Overexpression of a cytosolic pyrophosphatase (TgPPase) reveals a regulatory role of PP$_i$ in glycolysis for *Toxoplasma gondii*

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PP$_i$, is a critical element of cellular metabolism as both an energy donor and as an allosteric regulator of several metabolic pathways. The apicomplexan parasite *Toxoplasma gondii* uses PP$_i$ in place of ATP as an energy donor in at least two reactions: the glycolytic PP$_i$-dependent PFK (phosphofructokinase) and V-H$^+$-PPase [vacuolar H$^+$-translocating PPyrase (pyrophosphatase)]. In the present study, we report the cloning, expression and characterization of cytosolic TgPPase (*T. gondii* soluble PPase). Amino acid sequence alignment and phylogenetic analysis indicates that the gene encodes a family I soluble PPase. Overexpression of the enzyme in extracellular tachyzoites led to a 6-fold decrease in the cytosolic concentration of PP$_i$, relative to wild-type strain RH tachyzoites. Unexpectedly, this subsequent reduction in PP$_i$ was associated with a higher glycolytic flux in the overexpressing mutants, as evidenced by higher rates of proton and lactate extrusion. In addition to elevated glycolytic flux, TgPPase-overexpressing tachyzoites also possessed higher ATP concentrations relative to wild-type RH parasites. These results implicate PP$_i$ as having a significant regulatory role in glycolysis and, potentially, other downstream processes that regulate growth and cell division.

Key words: Apicomplexa, cellular metabolism, glycolysis, parasite, PP$_i$, pyrophosphatase (PPase), *Toxoplasma gondii*.

INTRODUCTION

PP$_i$, is a byproduct of many biosynthetic reactions (the synthesis of nucleic acids, coenzymes, proteins and isoprenoids, and the activation of fatty acids), and it has been proposed that the removal of PP$_i$, by PPases (pyrophosphatases) makes biosynthetic reactions thermodynamically favourable [1]. In addition, bioenergetic and regulatory roles of PP$_i$, have been suggested [2]. PP$_i$, can be generated by photophosphorylation, oxidative phosphorylation and glycolysis, and can be used in a number of reactions to replace ATP [3].

The cytosolic concentration of PP$_i$, is regulated in higher organisms, predominantly through the activity of soluble cytosolic PPases [4]. Inorganic PPases include membrane-bound V-H$^+$-PPases (vacuolar H$^+$-translocating PPases) and soluble PPases. The membrane-bound V-H$^+$-PPases utilize the energy released by hydrolysis of PP$_i$, to transport protons across the membrane of cells or organelles [5–8]. The soluble inorganic PPases that hydrolyse PP$_i$, to P, are essential enzymes and have high activity in the cytoplasm. The absence of these PPases would lead to the build-up of toxic levels of PP$_i$, accounting for the essential nature of the enzymes. Two families of non-homologous soluble inorganic PPases have been described: family I PPases, which are widespread in all types of organisms and prefer Mg$^{2+}$ as a cofactor [9,10], and family II PPases, which are exclusive to bacteria and prefer Mn$^{2+}$ as a cofactor [9–11]. One of the most studied family I PPases is that from *Saccharomyces cerevisiae* [12]. In addition to its PPase activity, this enzyme displays polyphosphatase activity in the presence of transition metal ions such as Zn$^{2+}$, Mn$^{2+}$ and Co$^{2+}$ as cofactors [13–16], and it also can hydrolyse organic tri- and di-phosphates, such as ATP and ADP [16–18].

An unusual characteristic of *Toxoplasma gondii*, a major opportunistic pathogen of fetuses from recently infected mothers and of patients with AIDS, is that it possesses cellular levels of PP$_i$, that are higher than those of ATP [19]. In addition, it stores PP$_i$, and poly P (polyposphate) in acidic organelles named acidocalcisomes [20–22]. Acidocalcisomes have been found in a number of organisms from bacteria to humans [23]. Acidocalcisomes from *T. gondii* are characterized by their electron density, high content of cations bound to PP$_i$, and poly P, and a number of pumps in their membranes, among them a V-H$^+$-PPase, which contributes to their acidification [20–22,24]. Incubation of fixed *Trypanosoma cruzi* [25] or *Trypanosoma evansi* [26] cells with a PPase removes the electron-dense matrix of acidocalcisomes, which indicates that PP$_i$, is an important component of this organelle’s structure. In addition to its use by the acidocalcisomal V-H$^+$-PPase [21,27], *T. gondii* PP$_i$, can also be used in place of ATP as an energy donor in the PP$_i$-dependent PFK (phosphofructokinase) reaction [28].

In the present study, we characterized biochemically a soluble PPase and named it TgPPase (*T. gondii* PPase). By overexpressing this enzyme in *T. gondii* tachyzoites we were able to isolate clones with up to ten times higher enzymatic activity than wild-type cells. This high cytosolic PPase activity altered the cytosolic concentration of PP$_i$, which was significantly reduced when compared with the cytosolic level in wild-type RH tachyzoites. These mutant cells showed alterations in their glycolytic pathway,
leading us to propose a regulatory role for PP, in the glycolytic pathway of these parasites.

**EXPERIMENTAL**

**Chemicals and reagents**

AMDP (aminomethylenediphosphonate) was synthesized by Professor Michael Martin (Department of Biophysics, University of Illinois at Urbana-Champaign, Urbana, IL, U.S.A.). Restriction enzymes, T<sub>4</sub> DNA ligase, reverse transcriptase, Taq polymerase, DNA ladder, TRizol<sup>®</sup> reagent and goat serum were from Gibco. The pET28a<sup>+</sup> expression system, Nitro-TA (Ni<sup>2+</sup>-nitrotriacetate) His·Bind<sup>®</sup> resin and benzonase nuclease were from Novagen. The pCR2.1-TOPO cloning kit, secondary antibodies, BCECF [2,7-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein] and BCECF-AM (where AM is acetoxyethyl ester) were from Invitrogen. Hybond-N nylon membrane, HiTrap desalting columns and the ECL (enhanced chemiluminescence) kit were obtained from Amersham Pharmacia Biotech. All other reagents were of analytical grade.

**Culture methods**

*T. gondii* tachyzoites (RH) were grown in hTERT (human telomerase reverse transcriptase) host cells using protocols described previously [29]. Transgenic fluorescent *T. gondii* tachyzoites expressing a YFP (yellow fluorescent protein)–YFP fusion gene were a gift from Dr Boris Striepen (Department of Cellular Biology, University of Georgia, Athens, GA, U.S.A.) [30].

**T. gondii growth measurements**

[<sup>1</sup>H]Uracil incorporation was conducted in hTert cells that were cultured in 12-well plates for 24 h before they were challenged with 1×10<sup>6</sup> tachyzoites per well. [<sup>1</sup>H]Uracil incorporation was measured 24 h later by measuring the amount of [<sup>1</sup>H]uracil incorporated into each well during the last 4 h [31,32]. *T. gondii* plaque assays were performed as described previously [33]. Assays were conducted in six-well plates each containing a confluent layer of hTERT host cells. Parasites (200 per well) were incubated for 9 days to allow invasion and replication (formation of plaques). Plaque number and relative plaque area (i.e., the percentage of total area occupied by a plaque-forming unit) were determined using ImageJ software (http://rsbweb.nih.gov/ij/).

**TgPPase cDNA cloning by 5′ and 3′ RACE (rapid amplification of cDNA ends)**

The protein sequence of the *Trypanosoma brucei* soluble inorganic PPase (GenBank<sup>®</sup> accession number AAP74702) was used to search the *T. gondii* genome database, revealing three polypeptides sharing high similarity to known soluble inorganic PPases. The 5′ and 3′ RACE technique was used to sequence the 5′ and 3′ ends of the *TgPPase* cDNA, using information from known contigs and EST (expressed sequence tag) sequences. The Invitrogen Life Technologies kit for 5′ and 3′ RACE was used.

**Database search and phylogenetic analysis**

BLAST analysis of the *TgPPase* protein against the OrthoMCL database (version 2) (http://www.orthomcl.org/cgi-bin/OrthoMclWeb.cgi) was performed to search for putative PPase orthologues. A multiple sequence alignment from the sequences obtained was created by ClustalW [34] and optimized using GeneDoc (version 2.6.002) [35] and MacClade (version 4.06) [36]. Phylogenetic trees were built on the basis of the optimized alignment using the parsimony and distance methods from the Felsenstein PHYLIP package (version 3.65). Briefly, 100 alignment replicates were created by the bootstrap method using the SEQBOOT program [37,38]. The bootstrapped dataset was then used to generate trees by the parsimony method using the PROTPARS program. For calculating distance, PROTDIST was used to generate 100 matrices from the bootstrapped dataset, by applying the Dayhoff PAM (point accepted mutation) evolutionary model of amino acid substitutions [39]. A total of 100 distance trees were built by the neighbour-joining method [40]. The final consensus trees were built using the majority rule and were visualized using TreeViewX (version 0.4) and Mega 3.1 (version 3.1) [41].

**Isolation of cells overexpressing TgPPase**

The *TgPPase* gene was amplified using primers (5′-AGATCT-ATGGAGTCGACCTTGCGC-3′ and 5′-CTAGGGTAA-GCCACAACCTTTCG-3′) containing BglII and AvrII restriction enzyme sites (underlined) by RT (reverse transcription)–PCR. The PCR products were cloned into the expression vectors pTubP30-FLAG/sagCAT (a gift from Dr William Sullivan, Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, IN, U.S.A.) [42], replacing the P30 gene. The resulting construct pTub-TgPPase–FLAG/sagCAT was sequenced and used for transfection of *T. gondii* tachyzoites using published protocols [33]. Selection was performed with 20 μM chloramphenicol. Cloning was performed by limited dilution and one clone was selected for further analysis and named TgPPase-OE (*TgPPase*-overexpressing cells). A clone expressing two copies of the gene encoding YFP was used for immunofluorescence co-localization assays [30].

**Preparation of recombinant TgPPase**

The whole open reading frame of *TgPPase* was amplified by RT–PCR with the primers 5′-CATATGCGATCTTGACCTTGCGC-3′ and 5′-GTCGAGCGATGCAACCTTTTCG-3′, containing the restriction sites for NdeI and Sall (underlined). The PCR product was cloned into the pCR 2.1-TOPO TA vector and subcloned into the expression vector pET28a<sup>+</sup>. The recombinant construct *TgPPase*/pET28a was transformed into *Escherichia coli* BL21-CodonPlus(DE3)-RIPL (Stratagene), and protein expression was induced with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside). rTgPPase (recombinant *TgPPase*) was purified with a His·Bind Quick 900 cartridge according to the manufacturer’s instructions (Novagen). The eluted fractions were pooled and desalted using a HiTrap column.

**Antibody generation and purification**

The purified rTgPPase protein was sent to Cocalico Biologicals for production of guinea pig polyclonal antiserum. The antiserum was affinity-purified with the cyanogen-bromide-activated resin [43].

**SDS/PAGE and Western blot analysis**

Proteins were separated by SDS/PAGE using standard protocols. Western blot analysis was performed as described previously [44] using an affinity-purified anti-*TgPPase* polyclonal antibody (1:10000 dilution) or anti-(α-tubulin) monoclonal antibody (1:15000 dilution).
Fluorescence microscopy

Immunofluorescence assays were performed as described previously [44] by using as primary antibody the purified anti-TgPPase at 1:100 dilution. The secondary antibody was Alexa Fluor® 555-conjugated goat anti-guinea pig IgG (H+L) (1:1000 dilution). Images were collected using an Olympus IX-71 inverted fluorescence microscope with a Photometric-cooled charge-coupled-device camera (CoolSnapHQ), and deconvolved for 15 cycles using Softworx deconvolution software (Applied Precision).

PPase activity measurement

rTgPPase activity was assayed by measuring the release of P_i, using a method described previously [45]. The standard assay mixtures varied based on what was specifically being tested. For pH-optimum experiments, assay conditions were 20 mM Tris/Hepes, 1.36 ng of purified rTgPPase protein and 23 μM PP_i, with 3 mM MgCl_2 (as a cofactor) for experiments using PP_i, as a substrate. The same buffer and amount of protein were used with 9 μM poly P_3 (tripolyphosphate) and 3 mM CoCl_2 (as a cofactor) for experiments using poly P_3 as a substrate. Experiments testing inhibition of rTgPPase activity used 50 mM Tris/HCl (pH 8.5) when PP_i was the substrate and 50 mM Mes (pH 6.0) when poly P_3 was the substrate. For experiments testing divalent cation cofactor preference, the assay conditions were similar, but cofactors were used as stated in the Figure legends at a final concentration of 3 mM. Experiments testing alternative substrates [100 μM GTP (guanosine triphosphate), 100 μM ATP or 100 μM poly P_3 (as a cofactor)] used assay mixtures containing 50 mM Tris/HCl (pH 8.5) or 50 mM Mes (pH 6.5) and 3 mM MnCl_2 and 45 ng of purified rTgPPase. After a 10 min incubation at 37 °C, reactions were stopped by the addition of an equal volume of a mixture of three parts of 0.045% Malachite Green and one part of 4.2% ammonium molybdate [46]. The absorbance at 660 nm was measured with a SpectraMax M2® plate reader (Molecular Devices). The specific activity of rTgPPase was defined as μmol of P_i released min⁻¹ per mg of protein. For determination of optimal pH conditions, a 20 mM Tris/Hepes mixed buffering system was employed to ensure differences in activity were only due to pH and not ionic conditions. Inhibitor experiments were conducted at K_i concentrations for both PP_i and poly P_3.

To measure PPase activity in total cell lysates, purified tachyzoites were washed twice with BAG [buffer A plus glucose: 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4, 50 mM Hepes (pH 7.2) and 5.5 mM glucose], and resuspended in a small amount of the same buffer. The cells were broken by sonication in a Branson Sonifier 450 instrument (10% amplitude, two 5 s pulses).

The activity was also measured in subcellular fractions obtained after lysis of tachyzoites with silicon carbide as described previously [24]. The homogenate was centrifuged at 100 000 g for 20 min at 4 °C, and these occupied 1% of the total cell volume. Acidocalcisomes volume was estimated assuming a spherical shape with a diameter of 200 nm and an average of ten acidocalcisomes per extracellular tachyzoite.

ATP was measured using an ATP determination kit (Molecular Probes). For calculation of the intracellular concentrations of ATP in tachyzoites, a cell volume of 16.5 ± 3 μl for 10^6 cells was used [24].

Measurement of proton extrusion in wild-type (RH) and TgPPase-OE cells

Rates of proton extrusion were measured in RH and TgPPase-OE cells following basic procedures [48]. Freshly egressed tachyzoites were washed with BAG and split into two portions: cells washed further and suspended in BAG, and cells washed further and suspended in buffer A without glucose. Both cell preparations were kept on ice at a concentration of 1×10^6 cells·ml⁻¹ until analysis. Proton extrusion was measured as described previously [48]. The fluorescence was measured using a PL-4500 fluorescence spectrophotometer (Hitachi). Calibration of fluorescence measurements to specific pH values was performed as described previously [48].

Measurement of intracellular pH in wild-type (RH) and TgPPase-OE cells

Loading of RH and TgPPase-OE cells with BCECF-AM for pH measurements was performed as described previously [49]. Intracellular pH was measured in a SpectraMax M2® plate reader at excitation wavelengths of 505/440 nm and emission at 530 nm. The calibration of the pH units/fluorescence ratio was as described previously [49,50].

Measurement of lactate extrusion in wild-type (RH) and TgPPase-OE cells

Freshly egressed tachyzoites were washed three times with BAG and resuspended in the same buffer at a cell concentration of 5×10^6 cells·ml⁻¹. Reactions were terminated at different
times (0–10 min) by spinning the cells down and removing the supernatant for analysis of the extruded lactate. This metabolite was quantified by the enzymatic conversion of lactate into pyruvate using LDH (Sigma) and measurement of the concomitant production of NADH. Enzymatic assay conditions were set up following published conditions [51,52]. A 70 μl aliquot equivalent to 3.5 × 10⁷ cells was added to 200 μl of reaction mixture (600 mM glycine, 346 mM hydrazine, 17 mM EDTA, 15 mM NAD and 10 units ml⁻¹ LDH; pH = 9.2) and fluorescence was monitored on the SpectraMax M2e plate reader for 20 min. NADH was measured using excitation and emission wavelengths of 340 nm and 460 nm respectively. Fluorescence values were converted into units of lactate using a standard curve where known amounts of L-lactate were added to the reaction mixture and NADH production was monitored.

RESULTS

Cloning the gene encoding TgPPase

The gene encoding a soluble PPase was identified by a BLASTN search with the T. brucei soluble inorganic PPase sequence (GenBank® accession number AAP747002). The T. gondii gene encodes a protein of 381 amino acids (TgPPase) with a predicted molecular mass of 42 kDa. The TgPPase sequence has an overall identity with Y-PPase (yeast PPase) of only 28% , but the 13 essential amino acids that form the active-site structure of the Y-PPase [inferred from the crystal structure (PDB code 1WGU)] are conserved [53] (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/440/bj4400229add.htm). The gene is expressed in both tachyzoites and bradyzoites (http://toxodb.org/toxo/, TGME49_083830).

Phylogenetic analysis of the TgPPase gene

The TgPPase amino acid sequence (AAU88181) was used as a query to search for orthologues in the OrthoMCL database (version 2). Our phylogenetic analysis shows two main branches with high bootstrap support (100%/72%) when kinetoplastid inorganic PPases are used as the outgroup (Figure 1). The two groups are (i) bacterial and bacterial-like plant soluble PPases, and (ii) plant chloroplast and fungal/animal cytosolic and mitochondrial PPases (Figure 1). In addition, our phylogenetic tree supports the notion that the T. gondii enzyme is a type I inorganic PPase since it clusters with fungal/animal and plant chloroplast soluble inorganic PPases with high bootstrap confidence levels using both distance and parsimony methods (74%/74%) (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/440/bj4400229add.htm for the alignment of the sequences used for the tree in Figure 1).

Expression, purification and biochemical characterization of TgPPase

The expression of TgPPase and purification by affinity chromatography produced a single protein band (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/440/bj4400229add.htm). The purified protein was characterized biochemically.

The enzymatic activity of the rTgPPase showed an absolute requirement for divalent cation cofactors, with Mg²⁺ being the most efficient at a concentration of 1 mM (238.17 ± 6.51 μmol Pₗ · min⁻¹ per mg of protein). The optimum pH for the hydrolysis of PP was 8.5 (Figure 2A). At this pH, other cations such as Co²⁺, Zn²⁺, Ca²⁺ and Mn²⁺ marginally stimulated the activity over basal levels (Figure 2C). The enzyme was also able to hydrolyse poly Pₗ, although with lower efficiency (Table 1). For this polyphosphate activity the preferred cofactor was Co²⁺ (Figures 2B and 2D), as occurs with Y-PPase [13,16], and the optimum pH range was 6–6.5. The enzyme had low hydrolysing activity of long-chain poly P (poly Pₗ), ATP and GTP, in the presence of Mn²⁺ as the divalent cation cofactor (see Supplementary Figures S4A and S4C at http://www.BiochemJ.org/bj/440/bj4400229add.htm).

Table 1, Figures 2(E) and 2(F) and Supplementary Figures S4(B) and S4(D) show the kinetic properties of the enzyme for the hydrolysis of PPₗ, poly Pₗ, poly Pₗ, ATP and GTP. At the optimal conditions, it is clear that the catalytic efficiency for the hydrolysis of PPₗ (measured as kcat/Km) is significantly higher than for the hydrolysis of the other substrates.

Inhibition of the rTgPPase

AMDP, a specific inhibitor of V-H⁺-PPase, did not show any effect on the activity of this enzyme at a concentration of 40 μM
Table 1  Kinetic parameters of rTgPPase with different substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>Cation</th>
<th>$V_{max}$ ($\mu$mol·min$^{-1}$ per mg)</th>
<th>$K_m$ ($\mu$M)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$·M$^{-1}$)</th>
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<tr>
<td>PP$_i$</td>
<td>8.5</td>
<td>Mg$^{2+}$</td>
<td>264.45 ± 8.33</td>
<td>23.10 ± 0.03</td>
<td>194.40</td>
<td>8.42 × 10$^6$</td>
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<tr>
<td>Poly P$_3$</td>
<td>6.0</td>
<td>Co$^{2+}$</td>
<td>6.41 ± 0.25</td>
<td>9.01 ± 1.21</td>
<td>0.14</td>
<td>1.57 × 10$^4$</td>
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<tr>
<td>Poly P$_{35}$</td>
<td>6.5</td>
<td>Mn$^{2+}$</td>
<td>6.16 ± 0.18</td>
<td>6.57 ± 0.95</td>
<td>0.28</td>
<td>4.31 × 10$^4$</td>
</tr>
<tr>
<td>ATP</td>
<td>6.5</td>
<td>Mn$^{2+}$</td>
<td>5.27 ± 0.23</td>
<td>51.42 ± 6.89</td>
<td>0.24</td>
<td>4.71 × 10$^3$</td>
</tr>
<tr>
<td>GP$_4$</td>
<td>6.5</td>
<td>Mn$^{2+}$</td>
<td>5.22 ± 0.40</td>
<td>98.66 ± 19.55</td>
<td>0.24</td>
<td>2.43 × 10$^3$</td>
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Figure 2  Enzymatic characterization of rTgPPase

PPase activity was determined as described in the Experimental section using 23 $\mu$M PP$_i$ (A and C) or 9 $\mu$M poly P$_3$ (B and D). (A and B) Optimum pH measurements with PP$_i$ (A) or poly P$_3$ (B) as substrate. A 20 mM Tris/Hepes mixed buffering system was used to manipulate the pH of the reaction buffer. (C and D) Optimum cation concentration measurements with PP$_i$ (C) or poly P$_3$ (D) as substrate. Results are expressed as percentage of maximum activity, taken as 100%. Where indicated, 5 mM EDTA was used. (E and F) Enzymatic activity measured with different concentrations of PP$_i$ (E) or poly P$_3$ (F). Experiments were performed using 3.0 mM MgCl$_2$, (E) or 3 mM CoCl$_2$ (F). Insets represent the linear transformation, by double reciprocal plot, of each curve. Experiments were repeated three times, each one in triplicate, with similar results. Results are means ± S.E.M. for three experiments.

Subcellular localization of TgPPase

To investigate the localization of TgPPase, we performed indirect IFA (immunofluorescence analysis) in tachyzoites expressing cytosolic YFP using antibodies against the TgPPase enzyme. As evidenced by the colocalization with YPF, the endogenous localization of the TgPPase enzyme was determined to be in the cytosol in tachyzoites of *T. gondii* (Figure 3A). Western blot analysis of cytosolic and membrane fractions of tachyzoites confirmed the cytosolic localization of the protein (Figure 3B). Both the specific activity and the total activity of the enzyme were higher in the soluble fractions (Figures 3C and 3D).

Effect of overexpression of TgPPase on cell growth and metabolism

A clone of *T. gondii* tachyzoites was isolated after limited dilution of cells transfected with the pTubTgPPase-FLAG/sagCAT plasmid and selection with chloramphenicol. These cells showed significantly higher levels of TgPPase protein (Figure 4A) and enzymatic activity (Figure 4B; $P < 0.05$). Further IFA localization
Figure 3  TgPPase localizes to the cytosol of wild-type RH T. gondii tachyzoites

RH (wild-type) tachyzoites expressing YFP were used to test for colocalization of endogenous expression of TgPPase. (A) TgPPase localized to the cytoplasm of RH tachyzoites. Parasites were fixed and stained with an anti-TgPPase antibody (1:100 dilution) or observed by direct YFP fluorescence where indicated. The overlay image shows co-localization of both proteins in the cytosol. Scale bars, 5 μm. (B) Western blot analysis of subcellular fractions of T. gondii tachyzoites. Equal protein amounts (5 μg) from supernatant (S) and pellet (P) fractions were loaded. The molecular mass standards (in kDa) are shown on the left-hand side. The blue arrow indicates the band corresponding to the TgPPase. (C and D). Specific and total enzymatic activity, respectively, in supernatant (S) and pellet (P) fractions after centrifugation of lysates at 100 000 g for 1 h. Other experimental details are as described in the Experimental section. DIC, differential interference contrast.

studies with cytosolic YFP determined that the TgPPase protein in OE (overexpressing) mutants maintained a cytosolic localization (results not shown). Rates of growth in OE mutants using uracil incorporation were found to be significantly lower (P < 0.05) when compared with the growth rate of wild-type RH parasites (Figure 4C). The lower growth rates were confirmed using plaque assays to measure growth/invasion efficiency in wild-type and OE mutants (Figures 4D–4F). OE mutants had significantly lower plaque numbers (P < 0.001) as well as lower relative size of plaque-forming units (P < 0.05).

To understand the possible consequences of elevated PPass activity in OE mutants, we investigated the cellular amounts of phosphate-related molecules. Cellular levels of PPi, were 40% lower in wild-type RH cells (Figure 5A; P < 0.01). Cellular concentrations of short-chain poly P in OE mutants and RH cells were similar at ∼5 mM in phosphate equivalents (Figure 5B). Under identical conditions as above, cellular concentrations of ATP were significantly elevated (P < 0.05) by 75% in extracellular tachyzoites overexpressing TgPPase (Figure 5C). However, under conditions in which no glucose was present, the difference in ATP concentration was abolished.

In order to rule out the possibility that the PPase OE phenotype was only a consequence of genetic manipulation and therefore non-specific in nature, we measured cellular PP, concentration and growth in mutant parasites expressing an unrelated gene, the fluorescent tomato protein (hereafter referred to as Tomato OE mutants). The Tomato OE mutants were derived from the same genetic background as the PPase OE mutants and underwent similar genetic manipulation. The cellular PP, concentration in the Tomato OE parasites was similar to that measured in wild-type RH parasites (see Supplementary Figure S6A at http://www.BiochemJ.org/bj/440/bj4400229add.htm). Likewise, growth rates in these unrelated expression mutants were similar to those of wild-type RH parasites as measured by plaque assays (Supplementary Figure S6B). These results indicate that the phenotype observed in the PPase OE mutants is not an artefact of genetic manipulation and bolster the relationship between PP, concentration and ATP and growth.

Since most PP, in T. gondii is localized in acidocalcisomes [24], we investigated whether cytosolic or organellar PP, was affected in cells overexpressing the PPase. The distribution of PP, in the cytosolic and organellar fractions of extracellular tachyzoites was determined by lysis of the plasma membrane by freeze–thaw cycling in a hypotonic buffer. In order to determine the concentration of PP, in these fractions, we assumed that the cellular volume and the size and number of acidocalcisomes in the OE mutants were similar to those of wild-type parasites (i.e. 1×10⁹ parasites = 16.5 μl and acidocalcisomes occupy 1% of the cellular volume). This assumption was confirmed in our microscopic analyses and in our indirect immunofluorescence assays examining acidocalcisome markers (e.g. TgVP1; results not shown). The majority of PP, was found in the organellar fraction (∼360 mM; Figure 6B). A significant difference, however, was observed in the soluble fraction (Figure 6A). RH cells had more than six times the concentration of PP, than OE mutants (0.55 and 0.09 mM respectively; P < 0.01). The efficiency of this fractionation was verified by measuring LDH activity in both the supernatant (i.e. cytosolic) and the pellet (i.e. organellar) fractions. Over 95% of measured LDH activity was found in the cytosolic fraction (results not shown). In conjunction with the PP, distribution, these results demonstrate effective lysis of the plasma membrane without significant disruption of organelles containing PP, (i.e. acidocalcisomes). These results indicate that the total cellular differences in PP, are a result of different concentrations of PP, in the cytosol, which is in agreement with the cellular location of the TgPPase enzyme (Figure 3).
Metabolic changes in TgPPase-OE cells as measured by proton extrusion, lactic acid release and intracellular pH

*T. gondii* is known to preferentially metabolize glucose to lactic and acetic acids and excrete them into the medium, accompanied by protons [54]. The large glucose-dependent differences observed in ATP concentration in conjunction with the cytosolic differences in PP concentration between RH and OE mutant parasites prompted us to investigate the glycolytic flux of extracellular tachyzoites. Proton extrusion and lactate extrusion were measured in extracellular tachyzoites in the presence and absence of glucose. In cells that rely on glycolysis for energy production, these processes have been shown to be reliable metrics of glycolytic flux [55–57].

To investigate whether the increase in ATP levels resulted as a consequence of an increased glycolytic rate, proton extrusion was measured in RH parasites and OE mutants, using the free-acid form of BCECF and quantifying the rate at which the pH (pH of extracellular medium) changed. Proton extrusion was measured in RH cells (Figures 7A, 7C and 7E) and OE mutants (Figures 7B, 7D and 7F) in the presence of glucose (5 mM) and compared with cells deprived of glucose for ∼ 2 h (Figures 7A and 7C, RH cells; Figures 7B and 7D, OE mutants). Furthermore, 5 mM glucose was added to the cells deprived of glucose during the proton extrusion measurement to evaluate the immediate response in cellular metabolism (Figures 7A, trace b, and 7E, RH cells; Figures 7B, trace b, and 7F, OE mutants). RH cells showed a small, but significant, increase (ANOVA: df = 1,11; *P* < 0.01) in proton extrusion when glucose was present (Figure 7A, trace c) or when glucose was added during the experimental incubation period (Figure 7A, trace b) relative to rates when cells were glucose-deprived (Figure 7A, trace a). Proton extrusion rates in OE mutants exhibited a greater sensitivity to the presence or absence of glucose. In the absence of glucose, (Figure 7B, trace a) rates of proton extrusion in OE mutants were slightly lower, although statistically similar to rates in glucose-deprived RH cells (average glucose-deprived rates were $-4.3 \times 10^{-4}$ and $-3.1 \times 10^{-4}$ pH units s$^{-1}$ for RH and OE cells respectively). When glucose was present in the experimental buffer, rates of proton extrusion in OE cells exhibited a significant 3.2-fold increase (ANOVA: df = 1,11; *P* < 0.001) from $-3.1 \times 10^{-4}$ to $-9.8 \times 10^{-4}$ pH units s$^{-1}$ (Figure 7B, traces b and c). Average rates of proton extrusion while glucose was present were significantly higher (ANOVA: df = 1,11; *P* < 0.001) in OE cells by 1.6-fold, relative to RH cells ($-9.8 \times 10^{-4}$ and $-6.0 \times 10^{-4}$ pH units s$^{-1}$ for TgPPase-OE and RH cells respectively; compare Figures 7D and 7F with 7C and 7E, grey columns). Despite the large differences in proton extrusion, there was no significant difference in pH$_i$ (intracellular pH) between RH parasites and OE mutants (ANOVA: df = 1,5; *P* > 0.05; results not shown). The average pH$_i$ was 7.18 and 7.23 for RH and OE cells respectively. Rates of proton extrusion in the unrelated Tomato OE mutants were similar to those of wild-type parasites (Supplementary Figure S7 at http://www.BiochemJ.org/bj/440/bj4400229add.htm), providing further support for the relationship between cellular PP concentration and glycolytic flux in *T. gondii*.

To better assess the link between PP, and glycolytic flux, rates of lactate extrusion were measured in RH and soluble PPase OE mutants as an independent verification of the rate of glycolysis. Rates of lactate extrusion were measured in RH cells and OE mutant cells in which glucose had been added to the experimental buffer (5 mM). Duplicate measurements of the amount of lactate extruded to the extracellular medium over the course of 10 min (measurements were taken at 0, 1, 2, 5 and 10 min) showed a significant linear increase with time for both cell types (Figure 8). The lactate extrusion rate for RH cells was 1.07 ± 0.03 (S.E.M.) fmol·h$^{-1}$·cell$^{-1}$, and for the OE cells (Figure 8, inset) it was 1.90 ± 0.04 (S.E.M.) fmol·h$^{-1}$·cell$^{-1}$ (ANOVA: df = 1,18; *P* < 0.001). The difference in lactate extrusion between RH and OE cells was 1.8-fold, a value similar to the difference in proton extrusion rates for both cell types (1.6-fold, see above).
Figure 5  *T. gondii* parasites overexpressing TgPPase contain lower PPi, and higher ATP levels

(A) Overexpression of TgPPase leads to a significant reduction in total PPi levels as compared with wild-type RH tachyzoites (ANOVA: df = 1,16; *P* < 0.01). Results are averages ± S.E.M of nine quantifications for each cell type. Values are in phosphate equivalents of hydrolysed short-chain poly P groups. (B) No difference in short-chain poly P levels between wild-type RH and OE mutants for TgPPase. Results are averages of nine determinations for each cell type. (C) Overexpression of TgPPase leads to an increase in the intracellular levels of ATP in the presence of glucose. Newly released tachyzoites were filtered and washed twice with BAG with or without glucose and incubated in the same buffer for 1 h. ATP was extracted and measured as described in the Experimental section. The results are representative of three independent experiments, each one performed in triplicate. Results are means ± S.D. *P* < 0.05 (Student’s t test).

Figure 6  PPi content of cytosolic and acidocalcisome fractions

PPi was determined in cytosol (A) and 14 000 g pellet (acidocalcisome) (B) fractions obtained as described in the Experimental section from wild-type (RH) and TgPPase-OE parasites. Results represent the average concentration ± S.E.M. of three quantifications for each cell type. Concentration was determined by dividing the total PPi (nmol) in the cytosolic and organelar fractions by their respective estimated volumes, as described in the Experimental section. The cytosolic PPi concentration was significantly lower in OE mutants (ANOVA: df = 1,4; *P* < 0.01).

The difference in the proton and lactate extrusion rates between RH and OE cells might be due to an inhibitory effect of PPi on the glycolytic pathway. The *T. gondii* PK (pyruvate kinase) is an allosteric enzyme and is activated by glucose 6-phosphate [58]. We investigated whether PPi, could be inhibiting this enzyme by measuring its activity in a *T. gondii* lysate in the presence of various PPi concentrations. For the range of PPi concentrations that we measured in the cytosol (i.e. up to 1 mM), we found no detectable inhibition of PK (results not shown).

**DISCUSSION**

The goals of the present study were to characterize the enzyme that directly regulates cytosolic levels of PPi in the apicomplexan parasite *T. gondii* and employ its expression as a means to manipulate cytosolic PPi concentrations in this medically relevant parasite. Bioinformatic searching (e.g. BLASTN searching and querying http://toxodb.org/toxo/) for soluble PPase yielded TgPPase as the only soluble PPase found in the *T. gondii* genome. PPi, is both an important substrate and product for several metabolic reactions, and its generation in the cytosol is highly regulated so as to avoid a build-up of toxic concentrations. For these reasons, we chose to use the overexpression of TgPPase as a means to manipulate the cytosolic concentration of PPi. It was our expectation that, given the placement of PPi, in so many key metabolic reactions, its manipulation would have far-reaching consequences on the metabolism of *T. gondii* tachyzoites.

**Figure 7**  Proton extrusion by wild-type (RH) and TgPPase-OE parasites

Rates of proton extrusion were measured using the free-acid form of BCECF. (A, C and E) Summary of proton extrusion in RH cells. (B, D and F) Summary of proton extrusion in OE mutant cells. (A) Representative tracings depicting changes in pH e in RH tachyzoites in the presence of 5 mM glucose (black, trace c), no glucose (light grey, trace a) and in cells where 5 mM glucose was added 300 s into recording (dark grey, trace b). (B) Same tracing as in (A) using OE mutants. (C) Resultant slope values (change in pH e per s) in the presence (grey) or absence (white) of glucose for proton extrusion in RH cells using data represented in traces a and c in (A). (D) Resultant slope values (change in pH e per s) in the presence (grey) or absence (white) of glucose for proton extrusion in OE mutant cells using data represented in traces a and c in (B). (E) Rate of pH change before (white) and after (grey) addition of 5 mM glucose to medium in RH cells using data represented in trace a in (A). (F) Rate of pH change before (white) and after (grey) addition of 5 mM glucose to medium in OE mutant cells using data represented in trace b in (B). All estimates shown in panels (C–F) were determined from three independent experiments, and error bars represent the S.E. of the slope.
The alignment of the predicted TgPPase protein sequence shows conservation of amino acids present in other PPases. Considering its phylogenetic position, its Mg$^{2+}$ requirement and its fluoride sensitivity, the TgPPase belongs to the family I PPases. Phylogenetic analysis shows that apicomplexan PPases cluster with the sequences belonging to mitochondria and chloroplasts, suggesting a proteobacterial origin. The cytosolic localization of TgPPase could be due to specific requirements of this enzyme in T. gondii. One hypothesis could be that the TgPPase gene was acquired through horizontal gene transfer, a mechanism that has been shown to occur in apicomplexan parasites [59–62]. Since the kinetoplastid PPases are highly divergent but still related to other type I inorganic PPases, we selected them as an outgroup (Figure 1). In a study by Gómez-García et al. [63], the Leishmania major inorganic PPase was suggested to be an ancestral type I eukaryotic soluble PPase with a calcium-dependent activity.

The catalytic efficiency of the purified recombinant TgPPase enzyme, as shown by the $k_{cat}/K_m$ values in Table 1, indicates that the enzyme is highly efficient when hydrolysing PP$\_i$. The enzyme is also able to hydrolyse poly P$_5$, poly P$_8$, ATP and GP$\_i$ with lower efficiency (Table 1). It is surprising that the recombinant enzyme shows an optimum pH of 8.5 and at the same time is cytosolic (Figure 3). On analysing Figure 2(A) it can be observed that the activity is still high at cytosolic pH 7.1–7.4. The enzyme preference for Mg$^{2+}$, when hydrolysing PP$\_i$, and for other non-physiological cations such as Co$^{2+}$ or Mn$^{2+}$, when hydrolysing other substrates, is similar to what has been found for Y-PPase [16].

Given that the phylogenetic analysis and biochemical characterization fully supported the identity of TgPPase as a Type I PPase with a high specificity for PP$\_i$, and only much lower affinities for alternate substrates, we employed the overexpression of this enzyme as a means to decrease the cytosolic concentration of PP$\_i$. Overexpression successfully increased the production of TgPPase protein as well as its activity. This increased activity was still maintained in the cytosol of OE mutants, as measured by fractionation experiments and indirect immunofluorescence assays. In conjunction with this, we observed a significant decrease in the substrate of TgPPase, PP$\_i$, which was localized to the cytosol of OE mutants. These results confirm that the overexpression of TgPPase is a reliable tool for understanding the metabolic significance of PP$\_i$.

The phenotype of the T. gondii clone overexpressing the gene encoding TgPPase (OE mutant) was somewhat unexpected. Relative to the wild-type parasites, OE mutants had higher levels of ATP. T. gondii tachyzoites rely on glycolysis for energy production [64,65]. Unlike glycolysis in mammalian cells, T. gondii possesses a PFK that utilizes PP$\_i$ to drive the rate-limiting conversion of fructose 6-phosphate into fructose 1,6-bisphosphate [28]. Our initial expectation was that the decrease in cellular PP$\_i$ concentration would cause a decrease in glycolysis, since a key energetic substrate had been significantly reduced. Measurements of ATP concentration showed that, contrary to this expectation, energy metabolism had increased in the OE mutants. To ensure that the increase in ATP was a specific outcome of increased energy metabolism, we measured rates of glycolytic flux.

TgPPase OE mutants displayed a significant increase in glycolysis, as measured by both proton and lactate extrusion from extracellular tachyzoites. Rates of proton and lactate extrusion have been shown to be tightly linked to glycolytic flux in a wide range of prokaryotes and eukaryotes, including bacteria [56,66], yeast [67] and human platelets [55]. T. gondii, like other apicomplexans, obtains most of its energy through glycolysis [64,65], with lactate representing a dominant end-product of glycolysis [54,68]. Recently, Lin et al. [65] used lactate extrusion to measure glycolysis in extracellular tachyzoites of T. gondii. These reasons compelled us to use rates of proton and lactate extrusion as metrics of glycolytic flux for direct comparison between RH parasites and OE mutants. Both cell types responded to the addition of 5 mM glucose with increased rates of proton extrusion. However, the rate of extrusion was $\sim$ 60% higher in OE mutants when in the presence of glucose. Similarly, rates of lactate extrusion in the presence of 5 mM glucose were significantly higher ($\sim$ 80%) in the OE mutants. The high sensitivity and immediate response to the addition of glucose in both cell types is not surprising given that the localization of glycolytic enzymes in extracellular tachyzoites is at the pellicle region, just below the plasma membrane [47]. This stimulation in glycolytic flux resulted in a quantitatively similar increase in ATP concentration in the OE mutants (i.e. ATP concentration increased by 75%). To show that these phenotypic traits observed in the TgPPase OE mutants are specific to the reduction in PP$\_i$, we compared our results from wild-type RH cells and TgPPase OE mutants with a completely unrelated expression mutant. Genetically modified parasites expressing the fluorescent tomato protein had similar levels of PP$\_i$ to RH and, likewise, similar rates of growth and glycolysis (as measured by proton extrusion).

The regulatory mechanism by which cytosolic concentrations of PP$\_i$ can be related to higher glycolytic flux in T. gondii remains undetermined. Regulation of glycolysis has been studied in several organisms from bacteria to higher eukaryotes. It has been demonstrated in other cell types that PP$\_i$ inhibits several glycolytic enzymes, such as hexokinase in T. brucei [69] and PEPCK (phosphoenolpyruvate carboxykinase) in T. cruzi [70]. In animal cells, PP$\_i$, either administered directly or produced intracellularly by activation of short-chain fatty acids, produces effects that mimic glucagon injection in that liver glucose is increased, and there is a change in the 3-phosphoglycerate/pyruvate ratio, suggestive of PK inhibition [4]. Three enzymes that typically are observed to be critical regulators of glycolytic flux are hexokinase, PFK and PK. In T. gondii, however, very little is known about the regulation of glycolysis [71]. T. gondii possesses one annotated hexokinase, but it does not use PP$\_i$ as a substrate and is not
inhibited by it either [71]. Our present analysis of PK in *T. gondii* wild-type and TgPPase-overexpressing cells found no significant difference in PK activity for the range of PP, differences between the two cell types.

As mentioned earlier, *T. gondii* tachyzoites possess a plant-like PFK that uses PP, as the high-energy phosphoryl donor rather than ATP (PP, PFK). Therefore a decrease in PP, would be expected to result in a corresponding decrease in the rate-liming conversion of fructose 6-phosphate into fructose 1,6-bisphosphate by PP, PFK. However, the *Km* for PP, PFK is 33 μM [28]. The cytosolic concentrations of both wild-type and OE mutants may be high enough (550 and 90 μM respectively) to result in only minimal differences in PP, PFK activity. An alternative explanation that has been proposed in plant cells [72] is that the increase in Pi from the breakdown of PP results in activation of fructose-6-phosphate 2-kinase, the product of which is fructose 2,6-bisphosphate, a strong activator of PP, PFK [72]. However, unlike plant cells, the PP, PFK of *T. gondii* does not appear to be activated by fructose 2,6-bisphosphate [28].

The up-regulation of glycolysis and increase in ATP levels due to decreases in cytosolic PP, has also been observed in plant cells [72,73]. In developing tubers of potato, cytosolic overexpression of PPhase causes a decrease in cytosolic PP, as well as an unexpected increase in sucrose degradation and subsequent increases in glycolysis and starch production [73]. The up-regulation of these pathways is thought to be due to the PP-, dependent sucrose-degradation pathway and downstream activation of starch synthesis. The cytosol of *T. gondii* possesses crystalline, water-soluble, polysaccharide particles called amylopectin granules, which are similar to those of plant starch [74]. Although these granules are typically associated with the bradyzoite and sporozoite stages, it has been shown that acidification of the medium for tachyzoites can cause a significant increase in the presence of amylopectin granules [74]. The increase that we observe in glycolysis and concomitant proton extrusion may be related to the activation of amylopectin synthesis and therefore analogous to stimulation of starch synthesis seen in plant cells. In addition, this effect could lead to the observed decrease in uracil incorporation and plaque formation, demonstrating a decrease in growth rates in overexpressing parasites.

It is a widely held concept that soluble PPases play an important metabolic role by driving reactions such as protein, RNA and DNA synthesis to completion [1] and that the PP, produced in anabolic reactions is instantly hydrolysed. However, a potential role of OE mutants may be high enough (550 and 90 μM respectively) to result in only minimal differences in PP, PFK activity. An alternative explanation that has been proposed in plant cells [72] is that the increase in Pi from the breakdown of PP results in activation of fructose-6-phosphate 2-kinase, the product of which is fructose 2,6-bisphosphate, a strong activator of PP, PFK [72]. However, unlike plant cells, the PP, PFK of *T. gondii* does not appear to be activated by fructose 2,6-bisphosphate [28].

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SUPPLEMENTARY ONLINE DATA

Overexpression of a cytosolic pyrophosphatase (TgPPase) reveals a regulatory role of PP_i in glycolysis for *Toxoplasma gondii*

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Figure S1  Multiple sequence alignment of TgPPase and type I inorganic PPases

The multiple sequence alignment was created using Clustal W and visualized by GeneDoc. Identical residues (>90%) among PPases are in black. The 13 essential active-site residues conserved in all type I soluble PPases are indicated by numbers above the alignment. Other conserved active-site residues are shown by asterisks. The four specific conserved interface residues of fungal/animal PPases are shaded in light grey and labelled 'i' above the alignment. Sequence accession numbers as provided by OrthoMCL DB or GenBank® are: *Toxoplasma gondii* (AAU88181), *Plasmodium falciparum* 3D7 (PFC0710w), *Cryptosporidium parvum* (cgd4_1400), *Escherichia coli* W3110 (NP_418647.1), *Saccharomyces cerevisiae* cytosolic PPase (YBR011C) and *Homo sapiens* cytosolic PPase (ENSP00000317687). CYT, cytosolic PPase; MIT, mitochondrial PPase.

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The nucleotide and protein sequence data reported will appear in GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession numbers AY619688 and AAU88181 respectively.
The multiple sequence alignment was created using Clustal W and visualized by GeneDoc. Identical residues (>70%) among PPases are shown in white lettering on a black background. The 13 essential active-site residues conserved in all type I soluble PPases are shaded in orange and indicated by the numbers above the alignment. Other conserved active-site residues are shaded in blue and indicated by asterisks. The four specific conserved interface residues of fungal/animal PPases are shaded in yellow and labelled as ‘i’ above the alignment. Sequence accession numbers from GenBank® are indicated in parentheses as follows: *Aquifex aeolicus* VF5 (NP_214066.1), *Arabidopsis thaliana* chloroplast PPase (F17114.160), *A. thaliana* (T25K16.5), *Chlamydomonas reinhardtii* mitochondrial PPase (AJ398232), *C. reinhardtii* chloroplast PPase (AJ398231), *Cryptosporidium parvum* (cgd4_1400), *Escherichia coli* W3110 (NP_418647.1), *Homo sapiens* cytosolic PPase (Q15181), *H. sapiens* mitochondrial PPase (Q9H2U2), *Kluyveromyces lactis* cytosolic PPase (Tb927.3.2840/Tb03.27C5.190), *Leishmania major* putative mitochondrial PPase (LmjF03.0910), *Mus musculus* cytosolic PPase (BAB25754.1), *M. musculus* mitochondrial PPase (BAB22922), *Nostoc sp.* PCC7730 (P80629), *Oryza sativa* chloroplast PPase (BAD16934.1), *O. sativa* (BAD87839), *Plasmodium falciparum* 3D7 (PFC0710w), *Rhodospirillum rubrum* (Q9RGQ1), *Saccharomyces cerevisiae* cytosolic PPase (YMR267W), *S. cerevisiae* mitochondrial PPase (YMR267W), *Solanum tuberosum* (CAA12415), *Synechocystis PCC6803* (P80507), *Thermus thermophilus* (BA924521), *Thermoplasma acidophilum* (P37981), *Toxoplasma gondii* (AAU88181), *Trypanosoma cruzi* putative PPase (t000.1047053508181.140), *Trypanosoma brucei* putative PPase (t0927.3.2840/t0927.27C5.190) and *Zea mays* (O48556).
Figure S3  Purification of recombinant TgPPase protein

The TgPPase gene was cloned into the bacterial expression vector pET28a+ and was expressed in E. coli BL21-CodonPlus(DE3)-RIPL as a fusion protein with an N-terminal poly-His tag. Affinity chromatography purification produced a single protein band, indicating isolation of a pure enzyme. Proteins were separated by SDS/PAGE (10% gel) and stained with Coomassie Brilliant Blue. Lane 1, crude extract from non-induced cells transformed with TgPPase/pET28a vector; lane 2, crude extract from induced cells transformed with TgPPase/pET28a recombinant vector; lane 3, supernatant fraction of whole-cell lysate clarified by centrifugation; and lane 4, the eluted protein, after purification through an Ni-NTA column. The band that corresponds to the purified TgPPase is indicated. Molecular mass in kDa is shown on the left-hand side.

Figure S4  Enzymatic activity of rTgPPase at various pHs (A), or with different metal cofactors (C) and poly P₇₅, ATP and GP₇₅ concentrations (B and D)

PPase activity was determined as described in the Experimental section in the main paper using 100 μM GP₇₅, 100 μM ATP and 100 μM poly P₇₅ (A and C). (C) Results are expressed as percentage of maximum activity, taken as 100%. Where indicated, 5 mM EDTA was used. The experiments were performed using 3.0 mM MnCl₂ (A, B and D) and 50 mM Tris/HCl at different pHs (A) or at pH 6.5 (B and D). (D) Linear transformation, by double reciprocal plot, of the curve in (B). Results are means ± S.E.M. for three independent experiments.
Figure S5  Effect of phosphatase inhibitors on rTgPPase activity

PPase activity with PPi (A and B) or poly P3 (C and D) as substrate was determined as described in the Experimental section in the main paper. All assays were performed using standard conditions for each substrate as indicated in the legend to Figure 2 in the main paper, but with substrate concentrations at Km values for PPi (23 μM) and poly P3 (9 μM). Various concentrations of sodium molybdate or sodium fluoride were added where indicated.

Table S1  Summary of IC50 calculations for TgPPase inhibition data shown in Supplementary Figure S5

Maximum inhibition and IC50 values are shown (± S.E.M.).

<table>
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Figure S6  PPi (A) and growth rates (B) in Tomato OE mutants, relative to wild-type RH parasites and PPase OE mutants

(A) Cellular concentration of PPi in RH, PPase OE mutants and Tomato OE mutants. Data for RH and pyrophosphatase OE mutants are taken from Figure 5(A) in the main paper. Data for the Tomato OE mutant represents mean ± S.E.M. of three determinations. (B) Growth in wild-type RH and Tomato OE mutants as measured by plaque assays. Values represent the average ± S.E.M. of six determinations of the number of plaque units that formed after addition of 200 parasites to each well in a six-well plate. Parasites were allowed to grow for 7 days, at which time the experiments were terminated and the plaques were counted using counter-staining techniques.

Figure S7  Proton extrusion by wild-type (RH) and Tomato OE mutant parasites

Rates of proton extrusion were measured using the free-acid form of BCECF. (A and C) Summary of proton extrusion in RH cells. (B and D) Summary of proton extrusion in Tomato OE mutant cells. (A) Representative traces depicting changes in pH, in RH tachyzoites in the presence of 5 mM glucose (black, trace b) or with no glucose (light grey, trace a). (B) Same traces as in (A) but using Tomato OE mutants. (C) Resultant slope values (change in pH per s) in the presence (grey bars) or absence (white bars) of glucose for proton extrusion in RH cells using data represented in traces a and b in (A). (D) Resultant slope values (change in pH per s) in the presence (grey bars) or absence (white bars) of glucose for proton extrusion in OE mutant cells using data represented in traces a and b in (B). All estimates shown in (C and D) were determined from two independent experiments, and error bars represent the S.E. of the slope.