Structure-Function Analysis of Trypanosoma brucei RNA Triphosphatase and Evidence for a Two-metal Mechanism*

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Trypanosoma brucei RNA triphosphatase TbCet1 is a 252-amino acid polypeptide that catalyzes the first step in mRNA cap formation. By performing an alanine scan of TbCet1, we identified six amino acids that are essential for triphosphatase activity (Glu-52, Arg-127, Glu-168, Arg-186, Glu-216, and Glu-218). These results consolidate the proposal that protozoan, fungal, and Chlorella virus RNA triphosphatases belong to a single family of metal-dependent NTP phosphohydrolases with a unique tunnel active site composed of eight β strands. Limited proteolysis of TbCet1 suggests that the hydrophilic N terminus is surface-exposed, whereas the catalytic core domain is tightly folded with the exception of a protease-sensitive loop (WKGRARKT) between two of the putative tunnel strands. The catalytic domain of TbCet1 is extraordinarily thermostable. It remains active after heating for 2 h at 75 °C. Analysis by zonal velocity sedimentation indicates that TbCet1 is a monomeric enzyme, unlike fungal RNA triphosphatases, which are homodimers. We show that triphosphatase is a potent competitive inhibitor of TbCet1 (Ki, 1.4 μM) that binds more avidly to the active site than the ATP substrate (Km, 25 μM). We present evidence of synergistic activation of the TbCet1 triphosphatase by manganese and magnesium, consistent with a two-metal mechanism of catalysis. Our findings provide new insight to the similarities (in active site tertiary structure and catalytic mechanism) and differences (in quaternary structure and thermal stability) among the different branches of the tunnel enzyme family.

The m7GpppN cap structure (cap 0) is a defining feature of eukaryotic mRNA that is required for mRNA stability and efficient translation. The cap is formed by three enzymes. The 5′-triphosphatase end of the nascent pre-mRNA is hydrolyzed to a diphosphate by RNA triphosphatase. The diphosphate end is capped with GMP by RNA guanylyltransferase, and the GpppN cap is methylated by RNA (guanine-N7) methyltransferase (1, 2). The mRNAs of kinetoplastid protozoa such as Trypanosoma and Leishmania contain a unique hypermodified “cap 4″ structure, which is derived from the m7GpppN cap by co-transcriptional methylation of seven sites within the first four nucleosides of the spliced leader RNA (3–6). Kinetoplastid mRNAs acquire their 5′ caps via trans-splicing of an RNA leader sequence containing the cap 4 structure. The hypermethylated cap is required for trans-splicing (7–9).

RNA guanylyltransferase and RNA triphosphatase components of the kinetoplastid capping apparatus have been identified (10, 11), but the proteins that catalyze the several methylation steps have not. Whereas the Trypanosoma brucei guanylyltransferase is mechanistically and structurally related to the guanylyltransferases of all other eukaryal species (1, 10), the T. brucei RNA triphosphatase (TbCet1) bears no resemblance whatsoever, structurally or mechanistically, to the analogous enzyme of the human host (11, 12). RNA triphosphatase is thereby recommended as a potential drug target for treatment of trypanosomiasis (11).

TbCet1 belongs to the “triphosphate-tunnel” family of metal-dependent phosphohydrolasmes that encompasses the RNA triphosphatases of many unicellular eukaryotic organisms, including fungi (Saccharomyces cerevisiae, Candida albicans, and Schizosaccharomyces pombe), microsporidia (Encephalitozoon cuniculi), and the malaria parasite Plasmodium falciparum (1, 13–15). This family was defined initially by the presence of two conserved glutamate-containing motifs (β1 and β11 in Fig. 1) and the signature property of hydrolyzing NTPs to NDPs in the presence of manganese (16). Subsequently, the crystal structure of the S. cerevisiae RNA triphosphatase Cet1 revealed that the active site is located within a topologically closed β barrel (the triphosphate tunnel) composed of eight antiparallel strands (Fig. 1) (17). Each of the eight β strands contributes one or more functional groups to the Cet1 active site (16-19). A single sulfate in the tunnel, which is proposed to mimic the γ phosphate of the substrate, is coordinated by multiple basic side chains projecting into the cavity from the tunnel walls (17). A single manganese ion within the tunnel cavity is coordinated with octahedral geometry to the sulfate to the side chain carboxylates of the two glutamates in β1 and a glutamate in β11. It is not clear whether the enzyme-bound divalent cation seen in the Cet1 crystal structure is the sole metal cofactor for the triphosphatase activity.

The amino acid sequence alignment in Fig. 1 illustrates that the β strands that comprise the Cet1 triphosphate tunnel are conserved in the known RNA triphosphatases of fungi and protozoan and also in the RNA triphosphatase encoded by an algal virus, Paramecium bursaria Chlorella virus-1 (20). We have proposed that the active site folds of the fungal, microsporidian, protozoan, and Chlorella virus RNA triphosphatases are conserved as β barrels, implying a common evolutionary origin for these enzymes (21). Extensive mutational analyses of the C. albicans and Chlorella virus RNA triphosphatases indicates that their active site architecture and catalytic mechanism adhere closely to those of yeast Cet1 (21, 22).

S. cerevisiae Cet1 and the S. pombe RNA triphosphatase Pct1 are both homodimers (17, 23, 24). Available evidence
indicates that homodimeric quaternary structure is essential for Cet1 and Pct1 function in vivo but not for their catalytic activity (25, 26). The two tunnels are at opposite poles of the Cet1 homodimer in a parallel orientation. Each tunnel rests on a globular domain (the pedestal) composed of elements from both protomers of the homodimer. Disruption of the Cet1 dimer interface by a single alanine substitution is lethal in vivo and renders Cet1 activity acutely thermostable at physiological temperatures in vitro (26). Similarly, a catalytically inactive monomer of fusion yeast Pct1 is defective in vivo in S. pombe and hypersensitive to thermal inactivation in vitro (26). These findings suggested an explanation for the conservation of quaternary structure in fungal RNA triphosphatasas, whereby the delicate tunnel architecture of the active site is stabilized by the homodimeric pedestal domain.

The trypanosome RNA triphosphatase TbCet1 has been shown to function in cap formation in vivo in S. cerevisiae in lieu of Cet1 (11). However, the enzyme has not been characterized biochemically beyond the initial documentation of the manganese-dependent NTPase activity of a recombinant TbCet1 fusion protein (11). Here we present a physical characterization of untagged native TbCet1 including the following: (i) probing TbCet1 tertiary structure by limited proteolysis; (ii) assessment of quaternary structure by zonal velocity sedimentation; (iii) domain demarcation by N-terminal deletion; and (iv) an analysis of TbCet1 thermal stability. The results of these experiments reveal striking differences between TbCet1 and Cet1, insofar as TbCet1 is a monomeric enzyme and it is extremely resistant to thermal inactivation.

We also present a mechanistic characterization of TbCet1 that includes the following: (i) an alanine scan pinpointing six amino acids essential for catalysis in vitro; (ii) identification of triphosphosphate as a potent competitive inhibitor; and (iii) evidence of synergistic activation of the TbCet1 triphosphatase by manganese and magnesium, consistent with a two-metal mechanism of catalysis. Our findings provide further insight to the evolution of the metal-dependent RNA triphosphatase family and its utility as an anti-infective drug target.

Expression and Purification of Recombinant TbCet1—The bacterial expression vector pET-His10/Smt3-tagged TbCet1 (pET-His10/Smt3-TbCet1) was constructed by PCR amplification using sense primers that introduced a BamHI site on the 5’ end of the truncated coding region. The PCR products were digested with BamHI and HindIII and then inserted into pET28-His10/Smt3 that had been digested with BamHI and HindIII. The inserts were sequenced completely to confirm the in-frame fusion junction and exclude unwanted coding changes. The His10/Smt3-tagged NΔ22 and NΔ33 proteins were produced in E. coli and purified by nickel-agarose affinity chromatography as described above.

Alanine Mutagenesis of TbCet1—Silent diagnostic restriction sites and single alanine substitutions were introduced into the pET28-His10/Smt3 that had been digested with BamHI and HindIII. The presence of the desired mutations was confirmed in each case by sequencing the entire insert. The occurrence of PCR-generated mutations outside the targeted region was thereby excluded. The His10/Smt3-tagged TbCet1-Ala proteins were produced in E. coli and purified by nickel-agarose affinity chromatography as described above.

Removal of the His10-Smt3 Tag—Aliquots of the 200 mM imidazole fractions of wild-type His10-Smt3-tagged TbCet1 (2 mg), NΔ22 (2 mg), and NΔ33 (1 mg) were treated for 1 h on ice with purified recombinant His10-Stagged S. cerevisiae Ulp1, a Smt3-specific cysteine protease (27), at a 1:1000 ratio of Ulp1 to His10-Smt3-TbCet1. The digests were adjusted to 50 mM NaCl by 1:4 dilution with buffer C (50 mM Tris-HCl, pH 8.0, 0.25 M NaCl, 0.1% Triton X-100). The samples were loaded onto the phosphocellulose columns that had been equilibrated with 50 mM NaCl in buffer C. The bound material was eluted stepwise with 2-ml aliquots of 200, 500, and 1000 mM NaCl in buffer C. The His10-Smt3 polypeptide was recovered mainly in the flow-through and 200 mM NaCl eluate fractions, whereas the released native TbCet1 proteins were recovered predominantly in the 500 mM NaCl eluates. The 500 mM NaCl fractions were then applied to 0.5 ml column of Ni2+-nitrilotriacetic acid-agarose resin. The nickel-agarose flow-through fractions of TbCet1 (1 mg), NΔ22 (1 mg), and NΔ33 (0.3 mg) were used for biochemical and physical studies.

Nucleoside Triphosphate Phosphohydrolase Assay—Reaction mixtures (10 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 1 mM MnCl2, 0.2 mM γ-32P[ATP], and serial 2-fold dilutions of either wild-type or mutant TbCet1 proteins (0, 0.125, 0.25, 0.5, 1, 2, and 4 ng) were incubated for 15 min at 37 °C. The reactions were quenched by adding 2.5 μl of 5 M formic acid. Aliquots of the mixtures were applied to polyethylenimine-cellulose TLC plates, which were developed with 1 M formic acid, 0.5 M LiCl. 32P release was quantified by scanning the chromatogram with a Fujix phosphorimaging device. The specific activity values were calculated from the slopes of the titration curves. The activity values for the mutant enzymes were then normalized to the specific activity of wild-type TbCet1.

Limited Proteolysis of TbCet1—Reaction mixtures (20 μl) containing 10 μg of native TbCet1 and trypsin or chymotrypsin as specified were incubated at room temperature for 20 min. The reactions were quenched by adding SDS, and the digestes were resolved by SDS-PAGE. The 250 mM imidazole fractions were transferred electrophoretically to a polyvinylidene difluoride membrane and visualized by staining the membrane with Coomassie Blue dye. Membrane slices containing intact TbCet1 and individual proteinolytic products were excised and subjected to automated Edman sequencing.

Glycerol Gradient Sedimentation—Native TbCet1 (100 μg) was mixed with catalase (50 μg), BSA (50 μg), and cytochrome c (50 μg) in 200 μl of 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 10% glycerol. The gradient mixture was applied to a 4.8-ml 15–50% glycerol gradient containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.1% Triton X-100. The gradient was centrifuged in a Beckman SW50 rotor at 50,000 rpm for 18 h at 4 °C. Fractions (~0.21 ml) were collected from the bottom of the tube. Aliquots (15 μl) of odd-numbered gradient fractions were analyzed by SDS-PAGE.
RESULTS

Structure-based Alanine Scan of TbCet1—To test the structural and mechanistic relation of the T. brucei and fungal RNA triphosphatases, we conducted a limited alanine scan of Tb-Cet1 using the tunnel of Cet1 as a guide. We targeted eight amino acids of TbCet1 corresponding to side chains in five of the \( /H9252 \) strands of the Cet1 tunnel: Glu-52 and Arg-56 (\( /H9252 \) 1); Arg-217 (\( /H9252 \) 6); Glu-168 (\( /H9252 \) 8); Arg-186 (\( /H9252 \) 9); and Glu-214, Glu-216, and Glu-218 (\( /H9252 \) 11). The eight targeted residues are conserved in all of the members of the tunnel family (Fig. 1). The TbCet1-Ala proteins were expressed as His10-Smt3-tagged fusions in E. coli in parallel with wild-type TbCet1. The recombinant proteins were purified from soluble bacterial extracts by nickel-agarose chromatography. SDS-PAGE analysis of the polypeptide compositions of the nickel-agarose protein preparations revealed similar extents of purification (Fig. 2A). The 45-kDa His10-Smt3-TbCet1 polypeptide was the predominant species in every case.

The triphosphatase activities of the wild-type and mutant His10-Smt3-TbCet1 proteins were assayed by the release of \( ^{32}\)Pi from 0.2 mM \([/H9252^{32}\text{P}]\)ATP in the presence of 1 mM manganese. The extent of ATP hydrolysis by the wild-type enzyme was proportional to enzyme concentration, and the reaction proceeded to completion at saturating enzyme (data not shown). We calculated a specific activity of 0.88 nmol of \( ^{32}\)Pi formed per nanogram of His10-Smt3-TbCet1 during a 15-min reaction, which corresponded to a turnover number of \( 44 \text{s}^{-1} \). The specific activities of the eight Ala mutants normalized to the wild-type specific activity are shown in Fig. 2B. Alanine substitutions at six of the eight positions examined elicited at least a 50-fold decrement in catalytic activity. These residues were Glu-52, Arg-127, Glu-168, Arg-186, Glu-216, and Glu-218. We designated these six residues as essential for the phosphohydrolase activity of TbCet1 by applying a criterion for essentiality of a 20-fold activity decrement incurred by side-chain removal. One residue at which alanine substitution reduced activity between 6 and 20% of wild type (Glu-214 in ScCet1) was deemed to be important but not essential. Arg-56 was judged not to be important, because the R56A mutant retained 64% of wild-type activity.

Fig. 2B shows a comparison of the effects of alanine mutations at the eight “equivalent” amino acids of TbCet1 and yeast Cet1 on manganese-dependent ATP hydrolysis (16, 18, 19). The salient point is that the mutagenic effects are perfectly concordant at seven positions with the exception being the \( /H9252 \) arginine, which is deemed essential in TbCet1 and important
in Cet1. The results of this focused mutational analysis indicate that (i) at least six of the putative \( \beta \) strands of TbCet1 probably contribute to the triphosphatase active site and (ii) the active site of TbCet1 is likely to adopt a tunnel fold analogous to that seen in the Cet1 crystal structure.

**Purification and Characterization of Recombinant Native TbCet1**—The His\(_{10}\)-Smt3 domain was removed by treatment of the His\(_{10}\)-Smt3-TbCet1 protein with purified His\(_{4}\)-tagged Ulp1, a Smt3-specific protease that hydrolyzes the polypeptide chain at the junction between His\(_{10}\)-Smt3 and the fused downstream protein (27). The tag-free native TbCet1 was purified away from the His\(_{10}\)-Smt3 fragment by phosphocellulose and nickel-agarose chromatography. SDS-PAGE analysis of the native TbCet1 preparation revealed a predominant 32-kDa polypeptide fused to His\(_{10}\)-Smt3. The two truncated fusion proteins were isolated by phosphocellulose and nickel-agarose chromatography. The His\(_{10}\)-Smt3 tags were removed with Ulp1, and the native forms of N\(_{22}\) and N\(_{33}\) were purified from soluble lysates by nickel-agarose chromatography. SDS-PAGE analysis showed that the truncated enzymes were pure and that they displayed the expected incremental shifts in electrophoretic mobility (Fig. 3A). The ATPase activities of N\(_{22}\) and N\(_{33}\) were measured in the assay mixtures, which contained 5–40 \( \mu \text{M} \) ATP and were purified by automated Edman chemistry after transfer from a SDS gel to a polyvinylidene difluoride membrane. A \( K_m \) of 25 \( \mu \text{M} \) ATP was calculated from a double-reciprocal plot of the data (Fig. 3C).

**Structure Probing of Native TbCet1 by Limited Proteolysis**—Native TbCet1 was subjected to proteolysis with increasing amounts of trypsin or chymotrypsin. N-terminal sequencing of the undegraded TbCet1 polypeptide by automated Edman chemistry after transfer from an SDS gel to a polyvinylidene difluoride membrane confirmed that the N-terminal sequence (SMEATER) corresponded to that of the native TbCet1 (MEATER) plus a single N-terminal serine derived from the P1 position of the Smt3 cleavage site (Fig. 4). Initial scission of the 32-kDa TbCet1 protein by trypsin yielded two major products, an \( \sim 23 \text{kDa} \) doublet and a single lower molecular mass species of \( \sim 10 \text{kDa} \) (Fig. 4). The upper and lower components of the doublet had overlapping N-terminal sequences (\( ^{83}\text{KTRTEVM} \) and \( ^{84}\text{TRTEVM} \)) arising through trypsin cleavage at adjacent residues Arg-82 and Lys-83, respectively, which are located between the putative equivalents of Cet1 strands \( \beta_1 \) and \( \beta_5 \) (Fig. 1). The smaller species retained the intact N terminus (SMEATER). Thus, trypsin cleavage at two tightly clustered sites split the enzyme into a \( \sim 9 \text{kDa} \) N-terminal fragment and a \( \sim 23 \text{kDa} \) carboxyl domain. We surmise that the C-terminal domain fragments are themselves tightly folded, insofar as they were resistant to digestion by concentrations of trypsin sufficient to cleave all of the input TbCet1 (Fig. 4). In contrast, the N-terminal fragment was converted at higher trypsin concentration into a lower molecular weight peptide with an N-terminal sequence (\( ^{11}\text{ERNERND} \)) indicative of secondary tryptic cleavage at residue Arg-10.

Treatment of TbCet1 with limiting concentrations of chymotrypsin yielded a strikingly similar pattern comprising a doublet of \( \sim 23 \text{kDa} \) and a single peptide of \( \sim 9 \text{kDa} \) (Fig. 4). The 9-kDa chymotryptic digestion product retained the original N terminus (SMEATER). The upper and lower components of the chymotryptic doublet had identical N-terminal sequences (\( ^{26}\text{KRRARG}^{30} \)) derived from scission at residue Trp-76, which is situated just proximal to Arg-82 and Lys-83, the major sites accessible to trypsin. (The two components of the chymotrypsin doublet obviously differ at their C termini, implying the existence of a chymotrypsin-sensitive site close to the C terminus of native TbCet1.) The results of the proteolytic analysis suggest that TbCet1 is a compact protein punctuated by a surface-accessible loop between the two of the putative \( \beta \) strands of its tunnel.

**N-terminal Deletions of TbCet1**—The N-terminal segment of TbCet1 is extremely hydrophilic. 17 of the first 22 side chains are either positively or negatively charged (Fig. 3). The trypsin sensitivity of the N-terminal Arg-10/Glu-11 dipeptide suggested that the N-terminal segment might be flexible or surface-exposed, in which case it might not be critical for catalysis. To test this idea, we engineered a deletion mutant N\(_{22}\) containing the TbCet1-(23–301) polypeptide fused to His\(_{10}\)-Smt3. A more extensive truncation, N\(_{33}\), was also constructed as a His\(_{10}\)-Smt3 fusion. The two truncated fusion proteins were produced in bacteria and purified from soluble lysates by nickel-agarose chromatography. The His\(_{10}\)-Smt3 tags were removed with Ulp1, and the native forms of N\(_{22}\) and N\(_{33}\) were isolated by phosphocellulose and nickel-agarose chromatography. SDS-PAGE analysis showed that the truncated enzymes were pure and that they displayed the expected incremental shifts in electrophoretic mobility (Fig. 3A). The ATPase activities of N\(_{22}\) and N\(_{33}\) were measured in the assay mixtures, which contained 5–40 \( \mu \text{M} \) ATP and were purified by automated Edman chemistry after transfer from a SDS gel to a polyvinylidene difluoride membrane. A \( K_m \) of 25 \( \mu \text{M} \) ATP was calculated from a double-reciprocal plot of the data (Fig. 3C).
terminus is dispensable for TbCet1 function in vitro and as few as 19 amino acids upstream of the first catalytic glutamate (Glu-54) in the putative β strand suffice for catalysis.

Native TbCet1 Is a Monomeric Enzyme—The quaternary structure of native recombinant TbCet1 was investigated by zonal velocity sedimentation through a 15–30% glycerol gradient (Fig. 5). Marker proteins catalase, BSA, and cytochrome c were included as internal standards in the gradient. After centrifugation, the polypeptide compositions of the odd-numbered gradient fractions were analyzed by SDS-PAGE. TbCet1 (calculated to be a 29-kDa polypeptide) sedimented as a discrete peak between BSA (66 kDa) and cytochrome c (12 kDa). The triphosphatase activity profile paralleled the abundance of the TbCet1 polypeptide and peaked at fraction 18. A plot of the S values of the three standards versus fraction number yielded a straight line (data not shown). An S value of 3.1 was determined for TbCet1 by interpolation to the internal standard curve. These velocity sedimentation results are consistent with a monomer quaternary structure for TbCet1. Analytical sedimentation equilibrium ultracentrifugation also indicated that TbCet1 is a monomeric protein (data not shown).

TbCet1 Is Exceptionally Thermostable—The monomeric quaternary structure of TbCet1 contrasts with the homodimeric quaternary structure of fungal RNA triphosphatases Cet1 and Pct1. Because homodimerization ensures that the active site architecture of the fungal RNA triphosphatases is stable at physiological temperatures, it was of interest to evaluate the thermal stability of the monomeric trypanosome RNA triphosphatase. Initial heat inactivation experiments were performed by preincubating native TbCet1 for 10 min at 22, 37, 45, 55, 65, or 85 °C followed by quenching on ice. The protein samples were then assayed for ATPase activity at 22 °C. The data were expressed as the ratio of ATP hydrolysis by TbCet1 preincubated at a given test temperature to the activity of the unheated control. We found that pretreatment at 22 °C reduced activity by 40% and that higher temperature pretreatments (up to 85 °C) had no further deleterious effect on activity (data not shown). Thus, TbCet1 was surprisingly thermostable in vitro, although it displayed a biphasic response to heat treatment. To better gauge the thermal stability of TbCet1, we heated the enzyme at 75 °C for various times, quenched the heated proteins on ice, and then assayed for ATPase activity at 22 °C. Here again, we noted a biphasic pattern whereby the phosphohydrolase activity declined quickly to ~60% of the control level (after 5–10 min of heating) and then remained at this level even after the enzyme was treated for up to 2 h at 75 °C (Fig. 6A). The extreme stability of TbCet1 to heat treatment contrasted with the rapid inactivation of S. cerevisiae Cet1 at 75 °C (Fig. 6A). Yeast Cet1 lost 98% of its activity in 10 min. The kinetics of Cet1 inactivation fit well to a monophasic exponential decay with a half-life of 3 min.

To distinguish between thermal effects on enzyme stability versus enzyme activity, we assayed ATP hydrolysis at reaction temperatures ranging from 4 to 75 °C. In this experiment, the enzyme sample was briefly equilibrated at the intended assay temperature and then added to reaction mixtures pre-equilibrated to the intended temperature. TbCet1 displayed a bell-shaped dependence on reaction temperature whereby activity increased linearly from 4 to 37 °C, peaked at 45 °C, and declined linearly from 55 to 75 °C (Fig. 6B). The extent of P_i release at 75 °C was 5% of the value at 37 °C. We conclude that TbCet1 is not a thermophilic enzyme. Rather, TbCet1 is exceptionally thermostable, either because it does not unfold globally during prolonged heating at 75 °C or it readily renatures in an active conformation when the heated enzyme is quenched on ice.

The thermal stability of TbCet1 was unaffected by the removal of the hydrophilic 22-amino acid N-terminal segment. Indeed, the NΔ22 enzyme was even more stable than the full-length protein, suffering no loss of activity over a 2-h preincubation at 75 °C (Fig. 6A). Bearing in mind that the basal level of activity of NΔ22 is half of that of TbCet1, we propose that the 40% decrement in TbCet1 activity elicited by brief heat treatment is a consequence of unfolding of the N-terminal domain or disruption of contacts between the N-terminal domain and the thermostable core domain. The more extensive truncated NΔ33 enzyme was also thermostable, losing only 30% of its initial activity after a 2-h incubation at 75 °C (Fig. 6A). Note that NΔ33 did not display the early phase of partial activity loss that was seen with full-length TbCet1.
Tripolyphosphate Is a Potent Competitive Inhibitor of TbCet1—We tested the effects of inorganic phosphate derivatives on the ability of native TbCet1 to hydrolyze 0.2 mM [γ-32P]ATP in the presence of 1 mM manganese. Inorganic phosphate, which is a product of the phosphohydrolase reaction, inhibited activity by 50% at 1.8 mM P_i (Fig. 7A and data not shown). Inorganic tripolyphosphate was a significantly more potent inhibitor than phosphate, with 50% inhibition at 25 μM PPP (Fig. 7A). The fact that tripolyphosphate elicited 50% inhibition when present at a concentration 8-fold less than input ATP and 40-fold less than input manganese argues against the possibility that it inhibits by acting as a chelator to competitively inhibit with ATP (or enzyme) for the metal cofactor. A simple explanation for the potent inhibition is that PPP binds more avidly than ATP to the triphosphate-binding pocket within the putative active site tunnel of TbCet1.

The mechanism of inhibition was evaluated by analysis of the effects of increasing PPP concentrations on ATP hydrolysis at three different concentrations of the [γ-32P]ATP substrate (50, 100, and 200 μM ATP). The activity data for each concentration of ATP were transformed into a Dixon plot of 1/v versus reaction parameters on the magnesium-ATPase activity of native TbCet1. Hydrolysis of 1 mM ATP displayed a sigmoidal dependence on magnesium concentration. The activity increased in proportion to increasing MgCl_2 in the range of 2.5–10 mM and was optimal at 20–40 mM MgCl_2 (data not shown). No activity was detected at magnesium ≤12.5 mM MgCl_2. The release of P_i from 1 mM ATP in the presence of 20 mM MgCl_2 and Tris-HCl buffer was optimal at pH 8.0 (data not shown). Product formation under these conditions was proportional to input TbCet1. The calculated specific activity of 0.7 nmol P_i released/ng TbCet1 in 15 min corresponded to a turnover number of 22 s^{-1} (data not shown). Mg-ATPase activity was even higher when the ATP concentration was increased to 5 mM (turnover number ~42 s^{-1}). These findings indicate that there is no intrinsic difference in the ability of manganese and magnesium to support the TbCet1 phosphohydrolase reaction chemistry.

To explain the differences in the optimal reaction parameters for manganese versus magnesium, we considered the possibility that TbCet uses a two-metal mechanism requiring occupancy of distinct metal binding sites on the enzyme and/or the ATP substrate. A simple model would be that one site prefers...
Trypanosome RNA Triphosphatase

**DISCUSSION**

*Trypanosome Cet1* is a Tunnel Family NTPase—By performing a mutational analysis of the *Trypanosome* NTPase, we identified seven amino acids that are essential or important for triphosphatase activity. Glu-52, Glu-216, and Glu-218 are implicated in metal binding on the basis of their homology to the metal-binding glutamates in strands β1 and β11 of Cet1 (Fig. 1). We posit a role for the essential Glu-168 side chain as a general base catalyst to activate the nucleophilic water for its attack on the γ phosphorus, analogous to the general base function postulated for the essential Glu-433 in the β8 strand of Cet1 (19). We suggest that the side chain of Arg-127 interacts with the γ phosphate and stabilizes the transition state. The homologous Arg-345 side chain in the β6 strand of Cet1 makes a bidentate contact to the sulfate (= γ phosphate) in the Cet1 crystal structure (17). Cet1 residues Arg-186 and Glu-214 correspond to Cet1 residues Arg-454 (β9) and Glu-492 (β11), which form a bidentate salt bridge that tethers two of the strands of the tunnel wall (17). It has been suggested that Arg-454 of Cet1 interacts with either the β or α phosphate of the NTP substrate based on its location anterior to the sulfate in the tunnel and the finding that mutations of Arg-454 increase the *Km* for ATP (16, 18). Poising a direct contact between equivalent *Trypanosome* side chain Arg-186 and the NTP would account for the more severe effects of the R186A mutation on enzyme activity compared with the E214A change. The E214A mutation would eliminate the salt bridge but not the putative substrate contact.

These results, together with previous mutational studies of manganese while the other prefers magnesium. A prediction of such a model is that a mixture of suboptimal levels of magnesium and manganese would elicit a synergistic activation of the *Trypanosome* ATPase. Indeed, that is what we observed (Fig. 9A).

**FIG. 5.** Glycerol gradient sedimentation of Cet1. Sedimentation was performed as described under “Experimental Procedures.” Aliquots (15 μl) of the indicated gradient fractions were analyzed by SDS-PAGE. The Coomassie Blue-stained gel is shown in the top panel. The positions of Cet1 and the internal standards catalase, BSA, and cytochrome c (cyt c) are indicated by arrows on the left. Aliquots (1 μl of a 1/100 dilution) of every fraction were assayed for manganese-dependent ATP hydrolysis. The activity profile is plotted in the bottom panel.

**FIG. 6.** Cet1 is thermostable. A, wild-type native Cet1, Cet1 truncation mutants N222 and N333, and the homodimeric *S. cerevisiae* triphosphatase Cet1-(201–549) were heated at 75 °C. Aliquots (20 μl) of each protein were withdrawn at the times specified and quenched on ice. Control aliquots of the proteins were kept on ice with no heat treatment. ATPase reaction mixtures contained aliquots (1 μl) of control or preheated Cet1 (2 ng), N222 (4 ng), N333 (10 ng), or ScCet1 (5 ng). The amounts of unheated enzymes sufficed to hydrolyze between 40 and 58% of the input ATP during the 15-min ATPase reaction at 22 °C. The extent of ATP hydrolysis by preheated enzyme was normalized to that of the unheated control enzyme (defined as 1.0). The normalized activities are plotted as a function of pretreatment time at 75 °C. B, reaction mixtures (50 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 1 mM MnCl₂, and 0.2 mM [γ-32P]ATP were pre-equilibrated at 4, 22, 37, 45, 55, 65, or 75 °C. The ATPase reactions were initiated by adding an aliquot of Cet1 (5 ng) that had been pre-heated for 2 min at the same temperature. The ATPase reactions were quenched after a 15-min incubation at the specified temperature.
S. cerevisiae, C. albicans, and Chlorella virus RNA triphosphatases (16, 18–22), consolidate the proposal that protozoan, fungal, and Chlorella virus RNA triphosphatases belong to a single family of metal-dependent NTP phosphohydrolases with a tunnel active site architecture. These findings suggest that a mechanism-based inhibitor identified by screening against a fungal enzyme might display broad activity against RNA triphosphates from protozoa and vice versa. Tripolyphosphate, shown here to be potent competitive inhibitor of TbCet1 (21), also inhibits fungal and Chlorella virus RNA triphosphatases (21, 24). Thus, tripolyphosphate provides a platform on which to design and test new derivatives.

Two-metal Catalysis—The yeast Cet1 structure revealed a single enzyme-bound manganese with three of the six octahedral coordination sites being occupied by Glu1 and Glu11 glutamates, one site being taken by an enzyme-bound water and a fifth site being taken by a water not engaged by the enzyme (17). The sixth contact is to the sulfate ion that likely mimics the hydrolyzed phosphate product. The structure suggests that the enzyme-bound metal promotes catalysis by stabilizing the negative charge developed on the phosphate in the transition state. The numerous contacts between the phosphate and essential lysine and arginine side chains projecting from the tunnel walls also help to stabilize the transition state. Other potential catalytic roles for divalent cations in phosphoryl transfer chemistry might include activation of the nucleophile by lowering the pKa of a metal-coordinated water, protonation of the leaving group via a metal-coordinated water, or ensuring optimal substrate conformation for in-line chemis-

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**Fig. 7. Competitive inhibition of TbCet1 by tripolyphosphate.** A, reaction mixtures (10 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 1 mM MnCl₂, 0.2 mM [γ-³²P] ATP, 0.5 ng of TbCet1, and P₁ or PPP, as specified were incubated for 15 min at 37 °C. The extents of ATP hydrolysis in the presence of the phosphate-containing compounds were normalized to the control level of ATP hydrolysis in their absence (defined as 1.0) and then plotted as a function of inhibitor concentration. B, competitive inhibition of TbCet1 ATPase by tripolyphosphate. Reaction mixtures (10 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 1 mM MnCl₂, 0.15 ng of TbCet1, 50, 100, or 200 μM [γ-³²P] ATP, and varying concentrations of PPP, were incubated for 15 min at 37 °C. The reaction products were analyzed by TLC. The mechanism of inhibition and the inhibition constant (Kᵢ) was determined from a Dixon plot of the reciprocal of the reaction velocity (nmol³²P, released/min) versus the concentration of tripolyphosphate at each fixed concentration of the ATP substrate: 50 μM (○), 100 μM (●), or 200 μM (□).

**Fig. 8. Hydrolysis of tripolyphosphate by TbCet1.** A, reaction mixtures (50 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 1 mM MnCl₂, 0.2 mM PPP, P₁, or ATP, and TbCet1 as specified were incubated for 15 min at 37 °C. The extent of Pi release is plotted as the function of input protein. B, reaction mixtures (50 μl) containing 50 mM Tris-HCl, pH 7.5, 0.2 mM PPP, 1 μg of TbCet1 and MnCl₂ or MgCl₂ as specified were incubated for 15 min at 37 °C. Phosphate release is plotted as a function of divalent cation concentration.

versus ATP is that the former has an extra negative charge on the phosphate occupying the “α phosphate” site in the tunnel that enables an additional electrostatic or hydrogen-bonding contact with the enzyme that is normally not available to the bridging 5’-O of the nucleoside.
The present study reveals a striking difference in the quaternary structure of T. brucei RNA triphosphatase, a monomeric enzyme, compared with that of fungal RNA triphosphatases Cet1 and Pct1, both of which are homodimers. At least one function of the homodimerization of Cet1 and Pct is to ensure their stability at physiological temperatures. Mutations that convert Cet1 and Pct1 to monomeric enzymes do not affect catalysis but do render Cet1 and Pct1 hypersensitive to thermal inactivation in vitro (26). For example, monomeric Cet1 loses 85% of its initial phosphohydrolase activity after a 10-min incubation at 40 °C, whereas dimeric Cet1 resists inactivation at 40 °C (26). In contrast, the monomeric TbCet1 retains activity after heating for 10 min at up to 85 °C and it is relatively impervious to prolonged treatment at 75 °C.

In the case of yeast Cet1, it is proposed that the tunnel fold is delicate and requires support by the globular pedestal domain to achieve and maintain an active conformation (26). Because the pedestal is composed of secondary structure elements contributed by both Cet1 protomers, it is sensible that disruption of the dimer would adversely affect the thermal stability of Cet1. The implication of this study is that the active site tunnel of TbCet1 is supported by a distinctive and inherently more stable scaffold that does not self-associate.

A simple explanation for the monomeric structure of TbCet1 vis a vis Cet1 is that TbCet1 lacks the structural elements that comprise the homodimer interface of Cet1, which include: (i) a loop and an α helix (α1) immediately upstream of the first strand of the tunnel (β1); (ii) two strands (β2 and β3) and an α helix (α2) located in the segment between β1 and β3; and (iii) a long α helix (α4) downstream of the last strand of the tunnel (β11 in Cet1) (17). To our inspection, TbCet1 has little or no similarity to Cet1 in the region upstream of the first tunnel strand and it lacks an obvious equivalent of the C-terminal α4 helix of Cet1. Thus, the differences in quaternary structure between fungal and trypanosome members of the tunnel enzyme family suggest the existence of two evolutionary branches. Whether homodimers represent the ancestral state or the derived state of the family remains unclear. Insight into this question should emerge as the RNA triphosphatases of other protozoan organisms are identified and characterized.

The structural basis for the remarkable thermal stability of TbCet1 cannot be surmised at present. Our deletion analysis shows that thermal stability is a property of the catalytic domain extending from amino acids 34–252. The charged N-terminal segment of TbCet1, although dispensable for catalysis, contributes a modest enhancement (2-fold or less) of activity and stability. Limited proteolysis suggests that the N-terminus is surface-exposed, whereas the catalytic core is tightly folded with the exception of an exposed segment between two of the putative tunnel strands. Efforts to crystallize the TbCet1 catalytic domain are underway.

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