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A novel calcium-dependent soluble inorganic pyrophosphatase from the trypanosomatid *Leishmania major*

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Abstract **A single-copy gene *IPP* encoding a putative soluble inorganic pyrophosphatase (LmsPPase, EC 3.6.1.1) was identified in the genome of the parasite protozoan *Leishmania major*. The full-length coding sequence (ca. 0.8 kb) was obtained from genomic DNA by polymerase chain reaction (PCR) and cloned into an *Escherichia coli* expression vector, and was overexpressed for functional protein purification and characterization. The recombinant LmsPPase, purified to electrophoretic homogeneity by a two-step chromatography procedure, exhibited a predicted molecular mass of ca. 30 kDa. The enzyme has an absolute requirement for divalent cations, exhibits a pH optimum of 7.5–8.0 and does not hydrolyze polyphosphates or adenosine triphosphate (ATP). LmsPPase differs from previously studied soluble pyrophosphatases with respect to cation selectivity, Ca^{2+} being far more effective than Mg^{2+}. Comparisons to known sPPases show a short N-terminal extension predicted to be a mitochondrial transit peptide, and changes in active-site residues and the neighboring region. Subcellular fractionation of *L. major* promastigotes suggests a mitochondrial localization. Molecular phylogenetic analysis indicates that LmsPPase is a highly divergent eukaryotic Family I sPPase, perhaps an ancestral class of eukaryotic sPPases functionally adapted to a calcium-rich, probably mitochondrial, environment.**

Key words: Soluble inorganic pyrophosphatase; Ca^{2+}; Divalent cation cofactor; Mitochondrion; *Leishmania major*

1. Introduction

Inorganic pyrophosphatases (PPases, EC 3.6.1.1) catalyze the hydrolysis of the phosphoanhydride bond in inorganic pyrophosphate (PP_i), a compound produced in various reversible nucleoside 5′-triphosphate-dependent reactions. These proteins play an important role in cell anabolism, providing a thermodynamic sink for biosynthetic reactions such as protein, RNA, and DNA syntheses [1]. PPases fall into two major classes, soluble PPases (sPPases) and membrane-bound H^+-translocating PPases (H^+-PPases). Both categories are thought to control intracellular PP_i levels and balance otherwise thermodynamically unfavorable pyrophosphorolytic biosynthetic reactions in different ways. The H^+-PPases are primary pumps that couple PP_i hydrolysis to generation of transmembrane proton gradients, a versatile source of biological energy [2,3]. sPPases are essential in bacteria [4] and yeast [5]. The two best-studied sPPases are those from *Escherichia coli* and *Saccharomyces cerevisiae* which are the prototypes of bacterial-type and eukaryotic-type Family I sPPases, respectively [6–10]. Sequence alignment of these proteins indicates that the active-site residues are very well conserved, even though the overall level of sequence similarity is low [8–10]. All Family I sPPases studied to date require a divalent metal cation cofactor, Mg^{2+} being by far the most efficient one [10]. In contrast to that, sPPases from diverse sources (bacteria, yeast and animals) were reported to be strongly inhibited by Ca^{2+} at very low physiological concentrations [11–14]. The mechanism of Ca^{2+}-induced inhibition of *E. coli* sPPase has been investigated, both replacement of Mg^{2+} by catalytically incompetent Ca^{2+} at M2 metal binding site of the enzyme and competence with $MgPP_i$ of non-hydrolyzable $CaPP_i$ for active site were reported as possible reasons for the inhibitory effect [15,16].

Trypanosomatid protozoans of the genus *Leishmania* are the causative agents of leishmaniasis, a parasitic disease of a worldwide occurrence: 12 million cases in 88 countries [17]. Recent evidence in these and other parasitic kinetoplastids demonstrates a PP_i-based cell bioenergetics, in which H^+-PPases play a key role. Homologs of the well-characterized H^+-PPases from higher plants [18,19], bacteria [2,20] and archaea [21] have been identified in many widely diverse parasitic and free-living protists [3,22,23], and occur in the acidocalcisomal membranes of a number of human pathogenic protozoa, both trypanosomatids and apicomplexans, responsible for endemic tropical diseases [24–27]. However, virtually no studies have been conducted on the sPPases of these evolutionary ancient pathogenic protists. Also, therapy against leishmaniasis and many other diseases caused by parasitic protists is needed to develop novel chemotherapeutic agents against targets essential for survival of the parasite.

Here we report the cloning and expression of the single *Leishmania major IPP* gene encoding the single sPPase of this parasite (LmsPPase) and the characterization of the re-

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Abbreviations: PP_i, inorganic pyrophosphate; sPPase, soluble inorganic pyrophosphatase; H^+-PPase, membrane-bound proton-translocating inorganic pyrophosphatase

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combinant protein. Comparison with its orthologs from bacteria, other protists and metazoa shows that it is a highly divergent Family I sPPase in which one of the catalytically important active-site residues, Arg78, that are conserved in all functional members of this protein group has changed. LmsPPase is highly active and the only sPPase that favors Ca^{2+}. A mitochondrial localization of this unique enzyme that may represent an ancestral class of eukaryotic-type sPPase is suggested.

2. Materials and methods

2.1. Reagents

Analytical grade dicalcium pyrophosphate (Ca_2PP_i; Aldrich Cat. No. 40155-2), dimagnesium pyrophosphate (Mg_2PP_i; Aldrich Cat. No. 41552-7) and tetrasodium pyrophosphate (Na_4PP_i; Sigma Cat. No. P 8010) were purchased from Sigma-Aldrich. Restriction enzymes, T4 DNA ligase and *Taq* polymerase from Boehringer Mannheim were used as specified by the manufacturer. Aprotinin, trypsin inhibitor, and dithiothreitol were purchased from Sigma Chemical Co. Phenylmethylsulfonyl fluoride (PMSF) and leupeptin were from Boehringer Mannheim. Other chemicals used were of analytical grade and purchased from Sigma-Aldrich. The oligonucleotides LM-PPAF and LM-PPAR were synthesized at the Analytical Services of the Instituto de Parasitología y Biomedicina López-Neyra (CSIC), Granada, Spain.

2.2. DNA manipulation and sequencing

Genomic DNA from *L. major* strain 252 promastigotes was isolated using the CsCl method [28]. Transformation for vector cloning was carried out in *E. coli* NovaBlue Singles™ competent cells (Novagen), and expression studies were carried out in *E. coli* BL21(DE3) (Invitrogen). Heterologous gene expression was performed with double-strand sequenced constructs in *E. coli* BL21(DE3) cells.

The specific oligonucleotides LM-PPAF (sense) 5′-catATGCTGAGTCGAGCGTTG-3′ (nucleotides that do not match with genomic sequence are in lowercase) and LM-PPAR (antisense) 5′-CTACCGCAGCCAATAGCCCGG-3′ were constructed and used for polymerase chain reaction (PCR) amplification of the 792-bp full-length open reading frame (ORF) of the *L. major IPP* gene (contig Sanger5664, The Sanger Institute-*Leishmania major* Genome Project, http://www.sanger.ac.uk/Projects/L_major) with genomic DNA as a template. PCR mixtures containing 50 pmol of each primer, were performed with 35 thermal cycles consisting of 60 s denaturation at 94°C, a 60 s annealing period at 45°C and a 60 s extension period at 72°C. The single PCR-amplified band (ca. 0.8 kb) was inserted without prior treatment in the pETBlue-1 AccepTor™ Vector (Novagen) that is compatible with PCR products containing single 3′-dA overhangs. The ATG start codon was located after three nucleotides in the sense primer to generate a new *Nde*I restriction site and the required 11-bp spacing from the ribosome binding site of the vector for optimal protein expression. Several recombinant pETBlue-1 clones containing the 0.8-kb DNA insert were obtained, one of them, pETLmPPA, was selected and double-strand sequenced with the 'ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction' kit (PE Biosystems) at the Analytical Services of the Instituto de Parasitología y Biomedicina López-Neyra, CSIC, Granada, Spain. The insert sequence of this construct, identical to the *L. major IPP* genomic sequence, was sent to EMBL/GenBank databases and assigned the accession number AY364408.

The pETLmPPA plasmid was used to transform the *E. coli* expression host BL21(DE3). Transformed clones were grown in Luria broth medium containing ampicillin (100 μg/ml). At an A_{600} of 0.6, 1 mM isopropyl β-D-thiogalactoside was added, the cultures were then grown for 3.5 h and the cells were eventually collected by centrifugation and stored at −80°C until use.

2.3. Protein techniques

Inorganic pyrophosphatase activity was assayed by the colorimetric determination of Pi produced from the enzymatic hydrolysis of PP_i at room temperature [29]. The reaction mixtures contained 50 mM Tris–HCl (pH 7.5), 4 mM cation salt ($MgCl_2$ or $CaCl_2$) and 2 mM Na_4PP_i (standard assay conditions). The reaction was started by the addition of enzyme and the PP_i released after 10 min was determined. Reaction rate units are μmol of Pi produced per min. When the efficiencies of other divalent metal cations as cofactors were tested the corresponding chloride salts were used in the assays instead of the Mg^{2+} or Ca^{2+} salts. Mg_2PP_i or Ca_2PP_i was utilized as substrate for kinetic parameter estimations (three independent determinations) and for experiments on enzymatic activation/inhibition by the simultaneous presence of these two divalent cations. To test the stringency of divalent cation dependence the activity was assayed either without added cation or with 4 mM ethylenediamine tetraacetic acid (EDTA). Optimum pH value for LmsPPase activity was determined in the pH range from 3.0 to 10.0. The buffers used (50 mM) were acetic acid–potassium acetate (pH range 2.5–5.5), MES–KOH (pH range 5.0–7.0), HEPES–KOH (pH range 6.5–7.5), citric acid–potassium citrate (pH range 7.0–9.0) and glycine–KOH (pH range 8.5–10.0); the pH values were adjusted at room temperature. Protein concentrations were determined using the Coomassie blue binding assay with bovine serum albumin as a standard [30].

Cell-free crude extracts – soluble protein fractions of cells disrupted by sonication and prepared in buffer supplemented with protease inhibitors (see below) – were obtained by centrifugation at $12\,000\times g$ for 5 min at room temperature from either *L. major* promastigotes or induced and control BL21(DE3) bacterial cultures, and eventually used for electrophoretic protein analysis, sPPase activity assays and Western blotting. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), employed for protein analyses of crude and purified samples, was performed with 12% polyacrylamide slab gels using a Mini-Protean II unit (Bio-Rad) according to the manufacturer's instructions. The gels were stained by standard procedures with Coomassie brilliant blue R-250 to visualize the protein bands.

Two monospecific anti-sPPase polyclonal antibodies used in Western blotting analyses [31] were generated by rabbit immunization with purified sPPases from the cyanobacterium *Synechocystis* sp. PCC6803 [31,32] and the microalga *Chlamydomonas reinhardtii* (chloroplastic isoform) ([33], Gómez-García, M.R. and Serrano, A., unpublished) dissolved in incomplete Freund's adjuvant.

2.3.1. Purification of LmsPPase. A frozen pellet of *E. coli* BL21(DE3)/pETLmPPA cells from a 2-l culture overproducing *L. major* sPPase was thawed and resuspended in 50 mM Tris–HCl (pH 7.5), 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 1 mM dithiothreitol and 0.5 mM EDTA (standard buffer) supplemented with protease inhibitors (1 mM PMSF, 2 mM ε-aminocaproic acid, 0.5 mM benzamidine and 20 μg/ml leupeptin) at a ratio of 3 ml/g (fresh weight) of cells. All operations were performed at 4°C. Cells were disrupted by ultrasonic treatment using a Branson Sonifier 450 (60 W, 2 min). The resulting broken cell suspension was centrifuged at $40\,000\times g$ for 30 min and the supernatant (crude extract) was treated with 10 mM streptomycin sulfate for 1 h at 4°C with continuous stirring and was then centrifuged as above. The resulting supernatant was then applied to a DEAE-cellulose DE-52 (Whatman) column (3 by 12 cm). After a wash, anion exchange chromatography was performed with a NaCl linear gradient (0–0.3 M; total volume, 300 ml) in standard buffer. Fractions of 4 ml were collected and those with enzymatic activity, which eluted at approximately 0.2 M NaCl, were pooled and applied to a hydroxyapatite Bio-Gel™ hydroxy-tryptophan (HTP) gel column (Bio-Rad) (2 by 10 cm). The sPPase protein was eluted by a linear gradient of $(NH_4)_2SO_4$ (0–0.1 M; total volume, 50 ml) in standard buffer. The purified LmsPPase preparation thus obtained was analyzed with SDS–PAGE and stored at −80°C until use for biochemical characterizations.

2.3.2. Subcellular localization by digitonin cell fractionation. Differential extraction of proteins of *L. major* promastigotes in the presence of increasing concentrations of digitonin was performed as previously described [34]. A 300-ml culture from *L. major* 252, grown until 1.2×10^7 cells/ml in M199 medium supplemented with 10% fetal bovine serum (Gibco), was harvested by centrifugation at $2500\times g$ and washed twice with phosphate-buffered saline (PBS). The pellet was resuspended in PBS plus the protease inhibitor cocktail described above to a concentration of 2×10^8 cells/ml. Aliquots of 1 ml were treated with 0.1–0.9% (v/v) digitonin and incubated for 30 min at 30°C. Samples were then centrifuged at $14\,000\times g$ for 2 min and the following enzymatic markers were assayed in the supernatants: the mitochondrial enzyme citrate synthase [35]; the glycosomal enzyme hexokinase [36]; and the cytosolic enzyme pyruvate kinase [37]. sPPase activity was also assayed in all these fractions with either

$MgCl_2$ or $CaCl_2$ as the cation source for the reaction. A control cell preparation was treated in parallel with Triton X-100 (0.5%, v/v), the obtained enzymatic activity levels being considered as the 100% values of the corresponding marker and query enzymes.

2.4. Sequence comparison and molecular phylogenetic analyses

A multiple sequence alignment of the deduced amino acid sequence of the *L. major IPP* gene and other selected eukaryotic and prokaryotic Family I sPPases publicly available was performed using the CLUSTAL X v.1.8 program [38]. This alignment was used to construct a phylogenetic tree by the distance method (neighbor-joining, BLOSUM matrix) with the same program. Some preliminary sequence data were obtained by sequence similarity searches using BLAST algorithms [39] on diverse unfinished microbial genome projects at the web sites of the National Center of Bioinformatics (NCBI), USA (http://www.ncbi.nih.gov/PMGifs/Genomes/allorg.html), the Joint Genomic Institute (JGI), USA (http://spider.jgi-psf.org/JGI_microbial/html/), The Sanger Institute, UK (http://www.sanger.ac.uk/Projects/), or the Institute for Genomic Research (TIGR), USA (http://www.tigr.org/tdb/mdb/mdb.html).

Deduced eukaryotic sPPase sequences were analyzed for protein sorting motifs, N-terminal targeting sequences and protein localization sites using the following web-available computer programs: (i) MitoProt II 1.0a4 [40], (ii) PSORTII [41], (iii) Predotar (http://www.inra.fr/predotar/), and (iv) SignalP V2.0.b2/TargetP V1.0 [42].

3. Results

3.1. Identification, cloning and expression of the L. major IPP *gene, and purification of functional recombinant LmsPPase*

The soluble protein fraction of *L. major* promastigote homogenates exhibited high specific activity levels of an alkaline sPPase only in the presence of divalent metal cations. The specificity for Ca^{2+} was clearly different from all previously studied sPPases. Ca^{2+} at μM level gave the highest activities, 4–5 U/mg of protein, while other cations gave activities of 1–3 U/mg $Cu^{2+} > Mn^{2+} > Fe^{2+} > Mg^{2+} > Zn^{2+} > Co^{2+}$. This is in contrast to all previously studied sPPases, for which Ca^{2+} is virtually inactive for Family I and II members and Mg^{2+} is the preferred cofactor for all Family I members studied to date. This unusual biochemical feature prompted us to take a bioinformatics approach to identify the gene(s) encoding putative sPPase(s) in the publicly available *L. major* genome database.

Searches for sPPase amino acid homologs in 'The Sanger Institute-*Leishmania major* Genome Project' database with BLAST algorithms [39] using either bacterial or eukaryotic Family I sPPase sequences as queries invariably generated a single hit located in contig 5664 (chromosome 3) corresponding to a 792-bp coding region devoid of introns. No hits for Family II sPPases, these are reported to be present only in bacteria [43], were found using the same searching strategy. This putative *L. major IPP* gene encodes a predicted protein of 293 amino acids (LmsPPase), with theoretical molecular mass and p*I* values of 28.9 kDa and 5.99 respectively, which exhibits 35–40% sequence identity with other eukaryotic Family I sPPases. Analysis of multiple sequence alignments with other sPPases revealed that 12 out of the 13 catalytically important active-site residues that are strictly conserved in all functional Family I sPPases studied to date [10,44] are present in LmsPPase (Fig. 1) – including the three Asp residues of the PROSITE motif (PDOC00325) that is the Family I sPPase signature. Residue Arg78 (using the *S. cerevisiae* sPPase numbering) involved in substrate binding [10,45] is replaced by a Gly in the LmsPPase, and others changes were found in the region around this residue (see Fig. 1), which is located at the excursion II between barrel strands β1 and β4 [10]. This substitution in LmsPPase suggested the possibility of an atypical catalytic behavior. In addition, sequence alignments with other eukaryotic sPPases identified a short (ca. 20 residues) N-terminal extension in LmsPPase that several programs (MitoProt, PSORT II) predicted as a mitochondrial transit peptide, and also revealed that LmsPPase lacks the extra C-terminal region of fungal and animal sPPases (see Fig. 1). The N-terminus feature is common to organellar (mitochondrial- and plastid-located) nuclear-encoded eukaryotic sPPases while the C-terminus feature is shared with prokaryotic and most protist sPPases (Fig. 1).

The *L. major IPP* gene was cloned from genomic DNA by PCR giving a single DNA fragment of the expected size (ca. 0.8 kb) containing the full-length coding region (Fig. 2A), which was directionally cloned in *E. coli* expression plasmid vector. The fully sequenced construct pETLmPPA (Fig. 2B) was used for LmsPPase overproduction in *E. coli* BL21(DE3) transformed cells. Sequencing of the cloned *IPP* gene confirmed the unusual Arg78 replacement by Gly found in the publicly available genome sequence. A major protein band with the expected molecular mass of LmsPPase (29–30 kDa) was observed after SDS–PAGE of the soluble fraction from induced transformed cells (Fig. 2C, lane 1). Functional recombinant LmsPPase was produced in cell extracts with a 10-fold increase of sPPase specific activity, ca. 20 μmol/min/mg of protein, with added Ca^{2+} over the control levels (with Mg^{2+}) of non-transformed cells. The recombinant protein (rLmsPPase) was purified to electrophoretic homogeneity by a two-step chromatography procedure (anionic exchange and hydroxyapatite chromatographies) that typically gave a Ca^{2+}-dependent specific activity of ca. 150 μmol/min/mg of protein and a yield of near 90% (Table 1). The rLmsPPase showed a remarkable affinity for both matrices and eluted as a single symmetrical activity peak from both columns (data not shown), in accordance with the acidic character of the predicted protein and its high affinity for Ca_2PP_i as a substrate (see below). The single protein band detected in the purified preparation exactly matched the major 29–30 kDa band observed in crude extracts of the transformed *E. coli* cells (Fig. 2C, cf. lanes 1 and 3). However, neither in crude extracts of either *L. major* promastigotes or pETLmPPA-transformed *E. coli* cells, nor in purified preparations two specific antibodies against cyanobacterial and algal Family I sPPases failed to detect LmsPPase in Western blots. Since these anti-sPPase antibodies readily cross-react with other Family I sPPases from diverse bacteria and eukaryotes (algae, plants, protists and fungi) ([32]; Gómez-García, M.R. and Serrano, A., un-

Table 1

	Volume (ml)	Total activity (μmol/min)	Protein (mg/ml)	Specific activity (μmol/min/mg)	Yield (%)
Crude extract	20	12 360	30.0	21.1	100
DEAE-cellulose eluate	50	11 200	5.2	43.1	91
Hydroxyapatite eluate	20	10 800	3.5	154.3	87

published results), this result strongly suggests the existence of structural differences between LmsPPase and other members of the Family I sPPase group (see below).

3.2. Functional characterization of recombinant LmsPPase

The purified rLmsPPase has an absolute requirement for divalent cation cofactors, Ca^{2+} being most efficient at μM level. The activity level with Mg^{2+} was only about 20% of that of Ca^{2+}, in contrast to all previously studied Family I sPPases which are Mg^{2+}-dependent enzymes unable to use Ca^{2+} as cofactor [10]. Actually, Ca^{2+} is a strong inhibitor of bacterial, fungal and animal Family I sPPases [11–13], and was reported to inhibit both cytosolic and mitochondrial matrix mammalian enzymes at physiological micromolar concen-

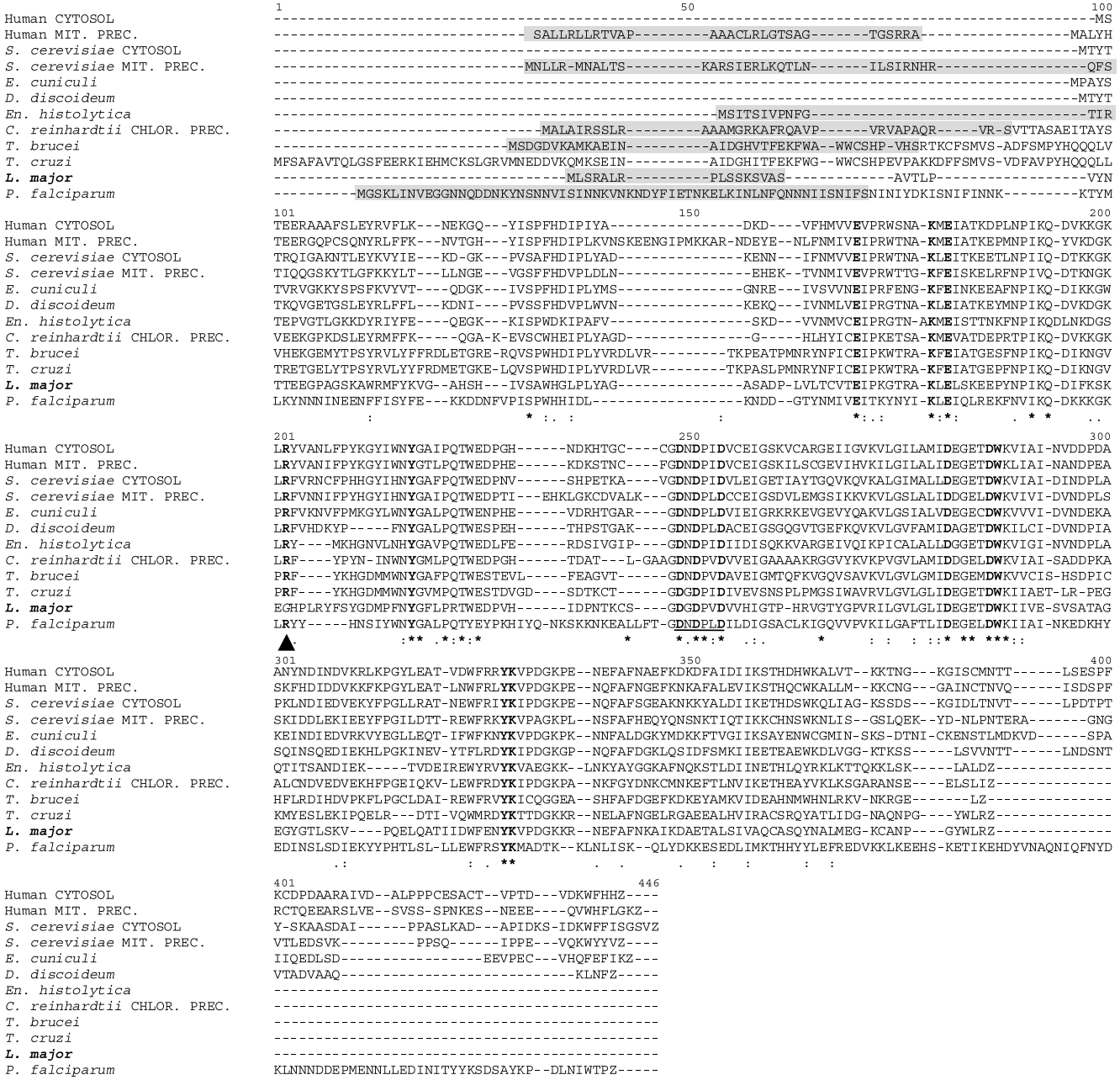

Fig. 1. CLUSTAL X sequence alignment of LmsPPase with other selected eukaryotic sPPases. Protein sequences derived from complete genes are shown in all cases. The 13 active-site residues reported to be functionally important for sPPase activity (Kalkare et al., 1994, 1996) are in bold; conserved residues are indicated by asterisks, conservative changes by semicolons and semiconservative changes by colons. The substitution of Gly for Arg at residue 78 in the yeast sPPase, indicated by a triangle, is italicized in LmsPPase. The PROSITE motif of active-site residues characteristic of Family I sPPases is underlined. N-terminal extensions suggested to be organellar (mitochondrial, hydrogenosomal or plastidial) transit peptides, or signal sequences (in the case of *Plasmodium falciparum* and *Trypanosoma brucei*) are shaded. Note the very long N-terminal regions of the predicted *P. falciparum* and *Trypanosoma cruzi* sPPase sequences. The sequences shown are: *S. cerevisiae* cytosolic sPPase (CAA31629) and mitochondrial sPPase precursor (NP_013994.1), human cytosolic sPPase (AAD24964) and mitochondrial sPPase precursor (AAG36781), *C. reinhardtii* chloroplastic precursor (CAC42762), *Encephalitozoon cuniculi* (AL590449), *P. falciparum* (NP_473275.1), *Entamoeba histolytica* (contig 318122, TIGR), *Dictyostelium discoideum* (AU061000 and AU034313), *T. brucei* (contigs 90C7TF and 81B5TF, TIGR), *T. cruzi* (contig TCKGB96TR, TIGR).

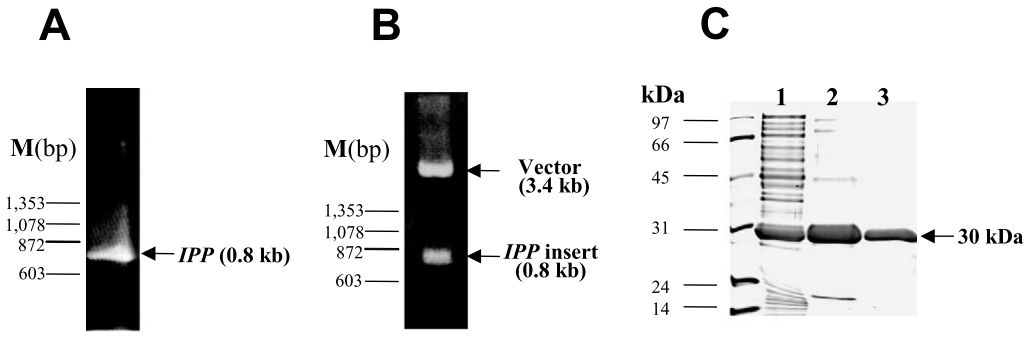

Fig. 2. A: Electrophoretic analysis of the PCR-amplified *L. major* DNA fragment containing the gene *IPP* run on a 0.7% agarose gel in 1/2×TBE using ΦX174 phage DNA cleaved with *Hae*III as a bp standard. A single band of ca. 0.8 kb was obtained. B: Electrophoretic analysis of the pETLmPPA construct cleaved with *Nde*I and *Eco*RI restriction enzymes. Two DNA fragments corresponding to the 3.4-kb pETBlue-1 vector and the excised 0.8-kb insert corresponding to the *IPP* gene were resolved by agarose gel electrophoresis as in A. C: Coomassie blue-stained SDS–PAGE of the recombinant LmsPPase on 12% polyacrylamide gel. Lane 1, cell-free extract of an induced LmsPPase *E. coli* BL21(D3) clone transformed with expressing plasmid pETLmPPA; lane 2, eluate (active pool) from the DEAE-cellulose DE-52 column; lane 3, eluate (active pool) from hydroxyapatite column. The arrow indicates the position of the 29–30 kDa protein band corresponding to the over-produced recombinant sPPase. The positions and molecular masses of protein standards (M; SeeBlue® pre-stained standard, Invitrogen) are indicated on the left. Approximately 25 μg of protein were applied in lanes 1 and 2 and 10 μg in lane 3.

trations [14,46]. Ca^{2+} is not simply an enzyme activator, as is Mn^{2+} for Family II sPPases [43], as Mg^{2+} is not required for Ca^{2+}-supported rLmsPPase activity. Several heavy metal cations, i.e. Zn^{2+} and Co^{2+}, also supported rLmsPPase activity, although less efficiently (see Fig. 3A). The recombinant enzyme exhibits therefore a metal cation specificity virtually identical to that of the native LmsPPase, suggesting no major structural modifications between them. The rLmsPPase exhibited maximum activities between pH 7.5 and 8.0, with similar Ca^{2+} and Mg^{2+} activity profiles (Fig. 3B). Negligible activity was detected below pH 6, suggesting that this enzyme cannot function in the acidocalcisomes, acidic cytosolic vesicles which contain most of the trypanosomatid cellular PP_i and polyphosphate [47].

The recombinant LmsPPase is very specific for PP_i as a substrate, no detectable activity being observed with adenosine triphosphate (ATP) and short-length polyphosphates, i.e. tri- and tetra-polyphosphate or a linear polymer of 13–18 phosphate residues (P_{13-18}). The strict preference for PP_i is not dependent upon the divalent cation, Ca^{2+} or Mg^{2+}, present in the reaction (data not shown). rLmsPPase has an almost 10-fold higher affinity for Ca_2PP_i than for Mg_2PP_i as indicated by their respective K_m values, 39 ± 2 and 370 ± 10 μM (means ± S.E.M. of three independent determinations) and is 10-fold more active with the first substrate (k_{cat} values 3693 s^{-1} and 369 s^{-1}, respectively). Thus, the catalytic efficiency, k_{cat}/K_m, of the enzyme with Ca^{2+} is about 100-fold higher than with Mg^{2+}. In the presence of both cations rLmsPPase activity was stimulated by increasing Ca^{2+} μM concentrations ($k_{0.5}$, 30–40 μM) in the presence of saturating Mg_2PP_i, whereas an inhibitory effect was observed by increasing Mg^{2+} mM concentrations ($k_{0.5}$, 1–2 mM) in the presence of saturating Ca_2PP_i (Fig. 4). This cation-dependent modulation of LmsPPase activity is produced at physiological concentrations of the effectors, possibly by competition for specific metal binding sites in the protein–substrate complex [10,15,16,48], suggesting that regulation of the enzyme by these two divalent cations may occur in vivo (see below).

3.3. *Subcellular localization of the sPPase of* L. major

Identification of the LmsPPase organelle targeting sequences and prediction of protein localization sites in the cells by

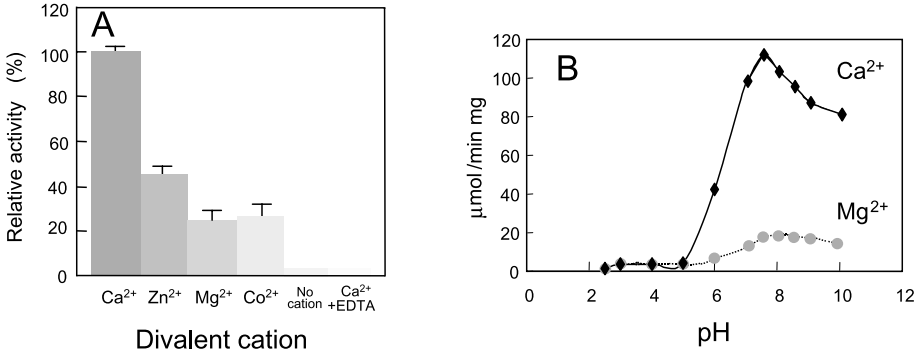

Fig. 3. A: Optimization of LmsPPase reaction conditions. Divalent cations (4 mM, chloride salts) were used as cofactors under standard assay conditions (ca. 20 μg of purified rLmsPPase). The assays without cation added or with 4 mM EDTA were used as controls of strict cation dependence. Data are means ± S.E.M. of four determinations and are expressed as percent of maximum activity obtained with Ca^{2+} defined as 100%. B: Effects of pH on LmsPPase activity with either Ca^{2+} (triangles) or Mg^{2+} (circles) as cation cofactor. About 20 μg of purified protein were assayed with the corresponding PP_i salts at the indicated pH values in the conditions described in Section 2.

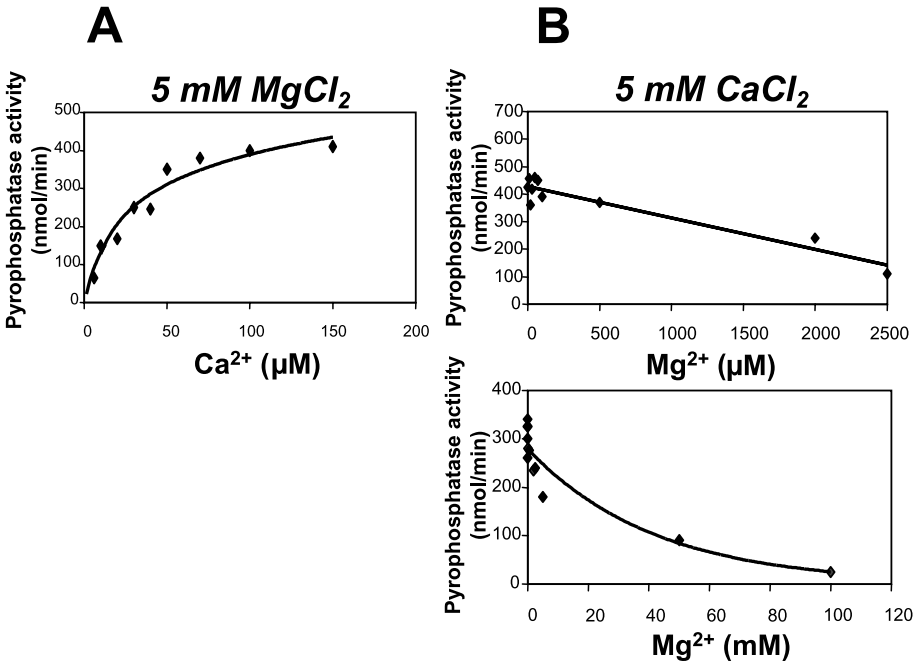

Fig. 4. Stimulating effect on LmsPPase activity of Ca^{2+} in the presence of excess Mg_2PP_i (A) and inhibitory effect of Mg^{2+} in the presence of Ca_2PP_i excess (B) with 4 mM PP_i salt and the varying concentrations of Ca^{2+} or Mg^{2+} (chloride salts). Data are means of three independent experiments. Lines correspond to the semilogarithmic curve (A), linear curve (B, top) and decreasing exponential curve (B, bottom) fits (R values, 0.95–0.97) obtained with the KaleidaGraph™ v.3.0 program.

computer programs suggest it is a mitochondrial targeted protein (probability indices were: 0.958 by MitoProt II, 0.877 by Predotar, and 78% by PSORT II). Both MitoProt II and PSORT II also predicted a 16-residue mitochondrial presequence or transit peptide in the N-terminal extension that aligned with those found in some mitochondrial and plastidic eukaryotic sPPase precursors (see Fig. 1). No signal peptide was identified in the LmsPPase sequence, implying that there is no protein sorting through secretion pathways. Differential digitonin solubilization experiments to determine the organellar localization were performed. The release profiles of sPPase activity with either Ca^{2+} or Mg^{2+} from *L. major* promastigotes were generated with increasing concentrations of digitonin [34,49] (Fig. 5). Various enzymatic activities were also assayed and used as cellular fraction markers. Cytoplasmic proteins are solubilized at rather low digitonin concentrations ($<0.1\%$) and sPPase activity was not released until 0.4% digitonin was present. A glycosomal location is compatible with this result, but it can be excluded since it has been reported that this organelle is devoid of sPPase activity [50] and no glycosomal targeting signals [51] were found in LmsPPase sequence. The sPPase activity release profile is very similar to that of the mitochondrial marker citrate synthase (Fig. 5), suggesting a localization of LmsPPase in this organelle.

3.4. Phylogenetic analysis reveals LmsPPase is a highly divergent eukaryotic-type sPPase

The phylogenetic protein analysis of LmsPPase with other Family I sPPases of various eubacteria and eukaryotes (Fig. 6) indicates that it belongs to the eukaryotic assembly of this family, which is consistent with the biochemical data presented in this work. However, it clusters with two other predicted sPPases of kinetoplastids in an evolutionary diverse deeply branching group adjacent to the prokaryotic enzymes. The trypanosomatid sPPases appear closely related to the orthologs of other evolutionary ancient protists (apicomplexans, amoebae) and more distantly related to the fungi–metazoa cluster, which includes the microsporidian sPPase. This is in agreement with recent molecular phylogenetic evidence that includes these amitochondriate protists in the fungal evolutionary lineage (secondary loss of mitochondria) [52]. The protein phylogenetic data clearly support the divergent and presumably ancient character of LmsPPase and are consistent with the peculiar catalytic properties of this novel eukaryotic-type Family I sPPase.

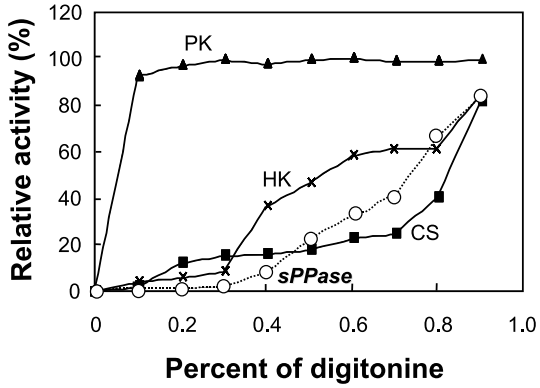

Fig. 5. Subcellular localization of LmsPPase in *L. major* promastigotes by digitonin treatment. Increasing digitonin concentrations were used to sequentially release LmsPPase and marker enzymes out of cellular compartments. PK, pyruvate kinase, cytosolic marker; HK, hexokinase, glycosomal marker; CS, citrate synthase, mitochondrial marker. Cytosolic enzymes are mainly solubilized by low (0.1%) digitonin concentrations. Data are from one representative experiment and are expressed as percent of maximum activities solubilized with Triton X-100 defined as 100%.

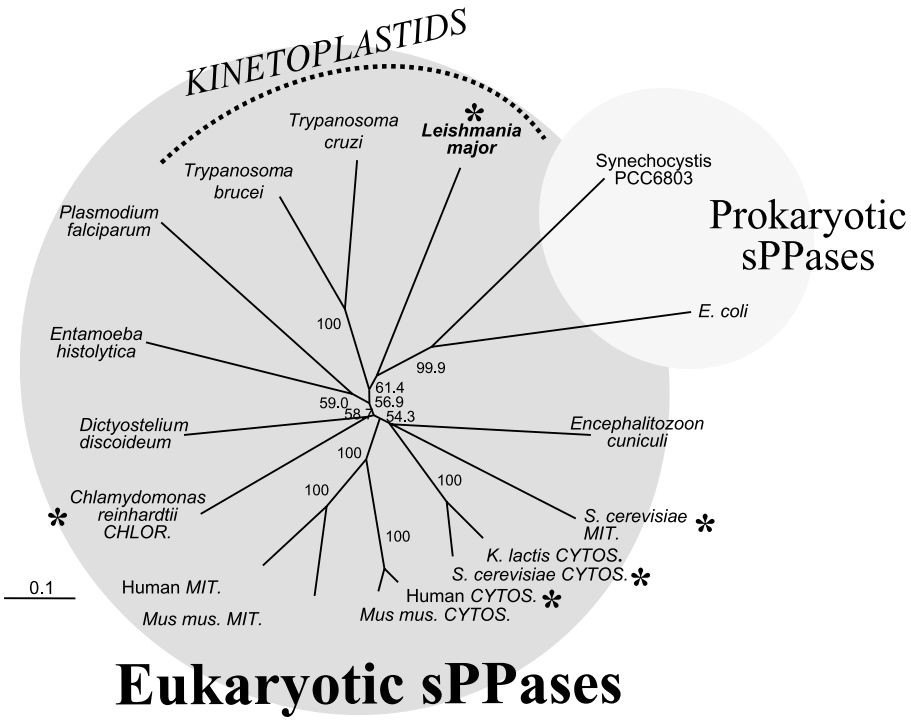

Fig. 6. Molecular phylogenetic analysis of sPPases from different eubacteria, parasitic and free-living protists, yeasts and metazoa. The amino acid sequence deduced from the full-length *L. major IPP* gene was aligned with sequences of selected eukaryotic and prokaryotic Family I sPPases using CLUSTAL X and subjected to phylogenetic analysis (neighbor-joining method, BLOSUM32 matrix) with the same program to generate an evolutionary distance tree. The numbers indicate the statistical support (bootstrap values from 1000 replicates) of selected associated groups on a percentage basis. Proteins clearly identified directly, or inferred by computer programs, to have organellar (mitochondrial or plastidic) localization and therefore synthesized as precursors are labelled accordingly. The 0.1 bar represents amino acid substitutions per site. Accession numbers for the sequences used are: *S. cerevisiae* cytosolic sPPase (CAA31629) and mitochondrial sPPase precursor (NP_013994.1); human cytosolic sPPase (AAD24964) and mitochondrial sPPase precursor (AAG36781); *Mus musculus* cytosolic sPPase (BAB25754) and mitochondrial sPPase precursor (BAB22922); *C. reinhardtii* chloroplastic precursor (CAC42762); *Kluyveromyces lactis* cytosolic sPPase (CAA32466); *E. cuniculi* (AL590449); *P. falciparum* (NP_473275.1); *E. histolytica* (contig 318122, TIGR); *D. discoideum* (AU061000 and AU034313); *T. brucei* (contigs 90C7TF and 81B5TF, TIGR); *T. cruzi* (contig TCKGB96TR, TIGR); *E. coli* (P17288); *Synechocystis* PCC6803 (AJ252207). Some putative full-length sPPase sequences were obtained by gene reconstruction from genomic DNA or EST overlapping sequences found by BLAST searches [34] in public databases or the microbial genome project web sites indicated in the text. Asterisks denote eukaryotic sPPases that have been biochemically characterized. Note the deep-branched position of the trypanosomatid group among the eukaryotic sPPases.

4. Discussion

We report here that the *IPP* gene in the trypanosomatid *L. major* encodes a functional sPPase, LmsPPase, with unique catalytic characteristics. The ORF (ca. 0.8 kb) encodes an acidic protein of 263 amino acids and a molecular mass of ca. 29 kDa which was overexpressed in a bacterial host and the recombinant protein purified. Unexpectedly, Ca^{2+} that was reported to be a very strong inhibitor of all Family I sPPases studied to date [11–14,46] is by far the most efficient metal cofactor for LmsPPase. It is interesting to note at this respect that Family II sPPases – a recently described novel type of bacterial sPPases structurally very different to the classical Family I enzymes – can use Ca^{2+} as well as Mg^{2+} as metal cofactors [43,53].

Sequences alignments revealed that there are some unusual features of the LmsPPase primary sequence. A short N-terminal extension is predicted to be a mitochondrial transit signal, in agreement with reports on mitochondrial targeting in kinetoplastids showing that nuclear-encoded mitochondrial proteins of these protists possess either short N-terminal presequences with several basic residues, as is the case of LmsPPase, or no presequences at all [54] In addition, the active-site residue in the consensus sequence Arg78, involved in substrate binding and strictly conserved in all previously studied functional sPPases [10,15,16], has been changed to Gly in LmsPPase. Whether the unique Ca^{2+}-supported activity of LmsPPase is directly related to this substitution is unknown. It is interesting to note at this respect that mechanistic and structural studies carried out on *E. coli* and yeast sPPases have shown that: (i) this Arg residue is directly involved in binding and optimum orientation for catalysis of one phosphate group of PP_i [16,55], and (ii) Ca^{2+} binding alters conformation of the Arg78 neighboring active-site region, resulting in inability of the enzyme to acquire the optimum for hydrolysis active-site conformation and substrate orientation [15,16]. In summary, the *L. major* sPPase described in this work appears to be a novel member of the eukaryotic assembly of Family I sPPases, although mechanistic and structural studies are required to clarify its peculiar catalytic features.

Kinetoplastid protozoa, together with the related group of euglenoids, represent one of the earliest extant group of eukaryotes containing mitochondria [52]. Molecular phylogenetic data suggest an ancient evolutionary character of the divergent LmsPPase. Sequence-predicted mitochondrial localization of the LmsPPase is supported by subcellular fraction-

ation experiments. The alkaline optimum pH of this protein suggests that it cannot be functional in acidocalcisomes, cytoplasmic vesicles in trypanosomatids [47].

Leishmania and other trypanosomatids have a single large mitochondrion containing a mass of organelle DNA in a special receptacle called a kinetoplast, located near the basal body of the flagellum. Location of the Ca^{2+}-dependent LmsPPase in the mitochondrion kinetoplast organelle is consistent with the key role of mitochondria in Ca^{2+} homeostasis. These organelles are capable of accumulating vast amounts of Ca^{2+} in response to a wide variety of physiological cell stimulations, including host cell invasion; levels of 500–800 μM, more than 200 times the cytoplasmic concentration, are transiently reached [56]. The in vitro regulation of LmsPPase activity by Ca^{2+} and Mg^{2+} at physiological concentrations similar to those found in mitochondria [57,58] suggests an important role for this enzyme in the energy metabolism of kinetoplastids and other parasitic protozoa. Therefore, the adaptation of LmsPPase in this Ca^{2+}-rich environment could be an important biochemical player during periods of intense energy demand.

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