Plant-like phosphofructokinase from *Plasmodium falciparum* belongs to a novel class of ATP-dependent enzymes

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**A B S T R A C T**

Malaria parasite-infected erythrocytes exhibit enhanced glucose utilisation and 6-phospho-1-fructokinase (PFK) is a key enzyme in glycolysis. Here we present the characterisation of PFK from the human malaria parasite *Plasmodium falciparum*. Of the two putative PFK genes on chromosome 9 (*PfPFK9*) and 11 (*PfPFK11*), only the *PfPFK9* gene appeared to possess all the catalytic features appropriate for PFK activity. The deduced PfPFK proteins contain domains homologous to the plant-like pyrophosphatase (PiP)-dependent PFK β and γ subunits, which are quite different from the human erythrocyte PFK protein. The *PfPFK9* gene β and γ regions were cloned and expressed as His-tagged recombinant enzymes in *Escherichia coli*. Complementation of PFK-deficient *E. coli* and activity analysis of purified recombinant proteins confirmed that *PfPFK9β* possessed catalytic activity. Monoclonal antibodies against the recombinant β protein confirmed that the PfPFK protein has β and γ domains fused into a 200 kDa protein, as opposed to the independent subunits found in plants. Despite an overall structural similarity to plant PPI-PFKs, the recombinant protein and the parasite extract exhibited only ATP-dependent enzyme activity, and none with PPI. Unlike host PFK, the *Plasmodium PFK* was insensitive to fructose-2,6-bisphosphate (F2,6-BP), phosphoenolpyruvate (PEP) and citrate. A comparison of the deduced PFK proteins from several protozoan PFK genome databases implicates a unique class of ATP-dependent PFK present amongst the apicomplexan protozoans.

**1. Introduction**

*Plasmodium falciparum*, the causative agent of severe malaria, is estimated to cause nearly 1 million deaths annually (WHO Report, 2008). It is the intraerythrocytic stages of the parasite that lead to the major clinical manifestations of the disease. During its intraerythrocytic growth phase, the malaria parasite relies mainly on glycolysis for its energy requirements (Lang-Unnasch and Murphy, 1998; Turner and Plaxton, 1990; Mertens, 1993; Alves et al., 2001; Muller et al., 2001). Recently, an additional ATP-PFK enzyme activity, and the gene coding for the same, has been demonstrated in *E. histolytica* (Chi et al., 2001). Thus, in plants and *E. histolytica*, the ATP- and the PPI-PFK activities co-exist.

The plant PPI-PFKs are composed of two individual subunits α and β and are generally present as a hetero-tetramer or hetero-octomer (α2β2 or α4β4), although enzymatically active oligomers with just the β subunit have been postulated in certain plant tissues (Yan and Tao, 1984; Wong et al., 1990; Turner and Plaxton, 2003). It has been reported through gene transcript analysis that different amounts of α and β subunits are present in different tissues and it is speculated that PPI-PFKs are regulated through differential α and β oligomerization (Wong et al., 1990; Suzuki et al., 2003). The plant PPI-PFKs are insensitive to the several allosteric regulators which modulate glycolysis in typical eukaryotic PFKs.

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However, plant PPI-PFKs exhibit allosteric behaviour towards very small concentrations of fructose-2,6-bisphosphate (F-2,6-bp) (Turner and Plaxton, 2003).

There seems to be a paucity of information regarding the structure and activity of the PFK protein amongst the apicomplexan parasites. Amongst Plasmodium species, activity of PFK has been reported only in the murine model of malaria Plasmodium berghei and was shown to be ATP-dependent (Buckwitz et al., 1990). However, activity assays showed a predominant PPI dependence in the coccidian organisms Toxoplasma gondii and Cryptosporidium (Peng and Mansour, 1992; Denton et al., 1996). The Plasmodium databases, PlasmoDB (http://plasmodb.org/plasmo/) (Aurrecoechea et al., 2009) as well as the Malaria Parasite Metabolic Pathways (MPMP) database (Ginsburg, 2006) defining all the genes encoding glycolytic enzymes, annotate two PFK genes, on chromosome 9 (PFPK9) (PlasmoDB gene ID: PF10755c) and 11 (PFPK11) (PlasmoDB gene ID: PF11_0294) as putative 6-phosphofructokinase and ATP-dependent PFK genes, respectively. No experimental information is available on the PFK proteins and activities in P. falciparum. In this paper we report the cloning and expression of the recombinant PFPFK9 domains as well as an analysis of the properties of the protein from the parasite extract. Despite the overall structural similarity with the PPI-dependent PFKs, the P. falciparum PFK protein exhibits ATP-dependent activity. Analysis of the deduced PFK sequences from several protozoan organisms demonstrates a novel class of ATP-dependent PFK present amongst the apicomplexan protozoa, distinct from the ATP-dependent genes of the protozoan kinetoplastids such as Leishmania and Trypanosoma (Michels et al., 1997; López et al., 2002).

2. Materials and methods

2.1. Plasmodium falciparum culture

Parasites were maintained in culture as described earlier (Goswami et al., 1997). Human blood, from healthy adults with 0+ blood group, was collected in acid citrate dextrose as the anticoagulant. After removing the leukocytes, the erythrocytes were washed and resuspended in complete RPMI (RPMI with 0.5% Albumax). Asexual stages of P. falciparum (3D7 strain) were cultured in vitro and maintained at 5% haematocrit in complete RPMI at 37°C in a humidified chamber containing 5% CO2.

2.2. Plasmodium falciparum genomic DNA preparation

pRBCs (~10% parasitemia) were taken and washed with cold PBS. The RBCs were lysed by incubating with 0.05% saponin in PBS at 37°C and the DNA was extracted using standard molecular methods (Sichthierle and Wahlgren, 2008). The DNA was visualised on 0.8% agarose gel to ensure that the preparation was unsheared and free of RNA.

2.3. Database searches and sequence analysis

The PFK sequences of plants (Ricinus communis, Arabidopsis thaliana, Solanum tuberosum, Oryza sativa), human (Homo sapiens), yeast (Saccharomyces cerevisiae), spirochaetes (Borrelia burgdorferi, Treponema pallidum), protozoa (T. gondii, Leishmania donovani, Trypanosoma brucei, Theileria parva, Cryptosporidium parvum, E. histolytica), were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). The sequences and the expression data of the Plasmodium genes were obtained from PlasmoDB (Aurrecoechea et al., 2009). Sequences were aligned and analyzed for similarity using Biology WorkBench (http://seqtool.sdsc.edu/CGI/BW.cgi/) or ClustaW (http://www.ebi.ac.uk/clustalw/).

2.4. Cloning and expression of PFPFK9 constructs in Escherichia coli

The PFKx, PFKβ and PFKβD fragments of 1.93, 1.98 and 1.0 kb, respectively, were amplified by PCR from P. falciparum genomic DNA using primers in which restriction enzyme sites were included to facilitate directional cloning. The primers used were:

(1) PFKxF1 ATGGATACCAAGAGTGG

(2) PFKxF2 GCCGGCATCACGATGATACCAAGAGTGG

(3) PFKxF3 GCCGGCATACGATACCAAGAGTGG

(4) PFKxF4 GCCGGCATACGATACCAAGAGTGG

(5) PFKxF5 GCCGGCATACGATACCAAGAGTGG

The PCR products were cloned in the expression vector pQE30 in the Sac and Sphl (PFKα) and Sphl and Psfl sites (PFKβ) and in the pGEX4T3 vector in the Sac and Notl sites (PFKβD). In both constructs the tags were N-terminal to the PFK protein domains. The constructs were confirmed by sequencing. These constructs were transformed into E. coli strain BL21DE3. Transformed bacterial cultures were grown in Luria–Bertani media supplemented with appropriate antibiotics to $A_{600}$ of ~0.6 at 37°C, and expression was induced with 0.3 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 28°C for 5 h.

2.5. Affinity purification of recombinant PFPFK9 protein domains

For the His tag-tagged proteins, cells from induced cultures were pelleted, resuspended in the His-purification base buffer (20 mM HEPES (pH 7.5), 500 mM NaCl, 20 mM imidazole, 5 mM MgCl2, 20 mM β-mercaptoethanol, 10% glycerol) with 1% Triton X–100 and bacteria protease inhibitors (Pepstatin, Leupeptin and phenylmethanesulphonylfluoride). For the GST-tagged proteins, the cell pellets from induced cultures were resuspended in the GST-purification base buffer (50 mM Tris–Cl (pH 8.0), 1 mM EDTA, 5 mM MgCl2, 150 mM KCl, 1 mM DTT and 25% glycerol) with 1% Triton X–100 and bacteria protease inhibitors. The cells were lysed by sonicating and the cellular debris removed by high speed centrifugation. Briefly, the supernatant, containing the tagged protein was incubated with Ni–NTA agarose beads (Qiagen, Germany) for the His-tagged proteins or Glutathione–agarose beads (Sigma, USA) for GST-tagged proteins at 4°C for 1 h. The beads were washed with the His- or GST-purification base buffer and the bound proteins were eluted with 250 mM imidazole or 5 mM glutathione, respectively.

2.6. Antibodies against PFPFK9

Recombinant purified protein His6-PFPFKβ was used for immunization of animals to raise the monoclonal antibodies. The hybridomas were generated with the service of Bioklon, Chennai, India. Briefly, about 50 μg of purified His6-PFPFKβ emulsified with FCA was administered i.p. into 6 weeks old, female BALB/c mice. After four weekly injections, the mice were immunized monthly for two months. Two sets of fusions were carried out, of which 12 clones showed reactivity to His6-PFPFKβ. Supernatants from these clones were tested for reactivity against the respective proteins using ELISA and the clone ZF4, which reacted with PFPFKβ but not PFPKx, was selected for subcloning.
2.7. Immunoblotting

Protein samples were prepared by boiling in SDS gel loading buffer for 10 min. To prepare Plasmodium samples, the pRBCs from an asynchronous culture were treated with 0.15% saponin in PBS for 10 min at 37 °C to liberate the parasites. The parasite pellet was washed at least three times with PBS until there was no visible trace of haemoglobin in the supernatant. The parasite pellets were lysed in reducing sample buffer (50 mM Tris-pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 10 min. The prepared samples were then run on SDS-PAGE gel of appropriate polyacrylamide concentration and transferred to methanol-activated Polyvinylidene Fluoride (PVDF) membrane (Millipore) in western trans-blot buffer using Trans-Blot Semi Dry Transfer Cell (Bio-Rad, USA). Membranes were blocked with 5% non-fat skim milk powder in PBS for 1 h and then probed with the desired antibodies. Primary antibody binding was detected by appropriate secondary antibodies conjugated to horseradish peroxidase (BD Biosciences, USA). The immunoblots were developed using 3,3′-Diaminobenzidine (Sigma) system or the ECL Plus™ (Amersham) system.

2.8. Immunofluorescence assay (IFA)

IFA was performed with *P. falciparum* (3D7 strain) as described earlier (Singh et al., 2002). The smears were fixed for 5 min using chilled methanol and permeabilized by 15 min of treatment with 0.05% saponin in PBS. Saponin (0.01%) in PBS was used as the washing buffer for the permeabilized IFA. The cells were blocked with 3% BSA in PBS for 1 h, followed by treatment with the monoclonal antibody culture supernatant. Secondary antibodies conjugated to Alexa-Fluor® 488 (Molecular Probes, Invitrogen) were used at 1:2,000 dilution. The smears were treated with DAPI (0.1 μg/ml) and were observed under an Axioplan Zeiss Epifluorescence microscope.

2.9. Enzyme activity assays

The ATP-PFK activity was followed spectrophotometrically by linking it to the oxidation of NADH to NAD⁺ in the presence of an excess of auxiliary enzymes (Beutler, 1984). The assay buffer was composed of 0.1 M Tris–HCl (pH 7.4), 0.5 mM EDTA (pH 8), 1 mM MgCl₂, 90 mM KCl, 1 mM DTT, 0.2 mM NADH, excess of the auxiliary enzymes-aldehyde dehydrogenase (200 μg), triose phosphate isomerase (40 μg), glyceraldehyde 3-phosphate dehydrogenase (80 μg) (Boehringer Mannheim) and 1 mM of one of the substrates-fructose 6-phosphate (F6P) or ATP in a total volume of 0.5 ml. After adding the cell lysate (total protein concentration 50 μg) or recombinant protein (pure protein concentration 4 μg), the decrease in absorbance was observed for 5 min at 340 nm to measure the background rate of conversion of NADH to NAD⁺. The reaction was then started by adding 1 mM of the missing substrate, typically F6P. The decrease in NADH was measured at 340 nm for 5 min with a Lambda 40 UV/Vis Spectrophotometer (Perkin Elmer Instruments). One unit of enzyme activity is defined as the amount of enzyme that would convert 1 μmole of F6P to product in 1 min (Massey and Deal, 1973). The effects of ADP, AMP, phosphoenolpyruvate (PEP), citrate, F-2,6-bP and Mg²⁺ on the enzyme preparations were checked at concentrations of the modulators ranging from 0.05 to 1 mM, with the exception of F-2,6-bP, where concentrations ranged from 1 to 11 μM and Mg²⁺, where 0.5–3 mM were used. The enzyme preparation was incubated with the assay buffer and modulator for 5 min at 37 °C, after which the reaction was started by the addition of 1 mM F6P. The activity was measured over the linear range within 3–5 min. The commercial ATP-PFK from rabbit muscle (Boehringer Mannheim) and also *Bacillus stearothermophilus* (BspFK) (kind gift of Prof. Gregory D. Reinhart, Texas A&M University, USA) were used as the positive controls for enzyme as well as modulator assays. The PPI-PFK activity was assayed as previously described (Van Schaftingen et al., 1982). The commercial PPI-PFK from potato tubers (Sigma) was used as a positive control. For statistical analysis of the results, the means of experimental groups were compared using one-way ANOVA (GraphPad InStat, San Diego, CA), and statistical significance was determined at P < 0.05.

2.10. Complementation of *E. coli* DF1020

*Escherichia coli* DF1020 strain (F-, pro-82, glv44; AS), LAM-, *Apfbd201*, recA56, endA1, (*A[malA-pfK]*200, thi-1, hisdK17) was obtained from the *E. coli* Genetic Stock Center, Yale University, USA. The cells were transformed with pQ3E0, pfKb/pQ3E0 and pfKb/pQ3E0 by the simple and efficient (SEM) method (Inoue et al., 1990). The primary inoculum of the transformants was made in LB broth and the secondary inoculum in M9 medium, each containing 100 μg/ml of ampicillin and supplemented with 0.4% glucose. The culture was induced by the addition of 0.3 mM IPTG at the time of inoculation and growth was monitored for 30 h at 37 °C by recording A600 every 4 h. *Escherichia coli* DF1020 transformed with pQ3E0 served as the negative control. The PFK gene from *B. stearothermophilus* (BspFK) (kind gift of Prof. Gregory D. Reinhart, TAMU, USA) was used as the positive control. The complementation study was similarly performed using *E. coli* RL257 (F-, [araD139], lacIp-4000 [lacF], e14-, *pKb205* [del-ins]: FRT, *flhD5301*, *A[frakK-yeiR]725* [frakA25], relA1, rpsL150 [strR], rbs22, *rPKa203* [del-ins]: FRT, *A[frakK-yeiR]725* [frakA25], relA1, rpsL150 [strR], rbs22) as the control strain.

2.11. Phylogenetic tree construction and analysis

The integrated phylogenetic analysis software, Bosque (Ramirez-Flandes and Ulloa, 2008) was used as the platform for analysis. For phylogenetic tree construction and inference, the PhyML algorithm using WAG as the substitution matrix was used.

3. Results

3.1. Deduced structures of the PfPFK genes

Multiple sequence analysis of the deduced PFK genes from apicomplexan parasite genomes revealed that in each of the coccidian organisms *T. gondii*, *C. parvum* and *Cryptosporidium hominis*, two putative PFK genes are present, while only one putative PFK gene was detected for each of the piroplasmic *T. parva*, *Theileria annulata* and *Babesia bovis*. A comparison between the deduced open reading frames (ORFs) from apicomplexan parasites and representative samples from other species was compiled. Fig. 1 shows the overall sizes and the approximate homologous positions of PFK genes from several organisms with respect to the *T. falciparum* PFK9 gene. *Escherichia coli* possesses the smallest PFK gene, while each of the apicomplexan organisms possesses large ORFs for the putative PFK genes. The apicomplexan single large ORFs contain the conserved domains orthologous to the putative plant PFKα and β gene subunits. Plants possess several paralogues of ATP- and PPI-PFK genes (Bapteste et al., 2003; Mustrof et al., 2007). A representative PPI- gene for each of the β and α subunit from *A. thaliana* is shown in Fig. 1. It has been documented earlier that the protozoan kinetoplastid organisms such as *Trypanosoma* and *Leishmania* possess single genes for PFK (Michels et al., 1997; López et al., 2002). These and the two *E. histolytica* PFK proteins are comparable in size to the β subunit of the plant PPI-PFKs (Deng et al., 2002).
indicate the approximate length of the gaps or extra insert sequences in the apicomplexan PFK sequences, respectively. The two regions of PfPFK9 that were cloned and expressed (pPFPKj and pPFKc) are indicated with double-arrowheads. The ATP-dependent PFK genes are shown for Trypanosoma, human and Escherichia coli. The size (in number of amino acids) of each PFK protein/subunit is shown in parenthesis. Toxoplasma gondii TgPFK: EEA99208, ToxoDB gene ID: 42.m00123; TgPFKb: EEA99567, ToxoDB gene ID: 45.m03242; Cryptosporidium parvum (Cp PFK: XP_626715, Cp PFKa: XP_626418); Theileria parva (TpPFK: EAN3260); Babesia bovis (BbPFK: XP_001610135); P. falciparum (Pf PFK9: CAD51837, PlasmodDB gene ID: PF100555; Pf PFK11: (AAN35878, PlasmodDB gene ID: PF11_0294); Arabidopsis thaliana (AtPFKj: QE7378, AtPFKc: BAF01310); Trypanosoma brucei (Tb: AA21036); Homo sapiens (Hs: Platelet PFK: Q01813); E. coli (Ec: E0A797).

Fig. 1. Schematic showing an orthologous-positional comparison of the deduced phosphofructokinase (PFK) protein structures present in different organisms with respect to the PFK9 protein of Plasmodium falciparum. The apicomplexan orthologues and the similar plant (Arabidopsis) pyrophosphate PFK regions are shown as while the thin lines and indicate the approximate length of the gaps or extra insert sequences in the apicomplexan PFK sequences, respectively. The two regions of PFPK9 that were cloned and expressed (pPFPKj and pPFKc) are indicated with double-arrowheads. The ATP-dependent PFK genes are shown for Trypanosoma, human and Escherichia coli. The size (in number of amino acids) of each PFK protein/subunit is shown in parenthesis. Toxoplasma gondii TgPFK: EEA99208, ToxoDB gene ID: 42.m00123; TgPFKb: EEA99567, ToxoDB gene ID: 45.m03242; Cryptosporidium parvum (Cp PFK: XP_626715, Cp PFKa: XP_626418); Theileria parva (TpPFK: EAN3260); Babesia bovis (BbPFK: XP_001610135); P. falciparum (Pf PFK9: CAD51837, PlasmodDB gene ID: PF100555; Pf PFK11: (AAN35878, PlasmodDB gene ID: PF11_0294); Arabidopsis thaliana (AtPFKj: QE7378, AtPFKc: BAF01310); Trypanosoma brucei (Tb: AA21036); Homo sapiens (Hs: Platelet PFK: Q01813); E. coli (Ec: E0A797).

The human PFK genes are an intermediate size, with the regulatory domain fused to the catalytic one (Kemp and Gunasekera, 2002), and the sequence homology between the apicomplexan genes and the human genes was found to be about 15%. The two special features noticed in the putative apicomplexan genes and the human genes was found to be only about 15%. The human PFK genes are an intermediate size, with the regulatory domain fused to the catalytic one (Kemp and Gunasekera, 2002), and the sequence homology between the apicomplexan genes and the human genes was found to be about 15%. The two special features noticed in the putative apicomplexan genes and the human genes was found to be only about 15%.

The specificity of the PFK protein towards preferential catalysis of PPI versus ATP as the phosphate donor has been assessed earlier and has been assigned mainly to the GGDD/G motif and the K/GT/SIDG/ND motifs (Fig. 2)(Shirakihara and Evans, 1988; Deng et al., 1999; Chi and Kemp, 2000). Through in vitro mutagenesis of the PPI–PFK gene of E. histolytica, it was shown that amino acids D/G at the fourth position of the GGDD/G motif and the starting K/G of the K/GT/SIDG/ND motif determined unambiguously as to whether an overall PPI-PFK protein will exhibit ATP- or PPI-dependent PFK activity (Chi and Kemp, 2000). However, the deciding amino acid appears to be the D/G at the fourth position of the GGDD/G motif, determined unambiguously as to whether an overall PPI-PFK protein will exhibit ATP- or PPI-dependent PFK activity (Chi and Kemp, 2000).

Sequence alignment of the apicomplexan PFK gene orthologues showed that they are closer to the plant PFK (PPi-PFK) genes (Supplementary Fig. S2). A larger collation of protozoan sequences showed that although the kinetoplastid PFK genes are generally compared with the E. histolytica PPI-PFK gene, kinetoplastid PFK genes cluster specifically with the EhPFKII (ATP-dependent) rather than the PPI-dependent EhPFK1 gene (Supplementary Fig. S3). The PfPFK9 gene ORF possesses orthologues in Plasmodium vivax, P. berghei and Plasmodium yoelii. However, a comparison of the important ATP/PPI, fructose 6-phosphate (F6P) and magnesium binding sites required for PFK activity (Carlisle et al., 1990) showed that neither the PfPFK11 gene nor its orthologues from other Plasmodium species contain these conserved features (Supplementary Fig. S3 and Table 1). Thus, it is unlikely that PfPFK11 protein or its orthologues in other Plasmodium species would be able to function as a PFK enzyme.

The specificity of the PFK protein towards preferential catalysis of PPI versus ATP as the phosphate donor has been assessed earlier and has been assigned mainly to the GGDD/ATP/G motif and the K/T/D/G/ND motifs (Fig. 2)(Shirakihara and Evans, 1988; Deng et al., 1999; Chi and Kemp, 2000). Through in vitro mutagenesis of the PPI–PFK gene of E. histolytica, it was shown that amino acids D/G at the fourth position of the GGDD/G motif and the starting K/G of the K/GT/SIDG/ND motif determined unambiguously as to whether an overall PPI-PFK protein will exhibit ATP- or PPI-dependent PFK activity (Chi and Kemp, 2000). However, the deciding amino acid appears to be the D/G at the fourth position of the GGDD/G motif, determined unambiguously as to whether an overall PPI-PFK protein will exhibit ATP- or PPI-dependent PFK activity (Chi and Kemp, 2000).

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3.2. Cloning, expression and characterisation of the PfPFK9 gene domains

Two fragments (1.98 and 1.93 Kbp) of the PfPFK9 gene, homologous to the plant β and the α subunits (Fig. 1), were amplified
Table 1
Comparison of catalytic residues of parasitic phosphofructokinases (PFKs).

<table>
<thead>
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<th>Residue numbers refer to their positions in E. histolytica PFKI.</th>
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<tbody>
<tr>
<td>Entamoeba histolytica PFKI</td>
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<td>Leishmania donovani PFK</td>
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<td>Plasmodium falciparum PFK9</td>
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<td>Plasmodium vivax PFK9 bo</td>
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<td>P. berghei PFK11 bo</td>
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<td>P. chabaudi PFK11 bo</td>
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Length (aa) PPi/ATP Mg²⁺ Fructose-6-phosphate

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<tr>
<th>D</th>
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<th>K</th>
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<td>249</td>
<td>310</td>
<td>420</td>
</tr>
</tbody>
</table>

Residue numbers refer to their positions in E. histolytica PFKI.
Plain text indicates the presence of residues which are identical to those found in E. histolytica pyrophosphate (PP)-PFK (PFK), while the bold text denotes ATP-PFK-specific residues.

NA, complete sequence not available for analysis; aa, amino acid.

and cloned in an His₆-tag expression vector. The recombinant fusion proteins were purified using an Ni-NTA column and observed to be at the expected sizes (75 and 73 kDa) in the SDS–PAGE (Fig. 3A). Monoclonal anti-PFPFKβ antibodies were generated, amongst which 2F4G10 was specific for PFPFK9 and did not cross-react with PFPFKα (Fig. 3A). However, it did recognise the orthologues of sizes 195 and 190 kDa from the rodent malaria parasites P. yoelii and P. berghei, respectively (Fig. 3B). The P. falciparum PFPFK9 appeared to be considerably labile and could not be detected at the expected sizes (75 and 73 kDa) in the SDS–PAGE as a result of the presence of lower protein bands in the immunoblots showed that the Plasmodium PFK proteins were synthesised as a whole and are not cleaved to produce α and β domains (Fig. 3B and C). The observed sizes of 210, 195 and 190 kDa are somewhat larger than the monomeric sizes of 159, 147 and 141 kDa as deduced for P. falciparum, P. yoelii and P. berghei, respectively, from the DNA sequence. Such discrepancies are often found with large proteins that are not as globular as the SDS–PAGE markers.

Results of the immunofluorescence assays of the asexual stages (rings, trophozoites and schizonts) of P. falciparum using anti-PFPFKβ monoclonal antibodies are shown in Fig. 3D. The antibodies showed no cross-reactivity with uRBCs. PFK was localised mainly in the cytoplasm and unlike enolase of P. falciparum (Pal-Bhomerick et al., 2007), no staining was observed in the nucleus. In several stages, especially in the trophozoite stages, punctate staining was observed, suggesting the presence of particulate complexes.

3.3. Assay of enzyme activity of the recombinant and native PFPFKs

Although in plants it is assumed that the β subunit is catalytic and α is regulatory, no cloning and recombinant protein study has been carried out to prove this. We carried out a complementation test, transforming E. coli strain DF1020, a PFK deletion mutant with PFPFKβ and α constructs. The deletion mutant was leaky and even the cells transformed with the vector grew slowly. However, a definitive growth advantage to cells containing PFPFKβ was observed indicating the β domain to be the more active domain (Fig. 4A). A complementation study was also performed with a tighter mutant, E. coli RL257, in which both pfkA and pfkB genes were deleted (Lovingshiner et al., 2006). The transformants showed no growth in minimal media, while the positive control, B. stearothermophilus PFK (BsPFK) showed a normal growth pattern. This suggested that the Plasmodium PFK gene domains are either not appropriately structured to function efficiently in a prokaryotic system or that these domains were not sufficient for the catalytic function. Therefore the PFK enzyme activities of the purified recombinant proteins were tested.

The purified recombinant His-tagged PFPFK β domain showed consistent but low enzyme activity. In order to obtain better folded domains of the protein, the β domain and a smaller jD region were expressed as GST-fusion proteins and purified using glutathione-agarose chromatography (Fig. 4B and C). A comparison between enzyme activities of the purified His- and GST-tagged-recombi-
nant proteins showed a distinct ATP-dependent activity in the β domain (Fig. 4D). The βD domain, containing all the active site residues, showed maximum activity amongst the recombinants, but it also showed the largest variations in different protein preparations. The His6-tagged β-pQE protein showed lower activity but with less variation (Fig. 4D). The His6-tagged α-pQE protein showed the least activity, which was still significant compared with the control purified GST protein. The recombinant PFK proteins were also assayed with PPI as substrate but they did not show any detectable activity.

These results suggest that the PIPFK9β domain is the catalytic subunit which uses ATP as substrate, and that it could function catalytically as an isolated domain. The PFKα domain also exhibited a very small, but distinct, amount of catalytic activity. A comparison of the substrate binding sites showed that the PFKα domain possessed most of the magnesium binding sites, but do not possess some of the canonical phosphate binding sites. In purified PPI-PFK activities in plants there is no unequivocal demonstration of the presence of an active α subunit, but the regulatory subunit α in yeast has been studied extensively (Klinder et al., 1998; Bär et al., 2000). It has been shown that in vivo, cells with single null mutations of the PFK1 (α) and PFK2 (β) genes are both viable and show glycolytic activity (Klinder et al., 1998), indicating that both subunits were capable of catalysis. However purification of just the active regulatory α subunit was very difficult and was possible only through severe denaturation and refolding steps (Bär et al., 2000). This indicated that in isolation, the conformation of the α-subunit is not stably oriented towards catalytic activity.

The activity results demonstrate that in Plasmodium the β domain constitutes the catalytic domain, while the regulatory properties might be conferred on PfPFK9 by the PFKα domain. We were not able to express the entire PfPFK9 in E. coli, nor did we observe any differentially enhanced or modulated activity by mixing the purified recombinant PIPFK9β and PfPFK9α proteins. We therefore assayed both the P. falciparum whole protein extract, containing the fused β and α domains in the native form, as well as the purified recombinant β-GST protein, for their kinetic properties and sensitivity to various modulators (Figs. 5 and 6, Tables 2 and 3).
Similar to the recombinant protein, only ATP-dependent and no PPi-dependent activity could be detected with the parasite preparation. The $K_m$ values for the PFK activity in the parasite extract were found to be 0.02 ± 0.007 mM for ATP and 0.014 ± 0.003 mM for F6P (Table 2). The recombinant β-GST protein also showed similar $K_m$ values for these substrates (Table 2). These values are comparable with those obtained for the P. berghei PFK and exhibit five- to 10-fold higher affinities compared with human RBC PFK values (Layzer, 1975; Buckwitz et al., 1988, 1990). Also, similar to the P. berghei enzyme, the P. falciparum enzyme showed biphasic behaviour towards ATP and was inhibitory at concentrations >1 mM ATP, while with F6P as the substrate, a hyperbolic activity was observed (Fig. 5). Although the β-GST protein showed very similar behaviour with F6P, at high concentrations of ATP the activity was extremely variable from preparation to preparation (data not shown), making it difficult to interpret the β-GST activity at higher ATP concentrations.

The effects of other known allosteric modulators of PFK enzyme, such as ADP, citrate, PEP and F-2,6-bP (Buckwitz et al., 1990; Uyeda, 1979) on native and recombinant PFK activities were studied (Fig. 6 and Table 3). It was observed that activity remained unaffected in the presence of most of these modulators with the exception of ATP and ADP. These were found to be inhibitory at concentrations >1.0 mM for ATP and >0.1 mM for ADP (Figs. 5 and 6). Thus the P. falciparum enzyme definitely exhibited allosteric behaviour for ATP and ADP, although it was insensitive to PEP, citrate and F-2,6-bP. There has been debate as to whether a Krebs cycle operates in Plasmodium species. Although TCA cycle intermediates have not been detected in the RBC stages of Plasmodium, some of the enzymes do exist, and it has been speculated that other stages of Plasmodium may have an operational TCA cycle (Mather and Vaidya, 2008). A recent study on the characterisation of the pyruvate kinase (PyK) enzyme from P. falciparum reported a definitive inhibitory effect of citrate on the PyK enzyme (Chan and Sim, 2005). Therefore we also assessed the effect of citrate on Plasmodium PFK activity. However, unlike PyK, citrate did not show any effect on PPFK activity. We have earlier reported the pH sensitivity of P. falciparum activity (Mehta et al., 2005), and similar to the P. berghei enzyme, it had shown a lower sensitivity to pH changes as opposed to the extreme sensitivity of the RBC enzyme to an acidic environment (Mehta et al., 2005; Buckwitz et al., 1988). Thus the P. falciparum enzyme was insensitive to several PFK modulators and exhibited kinetic properties similar to the partially purified preparation of the enzyme from P. berghei (Buckwitz et al., 1988, 1990). A more extensive kinetic analysis of the native and recombinant proteins will provide further insights into the allosteric nature of the P. falciparum enzyme.

3.4. Evolutionary relationship between protozoan PFKs

PFK is an important element of the glycolytic pathway, which exists in the eukaryotic, prokaryotic and archaeal life forms, and it has therefore been argued that the evolution of this protein ought to be quite constrained. However, studies of the PFK protein and the deduced genes from several organisms show quite the reverse (Müller et al., 2001; Baptiste et al., 2003). The most recent phylogenetic tree shows as many as seven subgroups of PFK (Baptiste et al., 2003). In this analysis, the apicomplexan organisms, certain protozoan organisms and plant genes were placed together in the ‘LONG’ clade. This analysis did not include several apicomplexan PFK genes such as B. bovis, T. gondii, C. hominis, T. parva and T. annulata. With a search through the current genome database for these organisms, we could identify two PFK genes each for T. gondii, C. parvum and C. hominis, while only one each from B. bovis, T. parva and T. annulata were found. A phylogenetic tree with 39 sequences of PFK genes, mainly from the apicomplexan parasites together with some representatives of the other groups is shown in Fig. 7. We have not included several bacteria and archaeabacteria spp. which constitute the groups III, B2 and SHORT clade (Müller et al., 2001; Baptiste et al., 2003).

We found that the ATP-dependent enzymes cluster together in three categories, two of which are canonical eukaryotic (E) and bacteria (B) sequences, and one is the X-category (Müller et al., 2001) of the ATP-dependent protist or plant PFKs. It is the LONG clade that gets clustered into two clades, that of PPI-dependent and the ATP-dependent genes (Fig. 6). The branching pattern is supported by high bootstrap values. It is observed that each of the apicomplexan organisms possesses at least one PFK, which clusters with PPK9 with key features of GGDG175, a sequence that signifies ATP-dependent activity (Chi and Kemp, 2000). Whenever two PFK genes are observed in the genome, these appear to split into PPI- and ATP-dependent structures, e.g. T. gondii, C. parvum and C. hominis. The large sizes of the proteins of the apicomplexan PFKs, PPI- or ATP-dependent proteins, separate them from PFKs from any other organism.

4. Discussion

The results presented in this paper demonstrate that the β domain of the P. falciparum PFK gene present on chromosome 9 codes for an active ATP-dependent PFK activity. The α domain, which shows greater homology with the regulatory γ-subunit of plant PPI-PFKs, showed marginal activity. Unlike the plant PFKs, where
the α and the β domains constitute separate subunits coded by separate genes, in *Plasmodium* species the *PFK9* gene orthologues contain the two domains fused and the entire large protein is expressed in the asexual stages. Plant PFK proteins have been characterised mainly through biochemical purification and no heterologous expression results are available. Very recently seven ATP-PFK proteins of *A. thaliana* have been characterised through heterologous cloning and expression (Mustroph et al., 2007; Winkler et al., 2007). However, no data exists for any plant PPI-PFK subunits to demonstrate unequivocally that the β domain is the catalytic domain. To our knowledge, the *P. falciparum* α and the β domains thus form the first of such PPI-PFK orthologues to be expressed in a heterologous system where we demonstrate that the β domain is indeed the catalytic domain.

Amongst all genomic databases assessed to date, the *PFK* ORFs are much larger in size in apicomplexan organisms. In *Plasmodium* species the existence of a large PFK protein of about 200 kDa is demonstrated in this paper. It has been hypothesised that gene fusion leading to the formation of multi-domain proteins is a major route of protein evolution, as it provides a mechanism for the physical association of different catalytic domains or catalytic and regulatory structures (Yanai et al., 2002). Mammalian PFK is itself an...
example of the catalytic and regulatory domains fused in the same gene, both apparently derived from the bacteria PFK (Kemp and Gunasekera, 2002). In Plasmodium, there have been reports of catalytic and regulatory gene fusions. For instance, the calcium-dependent protein kinase from P. falciparum (PfCPK) is a multi-domain protein composed of an N-terminal kinase domain fused to a C-terminal CaM-like calcium-binding domain (Zhao et al., 1994). While such fusions confer definitive association of the catalytic and regulatory regions, it could compromise the oligomerization regulation of the protein function. In plants, different expression levels of the α and β subunits have been observed in different tissues, indicating a control on the multimeric forms of the PFK protein (Wong et al., 1990; Suzuki et al., 2003).

**Fig. 5.** Dependence of phosphofructokinase activity from Plasmodium falciparum cell extracts on (A) F6P and (B) ATP concentrations. Assays were performed as described in Section 2 at a fixed concentration of 1 mM ATP and F6P in (A and B), respectively.

**Table 2**

<table>
<thead>
<tr>
<th>Organism</th>
<th>references</th>
<th>PO4 donor</th>
<th>K$_{\text{m}}$ ATP/PPi (μM)</th>
<th>K$_{\text{m}}$ F6P (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus stearothermophilus</td>
<td>a</td>
<td>ATP</td>
<td>120</td>
<td>23</td>
</tr>
<tr>
<td>Homo sapiens (RBC)</td>
<td>b</td>
<td>ATP</td>
<td>120</td>
<td>450</td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>c</td>
<td>ATP</td>
<td>8.7</td>
<td>16</td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>d</td>
<td>PPi</td>
<td>17</td>
<td>300</td>
</tr>
<tr>
<td>Entamoeba histolytica</td>
<td>e</td>
<td>PPi</td>
<td>17</td>
<td>40</td>
</tr>
<tr>
<td>Toxoplasma gondii</td>
<td>f</td>
<td>PPi</td>
<td>33</td>
<td>270</td>
</tr>
<tr>
<td>Plasmodium berghei</td>
<td>g</td>
<td>ATP</td>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>Plasmodium falciparum (parasite lysate)</td>
<td>h</td>
<td>ATP</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>Plasmodium falciparum (recombinant β protein)</td>
<td></td>
<td>ATP</td>
<td>25</td>
<td>13</td>
</tr>
</tbody>
</table>

The values in bold letters refer to those determined in the present study.

RBC, red blood cell; PO4, phosphate.

a Zhu et al. (1995).
b Layzer (1975).
c Knowles et al. (1990).
e Bruchhaus et al. (1996).
f Denton et al. (1996).
g Buckwitz et al. (1990).

**Table 3**

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Concentration (mM)</th>
<th>Percent activity$^a$ (standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEP</td>
<td>0.5</td>
<td>119 (28.7)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>123 (21.1)</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.5</td>
<td>69 (12.2)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>99 (28.5)</td>
</tr>
<tr>
<td>F-2,6-b-P</td>
<td>0.5</td>
<td>139 (24.3)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>112 (36.8)</td>
</tr>
<tr>
<td>AMP</td>
<td>0.5</td>
<td>111 (52.5)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>140 (47.6)</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>0.5</td>
<td>265 (79.8)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>498 (38.5)</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>377 (9.4)</td>
</tr>
</tbody>
</table>

$^a$ Activity without modulator is taken as 100%.

Examination of the group II PFK gene structures has been conducted recently with several PFK gene structures available in the NCBI database (Müller et al., 2001; Baptiste et al., 2003). It has been established that both eukaryotic and eubacteria spp. populate this group. The clade X contains the plant and kinetoplastid
Fig. 7. Phylogenetic tree showing phosphofructokinases (PFK) of different types from different organisms. Bootstrap values, based on 100 replicates, are indicated at the branch points. Ath PFPb: PFK\textsubscript{b} Arabidopsis thaliana, NP_172664; Rco PFPb: PFK\textsubscript{b} Ricinus communis (Castor bean), CAA83683; Tg PFKI: Toxoplasma gondii, EAE99208, ToxoDB gene ID: 42.m00123; Cpar PFKII: Cryptosporidium parvum, XP_626715; Chom PFK: Cryptosporidium hominis, EAL37572; Bb PFK: Borrelia burgdorferi, CAI11968; Eh PFK: Entamoeba histolytica, AAC044655; PyPFK\textsubscript{b}ho: Plasmodium yoelii, EAA20618, PlasmodDB gene ID: PY01321; Pb PFK\textsubscript{b}ho: Plasmodium berghei, CAI94255, PlasmodDB gene ID: PB000520.00.0; Pp PFK\textsubscript{b}ho: Plasmodium vivax, XP_001614690, PlasmodDB gene ID: Pp099200; Pf PFK: Plasmodium falciparum, CAD51837, PlasmodDB gene ID: PFI0755c; Tg PFKII: T. gondii, EEA99567, ToxoDB gene ID: 49.m03242; Cpar PFKII: C. parvum, XP_626418; Chom PFKII: C. hominis, EAL35989; Tpar: Theileria parva EAN32860; Bb PFK: Babesia bovis, XP_001610135; Tann: Theileria annulata, CAI74406; Py PFK11ho: P. yoelii, EAA18023, PlasmodDB gene ID: PY05918; Pb PFK11ho: P. berghei, CAI00470; Cpar PFK11: Cryptosporidium parvum, XP_001615400, PlasmodDB gene ID: Pb090200; Hsap P: Homo sapiens (PFK type C); Cfamil: Canis familiaris (Dog), P52784 (PFK, muscle type); Dmela: Drosophila melanogaster, P52034; Aoryz: Aspergillus oryzae, Q9HGZ0; ScPFKb: Saccharomyces cerevisiae, P16862; Dradio: Deinococcus radiodurans, Q9RWN1; Ecoli: Escherichia coli, P0A797 (PFK isozyme 1); Gotearo: Geobacillus stearothermophilus, P00512; Ath PFK: A. thaliana, AAL90928; Otat PFK: Oryza sativa, BA04187; Bb PFKII: B. burgdorferi, AC57070; Eh PFKII: E. histolytica EAL48335; Ldono: Leishmania donovani, AAK31633; Thru: Trypanosoma brucei, AAZ10161; Tmari PFK: Propionibacterium freudenreichii subspecies shermanii, P29495; Atum: Agrobacterium tumefaciens, AAK87863; Tmari PFKI: Thermotoga maritima, AAD35377.
PFKs which are distinctly ATP-dependent and quite different from the PPI-PFK structure. Thus the protozoan PFKs appear to derive both the ATP- and PPI-dependent structures from plants, distinct from other eukaryotic species. In *R. communis* (castor), the organisation of the α and β subunit genes was analyzed and it was concluded that these PPI-dependent PFK genes were not derived from the ATP-PFK gene, but that all the three evolved from a common ancestral gene (Todd et al., 1995). In the case of apicomplexans, it is not clear whether the basic PFK gene was subject to gene duplication and then diverged as the catalytic β and regulatory α domains, or whether the genes diverged first and then fused together to provide the larger protein. The presence of the large fused ORFs in all the apicomplexan organisms suggest that these must have originated from a fused ancestral PFK gene.

Our analysis demonstrates that *Plasmodium* contains only ATP-dependent activity. This fits with the mutational assignment of the ATP- versus PPI-PFK activity through the G175 motif (Chu and Kemp, 2000). The ATP-PFKs of the apicomplexan possess the G175N177A205 structure while those belonging to other protists or *Borrelia* exhibit a G175D177N205 motif (or G175N177L205 in the case of *E. histolytica*) motif (Table 1). All of the ATP-dependent structures showed a D175N177G205 motif except *B. burgdorferi*, which shows a D175N177A205 motif. Of all the apicomplexan G175–PFK gene structures, the *Plasmodium* protein is the only one which demonstrates ATP-dependent activity (Sander et al., 1982; this paper).

The phylogenetic tree (Fig. 7) shows that the apicomplexan PFK proteins seem to belong to a unique class of the Type II PFK proteins. Based on the sequence similarities and the G1/D175 characteristics of the deduced structures, we postulate that the PFKs from the other *Plasmodium* species, as well as *T. gondii*, *Pfi* and *C. parvum* PFKII, would exhibit ATP-dependent activity (Type IIb category). The Type IIb category thus seems to be populated exclusively by apicomplexan organisms, while Type IIa has representations from plants, amoeba, spirochaete *B. burgdorferi* and apicomplexans.

It is observed that in the second PFK gene, *PFKII1* in *P. falciparum*, almost all of the crucial catalytic motifs of this enzyme are missing (Table 1). It is possible that originally, together with other apicomplexan organisms, the two *PFK* genes served the function of PPI- and ATP-dependent activities and later the *PFKII1* gene lost this capability. The transcriptome and proteomic data indicate that this protein is not expressed in the asexual stages, but the mRNA is expressed relatively highly in the gametocytic stages, while the PPi-dependent PFK seems to be expressed at low levels (Aurrecoechea et al., 2009). The presence of homologous gene ORFs of *PFKII1* in other species of *Plasmodium* (percent identity scores of 38–46) indicates a possible function, which seems to pertain specifically to the gametocytes. Through over-expression studies of the *PFKII1* protein in *Plasmodium* asexual stages, it would be interesting to assess whether it plays a dominant negative role towards *PFKII* activity. It would also be of interest to generate reagents specific for PFKII and measure the PFK enzyme activity and the concentrations of each of PFKII and PFKII1 proteins in the sexual and asexual stages of *Plasmodium*.

Currently it appears that all living organisms possess at least one ATP-dependent PFK. The co-existence of PPI- and ATP-PFK occurs in plants, protists and in several bacteria species. Certain bacteria, which use multiple sources of carbon for growth, exhibit a very remarkable regulation of ATP-dependent versus PPI-dependent enzymes based on the carbon source. The immediate induction of ATP-dependent enzyme activity in the presence of glucose (Alves et al., 2001) indicates that in vivo glucose-metabolism is closely connected to ATP, and not PPI, as the phosphate donor for PFK. It is interesting to note that *Plasmodium, Babesia* and *Theileria* are obligatory parasites, which do not exist in a free form. These organisms would have access to glucose as a carbon source throughout their existence, while other protists which may spend some time in the environment as a cyst may not. For such free-living parasites it may be advantageous to possess both the ATP- and PPI-dependent PFKs. This is perhaps why *T. gondii*, *C. parvum* and *C. hominis*, which have a cyst form at some point in their life cycle, possess both of the PFKs, while *Plasmodium, Babesia* and *Theileria* species can make do with only one ATP-dependent PFK activity.

The plant PPI-dependent PFK enzymes catalyze a reversible reaction and operate near equilibrium (Nielsen et al., 2004). The 2,6-bp regulation of the eukaryotic ATP-PFKs is a hallmark of the regulation of glycolysis (Kemp and Gunasekera, 2002) but in plants this regulation is exerted only on the PPI-dependent protein and not on the cytosolic ATP-dependent protein (Nielsen et al., 2004). The enzyme activities in both directions are enhanced by very low (nanomolar) levels of F-2,6-bp and are supposed to regulate the flow of carbon through photosynthetic, catabolic and anabolic phases (Van Praag, 1997). The apicomplexan PPI-dependent enzymes of *T. gondii* and *C. parvum* are not sensitive to F-2,6-bp (Denton et al., 1996). It is hypothesised that the F-2,6-bp binds at the interface between the α and β subunits (Van Praag, 1997; Nielsen et al., 2004). In the cases of *T. gondii* and *C. parvum* the α and β units are fused, just as in *Plasmodium*; hence the interface may be very distinct from the plant heteroerotic structures. A database search failed to identify the fructose-6-phosphate, 2-kinase/fructose-2,6-bisphosphatase (FBPase) gene (responsible for the generation of F-2,6-bp) amongst all apicomplexans. The *Plasmodium* genome also does not seem to contain the gene for FBPase, which catalyzes the reverse reaction of PFK, leading to gluconeogenesis. It appears that glycolysis is a robust energy utilisation pathway in *Plasmodium* and is not subject to fine regulation as long as it receives glucose from its environment. The *T. gondii* database however, shows the presence of FBPase, indicating that the lack of the gene for FBPase in *Plasmodium* is not due to a problem with the search program. The lack of FBPase is consistent with the observation that gluconeogenesis is virtually non-existent in the asexual stages of *P. falciparum*.

The *Plasmodium* PFK protein is homologous to plant PFKs and shows only 15% similarity with human PFK proteins. With such major structural differences from the host PFK protein, the *Plasmodium* PFK protein is a potential drug target. Through heterologous expression, we have demonstrated that PFKK9β region from *P. falciparum* is the functional ATP-dependent catalytic domain of PFK. Elucidation of the structure of this important glycolytic PFK9 protein and a search for selective inhibitors to function as suitable drugs would enable us to better control this disease.

**Acknowledgements**

We thank the *E. coli* Genetic Stock Center, Yale University, USA, for providing us with the *E. coli* DH1020 strain and Prof. Gregory D. Reinhart, Texas A&M University, USA, for the *B. steatorrhophilus* PFK (BsPFK) gene construct and the *E. coli* RL257 strain. We are indebted to Prof. Nirbhay Kumar, Johns Hopkins School of Public Health, Baltimore, USA and Pavanee Neeraja, Tata Institute of Fundamental Research, Mumbai, India for inputs in discussions and experiments. We also wish to acknowledge Bioklone, Chennai, for the great interactive service provided for specific hybridoma selection for PFKβ.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2009.05.011.