 °C (Fig. 2A, lanes 8 and 9). The same results were obtained from the experiments performed at various other temperatures even for shorter reaction times (data not shown). However, with *E. coli* MazG (1 μg) the radioactive signal was observed only at the position of pyrophosphate (Fig. 2A, lane 2), and it shifted to the position of Pi only when the reaction mixture was treated with yeast inorganic pyrophosphatase (Fig. 2A, lane 3). Even if 10 μM of *E. coli* MazG was used, only PPi, and no Pi was detected in the products of *E. coli* MazG-catalyzed GTP hydrolysis (data not shown). These results indicate that *E. coli* MazG hydrolyzes GTP to GMP and PPi, whereas Tm-MazG is able to convert GTP to GMP and Pi.

The results above indicate that Tm-MazG has not only NTPase but also pyrophosphatase activity that effectively hydrolyzes pyrophosphate, a primary product of the NTPase activity, to Pi. In order to prove that pyrophosphate is a primary product of the NTPase activity, nonradioactive pyrophosphate was added into the Tm-MazG-catalyzed [γ-32P]GTP hydrolysis reaction mixture at various concentrations, since the degradation of newly formed radiolabeled pyrophosphate is expected to be inhibited in the presence of excess nonradioactive pyrophosphate. As shown in Fig. 2B, the accumulation of radiolabeled pyrophosphate was detectable in the presence of 2 mM pyrophosphate (Fig. 2B, lane 3), indicating that pyrophosphate is indeed produced in the Tm-MazG-catalyzed [γ-32P]GTP hydrolysis with 5 mM pyrophosphate in the reaction mixture, the amount of radiolabeled pyrophosphate was dramatically reduced (Fig. 2B, lane 4), and it was not detectable in the presence of 10 and 25 mM pyrophosphate (Fig. 2B, lanes 5 and 6), indicating that pyrophosphate does function as an inhibitor for the NTPase activity at higher concentrations. The inhibitory effect of pyrophosphate on the NTPase activity was further confirmed by another experiment, in which the GTP hydrolysis reaction was carried out with [α-32P]GTP (100 μM) as substrate in the presence of various concentrations of pyrophosphate (Fig. 3). It was found that GTP hydrolysis activity, as detected by the production of [α-32P]GMP, was decreased with higher pyrophosphate concentrations (Fig. 3, lanes 2–4), and the GTP hydrolysis was almost completely blocked at 5 mM pyrophosphate (Fig. 3, lane 4). With ATP as substrate for Tm-MazG, Pi release could be detected by the standard colorimetric assay, whereas almost no Pi production was detected with AMPPCP.
as substrate under the same reaction condition (Fig. 4A), indicating that AMPcpp, in which the linkage between α- and β-phosphates is not a phosphodiester bond, cannot be hydrolyzed by Tm-MazG. These results indicated that, in the Tm-MazG-catalyzed (d)NTP hydrolysis reaction, (d)NTP is first hydrolyzed between α- and β-phosphates, yielding (d)NMP and PPi, and subsequently the resultant PPi is hydrolyzed to Pi.

Pyrophosphatase Activity of Tm-MazG—In order to directly demonstrate that Tm-MazG has the pyrophosphatase activity, the purified Tm-MazG was incubated with pyrophosphate at 70 °C for 10 min, and then the amount of P_i resulting from pyrophosphate hydrolysis was measured by a colorimetric assay. As shown in Fig. 4A, Tm-MazG indeed did have pyrophosphatase activity that directly hydrolyzed PPi to Pi (Fig. 4A, column 3). Although the Tm-MazG preparation was purified from E. coli by heat treatment, one cannot exclude a possibility that the pyrophosphatase activity detected in Tm-MazG might be due to E. coli pyrophosphatase contamination. Therefore, we next examined the temperature dependence of the NTPase and pyrophosphatase activities. As shown in Fig. 5, both enzymatic activities increase as the reaction temperature increases, and the optimal temperatures are ~80 °C for both enzymatic activities, which is expected for a T. maritima protein. The NTPase activity of Tm-MazG increased more than 10 times from 30 to 80 °C (Fig. 5A), whereas the NTPase activity of E. coli MazG was the highest at 37 °C and dropped at 80 °C to only a few percent of that at 37 °C (data not shown). It has been reported that the activity of the E. coli pyrophosphatase is severely inhibited at higher temperatures (24). These results indicate that the pyrophosphatase activity is an intrinsic enzymatic activity of Tm-MazG.

To further support this notion, we tested the inhibitory effect of AMPcpp on the enzymatic activities of Tm-MazG. Tm-MazG was first incubated with various concentrations of AMPcpp at 70 °C for 2 min, and then pyrophosphate was added into the reaction mixture. The amounts of P_i released in the reaction
mixture were measured after another 10-min incubation at 70 °C. As shown in Fig. 4B, the pyrophosphatase activity was inhibited by AMPCPP; with increasing ratios of AMPCPP to pyrophosphate (1:1, 2.5:1, and 5:1), the amounts of released P, decreased to 97, 71, and 21%, compared with that without AMPCPP, which was taken as 100%. Next, Tm-MazG was incubated with a various concentration of AMPCPP at 70 °C for 2 min, and then [α-32P]GTP was added into the reaction mixture. After another 10-min incubation at 70 °C, the production of [α-32P]GMP was examined by thin layer chromatography followed by autoradiography. The GTP hydrolysis was inhibited with increasing AMPCPP concentrations (Fig. 4C). ATP hydrolysis was also inhibited by AMPCPP (data not shown). These results indicate that AMPCPP functions as an inhibitor for both NTPase and pyrophosphatase activities, and also confirm the notion that the pyrophosphatase activity is an intrinsic enzymatic activity of Tm-MazG protein. We also examined the effect of polyphosphate on the Tm-MazG pyrophosphatase activity and found that polyphosphate neither serves as substrate nor inhibits the pyrophosphatase activity under the reaction condition used.

Site-directed Mutation Analysis of the Conserved Amino Acid Residues in Tm-MazG—Tm-MazG is a member of the MazG protein family, which is highly conserved in bacteria. Sequence alignments of MazG proteins from E. coli, Yersinia pestis, Vibrio cholerae, Pasteurella multocida, Hemophilus influenzae, Caulobacter crescentus, Agrobacterium tumefaciens, and T. maritima are shown in Fig. 6A. The amino acid alignments reveal that there are a common motif duplicated in Tm-MazG N-terminal and C-terminal regions (Fig. 6B). There are six conserved Glu residues in the duplicated motif in Tm-MazG. In the NMR structure of E. coli MutT protein, another NTPase, there are four conserved Glu residues at its active site (25), suggesting that the Glu residues play an important role in the NTPase activity. Site-directed mutagenesis was performed to analyze the role of these highly conserved Glu residues in Tm-MazG in its enzymatic activities. Mutations, such as E41Q/E42Q, E45Q, and E61Q, in the N-terminal region significantly reduced the NTPase activity to ~10% of the wild-type activity (Fig. 7A). Interestingly, mutations, such as E173A, E176A, E185A/E186A, in the C-terminal region have little effects on the NTPase activity (Fig. 7A). Mutations at other conserved residues in the N-terminal region, such as R97A/R98A and K118E, also reduced the NTPase activity to about 10% of the wild-type activity (Fig. 7A). Surprisingly, the pyrophosphatase activity was substantially activated by all of the mutations described above (Fig. 7B). In particular, the E176A mutation showed the highest pyrophosphatase activity, which was about 3.7 times higher than the wild type activity (Fig. 7B, column 8). It should be noted that in Tm-MazG E41Q/E42Q, E45Q, E61Q, R97A/R98A, and K118E mutants, the pyrophosphatase activity increased while the NTPase activity decreased in comparison with the wild-type Tm-MazG protein. CD spectrums of purified E45Q and R97A/R98A mutant proteins were carried out at 80 °C. Both of their CD spectrums are similar to that of the wild-type Tm-MazG, indicating that they are properly folded (data not shown). However, there was no significant difference in cell growth between the cells overproducing the wild-type Tm-MazG and the cells overproducing the mutant proteins with limited NTPase activities. The overproduction of the E. coli MazG protein had also no significant inhibitory effect on cell growth.

DISCUSSION

The MazG protein family, consisting of the proteins homologous to E. coli MazG, is highly conserved in bacteria. The E. coli MazG homolog in T. maritima was encoded by the TM0931 gene. BLAST analysis of the sequence similarity between E. coli MazG and Tm-MazG revealed that their identity is 39% and their similarity is 59%. E. coli MazG had been characterized as an NTPase to hydrolyze (d)NTP to (d)NMP and PP; in the present study, we demonstrated that Tm-MazG has not only NTPase activity but also pyrophosphatase activity.

The fact that Tm-MazG has both NTPase and pyrophosphatase activities can be supported by the following experimental results: 1) Tm-MazG converts NTP/dNTP to NMP/dNMP without any detectable production of NDP/dNDP; 2) the production of radiolabeled PP; can be detected in the Tm-MazG-catalyzed [γ-32P]GTP hydrolysis in the presence of excess amounts of nonradioactive PP;; 3) Tm-MazG can hydrolyze ATP but not AMPCPP; 4) Tm-MazG can hydrolyze PP, to P; 5) both NTPase and pyrophosphatase activities increase at higher temperatures and have an optimal temperature at about 80 °C; 6) AMPCPP functions as an inhibitor for both NTPase and pyrophosphatase activities; 7) mutations of the highly conserved amino acid residues in Tm-MazG affect both enzymatic activities.

In the Tm-MazG-catalyzed (d)NTP hydrolysis reaction, (d)NTP is first hydrolyzed between α- and β-phosphates, yield-
ing (d)NMP and PPi, and then PPi is subsequently hydrolyzed to P1. Since PPi cannot be detected in the reaction mixture in the presence of Mg2+, PPi produced by the NTPase activity of Tm-MazG may remain at the same position for subsequent hydrolysis of the PPi to P1. At present, it is not certain whether two enzymatic activities share the same active site. However, since AMPCPPP inhibits both enzymatic activities, their active sites may be at least very close to each other or partially overlapping. It is important to note that a motif with highly conserved Glu residues is repeated in Tm-MazG, one in the N-terminal region and the other in the C-terminal region. Interestingly, site-directed mutations at the conserved amino acid residues in the N-terminal region severely disrupted the NTPase activity, whereas mutations at the conserved residues in the C-terminal region did not. Moreover, since all these mutations enhanced the pyrophosphatase activity, it appears that the N-terminal Tm-MazG motif is important for the NTPase activity, whereas both N- and C-terminal motifs may be involved in the pyrophosphatase activity.

It is also interesting to note that E. coli MazG has no detectable pyrophosphatase activity. The NTPase activity of E. coli MazG is significantly weaker than that of Tm-MazG, which may be due to the intrinsic pyrophosphatase activity of Tm-MazG that removes the pyrophosphate inhibitory effect on the NTPase activity. It remains to be determined how Tm-MazG has dual enzymatic activities. The determination of its three-dimensional structure may provide insights into the enzymatic mechanisms of Tm-MazG.

The cellular function of MazG is unknown at present. In E. coli, the mazG gene is located downstream of the mazEF addiction module, which has been proposed to be involved in the programmed cell death under stress conditions (26). However, T. maritima does not contain the mazEF homologues. Further studies are needed to elucidate whether MazG is functionally and/or physiologically related to the mazEF system in E. coli. Since the MazG protein is able to hydrolyze the phosphodiester bond between α- and β-phosphates in NTP/dNTP, other compounds such as ITP, xanthosine 5′-triphosphate, and the Nudix enzyme substrates Ap4A and Ap3A may serve as substrates for MazG protein. Analyses of the GenBank™ data base reveal that there are proteins containing MazG domain and another functional domain. The proteins containing the uroporphyrinogen-III methylase domain and MazG domain were found in Clostridium acetobutylicum, Bacillus anthracis, Staphylococcus aureus, Clostridium perfringens, Bacillus halodurans, and Thermoaerobacter tengcongensis, and on the other hand, a protein containing the helix-turn-helix XRE domain and MazG domain was found in Streptococcus thermophilus bacteriophage. The function of these domains may be related to the MazG function in the cell.

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REFERENCES

1. Zhang, J., and Inouye, M. (2002) J. Bacteriol. 184, 5323–5329
2. Bhattachar, S. K., Bullions, L. C., and Bessman, M. J. (1991) J. Biol. Chem. 266, 9050–9054
3. Akiyama, M., Maki, H., Sekiguchi, M., and Horiiuchi, T. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3945–3952
4. Cox, E. C. (1975) Genetics 73, (Suppl.), 67–80
5. Tajiri, T., Maki, H., and Sekiguchi, M. (1995) Mutat. Res. 336, 257–267
6. Maki, H., and Sekiguchi, M. (1992) Nature 353, 275–275
7. Mejean, V., Salles, C., Bullions, L. C., Bessman, M. J., and Claveyres, J. P. (1994) Mol. Microbiol. 11, 323–330
8. Kamath, A. V., and Yanofsky, C. (1993) Gene (Amst.) 134, 99–102
9. Mo, J. Y., Maki, H., and Sekiguchi, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11021–11025
10. Sakumi, K., Furuchi, M., Tsuzuki, T., Kakuma, T., Kawahata, S., Maki, H., and Sekiguchi, M. (1993) J. Biol. Chem. 268, 23524–23530
11. Cai, J. P., Kakuma, T., Tsuzuki, T., and Sekiguchi, M. (1995) Carcinogenesis 16, 2343–2350
12. Kakuma, T., Nishida, J., Tsuzuki, T., and Sekiguchi, M. (1995) J. Biol. Chem. 270, 20942–20948
13. Bullions, L. C., Mejean, V., Claveyres, J. P., and Bessman, M. J. (1994) J. Biol. Chem. 269, 12339–12344
14. Fujii, Y., Shimokawa, H., Sekiguchi, M., and Nakabeppu, Y. (1999) J. Biol. Chem. 274, 38251–38259
15. Bessman, M. J., Frick, D. N., and O’Handley, S. F. (1996) J. Biol. Chem. 271, 25655–25662
16. O’Handley, S. F., Frick, D. N., Bullions, L. C., Malden, A. A., and Bessman, M. J. (1996) J. Biol. Chem. 271, 24649–24654
17. Chung, J. H., Buck, J. H., Park, Y. I., and Han, Y. S. (2001) Nucleic Acids Res. 29, 3099–3107
18. Hwang, K. Y., Chung, J. H., Kim, S. H., Han, Y. S., and Cho, Y. (1999) Nat. Struct. Biol. 6, 691–696
19. Vanderheiden, G. S. (1970) Biochim. Biophys. Acta 215, 555–558
20. Lin, S., McLennan, A. G., Ying, K., Wang, Z., Gu, S., Jin, H., Wu, C., Liu, W., Yuan, Y., Tang, R., Xie, Y., and Mao, Y. (2001) J. Biol. Chem. 276, 18695–18701
21. Nelson, K. E., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Nelson, W. C., Ketchum, K. A., McDonald, L., Utterback, T. R., Malek, J. A., Linher, K. D., Garrett, M. M., Stewart, A. M., Cotton, M. D., Pratt, M. S., Phillips, C. A., Richardson, D., Heidelberg, J., Sutton, G. G., Fleischmann, R. D., Eisen, J. A., Fraser, C. M., et al. (1999) Nature 399, 323–329
22. Ames, B. N., and Duhon D. T. (1960) J. Biol. Chem. 235, 769–775
23. Schwemmle, M., and Stahl, P. (1994) J. Biol. Chem. 269, 11299–11305
24. Satoh, T., Takahashi, Y., Oshida, N., Shimizu, A., Shinozuka, H., Watanabe, M., and Samejima, T. (1999) Biochemistry 38, 1531–1536
25. Harris, T. K., Wu, G., Massiah, M. A., and Mildvan, A. S. (2000) Biochemistry 39, 1655–1674
26. Aizenman, E., Engelberg-Kulka, H., and Glaser, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6059–6063
