Properties of phosphoenolpyruvate mutase, the first enzyme in the aminoethylphosphonate biosynthetic pathway in *Trypanosoma cruzi*

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Running Title: Phosphoenolpyruvate mutase from *T. cruzi*

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Phosphoenolpyruvate mutase catalyses the conversion of phosphoenolpyruvate to phosphonopyruvate, the initial step in the formation of many naturally occurring phosphonate compounds. The phosphonate compound, 2-aminoethylphosphonate (AEP) is present as a component of complex carbohydrates on the surface membrane of many trypanosomatids including glycosylinositolphospholipids of *Trypanosoma cruzi*. Using partial sequence information from the *T. cruzi* genome project we have isolated a full-length gene with significant homology to PEP mutase from the free-living protozoan *Tetrahymena pyriformis* and the edible mussel *Mytilus edulis*. Recombinant expression in *Escherichia coli* confirms that it encodes a functional PEP mutase with a $K_m$ apparent of 8 µM for phosphonopyruvate and $k_{cat}$ of 12 s$^{-1}$. The native enzyme is a homotetramer with an absolute requirement for divalent metal ions and displays negative cooperativity for Mg$^{2+}$ ($S_{0.5}$ 0.4 µM; $n = 0.46$). Immunofluorescence and subcellular fractionation indicates that PEP mutase has a dual localisation in the cell. Further evidence to support this was obtained by western analysis of a partial sub-cellular fractionation of *T. cruzi* cells. Southern and Western analysis suggests that PEP mutase is unique to *T. cruzi* and is not present in the other medically important parasites, *T. brucei* and *Leishmania spp.*
INTRODUCTION

Phosphonates are widely distributed in nature as components of lipids, oligosaccharides and proteins, as well as microbial secondary metabolites with antibacterial (e.g. fosfomycin), antimalarial (fosmidomycin) and herbicidal (e.g. bialaphos) activities (1, 2). The presence of the carbon-phosphorus bond renders phosphonate compounds highly resistant to enzymatic hydrolysis and appears to be a key determinant to the function of these molecules (1, 3). The formation of the carbon-phosphorus bond in naturally occurring phosphonates is catalysed by the enzyme phosphoenolpyruvate mutase (PEP mutase1; systematic name phosphoenolpyruvate 2,3-phosphonomutase; EC 5.4.2.9 (4)). Phosphonopyruvate is subsequently converted into 2-aminoethylphosphonate (AEP or ciliatine), which is then incorporated into macromolecules such as phosphonolipids and proteoglycans (Figure 1A).

AEP was first identified as a minor component in acid hydrolysates of lipid and glycolipid extracts of the intracellular protozoan parasite Trypanosoma cruzi (5), the causative agent of Chagas’ disease. Although dismissed as a minor component of lipopeptidophosphoglycan (LPPG, now known as glycosylinositolphospholipid, GIPL), subsequent studies indicate that AEP may be a universal component of glycoconjugates (mucins and GIPLs) that cover the surface of different stages of the parasite (6-8). Mucins are highly glycosylated proteins that are anchored in the plasma membrane via a glycosylphosphatidylinositol (GPI) moiety. Although the lipid moiety of GPI anchors varies

1 The abbreviations used are: PEP mutase, phosphoenolpyruvate mutase; AEP, 2-aminoethylphosphonate; His6, hexahistidine; V-H+-PPiase, vacuolar type proton pyrophosphatase
throughout the life cycle, most GPIs and GIPLs share a common Man₄(AEP)GlcN-Ins-PO₄ core (6) (Figure 1B). AEP can also substitute for phosphoethanolamine in the linkage between the GPI anchor and the polypeptide chain of mucins, although this is not obligatory. In contrast, an additional AEP substituent on the O-6 of glucosamine of T. cruzi GPI anchors appears to be universal (6) and has also been identified in glycolipids from other members of the Kinetoplastida, including the dixenic bat trypanosome T. dionisii (9), and the monoxenic insect parasites Leptomonas samueli (10) and Herpetomonas samuelpessoaai (11). However, the most abundant surface glycoconjugate of Leishmania (lipophosphoglycan, a GPI-anchored polymer of the repeating disaccharide-phosphate units) does not contain AEP (12). Likewise, AEP is absent from the African trypanosome, T. brucei (5), where ethanolamine is an integral component of the variant surface glycoprotein (13).

Apart from the demonstration that ⁳²P can be incorporated into AEP (5), the biosynthesis of this intermediate has not been studied. The identification of pyruvate phosphate dikinase in T. cruzi (14), which catalyses formation of phosphoenolpyruvate from pyruvate, prompted us to search for other genes in the biosynthetic pathway to AEP. Here we report the isolation and functional expression of PEP mutase, some of its kinetic properties and its subcellular localisation in T. cruzi.
EXPERIMENTAL PROCEDURES

Organisms and reagents—Epimastigotes of *T. cruzi* (Y strain and Silvio clone X10-7) were cultured at 28 °C in RTH/FCS medium (RPMI, trypticase, haemin supplemented with 5% foetal calf serum) (15). Other trypanosomatids were cultured as described (15). The edible mussel, *Mytilus edulis*, was purchased from a local fishmonger in Dundee. Routine manipulations were conducted in *E. coli* strain JM109 and overexpression in strain BL21(DE3)pLysS (Stratagene). All chemicals were of the highest grade available from Sigma, BDH and Roche. Phosphonopyruvate (tris-cyclohexylammonium salt) was prepared as described (16, 17).

Cloning of PEP mutase gene from *T. cruzi* — A BLAST search of conserved sequence regions of PEP mutase from *M. edulis* and *Te. pyriformis* against the *T. cruzi* genome database yielded a partial sequence (Accession number AQ445225.2) with significant homology to other PEP mutases. This partial PEP mutase gene fragment was amplified using oligonucleotides (5-primed GGGGTTCGGCGACACAACGAAGCG, and 3-primed CTGCATGGCGGCAATGCAGGCCC), using the following conditions: denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min and followed by a final extension at 72°C for 10 min. The PCR product was cloned into the Novagen vector ZERO-BLUNT TOPO. The fluorescein-labelled PEP mutase PCR fragment was used as a probe in the initial isolation and characterization of this gene. A size-selected library (2-4kb) was constructed in pUC18 with gDNA digested with *Hind*III and *Sac*I. Positive clones were identified from a colony screen using the labelled probe
and found to contain the same size insert. One of these was sequenced using primers designed to the known partial PEP mutase gene sequence and the full-length gene was obtained.

For expression studies the open reading frame of PEP mutase was amplified by PCR as described above, using the following primers, PEPM.pET15bF

GGCTCGAGATGCGTCACTGCTGTGGTCTG and PEPM.pET15bR

GGCTCGAGTTATTTCTTCGGCAAGTACATTTCC, engineered with XhoI sites in the 5’ and 3’ primers respectively, for cloning into the Invitrogen E. coli expression vector pET15b.

Southern blot analysis — T. cruzi genomic DNA (5 µg) was digested with selected restriction endonucleases (BamHI, SacI, HindIII and NdeI), separated by gel electrophoresis using a 0.8% agarose gel and transferred to positively charged nylon membrane (Hybond N+, Amersham-Pharmacia Biotech). Hybridisation and signal detection was performed using the Gene Images labelling and detection kit (Amersham-Pharmacia-Biotech), following the manufacturer’s recommendations.

Western blot analysis — Total extracts of T. cruzi and other trypanosomatids (1x10⁶ parasites per lane) and M. edulis (5 µg) were fractionated by electrophoresis on 4-12% gradient SDS-PAGE gels (18). Immunoblot analysis was performed essentially as described (19), using polyclonal antisera to PEP mutase and GAPDH at a dilution of 1:50. Blots were developed by chemiluminescence following the manufacturers instructions (ECL kit, Amersham-Pharmacia Biotech).

Soluble expression of recombinant PEP mutase — A six-litre culture of BL21(DE3)pLysS/pET15b-PepM, derived from a single colony, was grown at 37°C with vigorous agitation in Terrific Broth containing 50 µg ml⁻¹ carbenicillin and 12.5 µg ml⁻¹ chloramphenicol. When the culture reached an OD₆₀₀ of approximately 0.8, the culture was
cooled to 25°C and agitation reduced to 100 rpm and the culture induced with a final concentration of 0.5mM isopropyl-β-D-thiogalactopyranoside. Cultures were grown for 16 h and harvested by centrifugation. Cells were washed with 20 mM Tris, pH 8.0 and lysed in 50 ml of breaking buffer (20 mM Tris, pH 8.0, 0.5 M NaCl, 5 mM MgCl₂, 100 µg ml⁻¹ DNAse I and protease inhibitor cocktail, Roche) by flash freezing in ethanol/dry ice bath followed by rapid thawing and bead beating. Cell debris was separated and discarded after centrifugation at 30,000 g for 30 min at 4°C.

Purification and properties of recombinant PEP mutase protein — The supernatant containing soluble protein was diluted two-fold with 20 mM Bis-Tris propane, 20 mM Tris (pH 7.4), 0.5 M NaCl, passed through a 0.2 µm Steriflip filter and loaded onto a nickel-chelating Sepharose High Performance column (Pharmacia) pre-equilibrated with the same buffer. Protein was eluted using a linear gradient from 0 to 1 M imidazole, active fractions pooled, dialysed against phosphate buffered saline (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) and digested with human thrombin (50 µg ml⁻¹) for 2 h at 25°C, to remove the hexahistidine (His₆) tag. The sample was then dialysed for 2 h in 20 mM BTP, 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT and loaded onto a 6-ml ResourceQ column (Pharmacia). Protein was eluted with a linear gradient from 0-0.5 M NaCl and the active fractions pooled, dialysed against 50 mM (K⁺) Hepes pH 8.0 containing 1 mM DTT, and 0.01% sodium azide. Aliquots of 50 µl were dispensed, rapidly frozen and stored at -80°C. Under these conditions the enzyme loses less than 10% of its activity over four months.

Native Mᵣ was determined by Superdex 200 column chromatography (Pharmacia) against gel filtration standards (Bio-Rad). Molecular mass was determined by MALDI-TOF in linear mode using sinapinic acid as a matrix on a Voyager-DE STR mass spectrometer (PerSeptive
Biosystems). Cross-linking experiments were conducted using the BS³ cross linker (bis[sulphosuccinimidy]suberate, Pierce), following the manufacturers instructions.

Kinetic analysis of PEP mutase — The pH optimum of the enzyme was determined over the pH range 5-10 using a mixed buffer system of 0.1 M CHES/0.1 M MES/ 0.1 M HEPPS. Enzyme activity was determined using a continuous spectrophotometric assay at 340 nm and 25°C, in which ATP synthesis is coupled through pyruvate kinase and lactate dehydrogenase to the oxidation of NADH. Each 1 ml assay contained 50 mM (K+) HEPES, pH 8.0, 0.5 mM DTT, 0.2 mM NADH, 1 mM ADP, 5 mM MgSO₄, 16 U of pyruvate kinase and 27 U of lactate dehydrogenase (both enzymes as solutions in 50% glycerol from Roche), 88 nM *T. cruzi* PEP mutase, and 1 mM phosphonopyruvate. The *Kₘ* for phosphonopyruvate was determined using this method by varying phosphonopyruvate concentration in the presence of 5 mM MgSO₄ or 5mM magnesium acetate. Concentrations of phosphonopyruvate were standardised by spectrophotometric assay in the presence of excess PEP mutase. Protein concentration was determined based on the calculated extinction coefficient at 280 nm (34850 M⁻¹ cm⁻¹). This method yields a 1.5-fold higher protein concentration than that determined by the bicinchoninic acid (BCA) method (Pierce). One unit of enzyme activity is defined as 1µmol NADH oxidised per minute. Since Mg²⁺ is required for the coupled assay, the requirement for divalent metal ions was determined using a direct assay following the formation of phosphoenolpyruvate. Each 1-ml assay contained 50 mM (K⁺) HEPES, pH 8.0, 0.5 mM DTT, 450 nM *T. cruzi* recombinant PEP mutase, 1mM phosphonopyruvate and varying amounts of MgSO₄. The production of phosphoenolpyruvate was assayed by the increase in absorbance at 233 nm using a ∆ε = 1.5 mM⁻¹cm⁻¹ (20). Data were fitted by non-linear regression analysis to either the Michaelis-Menten
equation (for phosphonopyruvate) or the Hill equation (for Mg$^{2+}$) using the computer programme GraFit.

*Sub-cellular fractionation* — The following procedures were performed at 4°C. *T. cruzi* epimastigotes were centrifuged and washed twice in STE buffer (0.32 M Sucrose, 25 mM Tris-HCl and 1 mM EDTA, pH 7.8). Cells were mixed with silicon carbide to form a paste and disrupted by grinding with a pestle and mortar (21). Grinding was continued until 90% of the cells were lysed as viewed by phase-contrast microscopy. The suspension was diluted 5-10 fold in STE buffer and briefly centrifuged 3 min at 100 g. The pellet was washed once in STE buffer and centrifuged. The combined supernatants were centrifuged for 10 min at 1000 g to remove nuclei and unbroken cells. The resultant supernatant was then centrifuged at 14,500 g for 10 min. The pellet obtained contains the large granule fraction. The supernatant was spun for 1 h at 139,000 g; the resulting supernatant contains the cytosolic fraction. The pellet was dissolved in STE buffer and contains the small granule or microsomal fraction.

*Production of T. cruzi PEP mutase antibody* — Antiserum was raised in mice against recombinant *T. cruzi* PEP mutase (100 µg). The initial injection was emulsified in complete Freund adjuvant and the second in incomplete Freund adjuvant.

*Immunolocalisation studies* — Mid-log phase epimastigotes were air-dried onto microscope slides and fixed in 4% paraformaldehyde in PBS (0.15 M NaCl/5 mM potassium-phosphate buffer, pH 7.4) for 10 min at room temperature, followed by methanol at -20°C for 2 min. Slides were then incubated in PBS/1% saponin and 1 mg ml$^{-1}$ heat-treated RNase for 30 min followed by blocking in 5% foetal calf serum/PBS for 5 min. The slides were then incubated in anti-*T. cruzi* PEP mutase diluted 1:50 in PBS for 1 hour at room temperature in a dark humid chamber. After washing in PBS, slides were incubated for 1 h in fluorescein isothiocyanate-
conjugated goat anti-mouse secondary antibody diluted 1:50 in PBS. Slides were washed again, incubated in 4,6-diamidino-2-phenylindole (DAPI) (1 µg ml⁻¹) for 2 min followed by a further wash in PBS and mounted in Mowiol containing phenylene diamine (1 µg ml⁻¹).

For double labelling experiments using the anti-GAPDH (rabbit) and anti PEP mutase (mouse) slides were treated as above except the primary antibody was a mixture of 1:100 and 1:50 dilution, respectively, of each antibody in PBS and the secondary was a mixture of anti-mouse FITC and anti-rabbit TRITC.

Double labelling experiments of monoclonal antibody to vacuolar type protein pyrophosphatase (V-H⁺-PPiase) and anti-PEP mutase used Zenon™ One mouse IgG1 labelling kit (Molecular probes) to label anti-V-H⁺-PPiase, as instructed by the manufacturers. Following staining with the anti-V-H⁺-PPiase, slides were stained with anti-PEP mutase as above.

Mitochondrial labelling of T. cruzi was conducted using MitoTracker Red 580 (Molecular Probes) as instructed by the manufacturers. Slides were double labelled with anti-PEP mutase as described above.
RESULTS

Cloning of the T. cruzi PEP mutase gene — A TBLASTN search of the EBI database with the Tetrahymena pyriformis protein sequence identified a T. cruzi genomic survey sequence (AQ445225.2) showing significant homology to PEP mutase. The partial sequence of the T. cruzi PEP mutase gene (PEPM) was amplified by PCR and used as a hybridisation probe for a southern blot analysis of genomic DNA digested with a range of restriction enzymes. The resulting restriction map indicated that PEPM is single-copy per haploid genome (not shown). A size-selected library was constructed with gDNA digested with HindIII/SacI and a 2.5-kb fragment cloned and sequenced. This contained a single full length open reading frame (GenBank/EMBL/DDBJ accession no. AJ414690) which showed significant homology to PEP mutase from other organisms (Fig. 2). The T. cruzi protein showed higher sequence identity to the eukaryotic proteins from M. edulis (65.4%) and Te. pyriformis (62%), than those from prokaryotes Mesorhizobium loti (51.3%) and Streptomyces hygroscopicus (42.7%). Based on crystallographic data for the M. edulis PEP mutase (22) the active site amino acids are conserved in all species including T. cruzi PEP mutase (Trp-47, Ser-49, Asp-61, Asp-88, Asp-90, Glu-117, Lys-123 and Arg-163). The residues forming the oxyanion hole are also conserved in all the PEP mutase homologues (Gly-50 and Leu-51). Furthermore, the Mg$^{2+}$-binding residue is Asp-88, which is also invariant amongst the PEP mutase homologues.

Expression and purification of recombinant T. cruzi PEP mutase — T. cruzi PEPM was sub-cloned into the expression vector pET15b containing an N-terminal His$_6$-tag and expressed in E. coli. The recombinant protein was purified on a nickel-chelating Sepharose High Performance column, digested with thrombin to remove the His$_6$-tag and further purified by
anionic exchange chromatography (Fig. 3A). The final yield of recombinant protein was approximately 3 mg l⁻¹ of culture. MALDI-TOF analysis of recombinant PEP mutase revealed a nominal molecular mass of 33,832 Da that correlates well with the predicted molecular mass of 33,482 Da after cleavage with thrombin. Migration on SDS-PAGE shows an Mr of ~36,800 (Fig. 3A) similar to that reported for *Te. pyriformis* (33,000) (4) and *M. edulis* (34,000) (20). On gel filtration chromatography *T. cruzi* PEP mutase migrates as a single symmetrical peak corresponding to a Mr of 86,000 Da (n = 2.6, mean of 2 experiments), suggestive of either a homodimer (as reported for the *Te. pyriformis* enzyme (23)) or a novel trimeric species. To test this possibility, the native enzyme was cross-linked with BS³ and analysed by SDS-PAGE (Fig 3C). The resulting gel indicates that the majority of the PEP mutase is recovered as a homotetramer with only trace amounts of trimer and dimer. Identical results were obtained when PEP mutase was held constant (1.5 mg ml⁻¹) and cross linker varied (0.25 – 2.0 mM) over 0.5 – 4 h (data not shown). Crystallographic studies on the *T. cruzi* enzyme also reveal a tetrameric arrangement (McNulty, Sarkar, Fairlamb and van Aalten, unpublished results) as reported for the enzyme from *M. edulis* (22). The reason for the anomalous behaviour on gel filtration is not known.

*Kinetic characterisation of recombinant PEP mutase protein* — The pH profile for PEP mutase enzyme activity follows a bell-shaped curve with pH optimum of 8 and apparent pKₐ values of 7.05 ± 0.09 and 9.12 ± 0.09 (data not shown). Under optimal conditions (pH 8.0, 1 mM phosphonopyruvate, 5 mM MgSO₄) the specific activity of the purified recombinant *T. cruzi* enzyme (54 U mg⁻¹) is similar to the enzymes from *M. edulis* (90 U mg⁻¹, (20)) and *Te. pyriformis* (22 U mg⁻¹ (23) or 113 U mg⁻¹, (24)). Using the coupled assay, the enzyme follows Michaelis-Menten kinetics with phosphonopyruvate (Figure 4A) yielding an apparent $K_m$ value
of $7.5 \pm 0.6 \, \mu M$, intermediate between $10 \, \mu M$ and $3.3 \, \mu M$ reported at pH 7.5 for *Te. pyriformis* and *M. edulis*, respectively (20, 25). A similar $K_m$ value ($8.4 \pm 1.1 \, \mu M$) was obtained with $5 \, mM$ magnesium acetate replacing $5 \, mM$ MgSO$_4$ (not shown). The $k_{cat}$ value for the *T. cruzi* enzyme ($12.4 \pm 0.2 \, s^{-1}$ with MgSO$_4$; $11 \pm 0.3 \, s^{-1}$ with magnesium acetate) is somewhat lower than those for *Te. pyriformis* ($150 \, s^{-1}$) and *M. edulis* ($34 \, s^{-1}$).

The requirement for divalent metal ions (1mM, all as chloride salts) was determined by measuring the thermodynamically favoured direction of the reaction, i.e. formation of phosphoenolpyruvate at 233 nm. The enzyme displayed a pronounced requirement for divalent metal ions with $< 1\%$ activity in the absence of Mg$^{2+}$. The extent of activation was identical to that reported for the *M. edulis* enzyme (20), with the order being Mg$^{2+} > $ Co$^{2+} > $ Mn$^{2+} > $ Zn$^{2+} > $ Ni$^{2+}$ with Ca$^{2+}$ showing no activation (not shown). Sulphate and other oxyanions have been reported to be inhibitory with the *Te. pyriformis* enzyme (25). However, with the *T. cruzi* PEP mutase SO$_4^{2-}$ is not inhibitory when compared to Cl$^-$ anion. In kinetic studies where MgSO$_4$ was the variable substrate, double reciprocal plots were non-linear (not shown). However, the data fitted well to the Hill equation yielding an $S_{0.5}$ of $0.40 \pm 0.04 \, \mu M$, $n = 0.46 \pm 0.03$, $k_{cat} 19 \pm 0.4 \, s^{-1}$ consistent with a negative co-operative effect between subunits (Figure 4B). Co-operative behaviour has not been reported with Mg$^{2+}$ for PEP mutase from *M. edulis* ($K_m 4 \, \mu M$) (20) or *Te. pyriformis* ($K_m 6 \, \mu M$) (23).

*Species distribution and intracellular location* — Western analysis using antibodies raised against the *T. cruzi* recombinant protein indicated that PEP mutase is constitutively expressed in the insect epimastigote, trypomastigote and mammalian amastigote stages of the life cycle (data not shown). The antiserum also detects a 34-kDa band in *M. edulis* extracts, but not in more closely related trypanosomatid species suggesting that PEP mutase is absent in *T. brucei*, *C.
fasciculata, L. major and L. donovani (Figure 5A). Two other trypanosomatids, T. dionisii and H. muscarum, that contain AEP-glycolipids also show a band corresponding to T. cruzi PEP mutase (Figure 5A).

Subcellular fractionation studies and immunolabelling experiments indicate that PEP mutase has a dual location in the cell. Western blots of subcellular fractions show that the enzyme is present in the cytosol and large granule fraction, which is enriched in mitochondria, glycosomes and other large vesicular organelles (Fig. 5B). This dual location is unlikely to be a preparative artefact since antiserum to T. cruzi trypanothione synthetase (26) showed a largely cytosolic location.

Immunofluorescence staining shows a diffuse cytosolic staining together with a punctate pattern in the cells (Fig 6). This distribution is reminiscent of glycosomal staining, even though the amino acid sequence does not show a type-I or type-II glycosomal targeting signal (27). Staining with anti-serum to glycosomal GAPDH is also punctate, but examination of the merged image indicates that anti-PEP mutase does not co-localise exactly with the glycosome (Figure 6A). Double labelling with anti-PEP mutase antibody and Mitotraker indicates that PEP mutase is not located to the mitochondrion either (Figure 6B). The presence of glycoconjugates in acidocalcisomes has been reported recently (Salto, M.L. et al (2002) Molecular Parasitology Meeting, Woods Hole, MA, abstract 234A). However, double labelling of slides with anti-PEP mutase and anti-H⁺-PPiase, an acidocalcisome marker, clearly do not co-localise (Figure 6C). These results suggest that PEP mutase is not specifically localised to the glycosome, mitochondria or acidocalcisome, but to some other unidentified organelle.
DISCUSSION

Although AEP was first identified in *T. cruzi* by $^{32}$P-labelling studies (5), the biosynthetic pathway has not been investigated in detail. Our current study has identified a functional PEP mutase in *T. cruzi*, which is expressed in all stages of the life cycle. The kinetic properties closely resemble those of *M. edulis* and *Te. pyriformis*, suggesting that the biosynthetic pathway is similar to other microorganisms (Figure 1). Attempts to identify candidate genes for the subsequent step in the pathway (phosphonopyruvate decarboxylase) in the partially completed *T. cruzi* genome database proved negative. Pyruvate phosphate dikinase, which could catalyse formation of PEP from pyruvate, has been localised to the glycosome (28), but PEP mutase does not perfectly co-localise with this organelle. Furthermore, pyruvate phosphate dikinase must serve additional roles in *T. brucei* since AEP and PEP mutase appear to be absent from this parasite (5). Additional studies are required to resolve the unusual subcellular localisation of this enzyme.

Phosphonolipids constitute 23% of total phospholipids in *Te. pyriformis* and it has been proposed that surface phosphonolipids are important for protection against phospholipases secreted by itself or by other organisms (29). However, this is unlikely to be the case for *T. cruzi* since phosphonolipids constitute a minor fraction (0.34%) of total phospholipids (5). However, the occurrence of PEP mutase in trypanosomatids correlates exactly with those parasites that have been reported to possess AEP-glycolipids, and thus one function of the pathway could be to supply AEP for glycolipid synthesis. Although AEP and ethanolamine phosphate can be used interchangeably to link the GPI-anchor to the polypeptide chain of mucins, the glucosamine moiety is exclusively substituted by AEP (6). Thus, the universality of AEP modification of the 6-O position of glucosamine in GIPLs and mucins suggests an essential role for this moiety in
these surface glycoconjugates. Since they are thought to play a role in attachment, invasion and intracellular survival in the parasite, the AEP moiety may be an important determinant in one or more of these functions. Likewise, GPI-anchors from *T. cruzi*, which show pronounced pro-inflammatory activity, play an important role in activation of innate immunity during infection (7, 8). Conceivably, the AEP-substituent could protect these molecules from degradation in the gut of the insect vector or the cytoplasm of vertebrate host cells. Gene knockout studies are planned to examine these possibilities and to evaluate whether this pathway could represent a therapeutic target since it is absent in the mammalian host.

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FIGURE LEGENDS

**Fig. 1.** Panel A: biosynthesis of aminoethylphosphonate in microorganisms. PEPM (phosphoenolpyruvate mutase) catalyses the initial step in this pathway. Panel B: common glycan core of *T. cruzi* GIPLs and mucins. AEP is present in all structures from all isolates analysed to date.

**Fig. 2.** Sequence alignment of PEP mutases. *T. cruzi* PEP mutase (accession number AJ414690), *M. edulis* (P56839, gi6831593), *Te. pyriformis* (P33182, gi417464). Breaks in alignments are indicated as dashes. Identical amino acid residues are marked with an asterisk and conserved residues with a dot. Active site residues in the *M. edulis* structure are in bold and those forming the oxyanion hole are underlined.

**Fig. 3.** Purification and properties of *T. cruzi* PEP mutase. Panel A: SDS-PAGE. *Lane 1*, soluble fraction of *E. coli* BL21(DE3)pLysS/pET15b-PEPM; *Lane 2*, flow through from nickel-chelating Sepharose High Performance column; *Lane 3*, pooled fractions after imidazole elution; *Lane 4*, pooled fractions from chromatography on Resource-Q, after removal of the His_{6}-tag with thrombin protease. Panel B: Native molecular mass determination by gel filtration chromatography. Open circles represent standards and the closed circle is PEP mutase (M_r 86,000, equivalent to 2.6 monomers). Panel C: Cross linking of recombinant PEP mutase. Samples of PEP mutase were incubated at room temperature for 1 h with 1 mM BS{sup 3} before quenching the reaction with 5 mM ethanolamine. Five micrograms of each sample was analysed by SDS-PAGE. *Lane 1*, PEP mutase (2 mg/ml) without cross linker; *Lanes 2-5*, PEP mutase
(0.25, 0.5, 1 and 2 mg/ml, respectively) plus cross linker; Lane 6, BSA (negative control) plus cross linker; Lane 7, aldolase (positive control) plus cross linker; M, size markers.

**Fig. 4.** Kinetic analysis of *T. cruzi* PEP mutase. Panel A: Lineweaver-Burk plot of rate versus phosphonopyruvate concentration. Panel B: Effect of Mg$^{2+}$ on enzyme activity expressed as a Hill plot. The fits are determined by non-linear regression using GraFit.

**Fig. 5.** Western blot analysis and subcellular distribution of PEP mutase. Panel A: Protein extracts (5 µg) from different trypanosomatids or from *M. edulis* were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane and probed using anti-PEP mutase antibody. Tc, *T. cruzi* epimastigote stage; Tb, *T. brucei* procyclic stage; Ld, *L. donovani* promastigote stage; Lm, *L. major* promastigote stage; Cf, *C. fasciculata*; Me, *M. edulis*; Td, *T. dionisii* epimastigote stage; Hm, *H. muscarum*. Panel B: subcellular distribution of *T. cruzi* PEPM. The large granule (LG), cytosol (C) and microsomal fraction (MF) of *T. cruzi* were prepared as described in the methods. Left panel protein blot probed with anti-serum to *T. cruzi* PEP mutase; right panel probed with antibody to *T. cruzi* trypanothione synthetase.

**Fig 6.** Immunofluorescence staining of *T. cruzi* cells with anti-PEP mutase. (A) Double-stained with antiserum to glycosomal GAPDH (glycosomal marker); (B) double labelling with Mitotracker (mitochondrial marker); (C) double labelling with a monoclonal antibody against *T. cruzi* V-H$^+$-PPiase (acidocalcisome marker). Size bar represents 5 µm.
A

\[
\begin{align*}
\text{Phosphoenolpyruvate (PEP)} & \rightarrow \text{Phosphono pyruvate} \\
& \rightarrow \text{2-Aminoethyl Phosphonate (AEP)} \\
& \rightarrow \text{Phospholipids} \\
& \rightarrow \text{GIPLs} \\
& \rightarrow \text{Mucins}
\end{align*}
\]

B

\[
\text{Man-\(\alpha\)-(1-2)-Man-\(\alpha\)-(1-6)-Man-\(\alpha\)-(1-4)-GlcN-\(\alpha\)-(1-6)-Ins-1-PO}_4
\]

Sarkar et al Figure 1
Sarkar et al Figure 2
\[ \frac{1}{v, \text{s}^{-1}} \]

\[ \frac{1}{\text{[Phosphonopyruvate], } \mu\text{M}^{-1}} \]

A

\[ \frac{v - \text{max}}{N(\text{max})/A} \]

B

Sarkar et al Figure 4
Sarkar et al Figure 5
Sarkar et al Figure 6
Properties of phosphoenolpyruvate mutase, the first enzyme in the
aminoethylphosphonate biosynthetic pathway in trypanosoma cruzi
Mitali Sarkar, Christopher J. Hamilton and Alan H. Fairlamb

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