Published in final edited form as:

Biochem J. 2011 December 1; 440(2): 229–240. doi:10.1042/BJ20110641.

Overexpression of a Cytosolic Pyrophosphatase (TgPPase) Reveals a Regulatory Role of Pyrophosphate in Glycolysis for *Toxoplasma gondii**

Douglas A. Pace, Jianmin Fang, Roxana Cintrón, Melissa D. Docampo, and Silvia N. J. Moreno[#]

Center for Tropical and Emerging Global Diseases and Department of Cellular Biology, University of Georgia, Athens, GA 30602

Abstract

Pyrophosphate (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 phosphofructokinase (PFK), and the proton translocating vacuolar pyrophosphatase (V-H⁺-PPase). In the present work, we report the cloning, expression, and characterization of a cytosolic pyrophosphatase from *T. gondii* (TgPPase). 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 RH wild type tachyzoites. Unexpectedly, this subsequent reduction in pyrophosphate 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.

INTRODUCTION

Pyrophosphate (PP_i) is a byproduct of many biosynthetic reactions (synthesis of nucleic acids, coenzymes, proteins, isoprenoids, and activation of fatty acids), and it has been proposed that the removal of PP_i by pyrophosphatases (PPases) makes biosynthetic reactions thermodynamically favorable [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].

*This work was supported in part by U.S. Public Health Service Grant AI-079625 to S.N.J.M.

[#]To whom correspondence should be addressed: Center for Tropical and Emerging Global Disease and Department of Cellular Biology, 350A Paul D. Coverdell Center, University of Georgia, Athens, GA 30602. Tel.: 706-542-4736; Fax: 706-542-9493; ; Email: smoreno@cb.uga.edu.

The nucleotide and protein sequences reported in this paper have been submitted to GenBank with the accession numbers AY619688 and AAU88181 respectively.

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 H^+ -pumping PPases (V- H^+ -PPases) and soluble form 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 hydrolyze PP_i to inorganic phosphate (P_i), are essential enzymes, and have high activity in the cytoplasm. The absence of these PPases would lead to the buildup of toxic levels of PP_i , accounting for the essential nature of the enzymes. Two families of nonhomologous soluble inorganic PPases have been described: family I PPases, which are widespread in all types of organisms and prefer Mg^{2+} as cofactor [9, 10], and family II PPases, which are exclusive to bacteria and prefer Mn^{2+} as 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 hydrolyze organic tri- and diphosphates, 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 in acidic organelles named acidocalcisomes [20–22]. Acidocalcisomes have been found in a number of organisms from bacteria to man [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 *T. 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 phosphofructokinase (PFK) reaction [28].

In this work we characterized biochemically a soluble PPase and named it TgPPase. By overexpressing this enzyme in *T. gondii* tachyzoites we were able to isolate clones with up to 10 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 to the cytosolic level in RH wild type tachyzoites. These mutant cells showed alterations in their glycolytic pathway leading us to propose a regulatory role of PP_i on the glycolytic pathway of these parasites.

EXPERIMENTAL

Chemicals and Reagents

Aminomethylenediphosphonate (AMDP) was synthesized by Michael Martin (University of Illinois at Urbana-Champaign). Restriction enzymes, T_4 DNA ligase, reverse transcriptase, *Taq* polymerase, DNA ladder, Trizol reagent, and goat serum were from GIBCO BRL, Life Technologies, Inc. (Gaithersburg, MD). The pET28a⁺ expression system, Ni-NTA HisBind resin, and benzonase nuclease were from Novagen Inc. (Madison, WI). pCR2.1-TOPO cloning kit, secondary antibodies, BCECF and BCECF-AM were from Invitrogen (Carlsbad,

CA). Hybond-N nylon membrane, HiTrap desalting column and ECL™ chemiluminescence kit were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were analytical grade.

Culture Methods

T. gondii tachyzoites (RH) were grown in hTERT host cells using described protocols and bradyzoites were obtained by differentiation of tachyzoites of the ME49 strain as described before [29]. Transgenic fluorescent *T. gondii* tachyzoites expressing a YFPYFP fusion gene were a gift from Dr. Boris Stripen (University of Georgia, Athens) [30].

T. gondii growth measurements

[³H]Uracil incorporation was conducted in hTert cells that were cultured in 12-well plates for 24 h before they were challenged with 1×10⁵ tachyzoites per well. [³H]uracil incorporation was measured 24 h later by measuring the amount of [³H]uracil incorporated into each well during the last 4 h [31] [32]. *T. gondii* plaque assays were performed as previously described [33]. Assays were conducted in 6-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., percent of total area occupied by a plaque forming unit) were determined using ImageJ software (NIH).

TgPPase cDNA Cloning by 5' and 3'-RACE

The protein sequence of the *T. brucei* soluble inorganic PPase (NCBI GenBank protein 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 (rapid amplification of cDNA ends) technique was used to sequence the 5' and 3' ends of the *TgPPase* cDNA, using information from known contigs and EST 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) (www.orthomcl.org/cgi-bin/OrthoMclWeb.cgi) was done to search for putative PPase orthologs. 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 based on 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 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 evolutionary model of amino acid substitutions [39]. One-hundred distance trees were built by the Neighbor-joining method [40]. The final consensus trees were built using the majority rule and 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 the primers (5'-AGATCTATGCAGTCTGCACCTCTGGC-3' and 5'-CCTAGGCGGTAGCCACAACCTTTTG-3') with the BglII and AvrII restriction enzyme sites (underlined) by RT-PCR. The PCR products were cloned into the expression vectors pTubP30-flag/sagCAT (a gift from Dr William Sullivan, Indiana University School of Medicine) [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 done with 20 μ M chloramphenicol. Cloning was performed by limited dilution and one clone was selected for further analysis and named TgPPase-OE. A clone expressing two copies of the YFP gene was used for immunofluorescence co-localization assays [30].

Preparation of Recombinant TgPPase

The whole ORF of *TgPPase* was amplified by RT-PCR with the primers 5'-CATATGCAGTCTGCACCTCTGGC-3' and 5'-GTCGACCGGTAGCCACAACCTTTTG-3' containing the restriction sites for NdeI and SalI (underlined). The PCR product was cloned into the pCR 2.1-TOPO TA vector and subcloned into the expression vector pET28a⁺. The recombinant construct TgPPase/pET28a was transformed into *E. coli* BL21-CodonPlus(DE3)-RIPL (Stratagene) and protein expression was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Recombinant TgPPase (rTgPPase) was purified with an His-Bind Quick 900 Cartridge following the manufacturer's instructions. The eluted fractions were pooled and desalted using a HiTrap column.

Antibody Generation and Purification

The purified rTgPPase protein was sent to Cocalico Biologicals, Inc (Reamstown, PA) 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 [44] using an affinity purified anti-TgPPase polyclonal antibody (1:10,000) or anti- α -tubulin monoclonal antibody (1:15,000).

Fluorescence Microscopy

Immunofluorescence assays were performed as described [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) (1:1,000). Images were collected on an Olympus IX-71 inverted fluorescence microscope with a Photometrix-cooled CCD camera (CoolSnapHQ), and deconvolved for 15 cycles using SoftWorx deconvolution software (Applied Precision, Inc).

PPase Activity Measurement

rTgPPase activity was assayed by measuring the release of P_i using a published method [45]. The standard assay mixtures contained 50 mM Tris-HCl (pH 8.5), 3 mM $MgCl_2$, 250 μM PP_i and 1.36 ng of purified rTgPPase protein or 50 mM MES (pH 6.0), 3 mM $CoCl_2$, 150 μM poly P_3 , or 50 mM MES (pH 6.5), 3 mM $MnCl_2$, and 100 μM GP_4 , 100 μM ATP, or 100 μM poly P_{75} and 45 ng of rTgPPase. After a 10 min incubation at 37 °C, reactions were stopped by the addition of an equal volume of a mixture of 3 parts of 0.045% malachite green and 1 part of 4.2% ammonium molybdate [46]. The absorbance at 660 nm was measured with a SpectraMax M2^e plate reader (Molecular Devices, Sunnyvale, CA). The specific activity of rTgPPase was defined as $\mu mol P_i$ released $min^{-1} mg^{-1}$ 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_m concentrations for both PP_i and Poly P_3 .

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

The activity was also measured in subcellular fractions obtained after lysis of tachyzoites with silicon carbide as described before [24]. The homogenate was centrifuged at 100,000 g for 60 min at 4 °C to obtain a supernatant and a pellet fractions. The enzymatic activity was measured as described above.

Extraction and Determination of PP_i , ATP

PP_i and ATP were extracted with ice-cold 0.5 M $HClO_4$ (PCA) as described previously [24]. PP_i measurements were done by measuring the amount of P_i released upon treatment with Thermostable Inorganic Pyrophosphatase (TIPP) (NEB). The reactions were performed in a 50 mM Tris-HCl buffer, pH 7.5, 6 mM $MgCl_2$, with 0.04 units/ml of inorganic pyrophosphatase. After 20 min incubation at 30 °C, the P_i released was measured with malachite green as described [45]. On the same PCA extraction, samples were used for the determination of short chain polyphosphate (poly P) by quantifying the amount of phosphate after treatment with *S. cerevisiae* polyphosphatase.

For the determination of cytosolic and organellar PP_i , purified tachyzoites ($4 - 8 \times 10^8$ cells) were washed once in an hypotonic lysis buffer (20 mM Hepes, pH 7.3, 100 mM sucrose, 10 mM sodium fluoride) by centrifugation at 1,000 $\times g$. The pellet was subjected to three cycles of freezing and thawing using a dry ice-ethanol bath (5 min), and a 37°C water bath (1 min), resuspended in hypotonic lysis buffer and centrifuged at 14,000 $\times g$ for 20 min at 4°C. The supernatant was taken as the cytosolic fraction and the pellet as the organellar fraction containing PP_i stored predominantly in acidocalcisomes. Efficiency of this fractionation was assessed by testing for lactate dehydrogenase (LDH) activity, a cytosolic enzyme in *T. gondii* [47] and therefore should not be detected in the organellar fraction. LDH activity was monitored fluorometrically by using the LDH present in the experimental

samples to oxidize NADH to NAD⁺ as the enzyme converted pyruvate (supplied in the reaction buffer) to lactate (excitation = 340 nm; emission = 460 nm). Both fractions were analyzed as described above for PP_i and short chain poly P. The intracellular and organellar concentrations of PP_i were calculated from the respective cell volume of $16.5 \pm 3 \mu\text{l}$ for 10^9 cells [24] and assuming that PP_i is mainly located in acidocalcisomes and these occupy 1% of the total cell volume. Acidocalcisome volume was estimated assuming a spherical shape with a diameter of 200 nm and an average of 10 acidocalcisomes per extracellular tachyzoite.

ATP was measured using an ATP determination kit (Molecular Probes, Catalog number A22066). For the calculation of the intracellular concentrations of ATP in tachyzoites a cell volume of $16.5 \pm 3 \mu\text{l}$ for 10^9 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 in two: cells further washed and suspended in the same buffer and cells further washed and suspended in the same buffer without glucose (BA). Both cell preparations were kept on ice at a concentration of 1×10^9 cells ml^{-1} until analysis. Proton extrusion was measured as described [48]. The fluorescence was measured by using a FL-4500 fluorescent spectrophotometer (Hitachi, Tokyo, Japan). Calibration of fluorescent measurements to specific pH values was done as described [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 done as described previously [49]. Intracellular pH was measured in the SpectraMax M2_e plate reader at excitation wavelengths of 505/440 nm and emission at 530 nm. The calibration of pH units to fluorescence ratio was as previously described [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 5×10^8 cells ml^{-1} . Reactions were terminated at different times (0–10 minutes) 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 to pyruvate using lactate dehydrogenase (LDH, Sigma, L-2500) and measurement of the concomitant production of NADH. Enzymatic assay conditions were setup following published conditions [51, 52]. A 70 μl aliquot equivalent to 3.5×10^7 cells was added to 200 μl of reaction mix (600 mM glycine, 346 mM hydrazine, 17 mM EDTA, 15 mM NAD, 10 U ml^{-1} LDH; pH = 9.2) and fluorescence was monitored on the SpectraMax M2_e plate reader for 20 min. NADH was measured using excitation and emission wavelengths of 340 nm and 460 nm, respectively. Fluorescent values were converted to units of lactate using a standard curve where known amounts of L-lactate were added to the reaction mix and NADH production was monitored.

RESULTS

Cloning the TgPPase Gene

The gene encoding a soluble PPase was identified by a BLAST search with the *T. brucei* soluble inorganic PPase sequence (NCBI GenBank protein accession number AAP74702). The *T. gondii* gene encodes for a protein of 381 amino acids (TgPPase) with a predicted molecular weight of 42 kDa. The TgPPase sequence has an overall identity with the yeast PPase (Y-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) are conserved [53] (Supplementary Fig. 1). Southern blot analysis was performed using a fragment covering the entire coding region of *TgPPase* as a probe, and showed only one band suggesting the presence of a single *TgPPase* gene in the *T. gondii* genome (Supplementary Fig. 2A). *TgPPase* transcript levels, as measured by RT-PCR, were similar for both tachyzoites and bradyzoites of the ME49 strain (Supplementary Fig. 2B).

Phylogenetic Analysis of the TgPPase Gene

The TgPPase amino acid sequence (AAU88181) was used as a query to search for orthologs 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 outgroup (Fig. 1). The two groups are: (1) bacterial and bacterial-like plant soluble PPases, and (2) plant chloroplast and fungal/animal cytosolic and mitochondrial PPases (Fig. 1). In addition, our phylogenetic tree supports 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 Fig. 3 for the alignment of the sequences used for the tree in Fig. 1).

Expression, Purification, and Biochemical Characterization of TgPPase

The expression of *TgPPase* and purification by affinity chromatography produced a single protein band (Supplementary Fig. 2C). The purified protein was characterized biochemically.

The enzymatic activity of the rTgPPase showed an absolute requirement for divalent cation cofactors with Mg^{2+} being the most efficient at a concentration of 1 mM ($238.17 \pm 6.51 \mu\text{mol P}_i \text{ min}^{-1} (\text{mg protein})^{-1}$). The optimum pH for the hydrolysis of PP_i was 8.5 (Fig. 2A). At this pH, other cations such as cobalt, zinc, calcium, and manganese, marginally stimulated the activity over basal levels (Fig. 2C). The enzyme was also able to hydrolyze tripolyphosphate (poly P_3) although with lower efficiency (Table 1). For this polyphosphatase activity the preferred cofactor was Co^{2+} (Figs. 2B and 2D), as occurs with the Y-PPase [13, 16], and the optimum pH range was 6 to 6.5. The enzyme had low hydrolyzing activity of long chain poly P (poly P_{75}), ATP and guanosine tetraphosphate (GP_4) in the presence of Mn^{2+} as the divalent cation cofactor (Supplementary Fig. 4A,C).

Table 1, Figs. 2E,F, and Supplementary Fig. 4B,D show the kinetic properties of the enzyme for the hydrolysis of PP_i , poly P_3 , poly P_{75} , ATP and GP_4 . At the optimal conditions, it is

clear that the catalytic efficiency for the hydrolysis of PP_i (measured as k_{cat}/K_m) is significantly higher than for the hydrolysis of the other substrates.

Inhibition of the rTgPPase

Aminomethylenediphosphonate (AMDP), a specific inhibitor of the vacuolar proton PPase did not show any effect on the activity of this enzyme at a concentration of 40 μ M (data not shown). However, sodium fluoride (NaF), a known inhibitor of soluble PPases, inhibited the enzyme with an IC_{50} of 223 ± 31.8 μ M (Supplementary Fig. 5A, Supplementary Table 1). Poly P_3 hydrolytic activity was also significantly inhibited by fluoride (Supplementary Fig. 5C). Sodium molybdate caused minor inhibition of pyrophosphate hydrolysis (maximum inhibition = 27%, Supplementary Fig. 5B). The small amount of activity for poly P_3 hydrolysis was effectively inhibited by sodium molybdate, a general phosphatase inhibitor. Imidodiphosphate (IDP) (40 μ M), a bisphosphonate derivative, did not have any effect on the activity of rTgPPase (data not shown).

Subcellular Localization of TgPPase

To investigate the localization of TgPPase, we performed indirect immunofluorescence analysis (IFA) in tachyzoites expressing cytosolic YFP using antibodies against the TgPPase enzyme. As evidenced by the colocalization with YFP, the endogenous localization of the TgPPase enzyme was determined to be the cytosol in tachyzoites of *T. gondii* (Fig. 3A). Western blot analysis of cytosolic and membrane fractions of tachyzoites confirmed the cytosolic localization of the protein (Fig. 3B). Both the specific activity and the total activity of the enzyme were higher in the soluble fractions (Figs. 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 (Fig. 4A) and enzymatic activity (Fig. 4B; $P < 0.05$). Further IFA localization studies with cytosolic YFP determined that the TgPPase protein in OE mutants maintained a cytosolic localization (data not shown). Rates of growth in overexpressing mutants (hereafter referred to as 'OE mutants') using uracil incorporation were found to be significantly lower ($P < 0.05$) when compared to the growth rate of RH, wild type parasites (hereafter referred to as 'RH') (Fig. 4C). The lower growth rates were confirmed using plaque assays to measure growth/invasion efficiency in wild type and over expressing mutants (Fig. 4D-F). 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 pyrophosphatase activity in OE mutants we investigated the cellular amounts of phosphate-related molecules. Cellular levels of PP_i were 40% lower than in wild type RH cells (Fig. 5A; $P < 0.01$). Cellular concentrations of short-chain poly P between OE mutants and RH cells were similar at about 5 mM in phosphate equivalents (Fig. 5B). Under identical conditions as above, cellular concentrations of ATP were significantly elevated ($P < 0.05$) by 75% in extracellular tachyzoites over-expressing TgPPase (Fig. 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 pyrophosphate 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. Cellular pyrophosphate concentration in the Tomato OE parasites was similar to that measured in RH, wildtype parasites (Supplementary Fig. 6A). Likewise, growth rates in these unrelated expression mutants were similar to those of RH, wild type parasites as measured by plaque assays (Supplementary Fig. 6B). These results indicate that the phenotype observed in the PPase OE mutants is not an artifact from genetic manipulation and bolster the relationship between pyrophosphate concentration and ATP and growth.

Since most PP_i in *T. gondii* is localized in acidocalcisomes [24] we investigated whether cytosolic or organellar PP_i was affected in cells over-expressing the pyrophosphatase. By lysis of the plasma membrane by freeze-thaw cycling in a hypotonic buffer, the distribution of PP_i in the cytosolic and organellar fractions of extracellular tachyzoites was determined. In order to determine the concentration of PP_i in these fractions we assumed that the cellular volume and the size and number of acidocalcisomes in the OE mutants were similar to that of wild type parasites (i.e., 1×10^9 parasites = 16.5 μ l 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, data not shown). The majority of PP_i was found in the organellar fraction (Fig. 6B, ~ 360 mM). A significant difference, however, was observed in the soluble fraction (Fig. 6A). RH cells had more than 6-times the concentration of PP_i 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., cytosol) and the pellet (i.e., organellar) fractions. Over 95% of measured LDH activity was found in the cytosolic fraction (Supplementary Fig. 6). In conjunction with the PP_i distribution, these results demonstrate effective lysis of the plasma membrane without significant disruption of organelles containing pyrophosphate (i.e., acidocalcisomes). These results indicate that the total cellular differences in PP_i are a result of different concentrations of PP_i in the cytosol, which is in agreement with the cellular location of the TgPPase enzyme (Fig. 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 to the medium, accompanied by protons [54]. The large, glucose-dependent differences observed in ATP concentration in conjunction with the cytosolic differences in PP_i 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 of the extracellular media (pH_e) changed. Proton extrusion was measured in RH (Fig. 7A, C, and E) and OE mutants (Fig. 7B, D, and F) in the presence of glucose (5 mM) and compared to cells deprived of glucose for ~ 2 h (Figs. 7A and C (RH cells); (Figs. 7B and D) (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 [Fig. 7A, *trace b* and 7E (RH); Fig. 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 (Fig. 7A, *trace c*) or when added during the experimental incubation time (Fig. 7A, *trace b and c*) relative to rates when cells were glucosedepived (Fig. 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, (Fig. 7B, *trace a*) rates of proton extrusion in OE mutants were slightly lower, though statistically similar to rates in glucosedepived RH cells (average glucose-deprived rates were -4.3×10^{-4} and -3.1×10^{-4} pH_e 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 -3.1×10^{-4} to -9.8×10^{-4} pH_e units s^{-1} (Fig. 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 (-9.8×10^{-4} and -6.0×10^{-4} pH_e units s^{-1} for TgPPase-OE and RH cells, respectively) (compare Figs. 7D and 7F with 7C and 7E, *gray columns*). Despite the large differences in proton extrusion, there was no significant difference in intracellular pH (pH_i) between RH parasites and OE mutants (ANOVA, $df = 1,5$; $P > 0.05$, data 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 Fig. 8A-D), providing further support for the relationship between cellular pyrophosphate concentration and glycolytic flux in *T. gondii*.

To better assess the link between pyrophosphate and glycolytic flux, rates of lactate extrusion were measured in RH and soluble pyrophosphatase 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 made at 0, 1, 2, 5, and 10 min) showed a significant linear increase with time for both cells types (Fig. 8). The lactate extrusion rate for RH cells was 1.07 ± 0.03 (SE) $\text{fmol h}^{-1} \text{cell}^{-1}$ and for the OE cells (Fig. 8, inset) 1.90 ± 0.04 (SE) $\text{fmol h}^{-1} \text{cell}^{-1}$ (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).

The difference in the proton and lactate extrusion rates between RH and the OE cells might be due to an inhibitory effect of PP_i on the glycolytic pathway. The *T. gondii* pyruvate kinase (PK) is an allosteric enzyme and is activated by glucose-6-P [58]. We investigated if PP_i could be inhibiting this enzyme by measuring its activity in a *T. gondii* lysate in the presence of varying PP_i concentrations. For the range of PP_i concentrations we measured in the

cytosol (i.e. up to 1 mM), we found no detectable inhibition of pyruvate kinase (data not shown).

DISCUSSION

The goals of this study were to characterize the enzyme that directly regulates cytosolic levels of pyrophosphate in the apicomplexan parasite *Toxoplasma gondii* and employ its expression as a means to manipulate cytosolic pyrophosphate concentrations in this medically relevant parasite. Bioinformatic searching (e.g., blast searching and querying Toxodb.org) for soluble pyrophosphatases yielded TgPPase as the only soluble pyrophosphatase found in the *T. gondii* genome. Pyrophosphate 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 over-expression of TgPPase as a means to manipulate the cytosolic concentration of pyrophosphate. It was our expectation that given the placement of PP_i in so many key metabolic reactions, its manipulation would have far-reaching consequences on the metabolism of *T. gondii* tachyzoites.

The alignment of the *TgPPase* gene sequence shows conservation of amino acids present in other PPases. Considering its phylogenetic position, its Mg²⁺ 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 outgroup (Fig. 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 hydrolyzing PP_i. The enzyme is also able to hydrolyze poly P₃, poly P₇₅, ATP and GP₄ 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 (Fig. 3). On analyzing Fig. 2A it can be observed that the activity is still high at cytosolic pHs 7.1–7.4. The enzyme preference for Mg²⁺, when hydrolyzing PP_i, and for other non-physiological cations such as Co²⁺ or Mn²⁺, when hydrolyzing other substrates, is similar to what has been found for the Y-PPase [16].

Given that the phylogenetic analysis and biochemical characterization fully supported the identity of TgPPase as a Type I pyrophosphatase with a high specificity for PP_i and only much lower affinities for alternate substrates, we employed the over-expression of this enzyme as a means to decrease the cytosolic concentration of PP_i. Over-expression 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 over-expression of TgPPase is a reliable tool for understanding the metabolic significance of pyrophosphate.

The phenotype of the *T. gondii* clone over-expressing *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 phosphofructokinase (PFK) that utilizes PP_i to drive the rate-limiting conversion of fructose-6-phosphate to 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 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 platelet cells [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 ~ 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 (~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 and TgPPase OE mutants with a completely unrelated expression mutant. Genetically modified parasites expressing the fluorescent tomato protein had similar levels of pyrophosphate 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* still 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 *Trypanosoma brucei* [69] and phosphoenolpyruvate (PEP) carboxykinase in *T. cruzi* [70]. In animal cells PP_i , either directly administered 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 3-phosphoglycerate/pyruvate ratio, suggestive of PK inhibition [4]. Three enzymes that typically are observed to be critical regulators of glycolytic flux are: hexokinase, phosphofructokinase (PFK), and pyruvate kinase (PK). In *T. gondii*, however, very little is known about 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 current analysis of pyruvate kinase (PK) in *T. gondii* wild-type and TgPPase over-expressing cells found no significant difference in PK activity for the range of PP_i differences between the two cell types.

As previously mentioned, *T. gondii* tachyzoites possess a plant-like phosphofructokinase that uses PP_i as the high-energy phosphoryl donor rather than ATP (PP_i -PFK). Therefore a decrease in PP_i would be expected to result in a corresponding decrease in the rate-limiting conversion of fructose-6-phosphate to fructose-1,6-bisphosphate by PP_i -PFK. However, the K_m for PP_i of PP_i -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_i -PFK activity. An alternative explanation that has been proposed in plant cells [72] is that the increase in orthophosphate (P_i) from the breakdown of PP_i results in activation of fructose-6-phosphate-2 kinase, the product of which is fructose-2,6-bisphosphate (F-2,6-BP), a strong activator of PP_i -PFK [72]. However, unlike plant cells, the PP_i -PFK of *T. gondii* does not appear to be activated by F-2,6-BP [28].

The up-regulation of glycolysis and increase in ATP levels due to decreases in cytosolic PP_i has also been observed in plant cells [72, 73]. In developing tubers of potato, cytosolic overexpression of pyrophosphatase causes a decrease in cytosolic PP_i 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_i -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]. While these granules are typically associated with the bradyzoite and sporozoite stages, it has been shown that media acidification in tachyzoites can cause a significant increase in the presence of amylopectin granules [74]. The increase we observe in glycolysis and the 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 over-expressing 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_i produced in anabolic reactions is instantly hydrolyzed. However, a potential role of PP_i as a regulator of metabolic processes has also been proposed [4]. Our results support this assertion. Our findings highlight the regulatory role of PP_i on a critical metabolic pathway of *T. gondii*. The regulatory role of PP_i requires further study. Metabolomic analysis in conjunction with the use of these over-expressing mutants to vary cytosolic concentration of PP_i should provide insight into further downstream consequences resulting from varying

cytosolic PP_i. Such analysis may have far reaching implications for the role of PP_i in metabolic regulation in a variety of cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Boris Striepen (UGA) for transgenic fluorescent *T. gondii* tachyzoites and the ptubYFP-YFP/sagCAT expression vector, William Sullivan (Indiana University School of Medicine) for the pTubP30-GFP-flag/sagCAT expression vector, and Takashi Asai for useful discussions. Nhu-y Nguyen provided assistance in proton extrusion measurements as part of an undergraduate research project. Cuiying Jiang and Allysa Smith provided excellent technical help.

Funding. This work was supported by U.S. National Institutes of Health Grant AI-079625 to S.N.J.M. D.A.P. was partially supported by an NIH T32 training grant AI-60546 to the Center for Tropical and Emerging Global Diseases. R.C. was supported in part by NIH research supplement 3R01AI068647-04S1.

Abbreviations Used

AMDP	aminomethylenediphosphonate
BCECF	2',7'-bis(2-carboxyethyl)- 5-(and-6)-carboxyfluorescein
GP₄	guanosine tetraphosphate
IDP	imidodiphosphate
LDH	Lactate dehydrogenase
MES	4-morpholineethanesulfonic acid
ORF	open reading frame
poly P	polyphosphate
poly P₃	tripolyphosphate
PPase	pyrophosphatase
PP_i	inorganic pyrophosphate
PK	pyruvate kinase
TgPPase	soluble inorganic pyrophosphatase 1 of <i>T. gondii</i>
TgPPase-OE	TgPPase overexpressing cells
YFP	yellow fluorescent protein.

References

1. Kornberg, A. On the metabolic significance of phosphorolytic and pyrophosphorolytic reactions. In: Kasha, HaPB., editor. Horizons in Biochemistry. New York: Academic Press, Inc.; 1962. p. 251-264.
2. Mansurova SE. Inorganic pyrophosphate in mitochondrial metabolism. Biochim Biophys Acta. 1989; 977:237–247. [PubMed: 2556179]

3. Lahti R. Microbial inorganic pyrophosphatases. *Microbiol Rev.* 1983; 47:169–178. [PubMed: 6135978]
4. Veech RL, Cook GA, King MT. Relationship of free cytoplasmic pyrophosphate to liver glucose content and total pyrophosphate to cytoplasmic phosphorylation potential. *FEBS Lett.* 1980; 117(Suppl):K65–K72. [PubMed: 6252049]
5. Baltscheffsky M, Schultz A, Baltscheffsky H. H⁺-PPases: a tightly membrane-bound family. *FEBS Lett.* 1999; 457:527–533. [PubMed: 10523139]
6. Maeshima M. Vacuolar H(+)-pyrophosphatase. *Biochim Biophys Acta.* 2000; 1465:37–51. [PubMed: 10748246]
7. Drozdowicz YM, Rea PA. Vacuolar H(+) pyrophosphatases: from the evolutionary backwaters into the mainstream. *Trends Plant Sci.* 2001; 6:206–211. [PubMed: 11335173]
8. Scott DA, de Souza W, Benchimol M, Zhong L, Lu HG, Moreno SN, Docampo R. Presence of a plant-like proton-pumping pyrophosphatase in acidocalcisomes of *Trypanosoma cruzi*. *J Biol Chem.* 1998; 273:22151–22158. [PubMed: 9705361]
9. Tammenkoski M, Benini S, Magretova NN, Baykov AA, Lahti R. An unusual, His-dependent family I pyrophosphatase from *Mycobacterium tuberculosis*. *J Biol Chem.* 2005; 280:41819–41826. [PubMed: 16239227]
10. Tammenkoski M, Moiseev VM, Lahti M, Ugochukwu E, Brondijk TH, White SA, Lahti R, Baykov AA. Kinetic and mutational analyses of the major cytosolic exopolyphosphatase from *Saccharomyces cerevisiae*. *J Biol Chem.* 2007; 282:9302–9311. [PubMed: 17215253]
11. Young TW, Kuhn NJ, Wadeson A, Ward S, Burges D, Cooke GD. *Bacillus subtilis* ORF yybQ encodes a manganese-dependent inorganic pyrophosphatase with distinctive properties: the first of a new class of soluble pyrophosphatase? *Microbiology.* 1998; 144(Pt 9):2563–2571. [PubMed: 9782505]
12. Oksanen E, Ahonen AK, Tuominen H, Tuominen V, Lahti R, Goldman A, Heikinheimo P. A complete structural description of the catalytic cycle of yeast pyrophosphatase. *Biochemistry.* 2007; 46:1228–1239. [PubMed: 17260952]
13. Hohne WE, Heitmann P. Tripolyphosphate as a substrate of the inorganic pyrophosphatase from baker's yeast; the role of divalent metal ions. *Acta Biol Med Ger.* 1974; 33:1–14. [PubMed: 4608770]
14. Josse J. Constitutive inorganic pyrophosphatase of *Escherichia coli*. I. Purification and catalytic properties. *J Biol Chem.* 1966; 241:1938–1947. [PubMed: 5329747]
15. Josse J. Constitutive inorganic pyrophosphatase of *Escherichia coli*. II. Nature and binding of active substrate and the role of magnesium. *J Biol Chem.* 1966; 241:1948–1955. [PubMed: 4287756]
16. Zyryanov AB, Shestakov AS, Lahti R, Baykov AA. Mechanism by which metal cofactors control substrate specificity in pyrophosphatase. *Biochem J.* 2002; 367:901–906. [PubMed: 12169093]
17. Schlesinger MJ, Coon MJ. Hydrolysis of nucleoside diand triphosphates by crystalline preparations of yeast inorganic pyrophosphatase. *Biochim Biophys Acta.* 1960; 41:30–36. [PubMed: 14442883]
18. Kunitz M. An improved method for isolation of crystalline pyrophosphatase from baker's yeast. *Arch Biochem Biophys.* 1961; 92:270–272. [PubMed: 13755147]
19. Moreno B, Bailey BN, Luo S, Martin MB, Kuhlenschmidt M, Moreno SN, Docampo R, Oldfield E. (31)P NMR of apicomplexans and the effects of risedronate on *Cryptosporidium parvum* growth. *Biochem Biophys Res Commun.* 2001; 284:632–637. [PubMed: 11396947]
20. Moreno SN, Zhong L. Acidocalcisomes in *Toxoplasma gondii* tachyzoites. *Biochem J.* 1996; 313(Pt 2):655–659. [PubMed: 8573106]
21. Rodrigues CO, Scott DA, Bailey BN, De Souza W, Benchimol M, Moreno B, Urbina JA, Oldfield E, Moreno SN. Vacuolar proton pyrophosphatase activity and pyrophosphate (PPi) in *Toxoplasma gondii* as possible chemotherapeutic targets. *Biochem J.* 2000; 349(Pt 3):737–745. [PubMed: 10903134]
22. Miranda K, de Souza W, Plattner H, Hentschel J, Kawazoe U, Fang J, Moreno SN. Acidocalcisomes in Apicomplexan parasites. *Exp Parasitol.* 2008; 118:2–9. [PubMed: 17761167]
23. Docampo R, de Souza W, Miranda K, Rohloff P, Moreno SN. Acidocalcisomes - conserved from bacteria to man. *Nat Rev Microbiol.* 2005; 3:251–261. [PubMed: 15738951]

24. Rodrigues CO, Ruiz FA, Rohloff P, Scott DA, Moreno SN. Characterization of isolated acidocalcisomes from *Toxoplasma gondii* tachyzoites reveals a novel pool of hydrolyzable polyphosphate. *J Biol Chem.* 2002; 277:48650–48656. [PubMed: 12379647]
25. Urbina JA, Moreno B, Vierkotter S, Oldfield E, Payares G, Sanoja C, Bailey BN, Yan W, Scott DA, Moreno SN, Docampo R. *Trypanosoma cruzi* contains major pyrophosphate stores, and its growth in vitro and in vivo is blocked by pyrophosphate analogs. *J Biol Chem.* 1999; 274:33609–33615. [PubMed: 10559249]
26. Mendoza M, Mijares A, Rojas H, Rodriguez JP, Urbina JA, DiPolo R. Physiological and morphological evidences for the presence acidocalcisomes in *Trypanosoma evansi*: single cell fluorescence and ³¹P NMR studies. *Mol Biochem Parasitol.* 2002; 125:23–33. [PubMed: 12467971]
27. Drozdowicz YM, Shaw M, Nishi M, Striepen B, Liwinski HA, Roos DS, Rea PA. Isolation and characterization of TgVP1, a type I vacuolar H⁺-translocating pyrophosphatase from *Toxoplasma gondii*. The dynamics of its subcellular localization and the cellular effects of a diphosphonate inhibitor. *J Biol Chem.* 2003; 278:1075–1085. [PubMed: 12411435]
28. Peng ZY, Mansour TE. Purification and properties of a pyrophosphate-dependent phosphofructokinase from *Toxoplasma gondii*. *Mol Biochem Parasitol.* 1992; 54:223–230. [PubMed: 1331784]
29. Luo S, Vieira M, Graves J, Zhong L, Moreno SN. A plasma membrane-type Ca(2+)-ATPase co-localizes with a vacuolar H(+)-pyrophosphatase to acidocalcisomes of *Toxoplasma gondii*. *EMBO J.* 2001; 20:55–64. [PubMed: 11226155]
30. Gubbels MJ, Li C, Striepen B. High-throughput growth assay for *Toxoplasma gondii* using yellow fluorescent protein. *Antimicrob Agents Chemother.* 2003; 47:309–316. [PubMed: 12499207]
31. Pfefferkorn ER, Pfefferkorn LC. Specific labeling of intracellular *Toxoplasma gondii* with uracil. *J Protozool.* 1977; 24:449–453. [PubMed: 21288]
32. Martin MB, Grimley JS, Lewis JC, Heath HT, Bailey BN, Kendrick H, Yardley V, Caldera A, Lira R, Urbina JA, Moreno SN, Docampo R, Croft SL, Oldfield E. Bisphosphonates Inhibit the Growth of *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondii*, and *Plasmodium falciparum*: A Potential Route to Chemotherapy. *Journal of Medicinal Chemistry.* 2001; 44:909–916. [PubMed: 11300872]
33. Roos DS, Donald RG, Morrisette NS, Moulton AL. Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Methods Cell Biol.* 1994; 45:27–63. [PubMed: 7707991]
34. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994; 22:4673–4680. [PubMed: 7984417]
35. Nicholas KB, Nicholas HB, Deerfield DW. GeneDoc: Analysis and Visualization of genetic variation. *EMBNEW.NEWS.* 1997; 4:14. ed.)^eds.).
36. Maddison WP, Maddison DR. Interactive analysis of phylogeny and character evolution using the computer program MacClade. *Folia Primatol (Basel).* 1989; 53:190–202. [PubMed: 2606395]
37. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution.* 1985; 39:783–791.
38. Efron B, Halloran E, Holmes S. Bootstrap confidence levels for phylogenetic trees. *Proc Natl Acad Sci U S A.* 1996; 93:7085–7090. [PubMed: 8692949]
39. Eck RV, Dayhoff MO. Evolution of the Structure of Ferredoxin Based on Living Relics of Primitive Amino Acid Sequences. *Science.* 1966; 152:363–366. [PubMed: 17775169]
40. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol.* 1987; 4:406–425. [PubMed: 3447015]
41. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform.* 2004; 5:150–163. [PubMed: 15260895]
42. Striepen B, He CY, Matrajt M, Soldati D, Roos DS. Expression, selection, and organellar targeting of the green fluorescent protein in *Toxoplasma gondii*. *Mol Biochem Parasitol.* 1998; 92:325–338. [PubMed: 9657336]
43. Fang J, Rohloff P, Miranda K, Docampo R. Ablation of a small transmembrane protein of *Trypanosoma brucei* (TbVTC1) involved in the synthesis of polyphosphate alters acidocalcisome

- biogenesis and function, and leads to a cytokinesis defect. *Biochem J.* 2007; 407:161–170. [PubMed: 17635107]
44. Fang J, Marchesini N, Moreno SN. A *Toxoplasma gondii* phosphoinositide phospholipase C (TgPI-PLC) with high affinity for phosphatidylinositol. *Biochem J.* 2006; 394:417–425. [PubMed: 16288600]
 45. Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA. An improved assay for nanomole amounts of inorganic phosphate. *Anal Biochem.* 1979; 100:95–97. [PubMed: 161695]
 46. Shatton JB, Ward C, Williams A, Weinhouse S. A microcolorimetric assay of inorganic pyrophosphatase. *Anal Biochem.* 1983; 130:114–119. [PubMed: 6135370]
 47. Pomel S, Luk FC, Beckers CJ. Host cell egress and invasion induce marked relocations of glycolytic enzymes in *Toxoplasma gondii* tachyzoites. *PLoS Pathog.* 2008; 4:e1000188. [PubMed: 18949028]
 48. Van Der Heyden N, Docampo R. Intracellular pH in mammalian stages of *Trypanosoma cruzi* is K⁺-dependent and regulated by H⁺-ATPases. *Mol Biochem Parasitol.* 2000; 105:237–251. [PubMed: 10693746]
 49. Moreno SN, Zhong L, Lu HG, Souza WD, Benchimol M. Vacuolar-type H⁺-ATPase regulates cytoplasmic pH in *Toxoplasma gondii* tachyzoites. *Biochem J.* 1998; 330(Pt 2):853–860. [PubMed: 9480901]
 50. Vanderheyden N, Benaïm G, Docampo R. The role of a H⁺-ATPase in the regulation of cytoplasmic pH in *Trypanosoma cruzi* epimastigotes. *Biochem J.* 1996; 318(Pt 1):103–109. [PubMed: 8761458]
 51. Hamilton SD, Pardue HL. Quantitation of lactate by a kinetic method with an extended range of linearity and low dependence on experimental variables. *Clin Chem.* 1984; 30:226–229. [PubMed: 6692526]
 52. Engel PC, Jones JB. Causes and elimination of erratic blanks in enzymatic metabolite assays involving the use of NAD⁺ in alkaline hydrazine buffers: improved conditions for the assay of L-glutamate, L-lactate, and other metabolites. *Anal Biochem.* 1978; 88:475–484. [PubMed: 29519]
 53. Heikinheimo P, Lehtonen J, Baykov A, Lahti R, Cooperman BS, Goldman A. The structural basis for pyrophosphatase catalysis. *Structure.* 1996; 4:1491–1508. [PubMed: 8994974]
 54. Ohsaka A, Yoshikawa K, Hagiwara T. ¹H-NMR spectroscopic study of aerobic glucose metabolism in *Toxoplasma gondii* harvested from the peritoneal exudate of experimentally infected mice. *Physiol Chem Phys.* 1982; 14:381–384. [PubMed: 7186134]
 55. Akkerman JWN, Gorter G, Sixma JJ. REGULATION OF GLYCOLYTIC FLUX IN HUMAN PLATELETS: RELATION BETWEEN ENERGY PRODUCTION BY GLYCO(GENO)LYSIS AND ENERGY CONSUMPTION. *Biochimica et Biophysica Acta.* 1978:241–250. [PubMed: 667125]
 56. Harold FM. Ion Transport and Electrogenesis in Bacteria. *Proceedings of the Biochemical Society.* 1979; 127:1971–1972.
 57. Ginsburg H. Abundant proton pumping in *Plasmodium falciparum*, but why? *Trends Parasitol.* 2002; 18:483–486. [PubMed: 12473363]
 58. Denton H, Brown SM, Roberts CW, Alexander J, McDonald V, Thong KW, Coombs GH. Comparison of the phosphofructokinase and pyruvate kinase activities of *Cryptosporidium parvum*, *Eimeria tenella* and *Toxoplasma gondii*. *Mol Biochem Parasitol.* 1996; 76:23–29. [PubMed: 8919992]
 59. Huang J, Mullapudi N, Lancto CA, Scott M, Abrahamsen MS, Kissinger JC. Phylogenomic evidence supports past endosymbiosis, intracellular and horizontal gene transfer in *Cryptosporidium parvum*. *Genome Biol.* 2004; 5:R88. [PubMed: 15535864]
 60. Huang J, Mullapudi N, Sicheritz-Ponten T, Kissinger JC. A first glimpse into the pattern and scale of gene transfer in Apicomplexa. *Int J Parasitol.* 2004; 34:265–274. [PubMed: 15003488]
 61. Striepen B, White MW, Li C, Guerini MN, Malik SB, Logsdon JM Jr, Liu C, Abrahamsen MS. Genetic complementation in apicomplexan parasites. *Proc Natl Acad Sci U S A.* 2002; 99:6304–6309. [PubMed: 11959921]
 62. Dziarszinski F, Popescu O, Toursel C, Slomianny C, Yahiaoui B, Tomavo S. The protozoan parasite *Toxoplasma gondii* expresses two functional plant-like glycolytic enzymes. Implications for

- evolutionary origin of apicomplexans. *J Biol Chem.* 1999; 274:24888–24895. [PubMed: 10455162]
63. Gomez-Garcia MR, Ruiz-Perez LM, Gonzalez-Pacanowska D, Serrano A. A novel calcium-dependent soluble inorganic pyrophosphatase from the trypanosomatid *Leishmania major*. *FEBS Lett.* 2004; 560:158–166. [PubMed: 14988016]
 64. Feagin, JE.; Marsons, M. The Apicoplast and Mitochondrion of *Toxoplasma gondii*. In: Weiss, LM.; Kim, K., editors. *Toxoplasma gondii. The Model Apicomplexan-Perspectives and Methods.* London: Academic Press; 2007. p. 207-244.
 65. Lin SS, Blume M, von Ahsen N, Gross U, Bohne W. Extracellular *Toxoplasma gondii* tachyzoites do not require carbon source uptake for ATP maintenance, gliding motility and invasion in the first hour of their extracellular life. *International Journal for Parasitology.* 41:835–841. [PubMed: 21515276]
 66. Hamilton IR, Martin EJS. Evidence for the Involvement of Proton Motive Force in the Transport of Glucose by a Mutant of *Streptococcus mutans* Strain DR0001 Defective in Glucose-Phosphoenolpyruvate Phosphotransferase Activity. *Infect. Immun.* 1982; 36:567–575. [PubMed: 6282753]
 67. Sigler K, Knotkov A, Kotyk A. Factors governing substrate-induced generation and extrusion of protons in the yeast *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta (BBA) - Biomembranes.* 1981; 643:572–582. [PubMed: 6264954]
 68. Denton H, Roberts CW, Alexander J, Thong KW, Coombs GH. Enzymes of energy metabolism in the bradyzoites and tachyzoites of *Toxoplasma gondii*. *FEMS Microbiol Lett.* 1996; 137:103–108. [PubMed: 8935663]
 69. Chambers JW, Kearns MT, Morris MT, Morris JC. Assembly of heterohexameric trypanosome hexokinases reveals that hexokinase 2 is a regulable enzyme. *J Biol Chem.* 2008; 283:14963–14970. [PubMed: 18387941]
 70. Acosta H, Dubourdieu M, Quinones W, Caceres A, Bringaud F, Concepcion JL. Pyruvate phosphate dikinase and pyrophosphate metabolism in the glycosome of *Trypanosoma cruzi* epimastigotes. *Comp Biochem Physiol B Biochem Mol Biol.* 2004; 138:347–356. [PubMed: 15325334]
 71. Saito T, Maeda T, Nakazawa M, Takeuchi T, Nozaki T, Asai T. Characterisation of hexokinase in *Toxoplasma gondii* tachyzoites. *Int J Parasitol.* 2002; 32:961–967. [PubMed: 12076625]
 72. Geigenberger P, Hajirezaei M, Geiger M, Deiting U, Sonnewald U, Stitt M. Overexpression of pyrophosphatase leads to increased sucrose degradation and starch synthesis, increased activities of enzymes for sucrose-starch interconversions, and increased levels of nucleotides in growing potato tubers. *Planta.* 1998; 205:428–437. [PubMed: 9640668]
 73. Farre EM, Tech S, Trethewey RN, Fernie AR, Willmitzer L. Subcellular pyrophosphate metabolism in developing tubers of potato (*Solanum tuberosum*). *Plant Mol Biol.* 2006; 62:165–179. [PubMed: 16915524]
 74. Guerardel Y, Leleu D, Coppin A, Lienard L, Slomianny C, Strecker G, Ball S, Tomavo S. Amylopectin biogenesis and characterization in the protozoan parasite *Toxoplasma gondii*, the intracellular development of which is restricted in the HepG2 cell line. *Microbes Infect.* 2005; 7:41–48. [PubMed: 15716062]

Figure 1. Molecular phylogenetic analysis of type I inorganic pyrophosphatases

The consensus phylogenetic tree was built from 100 replicates using the distance and parsimony methods as described in the “Experimental” section. Bootstrap values from 100 replicates are shown in *bold* and *italics* as obtained by the distance and parsimony methods, respectively. The 0.2 bar represents amino acid substitutions per site. The sequence accession numbers as provided by GenBank are indicated in parentheses: *Aquifex aeolicus* VF5 (NP_214066.1), *Arabidopsis thaliana* chloroplast PPase (At5g09650.1), *A. thaliana* (At1g01050.1), *Chlamydomonas reinhardtii* mitochondrial PPase (AJ298232), *C. reinhardtii*

chloroplast PPase (AJ298231), *Cryptosporidium parvum* (cgd4_1400), *Escherichia coli* W3110 (NP_418647.1), *Homo sapiens* cytosolic PPase (ENSP00000317687), *H. sapiens* mitochondrial PPase (ENSP00000343885), *Kluyveromyces lactis* cytosolic PPase (KLLA0E17721g), *K. lactis* mitochondrial PPase (KLLA0E11055g), *Leishmania major* putative mitochondrial PPase (LmjF03.0910), *Mus musculus* cytosolic PPase (ENSMUSP00000020286), *M. musculus* mitochondrial PPase (BAB22922), *Nostoc sp. PCC7120* (P80562), *Oriza sativa* chloroplast PPase (3698.m00155), *O. sativa* (12001.m13461), *Plasmodium falciparum* 3D7 (PFC0710w), *Rhodospirillum rubrum* (AF115341_1), *Saccharomyces cerevisiae* cytosolic PPase (YBR011C), *S. cerevisiae* mitochondrial PPase (YMR267W), *Solanum tuberosum* (CAA12415), *Synechocystis PCC6803* (P80507), *Thermoplasma acidophilum* (P37981), *Thermus thermophilus* (BAA24521), *Toxoplasma gondii* (AAU88181), *Trypanosoma brucei* putative mitochondrial PPase (Tb927.3.2840/Tb03.27C5.190), *T. cruzi* putative mitochondrial PPase (Tc00.1047053508181.140) and *Zea mays* (O48556). MIT, mitochondrial, CYT, cytosolic, CHL, chloroplastic.

Figure 2. Enzymatic characterization of the rTgPPase

PPase activity was determined as described under “Experimental” using 23 μM PP_i (A, C), or 9 μM poly P₃ (B, D). A, B Optimum pH measurements with PP_i (A) or Poly P₃ (B) as substrate. A 20mM Tris/HEPES mixed buffering system was used to manipulate the pH of the reaction buffer. C, D, Optimum cation concentrations measurements with PP_i (C) or Poly P₃ (D) as substrate. Results are expressed as % of maximum activity taken as 100%. Where indicated, 5 mM EDTA was used. E, F, enzymatic activity measured with different concentrations of PP_i (E) or PolyP₃ (F). These experiments were done using 3.0 mM

MgCl₂, (*E*), or 3 mM CoCl₂ (*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.

Figure 3. The TgPPase localizes to the cytosol of RH, wild type *T. gondii* tachyzoites
 RH (wildtype) tachyzoites expressing YFP were used [30] 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) or observed by direct YFP fluorescence where indicated. The overlay images show co-localization of both proteins in the cytosol. Scale bars = 5 μ m. (B) Western blot analysis of subcellular fractions of *T. gondii* tachyzoites. Equal protein amounts (5 μ g) from supernatant ("S") and pellet ("P") fractions were loaded. The molecular mass standards (in kDa) are

shown on the left. (*C, 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 described under “Experimental”.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Cells overexpressing TgPPase show higher levels of TgPPase protein and activity but grow at a slower rate

A, Western blot analysis. *RH* cells; *OE*, cells transfected with the expression vector ptubTgPPase-flag/sagCAT and selected with chloramphenicol as indicated under “Experimental” (TgPPase-OE cells). The blot was sequentially probed with anti-TgPPase antibody (*upper panel*) and anti- α -tubulin antibody as loading control (*bottom panel*). **B**, PPase activity of crude cell lysates obtained as described under “Experimental”. AMDP, a specific inhibitor of the vacuolar proton PPase (V-H⁺-PPase) at 40 μ M, was added to the lysate. **C**, OE mutant tachyzoites had significantly lower [³H]-uracil incorporation than wild-type cells after 24 h inoculation of hTERT host cells. The results are representative of three independent experiments, each one performed in triplicate. The bars are the means $\pm$ S.D. Asterisks (*) show P values < 0.05, determined by the Student’s *t*-test. **D-F**, Plaque assays comparing growth and invasion ability of wild type cells (*RH*) and TgPPase over expressing mutants (*OE*). **D**, Determination of plaque forming units in a monolayer of hTERT host cells. 200 parasites were incubated in each well for 9 days, washed away and remaining host cells were stained to determine the number of zones cleared of host cell due to parasite invasion and replication (i.e., plaques). Values shown are averages ($\pm$ SE), N = 6. **E**, Determination of relative area of plaques. Data taken from same experiments as shown in panel D. Area represents the percent of the total available area that the average plaque occupied. Singe factor ANOVA (df = 1, 11) showed significantly lower plaque numbers and

plaque area in OE mutant cells. * = $P < 0.05$; *** = $P < 0.001$. *F*, Representative wells used to determine plaquing efficiency for RH and TgPPase OE mutant parasites.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. *T. gondii* parasites overexpressing TgPPase (OE) contain lower PP_i and higher ATP levels

A, Over-expression of *TgPPase* leads to a significant reduction in total PP_i levels as compared with wild-type RH tachyzoites (ANOVA, $df = 1,16$; $P < 0.01$). Bars represent the average ($\pm$ SE) of 9 quantifications for each cell type. *B*, No difference in short chain poly P levels between wild type RH and OE mutants for *TgPPase*. Bars represent the average of 9 determinations for each cell type. Values are in phosphate equivalents of hydrolyzed short chain poly P groups. *C*, Over-expression 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 hour. ATP was extracted and measured as described under “Experimental”. The results are representative of three independent experiments, each one performed in triplicate. The bars are the means $\pm$ S.D. Asterisks (*) show P values < 0.05 , determined by the Student’s *t*-test.

Figure 6. PP_i content of cytosolic and acidocalcisome fractions

PP_i was determined in cytosol (A) and 17,000G pellet (acidocalcisome) fractions obtained as described under “Experimental” from wild type (RH) and TgPPase-OE parasites (OE). Bars represent average concentration ($\pm$ SE) of 3 quantifications for each cell type. Where no error bars are present the bars fall within the graphical representation of the average value. Concentration was determined by dividing the total nmol PP_i in the cytosolic and organellar fractions by their respective estimated volumes as described under “Experimental”. *Cytosolic PP_i concentration was significantly lower in OE mutants (ANOVA, $df = 1, 4$; $P < 0.01$).

Figure 7. Proton extrusion by wild type (RH) and TgPPase-OE (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 extracellular pH (pH_e) in RH tachyzoites in the presence of 5 mM glucose (black, trace c), no glucose (light gray, trace a), and in cells where 5 mM glucose was added 300 seconds into recording (dark gray, trace b). *B*, same tracing as in *A* using OE mutants. *C*, resultant slope values (change in pH_e per second) in the presence (gray bars) or absence of glucose (open bars) for proton extrusion in

RH cells using data represented in tracings 'a' and 'c' in panel A. *D*, resultant slope values (change in pH_e per second) in the presence (*gray bars*) or absence of glucose (*open bars*) for proton extrusion in OE mutant cells using data represented in tracings 'a' and 'c' in panel B. *E*, rate of pH_e change before (*open bars*) and after (*gray bars*) addition of 5 mM glucose to media in RH cells using data represented in tracing 'b' in panel A. *F*, rate of pH_e change before (*open bars*) and after (*gray bars*) addition of 5 mM glucose to media in OE mutant cells using data represented in tracing 'b' in panel B. All estimates shown in panels C-F were determined from three independent experiments and error bars represent the standard error of the slope.

Figure 8. Lactate extrusion in wild type (RH) and TgPPase-OE (OE) parasites

Lactate extrusion rates were measured using an enzyme-coupled reaction (LDH)

fluorescently monitoring the production of NADH as lactate is converted to pyruvate.

Lactate extrusion was monitored in RH (*open circles*) and OE mutant (*gray circles*)

tachyzoites with duplicate samples taken at 0, 1, 2, 5, and 10 minutes for each cell type.

Regression analysis was performed on each cell type and the resultant slope, depicting the

rate of lactate extrusion, is shown in the inset as the cell-specific rate of lactate extrusion.

Error bars are the standard error of the slope. * $P < 0.001$; ANOVA comparison of slopes

between RH and TgPPase-OE cells ($df = 1, 18$; F value = 253).

Table I

Kinetic parameters of rTgPPase with different substrates.

Substrates	pH	cation	V_{max}^* ($\mu\text{mole/min/mg}$)	K_m^* (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{M}^{-1}$)
PP _i	8.5	Mg ²⁺	264.45 $\pm$ 8.33	23.10 $\pm$ 0.03	194.40	8.42 $\times 10^6$
Poly P ₃	6.0	Co ²⁺	6.41 $\pm$ 0.25	9.01 $\pm$ 1.21	0.14	1.57 $\times 10^4$
Poly P ₇₅	6.5	Mn ²⁺	6.16 $\pm$ 0.18	6.57 $\pm$ 0.95	0.28	4.31 $\times 10^4$
ATP	6.5	Mn ²⁺	5.27 $\pm$ 0.23	51.42 $\pm$ 6.89	0.24	4.71 $\times 10^3$
GP ₄	6.5	Mn ²⁺	5.22 $\pm$ 0.40	98.66 $\pm$ 19.55	0.24	2.43 $\times 10^3$

* The K_m and V_{max} were calculated using SigmaPlot 10.0.