A gene encoding a putative GTP-binding protein, a TrmE homologue that is highly conserved in both prokaryotes and eukaryotes, was cloned from Thermotoga maritima, a hyperthermophilic bacterium. T. maritima TrmE was overexpressed in Escherichia coli and purified. TrmE has a GTPase activity but no ATPase activity. The GTPase activity can be competed with GTP, GDP, and dGTP but not with GMP, ATP, CTP, or UTP. \( K_m \) and \( k_{cat} \) at 70°C were 833 \( \mu \)M and 9.3 min\(^{-1} \), respectively. Our results indicate that TrmE is a GTP-binding protein with a very high intrinsic GTP hydrolysis rate. We also propose that TrmE homologues constitute a novel subfamily of the GTPase superfamily.

Proteins possessing GTP-binding and GTPase activities play essential roles in cell proliferation in both prokaryotes and eukaryotes. Their functions are very diversified and include involvement in protein translation (e.g., EF-Tu), signal transduction (e.g., small G proteins), cell growth (e.g., RAS), vesicular transport (e.g., RAB), and protein translocation across membranes (e.g., SRP), etc. (3, 4, 14). They all contain well-conserved motifs—G-1, G-3, and G-4—which are important for GTP-binding activity (4). Another motif, G-2, is not involved in binding to GTP but is involved in interaction with an effector molecule (3, 4). G-2 sequences are well conserved within each subfamily in the large GTPase family. Most GTP-binding proteins possess a very high intrinsic GTP hydrolysis rate, while their GTPase activities are highly stimulated by effector molecules through their binding to the G-2 motif. In general, the ratio of the active GTP-bound form to the inactive GDP-bound form of a GTP-binding protein is crucial for its functional regulation, and this process includes GTP hydrolysis that results from conformational changes of the GTP-binding protein (14).

Analysis of the Escherichia coli genome revealed that it contains a GTP-binding protein encoded by the gene originally designated \( \text{thdF} \) (1, 5). Recently, this gene was shown to be essential in \( E. \) coli and involved in tRNA modification and was thus redesignated \( \text{trmE} \) (6). The \( E. \) coli TrmE protein contains plausible GTP-binding motifs G-1 to G-4 consisting of 454 amino acid residues (6). To date, more than 20 proteins homologous to TrmE have been found in prokaryotes and eukaryotes (Fig. 1). Notably, all eubacteria, but not archaea, whose entire genomes have been sequenced contain TrmE; in addition, a TrmE homologue exists in expressed sequence tags from humans (data not shown). A TrmE homologue in Saccharomyces cerevisiae, MSS1, has been proposed to be a GTPase and is thought to be involved in translational regulation in mitochondria (9, 10). In this study we cloned the \( \text{trmE} \) gene, highly homologous to the \( E. \) coli \( \text{trmE} \) gene, from the hyperthermophilic bacterium Thermotoga maritima (19), and its product was highly purified. Its biochemical characterization revealed that \( T. \) maritima TrmE is indeed a GTP-binding protein having a very high intrinsic GTPase activity at 80°C.

### The \( \text{trmE} \) Gene Encodes a GTP-binding Protein

The \( \text{trmE} \) gene encodes a GTP-binding protein. GTP-binding motifs G-1 to G-4 are well conserved in all TrmE homologues (Fig. 1), strongly indicating that \( T. \) maritima TrmE is a GTP-binding protein and possesses a GTP hydrolysis activity. Motif G-2 is thought to be involved in binding to an effector molecule but not in GTP binding. The consensus sequence for G-2 is limited to TrmE homologues and cannot be applied to other subfamilies of GTP-binding proteins (data not shown), suggesting that TrmE homologues constitute a novel subfamily of the GTPase superfamily and that TrmE probably interacts with a specific effector molecule, which could regulate the GTPase activity of TrmE. The GTP-binding motifs are located within the third quarter of this protein from the N-terminal end (Fig. 1).

Besides GTP-binding motifs, at least four well-conserved regions (regions I, II, III, and IV) can be assigned (Fig. 1). Although their functional significance is unknown at present, these regions are likely to be related to the TrmE-specific function. Only the GTP-binding domains G-1 to G-4 are involved in GTP binding and GTPase activity in \( E. \) coli \( \text{TrmE} \) (6); therefore, regions I to IV are unlikely to be involved in GTP binding and GTPase activity in \( T. \) maritima. It is, however, possible that they are associated with the regulation of GTP binding and GTPase activity and/or that they might be involved in RNA modification, since \( \text{trmE} \) mutants exhibit deficiency in biosynthesis of 5-methylaminomethyl-2-thiouridine of tRNA (11). As three \( E. \) coli GTPases—Ffh, EF-G, and Era—are known to bind to RNA (8, 12, 13, 15, 17, 18, 21, 22) and their RNA binding is closely associated with their GTPase activities, the TrmE GTPase may also be closely associated with its RNA modification activity. It is interesting that the C-terminal sequence CVGK in region IV seems to be a consensus sequence, CAAX, where A represents an aliphatic amino acid residue and X represents any amino acid residue, for isoprenylation in the Ras protein (23), although no isoprenylation has been reported and no genes involved in isoprenylation have been identified in the prokaryotes so far. It is also interesting that \( E. \) coli \( \text{TrmE} \) has been shown to be localized in both the cytoplasm and the inner membrane (6).

During the process of a homology search for TrmE, we found another homologous protein, which has been registered as TM1446 in the \( T. \) maritima genome (19). This protein contains two tandem repeats of the GTP-binding domain of TrmE, but its homologues, unlike those of TrmE, are found only in the prokaryotes (data not shown).
To clone the trmE gene from T. maritima, the genomic DNA of T. maritima (a generous gift from Francis E. Jenney, Jr., University of Georgia) was used as a template for PCR. Primers 9541 (5'-AGACAACATATGGATACCATTGTCGCTGTAG-3' [an NdeI site is underlined]) and 9540 (5'-CCAAGCTTTCATTTTCCAACGCAAA-3' [a HindIII site is underlined]) were used. PCR was carried out with 30 cycles of amplification of 1 min at 95°C, 2 min at 55°C, and 3 min at 72°C.

FIG. 1. Amino acid sequence alignments of TrmE homologues. A homology search was carried out by the BLAST program (2). Residues identical to those of T. maritima TrmE are shown as dots, and gaps are indicated by dashes. The GTP-binding motifs G-1 to G-4 and well-conserved regions I to IV are indicated by bars above the sequences. Tm, T. maritima (National Center for Biotechnology Information [NCBI] protein database accession no. AAD35356.1); Ae, Aquifex aeolicus (AAC06992.1); Bs, Bacillus subtilis (AAK40226.1); Ec, Escherichia coli (AAC79694); Hi, Haemophilus influenzae (AAC22664.1); Pp, Pseudomonas putida (AAC44141.1); Ct, Chlamydia trachomatis (AAEU51391.1); Mp, Mycoplasma pneumoniae (AAQ50002.1); Rp, Rickettsia prowazekii (AAA15187); Ss, Synechocystis sp. strain PCC6803 (BAA17896); Se, Synechococcus elongatus (CAH63651.1); Cc, Cyanidium caldarium (chloroplast genome) (AAF12952); Sc, Saccharomyces cerevisiae (CAH63651.1); Sp, Schizosaccharomyces pombe (BAA17897).
The DNA sequences of the \( \text{ndk} \) containing \( 200 \) mM KCl, \( 5 \) mM MgCl\( _2 \), \( 1 \) mM DTT, \( 10 \) \( \mu \)M \( [\gamma-^32\text{P}] \)GTP, and \( 10 \) \( \mu \)g of purified TrmE protein at \( 70^\circ \text{C} \) for \( 10 \) min. The reaction was terminated by transfer of \( 5 \) \( \mu \)l of samples to \( 10 \) \( \mu \)l of ice-cold \( 20 \) mM EDTA. Portions of the terminated reaction mixture were spotted onto a polyethyleneimine-cellulose thin-layer chromatography plate, which was developed in \( 0.75 \) M KH\( _2\)PO\(_4\) (pH \( 3.65 \)). The plate was autoradiographed to identify hydrolyzed products of GTP. Peaks at 260, 160, and 52 kDa are designated a, b, and c, respectively, as shown. (C) Hydrolytic activity from GTP to GDP of TrmE. The GTPase assay was carried out in a 50-\( \mu \)l reaction mixture of 50 mM Tris-HCl (pH 9.0) containing 200 mM KCl, 5 mM MgCl\( _2 \), 1 mM DTT, 10 \( \mu \)M [\( \gamma-^32\text{P} \)]GTP, and 10 \( \mu \)g of purified TrmE protein at \( 70^\circ \text{C} \) for \( 10 \) min. The reaction was terminated by transfer of \( 5 \) \( \mu \)l of samples to \( 10 \) \( \mu \)l of ice-cold \( 20 \) mM EDTA. Portions of the terminated reaction mixture were spotted onto a polyethyleneimine-cellulose thin-layer chromatography plate, which was developed in \( 0.75 \) M KH\( _2\)PO\(_4\) (pH \( 3.65 \)). The plate was autoradiographed to identify hydrolyzed products of GTP. Spots corresponding to GTP, GDP, and GMP (lane 5) were identified by UV shadowing. Lanes 1 and 2, incubations without TrmE for 0 and 60 min, respectively; lanes 3 and 4, incubations with 10 \( \mu \)g of TrmE for 0 and 60 min, respectively.

FIG. 3. Effects of reaction temperature, pH, and salt concentration on the GTPase activity of TrmE. (A) The GTPase assay was carried out in a 50-\( \mu \)l reaction mixture of 50 mM Tris-HCl (pH 7.5) containing \( 5 \) mM MgCl\( _2 \), 1 mM DTT, 10 \( \mu \)M [\( \gamma-^32\text{P} \)]GTP, and 10 \( \mu \)g of TrmE for 10 min at different temperatures. The GTPase assay reaction was stopped by adding activated charcoal followed by centrifugation, and the release of \( ^32\text{P} \) in the supernatant was assayed using a liquid scintillation counter. (B) The GTPase assay was carried out in a 50-\( \mu \)l reaction mixture. Between pH 6.5 and 9.0, the mixture contained 50 mM Tris-HCl; at pH 9.5 and 10, the mixture contained 50 mM 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid. Both buffers contained 5 mM MgCl\( _2 \), 1 mM DTT, 10 \( \mu \)M [\( \gamma-^32\text{P} \)]GTP, and 10 \( \mu \)g of TrmE; and the reaction was carried out for 10 min at \( 70^\circ \text{C} \). (C) The GTPase assay was carried out in a 50-\( \mu \)l reaction mixture of 50 mM Tris-HCl (pH 7.5) containing 5 mM MgCl\( _2 \), 1 mM DTT, 10 \( \mu \)M [\( \gamma-^32\text{P} \)]GTP, 10 \( \mu \)g of TrmE, and different concentrations (0 to 500 mM) of KCl or NaCl for 10 min at \( 70^\circ \text{C} \). The reaction was carried out at least twice, and the average value for each point was used. Background values (without protein) were subtracted.

FIG. 2. Purification, oligomer formation, and GTPase activity of TrmE. (A) Purified TrmE was analyzed by sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis, and the gel was stained with Coomassie brilliant blue. Lane 1, bovine serum albumin (66 kDa) and trypsin inhibitor (21.7 kDa) as molecular mass standards; lane 2, purified TrmE (2 \( \mu \)g). (B) Purified TrmE was applied to a Superdex G200 (Pharmacia) column which had been equilibrated with \( 20 \) mM KPO\(_4\) buffer (pH 8.0) containing 50 mM NaCl. Thyroglobulin (669 kDa), apoferritin (443 kDa), \( \beta \)-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa) were used as molecular mass standards. Peaks at 260, 160, and 52 kDa are designated a, b, and c, respectively, as shown. (C) Hydrolytic activity from GTP to GDP of TrmE. The GTPase assay was carried out in a 50-\( \mu \)l reaction mixture of 50 mM Tris-HCl (pH 9.0) containing 200 mM KCl, 5 mM MgCl\( _2 \), 1 mM DTT, 10 \( \mu \)M [\( \gamma-^32\text{P} \)]GTP, and 10 \( \mu \)g of purified TrmE protein at \( 70^\circ \text{C} \) for \( 10 \) min. The reaction was terminated by transfer of \( 5 \) \( \mu \)l of samples to \( 10 \) \( \mu \)l of ice-cold \( 20 \) mM EDTA. Portions of the terminated reaction mixture were spotted onto a polyethyleneimine-cellulose thin-layer chromatography plate, which was developed in \( 0.75 \) M KH\( _2\)PO\(_4\) (pH \( 3.65 \)). The plate was autoradiographed to identify hydrolyzed products of GTP. Spots corresponding to GTP, GDP, and GMP (lane 5) were identified by UV shadowing. Lanes 1 and 2, incubations without TrmE for 0 and 60 min, respectively; lanes 3 and 4, incubations with 10 \( \mu \)g of TrmE for 0 and 60 min, respectively.
FIG. 4. Lineweaver-Burk plot of TrmE GTPase activity. The GTPase assay was carried out in a 50-μl reaction mixture of 50 mM Tris-HCl (pH 9.0) containing 200 mM KCl, 5 mM MgCl2, 1 mM DTT, 10 μg of TrmE, and different concentrations (0.001 to 3 mM) of GTP for 10 min at 70°C. Each point is the average of at least two experiments. Background values (without protein) were subtracted. Vo, initial rate of reaction.

Next, we determined the optimum conditions for GTPase activity. GTP hydrolysis occurred linearly with up to 30 μg of protein per reaction mixture (data not shown). With 10 μg of protein, GTP hydrolysis occurred linearly for up to 60 min of incubation (data not shown). To determine the optimal reaction temperature, GTP hydrolysis assays were carried out at 30, 40, 50, 60, 70, 80, and 90°C. As shown in Fig. 3A, maximum activity was found at 80°C. However, the higher temperature caused a higher background: 0.7 and 1.9% of input GTP was hydrolyzed without protein in 10 min at 80 and 90°C, respectively, while less than 0.3% was hydrolyzed at other temperatures. Therefore, all of the reactions described below were carried out at 70°C.

The effects of pH, salt, and Mg2+ on GTPase activity were subsequently examined. TrmE was found to prefer alkaline conditions, with the optimum activity at pH 9.0, while it had almost no activity at neutral and acidic pHs (Fig. 3B). The TrmE GTPase was found to be specifically activated by KCl and, as shown in Fig. 3C, at a sevenfold-higher rate at 200 mM KCl than at 200 mM NaCl. At present, we do not know whether these in vitro characteristics of pH and K+ concentration reflect in vivo conditions. The Mg2+ ion is also required for GTPase activity, which reached its highest level at greater than 1 mM Mg2+ (data not shown).

Thus, the optimum conditions for TrmE GTPase activity were determined to be as follows: the reaction is carried out at 70°C for 10 min in 50 mM Tris-HCl (pH 9.0) containing 200 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol (DTT), and 10 μM GTP with 10 μg of TrmE in a 50-μl reaction mixture. Biochemical parameters of the TrmE GTPase activity were determined under optimum conditions. Using GTP concentrations from 0.001 to 3 mM, the Lineweaver-Burk plot was determined (Fig. 4). Km and Vmax for the GTPase activity of TrmE were estimated to be 833 μM and 37 μM/min, respectively. kcat was calculated to be 9.3 min⁻¹. This indicates that T. maritima TrmE has a very high intrinsic GTP hydrolysis rate. Note that the concentration of GTP in exponentially growing bacterial cells is about 1 mM (20). E. coli TrmE also shows a very high intrinsic GTP hydrolysis rate (Km, 378 μM; kcat, 26 min⁻¹) (6), which is comparable to T. maritima TrmE, suggesting that the very high intrinsic GTP hydrolysis rate might be a common characteristic among TrmE proteins from vari-
ous species. It would be interesting to know if the hydrolysis rate is modulated by an effector molecule in the cells.

To determine the substrate specificity, competition experiments were carried out with various nucleotides added at a 300-fold excess over the GTP used in the reaction. As shown in Fig. 5, only GTP, GDP, and dGTP were found to compete GTP hydrolysis. The high substrate specificity of TrmE to guanine nucleotides was further confirmed by the fact that TrmE has no detectable ATPase activity under the conditions used for the GTPase assay (data not shown). Note that GTP binding of E. coli TrmE was shown to be compete with GTP, GDP, and dGTP (6).

Conclusions. We have demonstrated that T. maritima TrmE possesses a GTPase activity having a very high intrinsic GTP hydrolysis rate. Although its $K_m$ value is quite high (833 μM) in comparison with the $K_m$ value of E. coli Era (10 μM) (7), the TrmE GTPase activity is likely to be significantly regulated by its effector molecule(s) in the cells. The identification of such an effector molecule is important for our understanding of TrmE function, which is known to be essential for cell growth in E. coli (6).

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REFERENCES


