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Functional analysis, overexpression, and kinetic characterization of pyruvate kinase from *Plasmodium falciparum*☆

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Abstract

The important role of pyruvate kinase during malarial infection has prompted the cloning of a cDNA encoding *Plasmodium falciparum* pyruvate kinase (pfPyrK), using mRNA from intraerythrocytic-stage malaria parasites. The full-length cDNA encodes a protein with a computed molecular weight of 55.6 kDa and an isoelectric point of 7.5. The purified recombinant pfPyrK is enzymatically active and exists as a homotetramer in its active form. The enzyme exhibits hyperbolic kinetics with respect to phosphoenolpyruvate and ADP, with K_m of 0.19 and 0.12 mM, respectively. pfPyrK is not affected by fructose-1,6-bisphosphate, a general activating factor of pyruvate kinase for most species. Glucose-6-phosphate, an activator of the *Toxoplasma gondii* enzyme, does not affect pfPyrK activity. Similar to rabbit pyruvate kinase, pfPyrK is susceptible to inactivation by 1 mM pyridoxal-5′-phosphate, but to a lesser extent. A screen for inhibitors to pfPyrK revealed that it is markedly inhibited by ATP and citrate. Detailed kinetic analysis revealed a transition from hyperbolic to sigmoidal kinetics for PEP in the presence of citrate, as well as competitive inhibitory behavior for ATP with respect to PEP. Citrate exhibits non-competitive inhibition with respect to ADP with a K_i of 0.8 mM. In conclusion, *P. falciparum* expresses an active pyruvate kinase during the intraerythrocytic-stage of its developmental cycle that may play important metabolic roles during infection.

Keywords: Malaria; *Plasmodium falciparum*; Glycolysis; Citrate; Pyruvate kinase; cDNA cloning

Glycolysis has been recognized as a promising target for new drugs against *Plasmodium falciparum*, because the intraerythrocytic life-cycle of the parasite is highly dependent on glucose for energy [1]. During infection, the parasite utilizes as much as 100 times more glucose than uninfected erythrocytes [2], resulting in the formation of mainly lactate [3]. The levels of several glycolytic enzymes in infected cells are drastically elevated [2], as a result of increase in enzymes which are of parasitic origin [2]. Initial characterization of some glycolytic enzymes of *P. falciparum* have suggested that they may differ biochemically and structurally from their host counterparts [3]. For example, *P. falciparum* lactate dehydrogenase has been characterized in detail [4], and selective inhibitors against the enzyme have demonstrated in vitro antimalarial activities [5]. A functional *P. falciparum* malate dehydrogenase possessing a potential site for specific inhibitor design has also been reported [6]. Hexokinase, phosphofructokinase, and pyruvate kinase are important regulatory enzymes in the glycolytic pathway. In many protozoan parasites (*Trypanosoma brucei*, *Toxoplasma gondii*, and *Leishmania mexicana*), pyruvate kinase is highly regulated [7–9], suggesting that this enzyme may play important regulatory roles in glycolysis in these parasites. In *P. falciparum*, pyruvate kinase activity is detectable in the parasite's intraerythrocytic-stages [2]. Importantly, the enzyme appears to be increased drastically (>11-fold) upon infection [2]. Furthermore, recent studies have

☆ *Abbreviations:* pfPyrK, *Plasmodium falciparum* pyruvate kinase; PEP, phosphoenolpyruvate; PLP, pyridoxal-5′-phosphate.

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indicated that pyruvate kinase of parasite origin may play a role in decreasing the levels of 2,3-diphosphoglycerate in red blood cells [10], thereby contributing to osmotic fragility of host cells and hypoxia during malaria [10]. These observations indicate that *P. falciparum* pyruvate kinase may play a central role in malarial infection and deserves further detailed study.

Pyruvate kinase (EC 2.7.1.40) catalyzes the irreversible substrate level phosphorylation of ADP at the expense of phosphoenolpyruvate (PEP), yielding pyruvate and ATP. Almost all pyruvate kinases are homotetrameric enzymes. Among protozoans, the pyruvate kinases from *T. brucei*, *T. gondii*, *L. mexicana*, and *Entamoeba histolytica* have been characterized in detail [7–9,11]. Although pyruvate kinases of most species are activated by fructose-1,6-bisphosphate (F16BP), the *T. gondii* enzyme is insensitive to F16BP but markedly activated by glucose-6-phosphate (G6P) instead. The kinetics of *L. mexicana* and *T. brucei* pyruvate kinases are quite similar, both being activated predominantly by F26BP. *E. histolytica* pyruvate kinase is activated by F16BP but inhibited by citrate, malate, phosphate, and α-ketoglutarate. Although pyruvate kinase activity has been identified in *P. falciparum* [2,12] and *Plasmodium berghei* [13] infected red cells, little is known about their characteristics. We hence started our experiments by obtaining the cDNA of a predicted pyruvate kinase gene in the *P. falciparum* genome and determining whether the predicted gene encodes a functional pyruvate kinase enzyme. In this paper, the molecular cloning of a cDNA encoding *P. falciparum* pyruvate kinase (pfPyrK), overexpression of the active recombinant enzyme in *Escherichia coli*, and the detailed kinetic properties of the enzyme are reported.

Materials and methods

Cultivation conditions, RT-PCR, and cDNA cloning. The source of *P. falciparum* Tan strain was from the National University Hospital (Singapore). The parasites were cultured in vitro using treated human red blood cells and serum according to the method of Trager and Jensen [14]. Total *P. falciparum* RNA was extracted from a population of parasites exhibiting mixed-stages (ring, trophozoite, and schizont forms) using Rneasy Mini kit (Qiagen) according to manufacturer's instructions. The mRNA used in RT-PCR was purified from total parasite RNA using the Oligotex Mini kit (Qiagen) which employs an oligo(dT) matrix principle of purification. RT-PCR was performed using Sensiscript two-step RT-PCR (Qiagen) and a RT step at 37 °C for 1 h, followed by a PCR step using *pfu* polymerase (Strategene) and standard thermal cycling conditions. The forward and reverse primers for PCR contain the sequences 5′aaggatccagttcatttaagtacaaac3′ and 5′ttgtcgacttattcaatttgtacaa3′, respectively. The *Eco*RI and *Sal*I restriction sites incorporated are underlined. The amplified gene products were subjected to agarose gel electrophoresis using 0.8% agarose gels.

Cloning and heterologous expression. The target DNA was cloned into pCR-BluntII-TOPO (Invitrogen) according to manufacturer's instructions. DNA inserts in positive recombinant clones obtained were sequenced using the ABI PRISM BigDye terminator cycle sequencing kit (Applied Biosystems) and loaded onto an ABI PRISM 377 DNA sequencer for sequence determination. Finally, the target genes were excised from the cloning vector by appropriate double-digestion and subcloned into pGEX-6P1 for expression as a glutathione-*S*-transferase (GST)-tagged fusion protein. Cells for expression analysis were harvested after overnight induction of expression at 25 °C with 1 mM IPTG. Total protein was obtained by sonication using a Soniprep (Sanyo) sonicator set at an amplitude of 18 μm. Recombinant GST-fusion protein was purified by column glutathione elution method using the GST Purification Module (Amersham) according to manufacturer's instructions. SDS–PAGE was performed using a Hoefer Mighty Small (Amersham) electrophoresis system.

Biochemical and kinetic assays. The enzyme assay for pyruvate kinase was based on the formation of NAD in the presence of lactate dehydrogenase in an indirect assay [15]. The standard enzyme assay was adapted for kinetic measurement using a monochromator-based spectrophotometric plate-reader (Safire, Tecan Instruments, Austria). Two hundred microliters of the standard assay mixture contained 10 mM Hepes buffer, pH 7.5, 10 mM $MgCl_2$ (Merck), 50 mM KCl (Merck), 0.5 mM NADH, 1 mM PEP, and 1 mM ADP and 22 U lactate dehydrogenase. Each assay was carried out with a final concentration of 1.5 ng of enzyme/ml of assay mixture. Kinetic parameters for PEP under saturating conditions for ADP (3 mM) were carried out using PEP concentrations ranging from 0.1 to 2 mM. Those for ADP were carried out using ADP concentrations ranging from 0.1 to 2 mM ADP in the presence of 7 mM PEP (saturation). The reaction was started by the addition of pure enzyme and monitored at a wavelength of 340 nm at room temperature (26 °C) for up to 4 min. Enzyme activity was calculated by measurement of slope (A_{340}/min) under lineal time conditions. One unit (U) of pyruvate kinase activity is defined as the amount of enzyme required to produce 1 μmol of pyruvate from PEP in 1 min. Protein was quantified using Bradford assay reagent (Bio-Rad) according to manufacturer's instructions. Analytical gel filtration was carried out using a Superdex 200 HR 10/30 column run on the AKTA Purifier liquid chromatography system (Amersham Biosciences).

Results

cDNA cloning and sequence analysis of P. falciparum pyruvate kinase

As the putative *P. falciparum* pyruvate kinase gene deposited in the *P. falciparum* genome resource (PlasmoDB) [16] was predicted to contain two introns, the cDNA encoding pfPyrK was cloned by RT-PCR using mRNA purified from an in vitro culture (Fig. 1). Full DNA sequencing of the cDNA demonstrated a 1536 bp ORF encoding pfPyrK and confirmed the absence of introns in the cloned cDNA. The obtained pfPyrK ORF is identical in DNA sequence to that of strain 3D7 deposited in the PlasmoDB (Accession No. MAL6P1.160). BLAST analysis of the pfPyrK DNA sequence against *P. falciparum* genome sequence indicated that the pyruvate kinase gene exists as a single copy in *P. falciparum*. The ORF encodes a protein with a computed molecular mass of 55.6 kDa and an isoelectric point of 7.5, similar to those from other parasites such as *L. mexicana* (molecular mass of 54.3 kDa and isoelectric point of 6.5), and *T. gondii* (molecular mass of 57.5 kDa).

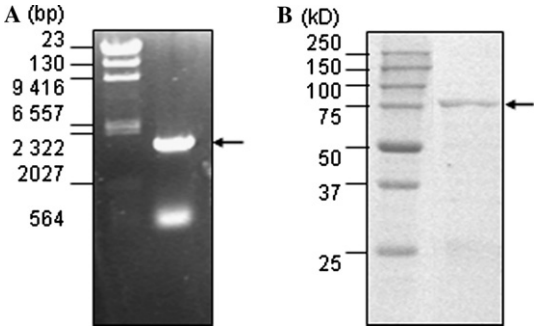

Fig. 1. RT-PCR amplification of cDNA encoding pfPyrK (A) and SDS-PAGE analysis of purified recombinant pfPyrK (B). The positive bands representing pfPyrK cDNA and protein are indicated by arrows.

In terms of amino acid sequence, pfPyrK exhibits the highest identity to pyruvate kinase of *Plasmodium yoelii* (88%) followed by that of *T. gondii* (64%). Interestingly, pfPyrK is almost as distant from human pyruvate kinases (42%) as from plant pyruvate kinases (46–47%). There are four domains in pyruvate kinase: N (a variable N-terminus), A, B, and C [17]. The active site is located in the A and B domains, whereas the C domain, and a cleft between the A and C domains, appear to contain the binding sites for effectors [17]. Multiple-alignment of amino acid sequences of protozoan pyruvate kinases with those of rabbit and human enzymes (Fig. 2) revealed that known binding sites for ADP, PEP, and cation, located in N, A, and B domains, are well conserved (76–100%); whereas sites for effector binding, located in domain C, are not conserved. In this dataset, the A domain is significantly more well conserved (43%) than the B and C domains (17% and 9%, respectively). This observation is in agreement with that on pyruvate kinases from a wide variety of organisms [18]. Phylogenetic analysis showed a wide divergence between pfPyrK and the isozymes in human (Fig. 3). These data, together with the observation of a low sequence identity between these genes, support the use of this enzyme as a drug target. In addition, some deletions in an effector binding site in the C domain which are unique to pyruvate kinases from *P. falciparum*, *P. yoelii*, *T. gondii*, and *Eimeria tenella* were observed (Fig. 2). This site could be a target for selective inhibitors to be designed or for new drugs that act against parasitic pyruvate kinases.

Kinetic characteristics of overexpressed pfPyrK

pfPyrK is expressed at high levels in *E. coli* as a GST-tagged protein. SDS–PAGE analysis of pfPyrK purified by affinity chromatography from lysates of *E. coli* expressing the recombinant protein showed that the molecular weight of the expressed protein is approximately 80 kDa (Fig. 1), in good agreement with the expected molecular weight for pfPyrK incorporated with a 26 kDa GST moiety. The purified pfPyrK is enzymatically active, indicating that the ORF encodes an authentic pyruvate kinase. As expected, no absorbance change was detectable from negative controls comprising of GST expressed from non-insert vector. Control assays in which one or the other co-substrate was omitted confirmed that there are no contaminating NADH consuming activities in the enzyme preparations. No net absorbance change was detected in these negative controls (<0.00 A_{340} nm/min). In order to determine the oligomerization state of pfPyrK, the purified enzyme was subjected to analytical gel filtration. The recombinant pfPyrK eluted from the column with an apparent molecular weight of approximately 316 kDa, as estimated using standard proteins of known molecular weights (Fig. 4). These data suggest that recombinant pfPyrK exists as a homotetramer. As expected, SDS–PAGE analysis of the eluted fractions containing pfPyrK recovered a single polypeptide with the expected molecular weight for pfPyrK. The eluted fractions corresponding to the tetrameric form of pfPyrK are enzymatically active (Fig. 4). Hence, like almost all pyruvate kinases from other organisms including those from *T. gondii* and *T. brucei*, active pfPyrK exists as a homotetramer. The specific activity of pfPyrK was 126 ± 4 U/mg protein at room temperature (26 °C) under optimal substrate concentrations in the presence of magnesium as the divalent cation. In terms of yield, 0.45–0.6 mg of recombinant pfPyrK could be reproducibly obtained from each fermentation batch comprising of 50 ml of *E. coli* culture. In separate experiments, enzymatic analysis of recombinant pfPyrK after proteolytic removal of the GST affinity-tag indicated that untagged pfPyrK is also enzymatically active with comparable specific activity (not shown). Hence, the presence of the GST-tag does not seem to influence the enzymatic activity or the quaternary configuration of the enzyme. However, because the recovery of pure untagged proteins after proteolytic cleavage was typically low, pure GST-tagged pfPyrK enzyme was used for further biochemical analyses.

The kinetic behaviors of pfPyrK for PEP and ADP were investigated (Fig. 5) and the obtained parameters are summarized in Table 1. Among protist pyruvate kinases, *L. mexicana* exhibits sigmoidal kinetics for both substrates [9], while for *T. gondii* kinetic behavior is sigmoidal for PEP but hyperbolic for ADP [7]. In terms of kinetics, pfPyrK resembles *E. histolytica* pyruvate kinase in that it exhibits hyperbolic kinetics with respect to both substrates under saturating conditions [11]. Although F16BP is an activator for many pyruvate kinases including those of *E. histolytica*, *T. gondii* pyruvate kinase is not sensitive to F16BP but instead is activated by G6P. In the case of *T. gondii* pyruvate kinase, the presence of G6P (1 mM) led to 16-fold

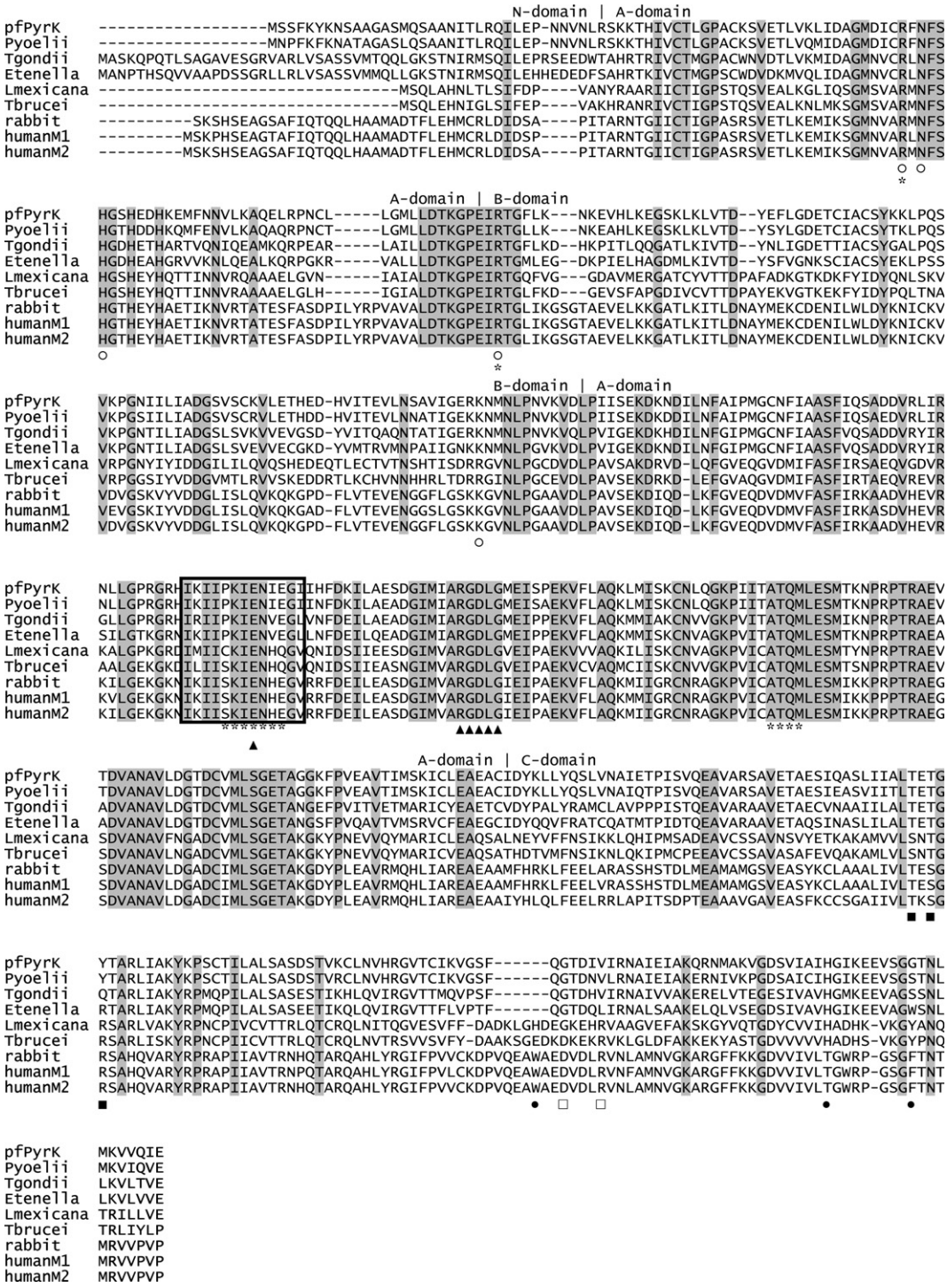

Fig. 2. Amino acid sequence alignment of *P. falciparum* PyrK with pyruvate kinases from other species. Accession Nos. are: pfPyrK, *P. falciparum* (this study; MAL6P1.160); Pyoelii, *P. yoelii* (EAA16536); Tgondii, *T. gondii* (BAB47171); Etenella, *E. tenella* (AF043910); Tbrucei, *T. brucei* (X17618); Lmexicana, *L. mexicana* (X74944); rabbit, rabbit muscle pyruvate kinase (15987970); humanM1, human muscle isozyme 1 (S64635); and humanM2, human muscle isozyme 2 (O18919). The 3D-domains (N/A/B/C) are indicated by vertical lines. A box indicates pyruvate kinase signature sequences. Open circles indicate ADP binding sites. The binding sites of the sugar, 1-phosphate, and 6-phosphate moieties of F16BP are indicated by closed circles, open squares, and closed squares, respectively. Asterisks and triangles indicate the PEP and divalent-cation binding-sites, respectively.

decrease in the K_m of PEP under non-saturation conditions [7]. Interestingly, pfPyrK did not respond significantly to either F16BP (1 mM) or G6P (1 mM) over a wide range of PEP and ADP concentrations, under both saturating as well as non-saturating conditions (Figs. 5A and C; Table 1). Pyridoxal-5′-phosphate (PLP) is a

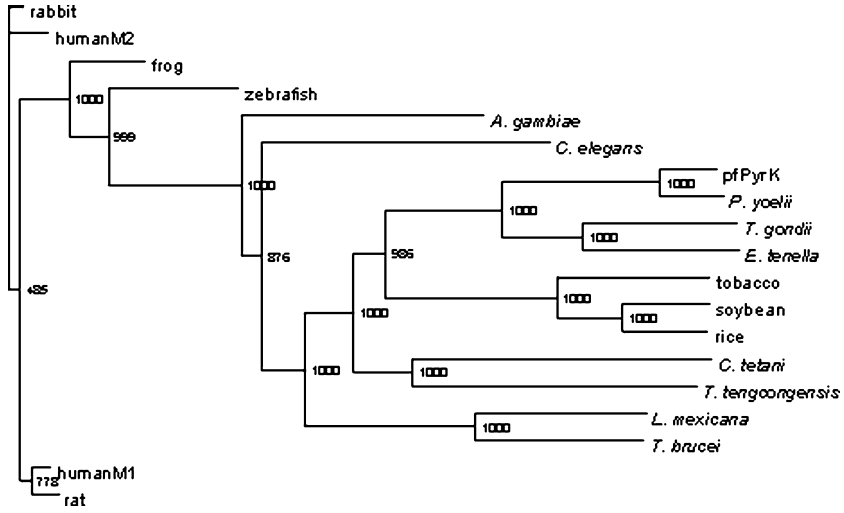

Fig. 3. Phylogenetic (neighbor-joining) tree constructed using pyruvate kinase amino acid sequences. The accession numbers, in addition to those indicated in Fig. 2, are as follows: soybean (AAM94349), rice (CAE05765), tobacco (CAA82628), zebrafish (NP_955365), *Caenorhabditis elegans* (CAA93424), *Anopheles gambiae* (EAA10555), frog (AAH44007), rat (NP_445749), *Clostridium tetani* (NP_783015), and *Thermoanaerobacter tengcongensis* (NP_623403).

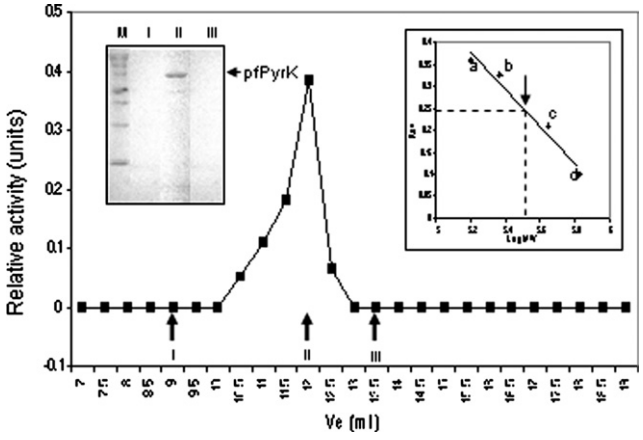

Fig. 4. Determination of oligomerization state of pfPyrK by gel filtration. Enzyme activity of eluted fractions (■) corresponds to elution of pfPyrK as a tetramer. Inset: calibration curve for determining the oligomerization state of recombinant PyrK. The column is calibrated using protein standards: (a) aldolase, (b) catalase, (c) ferritin, and (d) thyroglobulin. Elution fractions indicated were analyzed by SDS–PAGE electrophoresis and the results confirm the presence of pfPyrK in the enzymatically active fraction. M, molecular weight marker.

known inhibitor of rabbit muscle pyruvate kinase [19]. In an earlier study on rabbit muscle pyruvate kinase, treatment by PLP inactivates the enzyme by 90%. In order to assess the sensitivity of pfPyrK to inactivation by PLP, enzyme inactivation study was carried out together with rabbit muscle pyruvate kinase for comparison. For these experiments, the test enzymes were incubated with PLP at the indicated concentrations (Fig. 5F) for 15 min before they were employed in the enzyme assays. It was observed that pfPyrK was also sensitive to PLP. However, as compared to rabbit muscle pyruvate kinase, which could be inactivated up to 75% in this study, pfPyrK was less disposed to inactivation, being inactivated up to 22% maximally.

As there are almost no known inhibitors for pfPyrK, the effect of a wide array of metabolites was screened for their effect on pfPyrK activity (Fig. 6). Interestingly, among the compounds tested, 2 mM citrate was found to inhibit pfPyrK markedly (>97%) in a standard enzyme assay. The inhibition appears to be specific to citrate, and does not apply to all carboxylic acids, because the other carboxylic acids (α-ketoglutaric acid and isocitric acid) tested at identical concentrations did not show inhibitory effects. In addition, the enzyme was 64% inhibited by 2 mM ATP. Aspartate, the end-product of the PEP carboxykinase–aspartate aminotransferase pathway (which competes with pfPyrK for PEP) did not appear to regulate pfPyrK. Kinetic analysis in the presence of inhibitors (ATP and citrate) was subsequently carried out to ascertain the nature of inhibition with respect to each co-substrate under saturating conditions (Fig. 5 and Table 1). When present at a concentration of 1 mM, citrate appears to be a competitive inhibitor of PEP. Interestingly, results revealed a transition from hyperbolic to sigmoidal kinetics for PEP in the presence of 1.5 and 2 mM citrate, with Hill coefficients of 1.9 and 2.4, respectively (Fig. 5E). Significant competitive inhibitory behavior was observed for 2 and 2.5 mM ATP with respect to PEP, under saturating ADP concentrations (Fig. 5B). In addition, at concentrations of 2.5–3.5 mM, citrate appears to demonstrate non-competitive inhibition with respect to ADP under saturating PEP concentrations (Fig. 5D). The use of a Dixon plot at ADP concentrations of 0.2, 0.4, and 1.5 mM (not shown) indicated a K_i of 0.8 mM for citrate with respect to ADP.

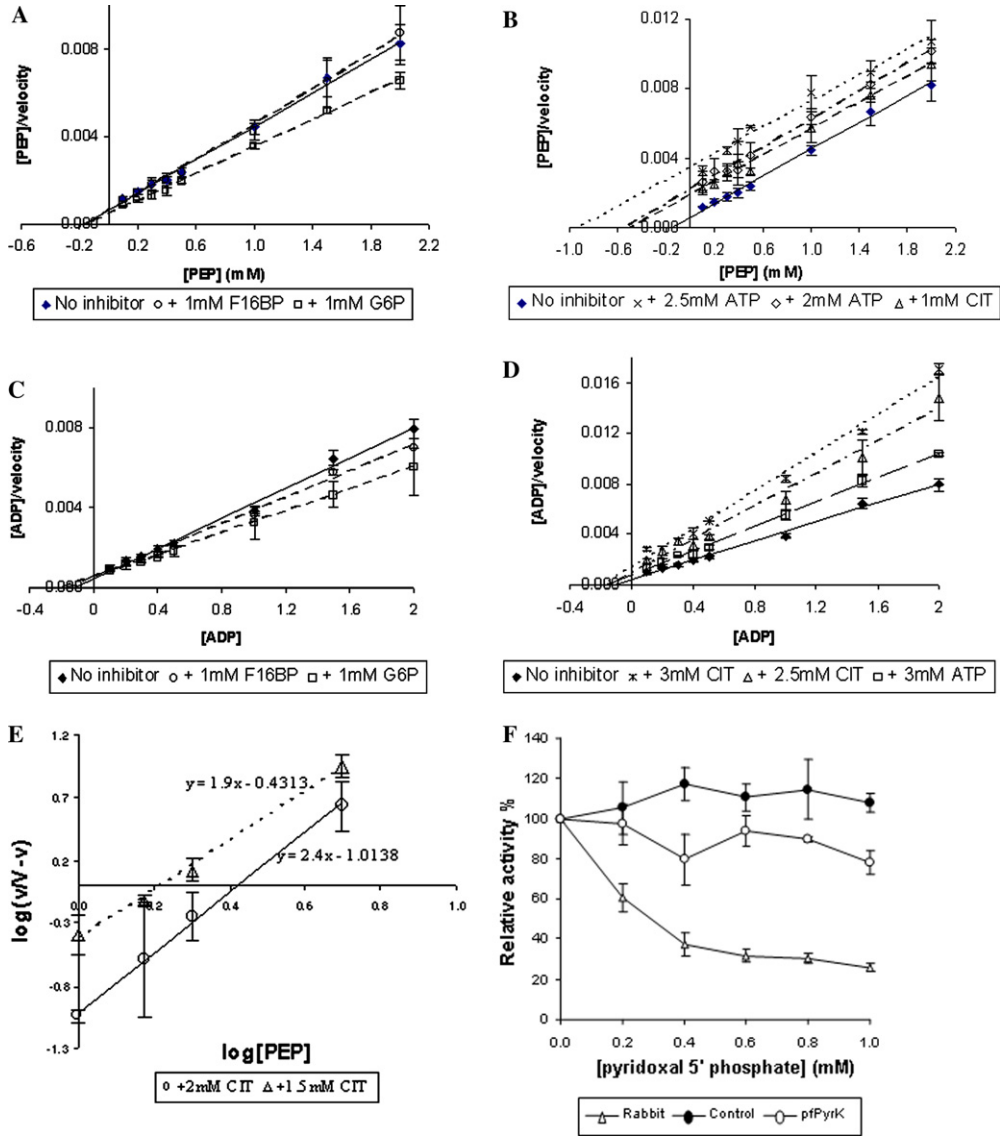

Fig. 5. Kinetic properties of pfPyrK. (A) Hanes plot using varying concentrations of PEP in the absence of effectors (closed lozenges ♦), or in the presence of 1 mM glucose-6-phosphate (open squares □), or 1 mM fructose-1,6-bisphosphate (open circles ○); (B) Hanes plot using varying concentrations of PEP in the absence of effectors (closed lozenges ♦), or 2.5 mM ATP (crosses ×), 2 mM ATP (open lozenges ◊), or 1 mM citrate (open triangles △); (C) Hanes plot using varying concentrations of ADP in the absence of effectors (closed lozenges ♦), or in the presence of 1 mM glucose-6-phosphate (open squares □), 1 mM fructose-1,6-bisphosphate (open circles ○); (D) Hanes plot using varying concentrations of ADP in the absence of effectors (closed lozenges ♦), or in the presence of 3 mM citrate (asterisks ∗), 2.5 mM citrate (open triangles △), or 3 mM ATP (open squares □); (E) Hill plot of $\log(v/V - v)$ vs log[PEP] in the presence of citrate (1.5 or 2 mM); (F) effect of enzyme inactivation by various concentrations of pyridoxal 5′ phosphate. Closed circles (●) indicate control experiments to confirm that pyridoxal phosphate has no effect on lactate dehydrogenase employed in the coupled enzyme assay for pyruvate kinase. Experiments using pfPyrK and rabbit muscle pyruvate kinase are indicated by open circles (○) and open triangles (△), respectively. Standard deviations from at least two independent experiments are shown.

Discussion

Because of the crucial role of pyruvate kinase in the metabolism of parasitic organisms, it has been indicated as a potential drug target against *Trypanosomes* and *Leishmania* [8,9]. The recombinant *T. brucei* pyruvate kinase is currently being crystallized as a step towards its use in designing selective inhibitors for development of anti-trypanosomal drugs [8]. Although the response of parasitic pyruvate kinase to phosphorylated sugars appears to be unusual in many parasitic organisms, relatively little is known about the pyruvate kinase from *Plasmodium*. Interestingly, it has been reported that in a mouse model, pyruvate kinase deficiency of host cells appears to protect against malaria, suggesting the importance of this pathway for the parasite's survival [19], and that pyruvate kinase may be considered as a potential target for the design of new drugs against malaria. Observations indicating the potential importance of pyruvate kinase in malaria [2,3,10,20] prompted us

Table 1
Enzyme kinetic parameters of pfPyrK

Substrate	$K_m \pm$ SD (mM)	$V_{max} \pm$ SD (U/mg)	n_H
Saturating conditions			
PEP (0.1–2 mM)			
No effector	0.190 ± 0.028 (100%)	276 ± 28 (100%)	
+F16P (1 mM)	0.214 ± 0.004 (112%)	291 ± 26 (105%)	
+G6P (1 mM)	0.143 ± 0.030 (75%)	326 ± 15 (118%)	
+citrate (1 mM)	0.507 ± 0.069 (266%)	275 ± 28 (99%)	
+citrate (1.5 mM)			1.9
+citrate (2 mM)			2.4
+ATP (2 mM)	0.497 ± 0.074 (261%)	246 ± 17 (89%)	
+ATP (2.5 mM)	1.205 ± 0.285 (634%)	283 ± 5 (102%)	
+ATP (3 mM)			1.2
ADP (0.1–2 mM)			
No effector	0.126 ± 0.043 (100%)	258 ± 15 (100%)	
+F16P (1 mM)	0.163 ± 0.027 (129%)	300 ± 3 (116%)	
+G6P (1 mM)	0.204 ± 0.085 (161%)	375 ± 93 (145%)	
+citrate (2.5 mM)	0.149 ± 0.060 (118%)	154 ± 18 (60%)	
+citrate (3 mM)	0.154 ± 0.002 (122%)	136 ± 2 (52%)	
+citrate (3.5 mM)	0.143 ± 0.057 (113%)	38 ± 8 (14%)	
+ATP (3 mM)	0.151 ± 0.060 (119%)	207 ± 12 (80%)	
Non-saturating conditions			
PEP (0.1–2 mM)			
No effector	0.337 ± 0.031 (100%)	96.8 ± 9.4 (100%)	
+F16BP (1 mM)	0.307 ± 0.070 (91%)	86.8 ± 4.7 (90%)	
+G6P (1 mM)	0.392 ± 0.076 (116%)	121.6 ± 12.3 (126%)	
ADP (0.1–2 mM)			
No effector	0.149 ± 0.022		

Kinetic analysis of PEP and ADP (saturating conditions) was carried out using co-substrate concentrations of 3 mM ADP and 7 mM PEP, respectively. Kinetic analysis of PEP and ADP (non-saturating conditions) was carried out in the presence of 1 mM ADP and PEP, respectively.

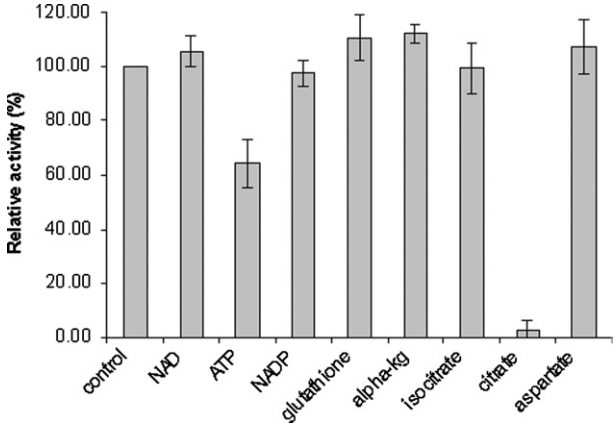

Fig. 6. Effect of various effector compounds on pfPyrK activity. All compounds were tested at a final concentration of 2 mM in the assay system. Relative activities were expressed in percentage with respect to the control reaction in which no effector was included.

to uncover the gene encoding for pfPyrK and to produce active recombinant pfPyrK for detailed characterization and comparison with its counterparts in other parasites, namely *T. brucei*, *L. mexicana*, and *T. gondii*. RT-PCR using mRNA extracted from *P. falciparum* infected cells indicated that pfPyrK is expressed during the intraerythrocytic-stage. DNA sequencing of the cloned pfPyrK cDNA validated the positions of introns predicted in PlasmoDB. With few exceptions, pyruvate kinases have been reported to be homotetramers. Our results also indicated that active recombinant pfPyrK exists as a homotetramer. A BLAST search against the complete *P. falciparum* genome sequence data (PlasmoDB) using pfPyrK amino acid sequence did not uncover any other ORF with significant similarity to pyruvate kinases. Hence, pyruvate kinase gene cloned in this study appears to exist as a single copy in the *P. falciparum* genome.

The affinity of pfPyrK for ADP and PEP appears to be higher than those of the corresponding enzymes from mammalian enzymes (K_m for ADP is 0.35 mM for cow and pig pyruvate kinases; K_m for PEP is 0.57 mM for rat pyruvate kinases). The affinity for PEP is also higher than that of pyruvate kinase from *T. gondii* ($K_m = 1$ mM). Similar to pyruvate kinases from *T. gondii*, rabbit muscle pyruvate kinase, and human M1 isozyme, pfPyrK is not activated by F16BP, the common activator of most pyruvate kinases [7]. In pyruvate kinases, the lysine at position 418 is thought to be involved in binding the 6-phosphate moiety of F16BP. However, similar to non-allosteric enzymes, pfPyrK has a glutamate at residue 418. In contrast, lysines are observed at position 418 in pyruvate kinases activated by F16BP,

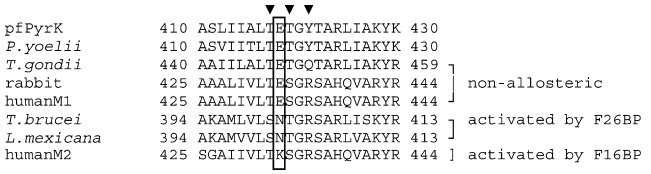

Fig. 7. Alignment of the binding site of the 6-phosphate moiety of F16BP within the C-domain. Box indicates the essential amino acids for binding within the binding site (indicated by arrows).

such as the human muscle isozyme 2 (Fig. 7). Hence, the observed properties of pfPyrK agree with amino acid sequence data. Unlike *T. gondii* pyruvate kinase, pfPyrK is not significantly activated by G6P. Hence, activation by G6P may not be a universal property related to parasitism as proposed earlier [7]. Interestingly, pfPyrK differs from *T. gondii* at three positions (K37, H40, and I473) identified by Ernest et al. [8] to be important in effector binding. These differences may account for the distinction in response to G6P between pfPyrK and *T. gondii* pyruvate kinase. The observation of sensitivity of pfPyrK to citrate, PLP, and ATP is interesting because no inhibitors have been described for pfPyrK so far. ATP, citrate, α-ketoglutarate, and a number of organic acids were known to inhibit *E. histolytica* pyruvate kinase [11]. However, it is interesting to note that while the *E. histolyica* enzyme is inhibited by 21 mM citrate and 6 mM ATP [10], pfPyrK is strongly inhibited at 2–2.5 mM citrate or ATP (Fig. 5), and is almost completely inhibited at 4 mM of these inhibitors (not shown). Also, inhibition of *E. histolyica* pyruvate kinase by citrate is not accompanied by a transition from hyperbolic to sigmoidal kinetics observed in pfPyrK. The binding sites for citrate are as yet unknown but have been suggested to be separate from those for phosphorylated sugars [11]. Nevertheless, the observation of distinct mechanisms of inhibition by citrate and ATP suggests independent binding sites for these inhibitors. Also, the physiological significance of inactivation by PLP has yet to be determined because the status of PLP in *P. falciparum* is unknown. Nevertheless, the difference in the susceptibility to PLP between pfPyrK and rabbit muscle pyruvate kinase may reflect structural differences in accessibility of binding sites to PLP.

In summary, the results in this study showed that *P. falciparum* genome encodes a biologically active pyruvate kinase that is expressed during the intraerythrocytic-stage. The inhibition of pfPyrK by citrate, PLP, and ATP, as well as its uniqueness in its insusceptibility to F16BP and G6P, were characterized in detail. These observations suggest that pfPyrK appears to be kinetically unique among protozoan pyruvate kinases and that pfPyrK is regulated by effectors that may be correlated with energy flow or metabolite levels in the cell. Molecular investigations to elucidate the binding sites for the uncovered inhibitors may be relevant to design of molecules that would inhibit the parasite's enzyme. Currently, the availability of copious amounts of recombinant pfPyrK facilitates the employment of this enzyme in drug-screening.

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