Novel Triphosphate Phosphohydrolase Activity of *Clostridium thermocellum* TTM, a Member of the Triphosphate Tunnel Metalloenzyme Superfamily*

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Triphosphate tunnel metalloenzymes (TTMs) are a newly recognized superfamily of phosphohydrolases defined by a unique active site residing within an eight-stranded β barrel. The prototypical members are the eukaryal metal-dependent RNA triphosphatases, which catalyze the initial step in mRNA capping. Little is known about the activities and substrate specificities of the scores of TTM homologs present in bacterial and archaeal proteomes, nearly all of which are annotated as adenylate cyclases. Here we have conducted a biochemical and structure-function analysis of a TTM protein (*Cth*TTM) from the bacterium *Clostridium thermocellum*. *Cth*TTM is a metal-dependent tripolyphosphatase and nucleoside triphosphatase; it is not an adenylate cyclase. We have identified 11 conserved amino acids in the tunnel that are critical for triphosphatase and ATPase activity. The most salient findings are that (i) *Cth*TTM is 150-fold more active in cleaving tripolyphosphate than ATP and (ii) the substrate specificity of *Cth*TTM can be transformed by a single mutation (K8A) that abolishes tripolyphosphatase activity while strongly stimulating ATP hydrolysis. Our results underscore the plasticity of *Cth*TTM substrate choice and suggest how novel specificities within the TTM superfamily might evolve through changes in the residues that line the tunnel walls.

RNA triphosphatases of fungi, protozoa, and several DNA viruses are the founding members of the triphosphate tunnel metalloenzyme (TTM) superfamily of phosphohydrolases (1). Members of this family that have been characterized biochemically include the triphosphatase components of the cellular mRNA capping systems of *Saccharomyces cerevisiae* (2, 3), *Schizosaccharomyces pombe* (4), *Candida albicans* (5), *Plasmodium falciparum* (1, 6, 7), *Trypanosoma brucei* (8, 9), *Encephalitozoon cuniculi* (10), and *Giardia lamblia* (11) plus the triphosphatases of the *Chlorella* virus, poxvirus, and baculovirus mRNA capping systems (12–17). The signature biochemical property of this branch of the TTM superfamily is the ability to hydrolyze NTPs to nucleoside diphosphates and *P*₁ in the presence of manganese (2). Their defining primary structure features are two glutamate-containing motifs that are required for catalysis by every family member and which comprise the metal-binding site.

The crystal structure of the *S. cerevisiae* RNA triphosphatase Cet1 bound to manganese and sulfate (a proposed mimic of the product complex with phosphate) revealed a then-novel tertiary structure in which the active site is situated within a topologically closed tunnel composed of eight antiparallel β strands (18). Extensive mutational analyses of yeast Cet1 identified a large ensemble of side chains essential for triphosphatase activity *in vitro* and *in vivo*, many of which make direct or water-mediated contacts with the divalent cation or the sulfate anion (3). It was thought initially that the Cet1-like RNA triphosphatases arose *de novo* in unicellular eukarya in tandem with the emergence of caps as the defining feature of eukaryal mRNA. This notion has been eclipsed by the finding that the heretofore unique tertiary structure and active site of yeast RNA triphosphatase are recapitulated in the crystal structures of archaeal and bacterial proteins of unknown biochemical function, including proteins from *Pyrococcus* (Protein Data Bank (PDB) accession codes 1YEM and 2DC4), *Vibrio* (2ACA), and *Nitrosomonas* (2FBL) (1). The Cet1-like archaeal/bacterial proteins are usually annotated as belonging to the so-called CYTH family (19), which is defined by its two biochemically characterized founding members, an *Aeromonas hydrophila* adenylate cyclase *CyaB* and a mammalian thiamine triphosphatase (20, 21). A crystal structure of a Cet1-like adenylate cyclase from *Versinia* (2FJT) has been reported recently (22). Given that the Cet1 clade, *CyaB*, and thiamine triphosphatase are all metal-dependent enzymes that act on triphosphate-containing substrates, we concluded that the tunnel fold first described for Cet1 is the prototype of a larger enzyme superfamily, which we named “tripolyphosphate tunnel metalloenzyme” (TTM) to reflect the defining structural features (1).

Although it is now clear that TTM proteins are distributed widely among bacterial, archaeal, and eukaryal taxa, virtually nothing is known about the presumptive enzymatic activities and substrate specificities of the dozens of TTM homologs present in bacterial and archaeal proteomes. Indeed, the majority of these TTM proteins are annotated as adenylate cyclases without any evidence that they have this activity. Here we have presented a biochemical characterization and structure-function analysis of a 156-amino-acid TTM pro-
Triphosphate Tunnel Metalloenzyme

A

| Cth  | MKEIEKFKPSGDAKSLAQKLVRQCYKSKDENK-DVRSRVFNKGYLTVKGTG | 60 |
| Neu  | MTEERKFLATFPPGEL-HAVPLRGQYLTPTDGLIEELRLQGTEYFMFLKS-BGGLSR | 58 |
| Cth  | LEYETEPVDGAEILEYLCERFVIKLYKOFQ-EGFTQVFEDLFGENELVTAIEILP | 119 |
| Neu  | QYETQQT-dV7QPEMLLPTEGRKVRKTYSGKLPDGQLPEQVAFQHLSPLMLV5EFL | 117 |
| Cth  | DENAV-FKFKDNGREVTGDPRVNLNLNNVNYKNNKE | 156 |
| Neu  | SEDAAQAFIPPMPEVETRDKKYKALALISP | 151 |

B

FIGURE 1. Structural similarities between CthTTM and NeuTTM. A, the amino acid sequence of C. thermocellum TTM is aligned to the homologous polypeptide encoded by N. europaea. Gaps in the alignment are indicated by dashes. Positions of amino acid side chain identity or similarity are indicated by dots above the alignment. The 14 conserved amino acids subjected to mutational analysis are indicated by single letter code. The secondary structure elements of NeuTTM are shown with β strands depicted as arrows and α helices as cylinders. B, stereo view of the tertiary structure of NeuTTM (PDB accession code 2FBL). The 14 conserved side chains targeted for alanine scanning are shown in stick representation; the side chain name (in single letter code) and number are given for NeuTTM (with the equivalent residue number in CthTTM beneath in parentheses).

dtein from the bacterium Clostridium thermocellum (CthTTM). Although annotated as an adenylate cyclase (GenBank\textsuperscript{TM} EAM47091), CthTTM had no detectable adenylate cyclase activity in our hands. Rather, CthTTM is a metal-dependent phosphohydrolase that specifically cleaves the β-γ phosphohydride bond of triphosphates and nucleoside triphosphates. We have determined the effects of 35 mutations of 14 amino acids within the putative triphosphate tunnel of CthTTM and thereby distinguished the functional groups required for global phosphohydrolase activity from ones selective for activity on nucleotide versus inorganic phosphohydride.

EXPERIMENTAL PROCEDURES

Recombinant CthTTM—The open reading frame encoding CthTTM was amplified from C. thermocellum genomic DNA (purchased from the American Type Culture Collection) with primers that introduced an Ndel site at the start codon and a BamHI site 3' of the stop codon. The PCR product was digested with Ndel and BamHI and inserted into pET16b to generate an expression plasmid encoding the CthTTM polypeptide fused to an N-terminal His\textsubscript{10} tag. Alanine and conservative substitution mutations were introduced by PCR using the two-stage overlap extension method (23). The inserts of all plasmids were sequenced to exclude the acquisition of unwanted coding changes during amplification or cloning. Wild-type and mutant pET-CthTTM plasmids were transformed into Escherichia coli BL21(DE3). Cultures (250 ml) were grown at 37 °C in Luria-Bertani medium containing 0.1 mg/ml ampicillin until A\textsubscript{600} reached ∼0.6. The cultures were adjusted to 0.1 mM isopropyl-β-D-thiogalactopyranoside and incubated at 37 °C for 3 h. Cells were harvested by centrifugation, and the pellet was stored at −80 °C. All subsequent procedures were performed at 4 °C. Thawed bacteria were resuspended in 25 ml of buffer A (50 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 10% sucrose). Lysozyme, phenylmethylsulfonyl fluoride, and Triton X-100 were added to final concentrations of 1 mg/ml, 0.5 mM, and 0.1%, respectively. The lysates were sonicated to reduce viscosity, and insoluble material was removed by centrifugation. The soluble extracts were applied to 1-ml columns of nickel-nitrilotriacetic acid-agarose (Qiagen) that had been equilibrated with buffer A. The columns were washed with 10 ml of the same buffer and then eluted stepwise with 4-ml aliquots of 50, 100, and 200 mM imidazole in buffer B (50 mM Tris-HCl, pH 8.0, 0.25 M NaCl, 10% glycerol, 0.05% Triton X-100). The polypeptide compositions of the column fractions were monitored by SDS-PAGE. The Hi\textsubscript{10}-CthTTM polypeptide was recovered predominantly in the 200 mM imidazole fraction. The 200 mM imidazole eluates were dialyzed against 50 mM NaCl in buffer C (50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.05% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol) and then applied to 1-ml columns of DEAE-Sephacel that had been equilibrated with 50 mM NaCl in buffer C. The columns were washed with 8 ml of the same buffer and then eluted stepwise with 4-ml aliquots of 100, 200, and 500 mM NaCl in buffer C. CthTTM was recovered in the flow-through. The proteins were stored at −80 °C. Protein concentrations were determined by using the Bio-Rad dye reagent with bovine serum albumin as the standard. The yield of wild-type CthTTM was 10 mg from a 250-ml bacterial culture.

Nucleoside Triphosphatase Assay—Reaction mixtures containing (per 10 μl) 50 mM Tris-HCl, pH 8.0, 2 mM MnCl\textsubscript{2}, and
100 μM [γ-32P]ATP or [α-32P]ATP (PerkinElmer Life Sciences), and CthTTM, as specified, were incubated for 30 min at 37 °C. An aliquot (1.5 μl) of the mixture was applied to a polyethyleneimine-cellulose TLC plate, which was developed with 0.5 M LiCl and 1 M formic acid. The radiolabeled material was visualized by autoradiography and 32P formation was quantified by scanning the TLC plate with a Fuji BAS2500 imager. Alternatively, reaction mixtures (50 μl) containing 50 mM Tris-HCl, pH 8.0, 2 mM MnCl2, 100 μM unlabeled NTP or dNTP, and CthTTM, as specified, were incubated for 30 min at 37 °C. The reactions were quenched by adding 1 ml of malachite green reagent (Biomol Research Laboratories, Plymouth Meeting, PA). Phosphate release was determined by measuring A620 and interpolating the value to a phosphate standard curve.

**Tripolyphosphatase Assay**—Reaction mixtures (50 μl) containing 50 mM Tris-HCl, pH 9.0, 10 mM MgCl2 or 0.5 mM MnCl2, 100 μM inorganic tripolyphosphate (PPP) (Sigma), and CthTTM, as specified, were incubated for 30 min at 37 °C. The reactions were quenched by adding 1 ml of malachite green reagent. Release of phosphate was determined by measuring A620 and interpolating the value to a phosphate standard curve.

**Glycerol Gradient Sedimentation**—An aliquot (100 μg) of CthTTM was mixed with catalase (50 μg), bovine serum albumin (50 μg), and cytochrome c (50 μg). The mixture was applied to a 4.8-ml 15–30% glycerol gradient containing 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.05% Triton X-100. The gradient was centrifuged at 50,000 revolutions/min for 17 h at 4 °C in a Beckman SW55Ti rotor. Fractions (~0.18 ml) were collected from the bottom of the tube. Aliquots of even-numbered fractions were analyzed by SDS-PAGE and assayed for nucleoside triphosphatase and tripolyphosphatase activities.

**RESULTS**

A TTM from C. thermocellum—The primary structure of the 156-amino-acid putative TTM from *C. thermocellum* (CthTTM) is aligned in Fig. 1A to a closely related TTM protein from *Nitrosomonas europaea* (NeuTTM; 151 amino acids) for which a crystal structure is available (PDB accession code 2FBL). The NeuTTM fold is distinguished by the fact that the eight-stranded antiparallel β barrel is not a closed tunnel but rather a C-shaped “cup” that is prevented from closing by virtue of the insertion of a broken C-terminal helix into one end of the tunnel (Fig. 1B). The position of the C-terminal segment in the tunnel aperture appears to be stabilized by a hydrogen bond from a conserved lysine in the β1 strand of the tunnel (Lys-7 in NeuTTM; Lys-8 in CthTTM) to a backbone carbonyl at the break in the C-terminal helix (Fig. 1B). The primary structures of CthTTM and NeuTTM have 73 positions of side chain identity/similarity. Especially striking is the identity of the constellation of acidic and basic residues that project into the tunnel (Fig. 1B) and are the equivalents of the essential constituents of the Cet1 active site.

**CthTTM Is a Nucleoside Triphosphate Phosphohydrolase**—Reaction of purified CthTTM with 100 μM [α-32P]ATP in the presence of a divalent cation resulted in quantitative conversion of the labeled substrate to ADP (Fig. 2A). No labeled product corresponding to 3',5'-cyclic AMP was detected. A parallel reaction of CthTTM with 100 μM [γ-32P]ATP resulted in conversion of nearly all of the labeled material to inorganic phosphate (Fig. 2A). The rate of release of 32P from [γ-32P]ATP was identical to the rate of conversion of [α-32P]ATP to [α-32P]ADP in parallel reaction mixtures containing the same enzyme concentrations (Fig. 2B), indicating that CthTTM catalyzed the hydrolysis of ATP to ADP and P_i.

ATP hydrolysis by CthTTM was optimal at pH 8.0–8.5 in Tris buffer and declined sharply as the pH was increased to 9.5 or decreased to ≈7.0 (Fig. 3A). No ATP hydrolysis was evident in the absence of a divalent cation (Fig. 2A). ATPase activity increased with manganese concentration up to an optimum of 2.5 mM and declined as the concentration was increased to 5, 10, and 20 mM (Fig. 3B). Other divalent cations (magnesium, cobalt, nickel, cadmium, calcium, copper, and zinc) were unable to support ATPase activity at 2 mM concentration (data not shown). CthTTM activity displayed a hyperbolic dependence on ATP concentration (Fig. 3C). From a double reciprocal plot, we calculated a K_m value of 26 μM ATP and a K_cat of 25 min⁻¹. NTP specificity was examined by colorimetric assay of the release of P_i from unlabeled ribonucleotides ATP, GTP, CTP, or UTP and deoxynucleotides dATP, dGTP, dCTP, and dTTP. CthTTM was active with all eight nucleoside triphosphates (Fig. 3D).

**Hydrolysis of Inorganic Tripolyphosphate Exceeds NTP Hydrolysis**—CthTTM displayed vigorous activity in releasing P_i from PPP, in the presence of a divalent cation cofactor, which could be either magnesium or manganese (Fig. 4A). The extent of P_i formation was proportional to CthTTM concentration and saturated at a level corresponding to 1 mol of P_i/mol of input PPP, substrate (Fig. 4A) and data not available.
Polyphosphatase activity with magnesium or manganese was indicated to hydrolysis of the nucleoside-linked. The specific activity in magnesium-dependent tripolyphosphate containing substrates, whether inorganic or nucleotide specificity. Reaction mixtures (50 µl) containing 50 mM Tris-HCl, pH 8.0, 2 mM MnCl₂, 30 nM CthTTM, and [γ-32P]ATP as specified were incubated for 15 min at 37 °C. D, nucleotide specificity. Reaction mixtures (50 µl) containing 50 mM Tris-HCl, pH 8.0, 2 mM MnCl₂, 100 µM NTP, or dNTP as specified, and 3.5 µM CthTTM were incubated for 30 min at 37 °C. Each datum in the bar graph is the average of two separate experiments. S.E. bars are shown.

CthTTM failed to release Pi from inorganic pyrophosphate (PPi) in the presence of magnesium or manganese, even at enzyme concentrations sufficient for maximal Pi release from triphosphosphate (Fig. 4A). We conclude that CthTTM converts PPI, to PPi, plus Pi. Thus, CthTTM is dedicated to hydrolysis of the β-γ phosphoanhydride linkage of triphosphate-containing substrates, whether inorganic or nucleoside-linked. The specific activity in magnesium-dependent triphosphosphate cleavage corresponded to a turnover number of ~3800 min⁻¹. This value is 150-fold higher than CthTTM ATPase activity.

When various metals were tested at 2 mM concentration, magnesium supported the highest triphosphatase activity, followed by manganese and cobalt, which were about one-half and one-fifth as effective as magnesium, respectively (Fig. 4B). Nickel, copper, calcium, cadmium, and zinc were ineffective (Fig. 4B). Magnesium supported optimal activity at 10–20 mM, whereas manganese-dependent activity was optimum at 0.31–0.62 mM and declined at higher concentrations (Fig. 4C). Triphosphatase activity with magnesium or manganese was optimal at pH 9.0–9.5 in Tris buffer (Fig. 4D). Activity was virtually nil at pH ≤ 6.5 (Fig. 4D).

CthTTM Is a Homodimer—The quaternary structure of CthTTM was gauged by zonal velocity sedimentation through a 15–30% glycerol gradient. Marker protein catalase (248 kDa), bovine serum albumin (66 kDa), and cytochrome c (13 kDa) were included as internal standards. CthTTM sedimented as a discrete peak in fractions 20–22 overlapping the “light” side of the bovine serum albumin peak (Fig. 5). A plot of the S values of the three standards versus fraction number yielded a straight line (data not shown). An S value of 3.6 was determined for CthTTM protein Cet1 (18, 24).
Structure-guided Alanine Scan of the CthTTM Tunnel—Fourteen conserved residues projecting into the tunnel were targeted for alanine scanning: Glu-4, Glu-6, and Lys-8 in /H9252; Arg-39 and Arg-41 in /H9252; Lys-52 in /H9252; Glu-62 and Glu-64 in /H9252; Lys-87 and Arg-89 in /H9252; Glu-100 and Asp-102 in /H9252; and Glu-115 and Glu-117 in /H9252 (Fig. 1).

CthTTM and the CthTTM-Ala proteins were produced in E. coli as His10 fusions and purified from soluble bacterial extracts by nickel-agarose chromatography (Fig. 6A). The His10-CthTTM polypeptide (calculated mass 20.5 kDa) migrated anomalously “heavy” during SDS-PAGE (Fig. 6A, middle panel). Tripolyphosphatase reaction mixtures (50 μl) containing 50 mM Tris-HCl, pH 9.0, 10 mM MgCl2, 100 μM PPPi, and 1 μl of a 1:20 dilution of the indicated gradient fractions were incubated for 30 min at 37 °C (bottom panel).

Several distinct classes of mutational effects were observed. Nine of the mutants were severely defective, with ATPase specific activities <1% of the wild-type value. These were E6A, R39A, R41A, K52A, E62A, K87A, R89A, D102A, and E117A. Three other mutants, E4A, E64A, and E115A, were 3% as active as wild-type CthTTM. Our operational definition of an essential residue is one at which side...
chain removal by alanine substitution reduces activity to ≤5% of wild-type CthTTM. By this criterion, Glu-4, Glu-6, Arg-39, Arg-41, Lys-52, Glu-62, Glu-64, Lys-87, Arg-89, Asp-102, Glu-115, and Glu-117 are essential residues and likely constituents of the phosphohydrolase active site. The E100A change had only a modest effect (22% of wild-type ATPase specific activity). Thus, Glu-100 was not essential for ATP hydrolysis.

The remarkable finding was that the K8A change elicited a ~16-fold increase in ATPase specific activity compared with wild-type CthTTM (Fig. 6B). We observed this effect with two independent preparations of the K8A protein. A substrate titration experiment (not shown) revealed that K8A resulted in a 21-fold increase in k_cat (530 min⁻¹) and a 3-fold increase in K_m (75 μM) relative to wild-type CthTTM. The gain-of-function effect can be rationalized by reference to the corresponding β1 lysine in NeuTTM (Fig. 1B), which forms a hydrogen bond to the main chain carbonyl at the break in the C-terminal helix that we presume impedes the adoption of the closed tunnel architecture seen in Cet1. The twelve side chains essential for CthTTM triphosphatase activity are splayed apart in the C-shaped cup conformation of NeuTTM compared with their positions in the closed Cet1 tunnel, which implies that the CthTTM β barrel must undergo a conformational change to trigger catalysis. We postulate that loss of the inhibitory hydrogen bond in the K8A protein favors the adoption of a catalytically competent tunnel conformation.

**Effects of Alanine Mutations on Triphosphatase Activity**—All 14 of the alanine mutations abolished or severely diminished magnesium-dependent triphosphatase activity (Fig. 7A). However, the triphosphatase activity of mutants E100A and D102A was restored (to 46 and 96% of wild-type, respectively) when manganese replaced magnesium as the metal cofactor (Fig. 7B). The relatively benign effect of the E100A change on the manganese-dependent triphosphatase was consistent with its modest effects on the manganese-dependent ATPase (Fig. 6B). The notable finding was that Asp-102 was essential for manganese-dependent ATPase, yet dispensable for manganese-dependent triphosphatase. Thus, the exact constellation of functional groups required for catalysis by CthTTM depends on the nature of the metal cofactor and the triphosphatase substrate. Perhaps the most striking instance of substrate dependence was the finding that the K8A change stimulated ATP hydrolysis but suppressed the hydrolysis of triphosphophosphate (Figs. 6B and 7). We speculate that Lys-8 makes a key ionic contact with the α phosphate dianion of PPP, that is not applicable to ATP, because its α phosphate is a monoanion.

**Probing Structure-Activity Relationships via Conservative Substitutions**—Twenty-one conservative substitutions were introduced at 12 of the residues defined as essential in the alanine scan: Glu-4, Glu-6, Arg-39, Arg-41, Lys-52, Glu-62, Glu-64, Lys-87, Arg-89, Asp-102, Glu-115, and Glu-117 (Fig. 8). As noted in the alanine scan, the magnesium-dependent triphosphatase activity was more sensitive to inactivation by conservative mutations than were either of the manganese-dependent hydrolytic reactions (Fig. 8). Based on the crystal structure of the Cet1-Mn⁴⁺ complex (18) and their clustering close together in the NeuTTM structure (Fig. 1B), Glu-4, Glu-6, Glu-115, and Glu-117 are likely metal-binding residues. These four glutamates were strictly essential for Mg⁴⁺-PPPase, insofar as their replacement with either aspartate or glutamine eliminated activity. We surmise that the carboxylate moiety is critical (i.e. the isosteric glutamine was ineffective) and that activity requires a critical distance from the carboxylate to the main chain of the tunnel strand that is not satisfied by the shorter Asp side chain. Yet, several of the conservative substitutions for these glutamates elicited significant gains of triphosphatase function (relative to the Ala mutants) in the presence of manganese. For example, the E6D change restored Mn⁴⁺-PPPase activity to a near wild-type level, whereas the E6Q change had no salutary effect (Fig. 8). Thus, CthTTM tolerates the shorter Asp side chain for triphosphatase hydrolysis with manganese as the cofactor. A plausible interpretation of this result is that Glu-6 directly coordinates the metal ion as part of an octahedral complex (as is the case for the equivalent glutamate of Cet1) and that the larger atomic radius of manganese (0.65 Å) versus magnesium (0.65 Å) allows Asp-6 to reach and engage a manganese coordination complex but not a magnesium complex. A similar ablation of magnesium-dependent phosphohydrolase activity, although preserving manganese-driven catalysis, was noted previously for a Glu→Asp mutation of baculovirus RNA triphosphatase (13), which is a
The putative member of the TTM family. The striking finding here is that the E6D change did not restore manganese-dependent ATPase activity (Fig. 8); this is another instance in which the enzymic requirements for catalysis by CthTTM differ for nucleoside versus inorganic triphosphate substrates.

The Cet1 equivalent of CthTTM Glu-4 coordinates the metal cofactor directly. Changing Glu-4 to either Asp or Gln resulted in restoration of Mn$^{2+}$-PPiPase activity to ~20% of wild-type (Fig. 8), a gain of function relative to the grossly defective E4A mutant (Fig. 7). Apparently, the role of this side chain in the Mn$^{2+}$-PPi complex can be fulfilled, in part, by the amide of Gln or the carboxylate of Asp. The Cet1 analog of CthTTM Glu-115 makes a water-mediated contact to the divalent cation (18). Replacing Glu-115 with Asp or Gln afforded a modest increase in Mn$^{2+}$-PPiPase activity (25 and 35% of wild-type, respectively) compared with the E115A mutant (Fig. 8). We speculate that CthTTM has residual activity when Glu-115 is mutated, because a water can still join the Mn$^{2+}$-PPi complex. The Cet1 equivalent of CthTTM Glu-117 coordinates the divalent cation directly. Here we find that the conservative E117D mutant was globally defective in triphosphate hydrolysis, implying a stringent requirement for the longer main chain-carboxylate linker. (We did not test the effects of a glutamine substitution for Glu-117.)

The essential positively charged residues Arg-39, Arg-41, Lys-52, Lys-87, and Arg-89 are good candidates to contact the anionic phosphates of the PPPi and NTP substrates, as suggested by the interactions of several of the equivalent side chains with a sulfate in the Cet1 tunnel (18). In CthTTM, Arg-39 in strand $\beta$ is strictly essential for all phosphohydrolase activities, insofar as replacement with lysine or glutamine phenocopied the global catalytic defect of the alanine mutant (Fig. 8). We speculate that Arg-39 makes an essential bidentate interaction via its guanidinium nitrogens that cannot be fulfilled by lysine. At CthTTM residue Lys-87 in strand $\beta$6, the glutamine mutant was globally defective (Fig. 8), indicating that positive charge is critical at this position (Fig. 8).
Arg-89 in strand β6 is strictly essential for Mg$^{2+}$-PPPase and Mn$^{2+}$-ATPase (neither lysine nor glutamine supported activity), but it could be functionally replaced by lysine (although not glutamine) for manganese-dependent triphosphosphate hydrolysis (Fig. 8). In the NeuTTM structure (Fig. 1B), the equivalent of CthTTM Arg-89 engages in a bidentate salt bridge with Asp-102. It is noteworthy that the strict essentiality of Asp-102 for Mg$^{2+}$-PPPase and Mn$^{2+}$-ATPase (neither glutamate nor asparagine sufficed) correlated with the requirement for its Arg-89 interaction partner, whereas the lack of a requirement for Asp-102 for Mn$^{2+}$-PPPase activity (alanine, glutamate, and asparagine mutants were active) correlated with the ability of lysine to replace Arg-89 for Mn$^{2+}$-PPPase. These data suggest that the salt bridge is required to properly orient Arg-89 for catalysis of Mg$^{2+}$-PPPase and Mn$^{2+}$-ATPase, whereas lysine is able to engage the Mn$^{2+}$-PPPase complex without forming an ion pair with another side chain.

According to the homologous NeuTTM structure, CthTTM Arg-41 in strand β3 makes a bifurcated contact from its terminal guanidinium nitrogens to the Oє atom of Glu-64 in strand β5. Although replacing Arg-41 with glutamine abolished activity and phenocopied the R41A mutant, we observed metal-specific effects of the R41K substitution whereby the lysine failed to support Mn$^{2+}$-PPPase but resulted in a partial restoration of function for Mn$^{2+}$-PPPase (Fig. 8). Conservative changes at the Arg-41-interacting Glu-64 position had either mild (Mn$^{2+}$-PPPase) or moderate (Mg$^{2+}$-PPPase, Mn$^{2+}$-ATPase) impact on activity, with glutamine being slightly superior to aspartate (Fig. 8).

Lys-52 in strand β4 could not be replaced by arginine for any of the activities tested (Fig. 8), perhaps because the bulkier arginine creates a steric clash. (We did not test the effects of glutamine at position 52). Lys-52 makes an ionic interaction with Glu-62 in strand β5. It is notable that Glu-62 could be replaced by glutamine with little impact on Mn$^{2+}$-PPPase, whereas aspartate was detrimental (Fig. 8). E62Q also supported residual levels of Mg$^{2+}$-PPPase and Mn$^{2+}$-ATPase activity (Fig. 8). These results indicate that, although a Glu at position 62 provided optimal activity, the relevant property of this residue was probably its capacity to accept a hydrogen bond.

**DISCUSSION**

We found that CthTTM is a metal-dependent triphosphatase and nucleoside triphosphatase. It was most assuredly not an adenylate cyclase in our hands, notwithstanding that it, and virtually every other TTM homolog in the NCBI data base, is annotated as an adenylate cyclase in the absence of corroborating functional data. Our survey of various substrates, although not exhaustive, established the chemical specificity of CthTTM for hydrolysis of the β-γ phosphoanhydride linkage of triphosphate-containing substrates. CthTTM displayed little or no discrimination of the sugar or base components of nucleoside triphosphate substrates, which were hydrolyzed to form a nucleoside diphosphate and P$_i$. The key finding was that CthTTM was at least two orders of magnitude more active in cleaving triphosphatase than ATP. This strong preference for an inorganic triphosphate is unprecedented among TTM proteins studied previously. For example, *T. brucei* Cet1 can hydrolyze PPP, but only 0.5% as well as it hydrolyzes ATP (9).

The ensemble of side chains important for CthTTM triphosphatase and ATPase activity, irrespective of metal cofactor choice, consists of 11 amino acids: Glu-4, Glu-6, Arg-39, Arg-41, Lys-52, Glu-62, Lys-87, Arg-89, Glu-115, and Glu-117. We discussed above the likely contributions of several of these side chains to metal and triphosphate binding based on structural and functional studies of other TTM proteins and the homologous NeuTTM crystal structure. A fuller appreciation of their catalytic contributions will hinge on obtaining crystal structures of this and other TTM proteins with genuine triphosphate substrates bound in the active site.

It is most remarkable that the substrate specificity of CthTTM can be transformed by single missense mutation. The K8A change virtually abolished triphosphatase activity while strongly stimulating the hydrolysis of ATP. This result highlights the plasticity of the CthTTM substrate choice and has implications for the rapid acquisition of novel specificities within the TTM superfamily through changes in the residues that line the tunnel walls. We discussed above the likely roles of tunnel conformational equilibria and substrate-specific contacts in mediating the changes in activity accompanying the K8A mutation. It is worth pointing out that the equivalent lysine→alanine change in the β1 strand of yeast Cet1 had no effect on ATPase or RNA triphosphatase activity (3), which is sensible, because Cet1 has no equivalent of the “inhibitory” C-terminal helix of NeuTTM that clogs one of the ends of the tunnel. Our studies of CthTTM emphasize that nothing can be taken for granted about the activity of any particular TTM protein, be it substrate specificity or structure-activity relationships, which need to be probed directly for each TTM family member of interest.

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