Polyphosphatase Activity of CthTTM, a Bacterial Triphosphate Tunnel Metalloenzyme*

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Triphosphate tunnel metalloenzymes (TTMs) are a superfamily of phosphotransferases with a distinctive active site located within an eight-stranded β barrel. The best understood family members are the eukaryal RNA phosphatases, which catalyze the initial step in mRNA capping. The RNA phosphatases characteristically hydrolyze nucleoside 5’-triphosphates in the presence of manganese and are inept at cleaving inorganic triphosphate. We recently identified a TTM protein from the bacterium Clostridium thermocellum (CthTTM) with the opposite substrate preference. Here we report that CthTTM catalyzes hydrolysis of guanosine 5’-tetraphosphate to yield GTP and P\textsubscript{i} (K\textsubscript{m} = 70 μM, k\textsubscript{cat} = 170 s\textsuperscript{-1}) much more effectively than it converts GTP to GDP and P\textsubscript{i} (K\textsubscript{m} = 70 μM, k\textsubscript{cat} = 0.3 s\textsuperscript{-1}), implying that a nucleoside interferes when positioned too close to the tunnel entrance. CthTTM is capable of quantitatively cleaving diadenosine hexaphosphate but has feeble activity with shorter derivatives diadenosine tetraphosphate and diadenosine pentaphosphate. We propose that the tunnel opens to accommodate the dumbbell-shaped diadenosine hexaphosphate and then closes around it to perform catalysis. We find that CthTTM can exhaustively hydrolyze a long-chain inorganic polyphosphate, a molecule that plays important roles in bacterial physiology. CthTTM differs from other known polyphosphatases in that it yields a 2:1 mixture of P\textsubscript{i} and PP\textsubscript{i} end products. Bacterial/archaeal TTM activities have a C-terminal helix located near the tunnel entrance. Deletion of this helix from CthTTM exerts pleiotropic effects. (i) It suppresses hydrolysis of guanosine 5’-tetraphosphate and inorganic PP\textsubscript{i}; (ii) it stimulates NTP hydrolysis; and (iii) it biases the outcome of the long-chain polyphosphatase reaction more strongly in favor of P\textsubscript{i} production. We discuss models for substrate binding in the triphosphate tunnel.

The RNA triphosphatase components of the mRNA capping systems of fungi, protozoa, and several DNA viruses are the founding members of the triphosphate tunnel metalloenzyme (TTM)\textsuperscript{2} superfamily of phosphohydrolases (1–21). The signature biochemical property of this branch of the TTM superfamily is the ability to hydrolyze NTPs to NDPs and P\textsubscript{i} in the presence of manganese. The crystal structure of the Saccharomyces cerevisiae RNA phosphatase Cet1 bound to manganese and sulfate (a proposed mimic of the product complex with phosphate) revealed what was then a novel tertiary structure in which the active site is situated within a topologically closed tunnel composed of eight antiparallel β strands (2). The Cet1-like TTM fold is conserved in the crystal structure of mimivirus RNA triphosphatase, which has an acetate anion bound in its tunnel (21). A plausible mechanism has been proposed whereby Cet1-like proteins catalyze direct attack of a nucleophilic water on the γ-phosphorus of NTP or pppRNA substrates (4). The putative water nucleophile is oriented by an essential glutamate contributed by the fifth β strand of the tunnel wall. A proposed pentacoordinate transition state is stabilized by interactions of the γ-phosphate oxygens with the divalent cation and a layer of essential lysine and arginine side chains that emanate from the third and sixth β strands of the tunnel walls. The octahedral metal coordination complex is formed by a set of essential conserved glutamates from strands β1 and β8 and an aspartate from β7 that are clustered on the floor of the tunnel.

The tertiary structure and active site architecture of yeast and mimivirus RNA triphosphatases are recapitulated in the crystal structures of several archaeal and bacterial proteins, of mostly unknown biochemical function, that have been solved as a part of structural genomics initiatives; these include proteins from Pyrococcus (Protein Data Bank codes 1YEM, 2DC4, and 2EEN), Vibrio (2ACA), Nitrosomonas (2FBL), and Yersinia (2FIT) (3, 22). Primary structure similarity searches against these proteins readily identify a large bacterial/archaeal TTM clade widely distributed among taxa (23). Given that bacterial and archaean RNAs do not have 5’ caps, it is reasonable to think that members of this clade might play novel and diverse roles in prokaryotic physiology, to which insights might be gained by examining the activity and substrate specificity of exemplary family members. This theme is underscored by the identification and structural characterization of a mammalian enzyme, thiamine triphosphatase, as a TTM protein with extreme specificity for metal-dependent hydrolysis of thiamine triphosphate to thiamine diphosphate and P\textsubscript{i} (Protein Data Bank codes 3BH4 and 2JMU) (24, 25).

Here we focus our attention on Clostridium thermocellum TTM (CthTTM), the first bacterial TTM-type phosphohydrolase.

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2. The abbreviations used are: TTM, triphosphate tunnel metalloenzyme; CthTTM, C. thermocellum TTM; NeuTTM, N. europea TTM; PhoTTM, P. horikoshii TTM; Gpppp, guanosine 5’-tetraphosphate; ApppppA, P\textsuperscript{1},P\textsuperscript{2},P\textsuperscript{3},P\textsuperscript{4},P\textsuperscript{5}-di(adenosine-5’)-tetraphosphate; ApppppA, P\textsuperscript{1},P\textsuperscript{2},P\textsuperscript{3},P\textsuperscript{4},P\textsuperscript{5}-di(adenosine-5’)-hexaphosphate; CIP, calf intestine phosphatase; PPK, polyphosphate kinase; PEI, polyethyleneimine.
lase to be subjected to detailed biochemical and structure-function analysis (26). Purified recombinant CthTTM is a vigorous inorganic triphosphatase that converts PPP, to PPI plus P₄ in the presence of manganese or magnesium. In fact, CthTTM is 2 orders of magnitude more active in cleaving PPP, than it is in hydrolyzing the β-γ phosphoanhydride of ATP (26). By contrast, the Cet1-like RNA triphosphatases are much more active with ATP than PPP. Indeed, PPP, is a potent competitive inhibitor of their ATPase activity (11, 14). We are interested in understanding the basis for the distinctive substrate preference of CthTTM and have been guided in this effort by crystal structures of homologous TTM proteins.

The primary structure of the 156-amino acid CthTTM protein is aligned in Fig. 1A to two related prokaryotic TTM proteins, from *Nitrosomonas europaea* (NeuTTM; 151 amino acids) and *Pyrococcus horikoshii* (PhoTTM; 165 amino acids), for which crystal structures have been solved (Fig. 1B), albeit without any functional characterization. As expected, the positions of side chain identity/similarity in the three proteins (indicated by dots) are located within the eight β strands that comprise the triphosphate tunnel of *Pho*TTM (Fig. 1A). The NeuTTM fold is distinguished by the fact that its antiparallel β barrel is not a closed tunnel, as in *Pho*TTM, but rather an open C-shaped “cup” that is prevented from closing by virtue of the insertion of a C-terminal helix into one end of the tunnel (Fig. 1B). In the open state, the essential amino acid side chains that comprise the active site are displaced from the catalytically active arrangement seen in the Cet1-Mn²⁺/SO₄²⁻ complex (2). We speculated previously that binding of the polyanionic substrate to the open conformation of the bacterial/archaeal-type TTM apoenzyme might trigger closure of the tunnel and the formation of a proper Michaelis complex (26). Similar inferences regarding substrate-induced conformational changes have been drawn for mammalian thiamine triphosphatase (25), based on documentation of alternative atomic structures analogous to the open and closed states depicted in Fig. 1B. By contrast, Cet1 has a topologically closed “O-shaped” tunnel that cannot open, because the crossover strands of the Cet1 tunnel penetrate into a globular pedestal domain upon which the tunnel rests (2). Unfortunately, because no TTM protein structure has been solved with a true substrate (NTP, RNA, or thiamine triphosphate) bound at the active site, we cannot confidently know the orientation of the substrate and the site of asymmetric hydrolysis of the phosphoanhydride linkage. Nevertheless, many TTM structures have been solved with anionic ligands in the tunnel (e.g. sulfate, phosphate, citrate, acetate, or chloride).

In this respect, it is striking that the closed tunnel conformation in the *Pho*TTM structure correlates with the presence of a linear array of three chloride anions in the tunnel cavity (Fig. 1B), which are putative mimetics of three phosphates of the enzyme’s substrate. A detailed stereo view of the *Pho*TTM tunnel is shown in Fig. 1C (albeit with the conserved amino acids of the tunnel numbered according to their positions in CthTTM). The tunnel is oriented in Fig. 1 so that the chloride anion closest to the viewer corresponds to the sulfate in Cet1; thus, we regard this atom as a potential analog of the γ-phosphate and, by extension, the two other chlorides serially deeper in the tunnel as rough analogs of the β- and α-phosphates, respectively. Many of the conserved polar side chains surrounding the chlorides have been shown by mutational analysis to be essential for CthTTM triphosphatase activity (26).

It is critical to note that the C-terminal α helix of PhoTTM sterically occludes the back opening of the tunnel even after closure has occurred (Fig. 1, B and C). This C-terminal “plug” helix is a conserved feature of all of the structurally characterized bacterial and archaeal TTM proteins as well as mammalian thiamine triphosphatase. By contrast, a plug helix is conspicuously absent from yeast and mimivirus RNA triphosphatases, which have unobstructed “entrance” and “exit” sides to their tunnels (2, 21). The plug helix in PhoTTM blocks the side corresponding to the putative entrance to the Cet1 tunnel; the open side closer to the viewer in Fig. 1C corresponds to the putative exit side of the Cet1 tunnel. We envision that the plug helix must move away from the tunnel entrance in order for an NTP to bind productively and for efficient hydrolysis and product release. This idea was supported by the initial finding that an alanine mutation of CthTTM residue Lys⁸, which in PhoTTM tethers the plug helix to the tunnel opening via a hydrogen bond to the main chain of the plug helix (Fig. 1C), stimulated the ATPase activity of CthTTM (26).

Here we delve further into the catalytic repertoire of CthTTM by studying its ability to cleave longer nucleotide 5’-polyphosphate and inorganic polyphosphate substrates. Our results suggest that opening of the tunnel is relevant to productive substrate binding, and they underscore the role of the C-terminal helix as a substrate filter.

**EXPERIMENTAL PROCEDURES**

**Materials**—Guanosine 5’-tetraphosphate (Gpppp), P₄,P₄-di(adenosine-5’) tetraphosphate (AppppA), P₅,P₅-di(adenosine-5’) pentaphosphate (ApppppA), P₆,P₆-di(adenosine-5’) hexaphosphate (AppppppA), and *Crotalus adamanteus* nucleotide pyrophosphatase were purchased from Sigma. [γ-³²P]ATP (3000 Ci/mmol) was obtained from PerkinElmer Life Sciences. Calf intestine phosphatase (CIP) was purchased from Roche Applied Science. Ni²⁺-nitrilotriacetic acid-agarose TLC plates were from Baker.

**Recombinant CthTTM**—Full-length CthTTM and the C-terminal truncation mutant CthTTM-(1–137) were produced in *Escherichia coli* BL21(DE3) as N-terminal His₁₀-tagged fusions and purified from soluble bacterial extracts by sequential Ni²⁺-agarose and DEAE-Sephadex column chromatography steps, as described previously (26). The protein concentrations were determined by using the Bio-Rad dye reagent with bovine serum albumin as the standard. The yields of CthTTM and CthTTM-(1–137) from a 250-ml bacterial culture were 11 and 9 mg, respectively. The protein preparations were stored at −80 °C.

**Recombinant Polyphosphate Kinase (PPK)**—The *E. coli* ppp open reading frame was amplified by PCR from genomic DNA with primers that introduced an NdeI site at the start codon and a BamHI site immediately after the stop codon. The *ppk* gene was inserted into pET16B so as to fuse the coding sequence to a
medium containing 0.1 mg/ml ampicillin until the $A_{600}$ reached ~0.6, at which time the culture was adjusted to 2% ethanol and 0.2 mM isopropyl-β-D-thiogalactopyranoside. The culture was then incubated at 17 °C for 16 h with continuous shaking. 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 50 ml of buffer C (50 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 10% glycerol, 0.05% Triton X-100) and then eluted stepwise with 2-ml aliquots of 50, 100, 200, and 500 mM imidazole in buffer A. The polypeptide compositions of the column fractions were monitored by SDS-PAGE. The recombinant His$_{10}$-PPK polypeptide was recovered predominantly in the 500 mM imidazole fraction, which was then dialyzed against 200 mM NaCl in buffer B (50 mM Tris-HCl, pH 8.0, 0.25 M NaCl, 10% glycerol, 0.05% Triton X-100). The yield was 0.2 mg of PPK from a 500-ml bacterial culture. The PPK preparation was stored at ~80 °C.

Preparation of $[^{32}P]$ Polyphosphate—A reaction mixture (0.5 ml) containing 50 mM Tris-HCl (pH 7.5), 40 mM (NH$_4$)$_2$SO$_4$, 10 mM MgCl$_2$, 4 mM ATP, 100 μCi of [$γ$-$^{32}$P]ATP, and 18 pmol of E. coli PPK was incubated at 37 °C for 45 min. The reaction was quenched by adding 50 μl of 0.5 M EDTA. Conversion of ATP to polyphosphate

5' leader encoding a His$_{10}$ tag. The pET-PPK plasmid was transformed into E. coli BL21(DE3). A 500-ml culture derived from a single transformant was grown at 37 °C in Luria-Bertani broth overnight. The following day, it was further grown at 30 °C for 6 h before being induced with 0.25 mM isopropyl-β-D-thiogalactopyranoside. The culture was then incubated at 17 °C for 16 h with continuous shaking. 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 50 ml of buffer C (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 10% sucrose). Lysozyme, phenylmethylsulfonyl fluoride, and Triton X-100 were added to final concentrations of 1 mg/ml, 0.2 mM, and 0.1%, respectively. The lysate was sonicated to reduce viscosity, and the insoluble material was removed by centrifugation. The soluble extract was applied to a 5-ml column of Ni$_2$-nitrilotriacetic acid-agarose (Qiagen) that had been equilibrated with buffer A (50 mM Tris-HCl, pH 8.0, 0.25 M NaCl, 10% glycerol, 0.05% Triton X-100). The column was washed with 15 ml of the same buffer and then eluted stepwise with 2-ml aliquots of 50, 100, 200, and 500 mM imidazole in buffer A. The polypeptide compositions of the column fractions were monitored by SDS-PAGE. The recombinant His$_{10}$-PPK polypeptide was recovered predominantly in the 500 mM imidazole fraction, which was then dialyzed against 200 mM NaCl in buffer B (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.05% Triton X-100). The yield was 0.2 mg of PPK from a 500-ml bacterial culture. The PPK preparation was stored at ~80 °C.
from the polymeric PPK reaction product that remained at the origin. Approximately 20% of input [γ-32P]ATP was converted to polyphosphate via this procedure. The [32P]polyphosphate was purified as described by Keasling et al. (27), with some modifications. The quenched reaction mixture was extracted once with an equal volume of phenol-CHCl3 and then twice with CHCl3. Isopropyl alcohol (0.7 volume) was then added to the aqueous phase, and the solution was left at room temperature for 1 h. The mixture was microcentrifuged at 15,000 rpm, and the supernatant was discarded. Cold 100% ethanol was added to the polyphosphate-containing precipitate, and the tube was kept on dry ice for 30 min. This was followed by centrifugation at 4 °C at 15,000 rpm. The supernatant was discarded and the precipitate was rinsed with cold 70% ethanol. After repeat centrifugation and removal of the ethanol supernatant, the precipitated [32P]polyphosphate was dried and then dissolved in water. PEI-cellulose TLC confirmed that all free radiolabeled ATP had been removed. The total phosphate concentration of the polyphosphate preparation was determined by digestion with CIP, which quantitatively liberated the radiolabel as 32P1, as verified by TLC. The phosphate content of the CIP hydrolysate was determined colorimetrically with the malachite green reagent. The concentrated [32P]polyphosphate preparation (11.7 mM phosphate) was stored at −20 °C and diluted prior to use in polyphosphatase assays.

RESULTS

Nucleoside Tetraphosphatase Activity of CthTTM—Prompted by our initial finding that CthTTM was ~150-fold more active with PPP, than ATP (26), we speculated that the nucleoside moiety might sterically hinder productive substrate binding in the active site. This hypothesis engenders a prediction that CthTTM should regain activity if the interfering nucleoside moiety were shifted away from the terminal phosphonohydride linkage that undergoes enzymatic hydrolysis. Thus, we queried whether CthTTM could catalyze phosphate release from a nucleoside 5′-tetraphosphate substrate, Gpppp. We found that CthTTM did cleave 100 μM Gpppp in the presence of 200 μM manganese and that the extent of P1 release as a function of input enzyme was acutely biphasic (Fig. 2A). P1 release was steeply proportional to the amount of enzyme added up to 1 ng of CthTTM, at which point the molar yield of phosphate was equal to the initial amount of Gpppp substrate. Further phosphate release displayed a much shallower dependence on increasing CthTTM from 50 to 400 ng. The specific activities calculated from the slope of the steep and shallow phases corresponded to turnover numbers of 150 and 0.07 s−1, respectively. We ascribe the initial steep phase to efficient and quantitative conversion of Gpppp to GTP and P1 at low enzyme concentrations, followed by a shallow phase of inefficient conversion of GTP to GDP and P1 at higher enzyme concentrations. Indeed, the enzyme dependence of P1 release from 100 μM GTP substrate under the same reaction conditions was 0.06 s−1 (Fig. 2A), a value virtually identical to that of the shallow phase of the Gpppp titration profile. Thus, CthTTM is 2100-fold more active on a nucleoside 5′-tetraphosphate than on a standard NTP under these conditions.

A different profile of enzyme dependence was observed when CthTTM was reacted with 100 μM Gpppp in the presence of magnesium (Fig. 2B). Here, P1 release was monophasic and proportional to enzyme (estimated turnover number 70 s−1). The reaction attained an insurmountable plateau when 1 mol of phosphate was released/mol of input Gpppp substrate, presumably corresponding to quantitative conversion of Gpppp to a GTP product. To verify this interpretation, the fate of the Gpppp nucleotide moiety in the presence of CthTTM and magnesium was monitored by PEI-cellulose TLC in buffer containing 1.25 M LiCl, 1 M HCOOH. This procedure revealed complete conversion of Gpppp to GTP, with no detectable GDP formation (not shown). Moreover, a parallel titration experiment with GTP as the substrate verified that the enzyme was unable to catalyze release of P1 from GTP in the presence of magnesium (Fig. 2B). These results agree with previous findings (26) that CthTTM performs ATP hydrolysis with manganese but not magnesium.

Further characterization of the tetraphosphatase showed that P1 accumulated steadily with time and that the rate of release of P1 from Gpppp was proportional to enzyme concen-
tration with either manganese or magnesium as the cofactor (Fig. 2, C and D). Hydrolysis of Gpppp by CthTTM required divalent metal ion and was optimal in Tris buffer at pH 8.5 with either manganese or magnesium (not shown). Various divalent metal ions were tested at concentrations ranging from 0.1 to 20 mM. As noted above, manganese supported the highest specific activity (150 s⁻¹) at its optimal concentration of 200 μM, whereas magnesium was half as effective (70 s⁻¹) at its optimum concentration of 20 mM. Cobalt supported much lower activity (5 s⁻¹) at its optimum of 200 μM. We detected no triphosphatase activity with calcium, cadmium, copper, nickel, or zinc.

The extent of Pi release in the presence of 0.2 mM manganese displayed a hyperbolic dependence on Gpppp concentration (not shown). From a double-reciprocal plot of the data, we calculated a \( K_m \) of 70 μM Gpppp and a \( k_{cat} \) of 170 s⁻¹. The steady-state kinetic parameters for hydrolysis of GTP to GDP and Pi under optimal reaction conditions (pH 8.0; 2 mM MnCl₂) were as follows: \( K_m = 70 \mu M \) GTP and \( k_{cat} = 0.3 \) s⁻¹. Thus, CthTTM discriminated between the nucleoside tetraphosphate and triphosphate by a factor of 570 at the level of reaction chemistry rather than initial substrate binding.

The kinetic parameters for Gpppp hydrolysis in 20 mM magnesium were as follows: \( K_m = 86 \mu M \) Gpppp and \( k_{cat} = 96 \) s⁻¹. The turnover number for magnesium-dependent Gpppp hydrolysis was similar to the value of 63 s⁻¹ reported for magnesium-dependent hydrolysis of PPP (26). The failure of CthTTM to hydrolyze GTP in the presence of magnesium was at least partly reflective of weak substrate binding, insofar as the addition of excess GTP had only a weak inhibitory effect on magnesium-dependent hydrolysis of Gpppp. Specifically, the extents of Pi release from 0.2 mM Gpppp in the presence of 0.4, 0.6, 0.8, and 1 mM GTP were 98, 90, 85, and 78% of the control reaction lacking GTP (not shown).

**Effects of Alanine Mutations on CthTTM Tetraphosphatase Activity**—We surveyed a collection of alanine mutants of CthTTM (26) for their ability to hydrolyze Gpppp (Fig. 3). Mutants E6A, R39A, R41A, K52A, E62A, K87A, R89A, and E117A were shown previously to have <1% of the specific activity of wild-type CthTTM in manganese-dependent ATP hydrolysis; mutants E4A and E115A were 3% as active as wild-type CthTTM in ATP hydrolysis (26). Here we find that each of these changes, involving amino acids in the tunnel active site (Fig. 1C), had similarly severe effects on the hydrolysis of Gpppp, no matter what the divalent cation cofactor (Fig. 3). E64A, shown previously to have 3% of wild-type ATPase activity and 7% of wild-type triphosphatase activity in the presence of manganese (26), was found here to be 12% (Mn²⁺) or 17% (Mg²⁺) as active as wild-type CthTTM in hydrolyzing guanosine tetraphosphate (Fig. 3). E100A, which was 22 and 40% as active as wild-type CthTTM in manganese-dependent cleavage of ATP and PPP, respectively (26), was seen here to have 20% of wild-type activity in hydrolyzing Gpppp in the presence of manganese (Fig. 3). These concordant results suggest that hydrolysis of nucleoside tri- and tetraphosphates and triphosphate requires the same core constellation of active site functional groups. The E100A mutant, which was reported previously to have lower triphosphatase activity in the presence of manganese (14% of wild type) and magnesium (<1%), was found here to have reduced activity in hydrolyzing Gpppp, again with the activity decrement in manganese (40% of wild type) being less than that in magnesium (9% of wild type) (Fig. 3). The concordant trend of K8A toward lower activity with Gpppp and PPP, contrasts with the 21-fold increase in \( k_{cat} \) for ATP hydrolysis by K8A compared with wild-type CthTTM (26). This disparity underscores that Lys⁸ is a determinant of substrate specificity, simultaneously making positive contributions to Gpppp and PPP, cleavage while suppressing nucleoside triphosphate hydrolysis. Reference to the PhoTTM structure shows that the equivalent of Lys⁸ makes two potentially significant contacts: (i) a hydrogen bond to the main chain of the plug helix and (ii) a
contact to the chloride anion proposed to mimic the α-phosphate of ATP (Fig. 1C). Additional experiments will be presented that address the contributions of the plug helix to the disparate effects of K8A on activity with different substrates and different divalent cations.

**Steric Restrictions on CthTTM Hydrolysis of Dinucleoside Polyphosphates**—The vigorous activity of CthTTM with triphosphate and a nucleoside tetraphosphate, but not nucleoside triphosphates, highlights the hindrance of catalysis by a nucleoside positioned too close to the ss-phosphate phosphohydrolase. In order to probe whether catalysis can occur when both ends of the phosphoanhydride bridge are “blocked,” we reacted CthTTM with a series of dinucleoside 5',5'-polyphosphates: AppppA, AppppA, and AppppppA. These dinucleotides yielded no inorganic phosphate when treated with CIP, an enzyme that requires a “free” (R–O–PO₃)₃ phosphate terminus (Fig. 4, CIP). However, treatment with snake venom nucleotide pyrophosphatase, which cleaves within the polyphosphate bridge, generates adenylates with free R–O–PO₃ ends that are sensitive to CIP digestion, so that the adenylate 5'-phosphates are converted nearly quantitatively to Pᵢ by treatment with nucleotide pyrophosphatase plus CIP (Fig. 4). No phosphate was released by nucleotide pyrophosphatase per se (Fig. 4).

The instructive findings were that CthTTM (added at 8 μM concentration) failed to hydrolyze the tetraphosphate bridge of 250 μM AppppA to form a CIP-sensitive product (Fig. 4A), and it displayed feeble activity in cleaving within the pentaphosphate bridge of AppppppA, such that only 10% of the available nucleotide phosphate was released as Pᵢ in the presence of CIP (Fig. 4B). By contrast, CthTTM did hydrolyze the hexaphosphate linker of AppppppA, resulting in the release of 89% of the available nucleotide phosphate groups as Pᵢ in the presence of CIP (Fig. 4C). Omission of CIP treatment reduced the extent of CthTTM-catalyzed Pᵢ release from AppppppA to a level corresponding to 1.9 nmol of Pᵢ/nmol of input AppppppA (Fig. 4C), indicating that one or more of the adenylate nucleotides formed by incision of the hexaphosphate bridge was itself a substrate for the CthTTM phosphohydrolase. These results highlight that the capacity of CthTTM to cleave a polyphosphate chain with nucleoside-blocked ends is strictly limited by the length of the phosphate linker. Whereas CthTTM cleaved ApppppA in the presence of 5 mM manganese, we detected no activity with this substrate in the presence of 5 mM magnesium or cobalt (not shown). The E6A mutant of CthTTM catalyzed no detectable AppppppA cleavage (not shown).

**Tracking the Outcome of ApppppA Cleavage**—The fate of the 250 μM AppppppA nucleotide during its reaction with 8 μM CthTTM in the presence of manganese was monitored by withdrawing aliquots at various times, quenching the reaction with EDTA, binding the nucleotides to an anion exchange column, and then following their elution profile (by UV absorbance) as the column was developed with a linear salt gradient (Fig. 5). Absent CthTTM, the substrate consisted of a nearly homogeneous UV-absorbing species eluting at high salt that corresponds to diadenosine hexaphosphate; trace amounts of AMP were seen eluting at lower salt concentration (Fig. 5; 0 min). The amount of AppppppA decayed steadily with time, concomitant with the appearance of a novel UV-absorbing product eluting at lower salt that corresponded to ADP. The reaction attained an end point at 75 min, at which time all of the ApppppppA had been converted to ADP; there was no change in the amount of the minor AMP contaminant at any point during the reaction. Low levels of ATP (eluting between ADP and ApppppppA) were detected at 5, 15, and 30 min (Fig. 5). The ATP level declined at 50 min and then disappeared at the 75 min reaction end point. This kinetic profile suggested that ATP might be an intermediate in the conversion of ApppppppA to ADP. In the simplest scheme, ApppppppA is cleaved symmetrically by CthTTM to yield two molecules of ATP, which are then hydrolyzed to the ADP end product. The low abundance of the putative ATP intermediate implies that the rate of ApppppppA hydrolysis must be significantly slower than the rate of ATP hydrolysis. By integrating the areas under the UV absorbance curves, we calculated a turnover number for the consumption of ApppppppA of 0.68 min⁻¹, a value that is indeed much less than the kcat of 25 min⁻¹ for ATP hydrolysis by CthTTM in the presence of manganese (26). Although we did not detect any UV-absorbing material eluting between ATP and ApppppppA that could correspond to ApppppA (Fig. 5), it is possible that the reaction proceeds via an alternative scheme in which ApppppA is cleaved asymmetrically to
yield ADP and Apppp, followed by very rapid conversion of Appppp to ATP and finally slower conversion of ATP to ADP.

It is striking that the observed rate of AppppppA cleavage is 4 orders of magnitude slower that the rate of hydrolysis of a Gpppp or tripolyphosphate. Although steric hindrance by the second nucleoside in AppppppA could account for this disparity, another contributing factor might be differential reactivity of CthTTM at a free terminal phosphate R–O–PO₃⁻ (present in Gpppp and PPPi) versus an internal phosphoanhydride linkage R–O–(PO₂⁻)–O–R (present in AppppppA). Note that CthTTM activity with longer dinucleoside polyphosphate substrates could not be assessed readily, because these molecules are not available from commercial sources.

Inorganic Polyphosphatase Activity of CthTTM—Inorganic polyphosphate is a ubiquitous intracellular macromolecule with an important role in the response of many bacterial species to environmental stress (28, 29). Therefore, we considered the prospect that long-chain polyphosphates might be a physiological substrate for CthTTM. We prepared [32P]polyphosphate via enzymatic synthesis from [6-32P]ATP by E. coli PPK (27). We found that reaction of CthTTM with [32P]polyphosphate in the presence of 10 mM manganese resulted in the release of 32Pi and 32PPi products that could be resolved from the [32P]polyphosphate substrate by PEI-cellulose TLC (Fig. 6A). The identity of the slower migrating product as PPi was verified by treating the

![Figure 5](image1.png) **FIGURE 5. Kinetics of CthTTM reaction with diadenosine hexaphosphate.** A reaction mixture (400 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM MnCl₂, 250 μM AppppppA, and 64 μg of CthTTM (8 μM enzyme) was incubated at 37 °C for 60 min (lane +). CthTTM was omitted from a control reaction mixture (lane −). Aliquots (1 μl) were applied to a PEI-cellulose TLC plate, which was developed in 0.75 M LiCl, 1 M HCOOH. An autoradiograph of the TLC plate is shown. The species corresponding to P₁, PP₁, and polyphosphate (polyP) are indicated on the right. B, a reaction mixture (80 μl) containing 50 mM Tris-HCl (pH 7.5), 10 mM MnCl₂, [32P]polyphosphate (59 μM phosphate groups; ~85 nm polyphosphate, assuming an average chain length of 700 phosphate groups), and CthTTM (3.2 μg; 2 μM enzyme) was incubated at 37 °C. Aliquots (10 μl) were withdrawn at the times specified and quenched immediately with 5 μl of loading buffer (270 mM Tris borate (pH 8.3), 26 mM EDTA, 30% sucrose). The time 0 sample was taken prior to adding CthTTM. The samples were analyzed by electrophoresis through a 40-cm 6% polyacrylamide gel containing 7 M urea in 90 mM Tris borate, 2.7 mM EDTA. The gel was prerun at 750 V for 30 min before loading the samples in the wells. A tracking dye mix containing bromphenol blue, xylene cyanol, and an aliquot of 32P was loaded in a separate well. Electrophoresis was continued at 750 V until the bromphenol blue had migrated ~14 cm into the gel. Radiolabeled material was visualized by autoradiography.
reaction products with yeast inorganic pyrophosphatase, which completely depleted the slower migrating species and elicited a concomitant increase in the level of $^{32}$P$_i$ (not shown).

We also monitored the progress of the polyphosphatase reaction by gel electrophoresis (Fig. 6B). Absent CthTTM, the $^{32}$PP$_i$ polyphosphate substrate migrated as a heterogeneous high molecular weight polymer near the top of the gel. Although we do not have polyphosphate standards to accurately calibrate polymer size, previous studies established that the product of the in vitro synthesis of $^{32}$PP$_i$ polyphosphate by PPK has a chain length of $\approx$700 phosphate groups (30). CthTTM catalyzed a time-dependent decrease in the size of the radiolabeled polyphosphate (reflected in its increased electrophoretic mobility), which was eventually converted into a rapidly migrating end product that corresponded to the mixture of $^{32}$PP$_i$ and $^{32}$P$_i$ observed in the TLC analysis. There is an apparent area of exclusion of the labeled polyphosphate about two-thirds of the way down the gel, with label compression above and below the excluded area. This electrophoretic anomaly for polyphosphates was seen previously by Wurst and Kornberg (31), who indicated that the excluded area corresponds to polyP$_{4-15}$.

To verify that the inorganic polyphosphatase activity is mediated by CthTTM, we subjected the enzyme to zonal velocity sedimentation through a 15–30% glycerol gradient (Fig. 7A). Marker proteins catalase (248 kDa), bovine serum albumin (66 kDa), and cytochrome c (13 kDa) were included as internal standards and were resolved accordingly, as gauged by SDS-PAGE analysis of the gradient fractions. The peak positions for the markers are denoted in Fig. 7A. Aliquots of even-numbered gradient fractions were assayed for hydrolysis of $^{32}$P$_i$ polyphosphate via the TLC method; the yields of $^{32}$P$_i$ and $^{32}$PP$_i$ products are plotted separately in Fig. 7A. The activity profiles for formation of the two products were identical, peaking in fractions 16–18, and they coincided with the sedimentation profile of the CthTTM polypeptide (not shown). We conclude that CthTTM has an inherent inorganic polyphosphatase activity.

Characterization of the CthTTM Polyphosphatase Reaction—Polyphosphatase activity was optimal in Tris-HCl buffer at pH 7.5–8.0. No activity was detectable in the absence of a divalent cation. Manganese supported optimal activity at 10–20 mM concentration; product formation at 2 and 5 mM manganese was 39 and 57% of the value at 10 mM (not shown). Cadmium (10 mM) was 40% as effective as manganese, whereas 10 mM magnesium, cobalt, copper, and nickel were ineffective (not shown). It was apparent that the molar yield of $^{32}$P$_i$ was consistently about twice that of the $^{32}$PP$_i$. To better evaluate this phenomenon, we examined product release from $^{32}$PP$_i$ polyphosphate as a function of time, using a different TLC buffer that allowed us to separate and quantify the $^{32}$PP$_i$, $^{32}$PP$_i$, and $^{32}$P$_i$ species, whereas larger polyphosphates remained at the origin. This experiment revealed simultaneous accumulation of $^{32}$P$_i$ and $^{32}$PP$_i$ products at rates of 0.27 and 0.14 min$^{-1}$, respectively (Fig. 7B). Both products plateaued at 60–90 min, at which time the $^{32}$P$_i$/$^{32}$PP$_i$ ratio was 1.7 (Fig. 7B). This kinetic profile is not consistent with a precursor-product relationship of $^{32}$PP$_i$ to $^{32}$P$_i$. Indeed, we showed previously that CthTTM has no detectable inorganic pyrophosphatase activity (26). A key finding here was that only trace levels of $^{32}$PP$_i$ were detected during the CthTTM polyphosphatase reaction (Fig. 7B). These results suggest that (i) CthTTM can directly liberate either PP$_i$, or P$_i$ from the end of the polyphosphate chain and that it prefers the option of PP$_i$ release to PP$_i$ release by about a factor of 2 (possible explanations for this preference will be discussed) and/or (ii) any $^{32}$PPP$_i$ that was liberated from the end of the polyphosphate chain was rapidly hydrolyzed to $^{32}$PP$_i$ plus $^{32}$P$_i$.

The standard polyphosphate assays in the preceding experiments were performed at a relatively low concentration of $^{32}$PP$_i$ polyphosphate (59 $\mu$M total phosphate groups; $\approx$85 $\mu$M polyphosphate chains, assuming an average chain length of 700 phosphate groups). Whereas the combined yield of the two reaction products ($^{32}$P$_i$ plus $^{32}$PP$_i$) from this amount of $^{32}$PP$_i$ polyphosphate was proportional to the amount of CthTTM added to the reaction (Fig. 7C), the calculated specific activity was only 6.6 pmol of product released per pmol of enzyme during the 60-min reaction. A series of enzyme titrations at increasing concentrations of $^{32}$PP$_i$ polyphosphate over a 64-fold range (from 0.085 to 5.4 $\mu$M polyphosphate chains)

FIGURE 7. Characterization of the polyphosphatase activity of CthTTM. A, velocity sedimentation. A mixture of CthTTM (100 $\mu$g) and internal standards catalase (50 $\mu$g), bovine serum albumin (50 $\mu$g), and cytochrome c (50 $\mu$g) 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 for 17 h at 4 °C at 50,000 rpm in a Beckman SW55Ti rotor. Fractions (0.2 ml) were collected from the bottom of the tube. Aliquots of even-numbered fractions were analyzed by SDS-PAGE and assayed for polyphosphatase activity. Reaction mixtures (10 $\mu$l) containing 50 mM Tris-HCl (pH 7.5), 10 mM MnCl$_2$, $^{32}$PP$_i$ (59 $\mu$M polyphosphate groups), and 2 $\mu$l of the glycerol gradient fractions were incubated at 37 °C for 60 min. The reaction products were analyzed by TLC as described in the legend to Fig. 6A. The $^{32}$P$_i$-labeled products were quantified by scanning the TLC plate with a FUJIX BAS2500 imager. The extents of release of $^{32}$PP$_i$, (A) and $^{32}$Pi, (B) are shown. B, reaction mixtures (80 $\mu$l) containing 50 mM Tris-HCl (pH 7.5), $^{32}$PP$_i$ polyphosphate (59 $\mu$M polyphosphate groups) were incubated for the specified time intervals at 37 °C with 10 mM MnCl$_2$ and 6.4 $\mu$g of CthTTM. Aliquots (10 $\mu$l) were withdrawn at the times specified and quenched immediately with EDTA. The amounts of $^{32}$PP$_i$, (gray circles), $^{32}$Pi, (black circles), and $^{32}$P$_i$ (gray triangles) formed (per 10 $\mu$l of reaction mixture) were determined by PEI-cellulose TLC in 1.25 M LiCl, 1 M HCOOH. C, reaction mixtures (10 $\mu$l) containing 50 mM Tris-HCl (pH 7.5); 10 mM MnCl$_2$; either 50 $\mu$M (●), 240 $\mu$M (■), 940 $\mu$M (○), or 3800 $\mu$M (▲) $^{32}$PP$_i$ polyphosphate (as total phosphate concentration; corresponding to $\approx$0.085, 0.34, 1.3, and 5.4 $\mu$M polyphosphate chains); and the specified amounts of CthTTM were incubated at 37 °C for 60 min. The extents of product formation ($^{32}$Pi plus $^{32}$PP$_i$) were plotted as a function of input CthTTM for each polyphosphate substrate concentration.
revealed a steady increase in specific activity, up to 90 pmol of product released per pmol of enzyme at the highest level tested (Fig. 7C).

Effect of Deleting the Plug Helix—We produced and purified a truncated protein, CthTTM-(1–137), that lacks the plug helix. CthTTM-(1–137) was then tested for its activity with a variety of substrates utilized by wild-type CthTTM. Fig. 8A shows manganese-dependent hydrolysis of Gpppp and GTP. Reaction mixtures (50 μl) containing 50 mM Tris-HCl (pH 8.5), 200 μM MnCl₂, 100 μM Gpppp, or 100 μM GTP (C) and CthTTM-(1–137) as specified were incubated for 30 min at 37 °C. When hydrolysis of tripolyphosphate. Reaction mixtures (50 μl) containing 50 mM Tris-HCl (pH 9.0), 0.5 mM MnCl₂, 100 μM PP, and wild-type CthTTM or CthTTM-(1–137), as specified, were incubated for 30 min at 37 °C. Pᵢ release was determined using the malachite green method and is plotted as a function of input enzyme. C and D, reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.5), 10 mM MnCl₂, [³²P]polyphosphate (59 μM phosphate), and either wild-type CthTTM (C) or CthTTM-(1–137) (D), as specified, were incubated for 60 min at 37 °C. The extents of release of ³²P₃, ³²P₂, and ³²P₁ were determined by TLC and are plotted as a function of input enzyme.

FIGURE 8. Phosphohydrolase activities of CthTTM-(1–137). A, hydrolysis of Gpppp and GTP. Reaction mixtures (50 μl) containing 50 mM Tris-HCl (pH 8.5), 200 μM MnCl₂, 100 μM Gpppp, or 100 μM GTP (C) and CthTTM-(1–137) as specified were incubated for 30 min at 37 °C. B, hydrolysis of tripolyphosphate. Reaction mixtures (50 μl) containing 50 mM Tris-HCl (pH 9.0), 0.5 mM MnCl₂, 100 μM PPi, and wild-type CthTTM or CthTTM-(1–137), as specified, were incubated for 30 min at 37 °C. Pᵢ release was determined using the malachite green method and is plotted as a function of input enzyme. C and D, reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.5), 10 mM MnCl₂, [³²P]polyphosphate (59 μM phosphate), and either wild-type CthTTM (C) or CthTTM-(1–137) (D), as specified, were incubated for 60 min at 37 °C. The extents of release of ³²P₃, ³²P₂, and ³²P₁ were determined by TLC and are plotted as a function of input enzyme.

Deletion of the plug helix reduced manganese-dependent inorganic tripolyphosphatase activity by 16-fold compared with wild-type CthTTM (Fig. 8B), again reprising the effects of the K8A change noted previously (26). We detected no hydrolysis of PPP, by CthTTM-(1–137) in the presence of magnesium (not shown). The specific activity of CthTTM-(1–137) in magnesium-dependent cleavage of AppppAppppApppp was 15-fold lower than that of wild-type CthTTM (not shown).

A comparison of the hydrolysis of inorganic polyphosphate by wild-type CthTTM (Fig. 8C) versus CthTTM-(1–137) (Fig. 8D) revealed little difference in total product formation (³²PPᵢ plus ³²P₂) as a function of input enzyme. However, the distribution of products was quite different. Whereas wild-type CthTTM liberated a 1.9-fold molar excess of ³²P₂ over ³²PPᵢ, the truncated enzyme displayed a stronger bias toward ³²PPᵢ, which was produced in 4.1-fold excess over ³²PP₂. A control experiment showed that CthTTM-(1–137) catalyzed no detectable conversion of PPᵢ to PP₂, under the same reaction conditions (not shown). Thus, we surmise that deleting the plug helix directly affects the outcome of the polyphosphate hydrolysis reaction, by favoring cleavage at the terminal phosphate.

DISCUSSION

By surveying a variety of nucleotidyl and inorganic polyphosphates as substrates for CthTTM, we gain a new appreciation of its catalytic potential and the constraints to substrate utilization imposed by steric hindrance and the choice of metal cofactor. Initial studies showed that CthTTM hydrolyzed NTPs to NDPs and Pᵢ, exclusively with manganese, without apparent preference for any of the standard purine or pyrimidine bases or deoxy versus ribo sugars. However, NTP hydrolysis was conspicuously feeble compared with hydrolysis of tripolyphosphate, which was supported by manganese and magnesium (26). Here we find that a nucleoside 5'-tetraphosphate is a far better substrate for CthTTM than the cognate NTP and that the reaction occurs with either manganese or magnesium. These findings engender a model for productive substrate binding whereby the nucleoside hinders the formation of a proper Michaelis complex when it is located too close to the terminal phosphate (Fig. 9).

Based on the structures of Cet1 and PhoTTM, we envision that three phosphates of the substrate bind within the tunnel so that the terminal scissile phosphoanhydride is poised over the catalytic metal. This establishes an asymmetry to the active site, even when the substrate is chemically symmetrical, as in the case of triphosphate. We and others (25) have proposed that NTPs and thiamine triphosphate, which are asymmetric chemically, bind the tunnel so that the NDP or ThDP leaving group is located on the “entrance” side of the tunnel. In the case of the bacterial/archaean TTMs and mammalian thiamine triphosphatase, this corresponds to the face of the tunnel that is occupied by the plug helix (which apparently must be displaced to some degree when substrate binds productively). The sharp increase in activity with nucleoside tetraphosphate versus NTP would thereby reflect the movement of the nucleoside further from the tunnel entrance when the β, γ, and δ phosphates of
G-Gpppp-G → GDP + P_i

G-Gpppp-G → GTP + P_i

A-ApppppA-A → ATP + ATP

P-P-P-P-P-P-P-P- ● P-P-P-P-P-P-P-P- ● P-P-P-P-P-P-P-P- ● P-P-P-P-P-P-P-P- ● P-P-P-P-P-P-P-P- → polyP_{n+1} + P_i

P-P-P-P-P-P-P-P- → polyP_{n+2} + P_i

The ability of CthTTM to degrade a long-chain inorganic polyphosphate is intriguing in light of the multiple roles imputed to polyphosphate in bacterial physiology (28, 29). Although evaluation of CthTTM in polyphosphate biology ultimately hinges on a genetic analysis via targeted gene disruption (to our knowledge, not currently practiced in C. thermocellum), the properties of the inorganic polyphosphatase activity reported here are quite interesting and distinct from other known polyphosphate catabolizing enzymes: Ppx1, Ppx, and Ppn1. Yeast Ppx1 and E. coli PPX are metal-dependent exopolyphosphatases that cleave the terminal phosphoanhydride linkage to yield P_i as the sole product of each catalytic cycle (31, 32). The structures of yeast Ppx1 (33) and E. coli PPX (34) have been solved; they are unrelated to each other and bear no resemblance to the TTM fold. Yeast Ppn1 is an endopolyphosphatase that cleaves long polyphosphate chains internally to yield shorter polyphosphates, ultimately forming a mixture of P_i and PPP_i, as the end products (35).

By contrast, CthTTM cleaves polyphosphate to yield a mixture of P_i and PPP_i, with P_i being formed in ~2-fold molar excess over PPP_i, with no significant accumulation of PPP_i, during the
reaction. This product distribution implies that CthTTM hydrolyzes polyphosphate in both exo mode when forming P_i and endo mode when generating PP_i. Several plausible substrate-binding scenarios are depicted in Fig. 9 to account for this pattern of product formation. In the exo mode, we propose that the polymer accesses the tunnel on the entrance side so that the terminal phosphate is over the metal and hydrolysis liberates P_i. The endo mode of PP_i hydrolysis could entail (i) polymer access through the tunnel entrance, albeit with the penultimate n-1 phosphate over the metal and the terminal phosphate protruding from the tunnel exit or (ii) polymer entry through the exit side of the tunnel, with the three terminal phosphates bound so that the cleavage occurs between the n-1 and n-2 phosphates. The balance between exo and endo modes is apparently flexible, because we find that deletion of the plug helix shifts the reaction outcome toward generation of P_i.

Finally, our studies of CthTTM emphasize the need for skepticism regarding the data base annotations of dozens of uncharacterized bacterial/archaeal TTMs as “adenylate cyclases.” CthTTM has no such activity (26). As shown here and previously, it exhibits the signature manganese-dependent phosphohydrolase function of TTM proteins (1) but has its own distinctive substrate specificity and reaction outcomes. Surveying the properties of additional bacterial and archaeal TTMs, especially those for which crystal structures are in hand, should provide important clues to the function and evolution of this enzyme superfamily.

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