

Molecular cloning and characterization of *Plasmodium falciparum* transketolase

Shweta Joshi^a, Alok Ranjan Singh^a, Ashutosh Kumar^b, Prakash Chandra Misra^c,
Mohammad Imran Siddiqi^b and Jitendra Kumar Saxena^{a,*}

^a *Division of Biochemistry, Central Drug Research Institute, Lucknow-226001 India*

^b *Division of Molecular and Structural Biology, Central Drug Research Institute, Lucknow-226001, India*

^c *Department of Biochemistry, Lucknow University, Lucknow-226001, India*

Abstract

The pentose phosphate pathway (PPP) is an important metabolic pathway for yielding reducing power in the form of NADPH and production of pentose sugar needed for nucleic acid synthesis. Transketolase, the key enzyme of nonoxidative arm of PPP, plays a vital role in the survival/replication of the malarial parasite. This enzyme in *Plasmodium falciparum* is a novel drug target as it has least homology with the human host. In the present study, the *P. falciparum* transketolase (PfTk) was expressed, localized and biochemically characterized. The recombinant PfTk harboring transketolase activity catalyzed the oxidation of donor substrates, fructose-6-phosphate (F6P) and hydroxypyruvate (HP), with K_m^{app} values of 2.25 and 4.78 mM, respectively. p-Hydroxyphenylpyruvate was a potent inhibitor of PfTk, when hydroxypyruvate was used as a substrate, exhibiting a K_i value of 305 μM . At the same time, noncompetitive inhibition was observed with F6P. The native PfTk is a hexamer with subunit molecular weight of 70 kDa, which on treatment with low concentrations of guanidine hydrochloride (GdmCl) dissociated into functionally active dimers. This protein was localized in the cytosol and nucleus of the parasite as studied by confocal microscopy. A model structure of PfTk was constructed based on the crystal structure of the transketolases of *S. cerevisiae*, *L. mexicana* and *E. coli* to assess the structural homology. Consistent with the homology modeling predictions, CD analysis indicated that PfTk is composed of 39% α -helices and 26% β -sheets. The availability of a structural model of PfTk and the observed differences in its kinetic properties compared to the host enzyme may facilitate designing of novel inhibitors of PfTk with potential anti-malarial activity.

Keywords: *Plasmodium falciparum*; Transketolase; Pentose phosphate pathway; Hexamer protein

Abbreviations: PfTk, *P. falciparum* transketolase; SEC, size exclusion chromatography; GdmCl, guanidine hydrochloride; pCMB, p-chloromercuribenzoate; NEM, N-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; HPP, p-hydroxyphenylpyruvate

*Corresponding author. Tel.: +91-522-2625932; fax: +91-522-2623405.

E-mail address: jksdri@yahoo.com (J. K. Saxena).

1. Introduction

Malaria is one of the leading causes of morbidity and mortality in the Tropics. Every year about 300-500 million people are infected with malaria causing 1-2 million deaths [1,2]. Considering the strong drug resistance in the parasite against commonly used drugs, an urgent need exists to identify new drug targets and develop new pharmacophores with unique structures and modes of action. Targeting the parasite's metabolic pathways, that lead to the formation of functional and structural components of the parasite, can be a good strategy for new anti-malarial development.

The availability of the *Plasmodium falciparum* genome sequence [3] has revealed the existence of a fundamental metabolic pathway in the parasite, namely the pentose phosphate pathway (PPP). The enzymes of PPP have been shown to be one of the most promising targets for the chemotherapy of parasitic protozoa [4]. The presence of PPP activity in *P. falciparum* infected erythrocytes has been reported [5-7] and biochemical studies have indicated that the non-oxidative arm of PPP is active in the malarial parasite [8]. However, the gene encoding transaldolase has not been identified in the genome of *P. falciparum* [3].

Transketolase (EC 2.2.1.1) is the key enzyme of the non-oxidative branch of PPP, which normally transfers two carbon units from xylulose-5-phosphate to ribose-5-phosphate or erythrose-4-phosphate thereby generating glyceraldehyde-3-phosphate (G3P), sedoheptulose-7-phosphate and fructose-6-phosphate (F6P) in the process [9,10]. Thiamine diphosphate (ThDP) is employed as a cofactor in the reactions catalyzed by transketolase. The expression profile of genes coding for different enzymes of PPP in *P. falciparum* has indicated that transketolase is maximally transcribed after the invasion of parasite into human RBC [8].

In a malarial parasite, transketolase plays an important role in the generation of ribose-5-phosphate which is required for nucleotide synthesis. This enzyme operates the non-oxidative arm of PPP in the reverse direction to utilize F6P and G3P produced by the glycolytic pathway [11]. The crucial role of transketolase in nucleic acid synthesis has been demonstrated by the incorporation of radiolabelled carbon from glucose into nucleotides [6,11]. These studies have shown that more than 80% of nucleic acid in the parasite is derived directly or indirectly from the non-oxidative part of PPP. In addition to playing an important role in nucleic acid synthesis, transketolase, in absence of transaldolase, seems to be responsible for the generation of erythrose-4-phosphate from F6P and G3P [3]. Erythrose-4-phosphate is a key metabolite required in the shikimate pathway for the production of the aromatic precursor chorismate, which is further metabolized into several aromatic compounds including folate [12]. The presence of shikimate pathway in the malarial parasite and its absence in mammals make the enzymes of this pathway potential novel drug targets [13].

Furthermore, the transketolase activity which is up-regulated in proliferative cells and tumor tissues serves as a target for therapeutic intervention in cancer and its inhibitors are also being developed as anti-tumor agents [14,15]. Oxythiamine, a potent inhibitor of transketolase, is reported to cause a dramatic decrease in tumor cell proliferation under *in vitro* and *in vivo* conditions [15]. However, the effect of transketolase inhibitors on the growth of the malarial parasite is yet to be investigated.

The present study reports the cloning, over-expression and biochemical characterization of *P. falciparum* transketolase (PfTk). Kinetic studies of the purified

recombinant PfTk has indicated significant differences between the parasitic and host enzyme. Due to the critical role of transketolase in the survival of the malarial parasite and its low homology with the host enzyme, PfTk may serve as a novel and promising target for designing inhibitors with anti-malarial activity.

2. Materials and methods

2.1. Parasite culture

P. falciparum (clone NF54) was cultured with human B⁺ erythrocytes (5% hematocrit) in RPMI 1640 media (Sigma) supplemented with 25 mM HEPES, 50 µg/ml gentamycin, 370 µM hypoxanthine and 0.5% (w/v) AlbuMax II as per protocol of Trager and Jensen [16].

2.2. Isolation of parasite from infected erythrocytes and DNA extraction

P. falciparum (clone NF54) in late trophozoite stage was harvested from erythrocytes showing approximately 10% parasitemia. Infected erythrocytes were separated by centrifugation at 800 × g for 5 min and parasites were liberated by incubating with an equal volume of 0.15% (v/v) saponin in phosphate-buffered saline (PBS) at 4 °C for 15 min. Parasites were sedimented by centrifuging at 1300 × g for 5 min and the resulting parasite pellet was washed four-five times with ice cold PBS. The washed parasites were stored at -80 °C until used. Genomic DNA was extracted from isolated parasites by phenol chloroform extraction [17].

2.3. PCR amplification and cloning of PfTk gene

The putative transketolase (PfTk) gene was PCR amplified from the genomic DNA of the parasite using gene specific primers 5'-ATGAACATTATGGATAACGAA ATAG-3' (forward) and 5'-TCATTTTCAGTTTATTTTTCATAAAGGC-3' (reverse) designed on the basis of sequence information available at www.PlasmoDB.org. The PCR amplification consisted of 35 cycles (30 sec at 94 °C, 1 min at 55 °C and 2 min at 68 °C), followed by an extension cycle (10 min at 68 °C) on a PTC 200 PCR system (MJ Research, USA). The amplified PCR product was subsequently cloned into the linearized pCR[®]T7/NT-TOPO[®] expression vector (Invitrogen, USA). *E. coli* DH5α cells were transformed with the ligation product and grown overnight on agar plates supplemented with 100 µg/ml ampicillin. Correct recombinants were identified by restriction digestion and sequencing.

2.4. Over-expression and purification of PfTk

BL21(DE3)Rosetta cells harboring the appropriate recombinant plasmid were grown at 37 °C in Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) to an OD_{600 nm} of 0.4-0.6. The expression of recombinant protein was induced with 1 mM isopropyl-β-thiogalactopyranoside (IPTG). The cultures were then allowed to grow further for 20 h at 18 °C with shaking and the cells were harvested by centrifugation. The cell pellet was suspended in lysis buffer [50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 10 mM imidazole and 10% (v/v) glycerol] containing protease inhibitor cocktail (Sigma) and the bacterial cells were lysed by sonication (Ultrasonic processor, Model-XL-2020, Germany). The lysate was centrifuged at 10,000 × g for 30 min at 4 °C and the supernatant was incubated with Ni-nitrilotriacetic acid (Ni²⁺-NTA) agarose resin (Qiagen, Germany), pre-equilibrated with the lysis buffer at 4 °C for 1 h. The suspension was applied to a column and washed with 10 bed volumes of lysis buffer containing 20 mM imidazole.

The bound protein was eluted with lysis buffer containing 250 mM imidazole. The fractions containing PfTk were further purified by 0–40% (w/v) ammonium sulfate precipitation. The precipitated protein was finally resuspended in 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl and used for further studies. Protein concentration was determined by the method of Lowry et al. [18]. SDS-PAGE and Western blotting using anti-His antibodies were employed to check the purity of the eluted protein. The subunit mass was determined by 10% (w/v) SDS-PAGE according to the method of Laemmli [19].

2.5. Size exclusion chromatography (SEC)

Gel filtration experiments were carried out on a Superdex 200HR 10/300 column (manufacturer's exclusion limit 600 kDa) with AKTA fast performance liquid chromatography (Amersham). The column was calibrated with various molecular weight standard markers (Amersham). The gel filtration column was run in 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl at a flow rate of 1 ml/min. For GdmCl-treated protein samples, the column was equilibrated and run with the same buffer containing the desired concentrations of GdmCl.

2.6. Glutaraldehyde cross-linking studies

Glutaraldehyde cross-linking studies were performed as described previously [20]. To native and GdmCl-treated (overnight at 4 °C) PfTk (0.1 mg/ml) was added an aliquot of 25% (v/v) glutaraldehyde to achieve a final concentration of 1% (v/v) glutaraldehyde. These samples were incubated at 25 °C for 5 min followed by quenching the cross-linking reaction by addition of 200 mM sodium borohydride. After 20 min incubation, 3 μ l of 10% (w/v) aqueous sodium deoxycholate was added. The pH of the reaction mixture was lowered to 2–2.5 by the addition of orthophosphoric acid that resulted in precipitation of the cross-linked proteins. After centrifugation (13,000 \times g, 4 °C, 10 min) the obtained precipitates were re-dissolved in 0.1 M Tris-HCl (pH 8.0), 1% SDS (w/v) and 50 mM DTT and heated at 90–100 °C. The molecular mass of the cross-linked products were determined by 6% (w/v) SDS-PAGE.

2.7. Enzyme assay and kinetics

Transketolase activity was measured by recording the oxidation rate of the α -carbanion intermediate in the presence of ferricyanide according to the method of Kochetov [21]. The reaction mixture in 1.0 ml contained 50 mM glycyl-glycine buffer (pH 7.6), 2 mM magnesium chloride, 0.1 mM thiamine pyrophosphate, 0.5 mM potassium ferricyanide, 3 mM fructose-6-phosphate (F6P)/hydroxypyruvate (HP) and 0.24 mg enzyme protein. The reaction was initiated by the addition of enzyme and the reduction of ferricyanide was monitored at 420 nm using UV-1601 PC spectrophotometer (Shimadzu, Japan). One unit of activity is defined as the amount of enzyme which catalyzes the oxidation of 1 μ mole of F6P/HP per minute at 37 °C. The K_m values of enzyme for F6P and HP were determined by measuring the rate of reaction at varying concentrations of respective substrates. The optimal pH for enzyme activity was determined by using buffers of different pH viz., Glycyl-glycine (7.0–9.0) and Glycine-NaOH (9.0–10.0). The effect of substrate analogue (p-hydroxyphenylpyruvate), -SH inhibitors [p-chloromercuribenzoate (pCMB) & N-ethyl maleimide (NEM)] and metal chelators [O-phenanthroline & ethylenediamine tetraacetic acid (EDTA)] on the enzyme activity was measured by pre-incubating the enzyme with these ligands for 10 min.

2.8. Generation of polyclonal antisera

Rabbit was immunized with the purified recombinant PfTk protein (140 μ g) emulsified in Freund's complete adjuvant (Sigma, USA). After four weeks a booster dose of

the protein emulsified in Freund's incomplete adjuvant was administered. The rabbit sera was collected 10 days after the booster dose and the antibody titer was measured by ELISA.

2.9. Western immunoblotting

To determine expression of PfTk in the parasite, antibodies generated in rabbit were used in a Western blot. Trophozoite stage of parasite was used to prepare lysate as described earlier [22]. The parasite lysate as well as recombinant PfTk were boiled in SDS sample buffer and resolved on 10% (w/v) SDS-PAGE. The separated proteins were transferred onto the nitrocellulose membrane (Amersham) and blocked in blocking buffer [1 × PBS, 5% (w/v) skimmed milk] for 2 h. The blot was washed and incubated for 1 h with primary antibody (anti-PfTk serum, 1:1000) diluted in blocking buffer. Later, the blot was washed and incubated for 1 h with secondary antibody conjugated to HRP [anti-rabbit IgG (Sigma), 1:10,000] diluted in blocking buffer. Bands were visualized by using ECL detection kit (Amersham).

2.10. Confocal microscopy

Confocal microscopy was employed to validate the expression and localization of PfTk in the parasite. The synchronized *P. falciparum* cultures at ring, trophozoite and schizont stages were processed for confocal microscopy according to the method of Tonkin et al. [23]. Briefly, cells were washed with PBS and fixed in solution containing 4% (w/v) para formaldehyde and 0.0075% (v/v) glutaraldehyde (Sigma) in PBS for 30 min. After one wash with PBS, fixed cells were permeabilized with 0.1% (v/v) TritonX-100 in PBS for 10 min. After another PBS wash, cells were treated with 0.1 mg/ml sodium borohydride in PBS for 10 min. Cells were washed once with PBS and blocked by 3% BSA treatment for 1 h. Cells were incubated overnight with anti-PfTk antibodies (1:50 dilution containing 3% BSA at 4 °C). Cells were washed 4-5 times with ice cold PBS and after incubation with anti-rabbit IgG coupled with FITC (3.6 µg/ml in 3% BSA) for 5-6 h at room temperature, the cells were allowed to settle onto coverslips coated with poly-L-lysine (100 µg/ml). Subsequently, nucleus of the parasite was stained with 20 µg/ml DAPI (MBI Fermentas, USA) for 45 min at room temperature followed by 4-5 washings with PBS. Finally, the coverslips were mounted on slides in a mounting media containing 0.025% (w/v) paraphenyldiamine (PPD) (Sigma) dissolved in 90% (v/v) glycerol. The slides were viewed in a confocal laser scanning microscope (Zeiss LSM 510 Meta) under a 60x oil immersion objective lens.

2.11. Molecular modeling

With an aim to study structure function relationship as well as to explore structural similarity with the existing transketolases, a homology model of PfTk was generated. All computational studies were performed on a SGI Origin server equipped with four 600-MH3-R-12000 processors. The structure of PfTk was constructed by homology modeling using the Modeller program [24] interfaced with Insight II 2000.1 [25]. Templates used for the modeling were transketolases from *Saccharomyces cerevisiae* (ID: 1TRK, 2.0 Å resolution) [26], *Leishmania mexicana* (ID: 1R9J, 2.22 Å resolution) [27] and *Escherichia coli* (ID: 1QGD, 1.90 Å resolution). Stereochemical evaluations were performed with the program PROCHECKv3.4.4 [28]. Energy minimizations were accomplished in the DISCOVER_3 interfaced with Insight II 2000.1 [25]. This model was subjected to energy minimizations, first by 200 steps of steepest descent and then by 1000 steps of conjugate gradient using CVFF force field.

2.12. CD analysis

Circular dichroism spectropolarimetry was performed with 0.15 mg/ml PfTk (4.28×10^{-6} M protein concentration) in 50 mM NaH_2PO_4 and 100 mM Na_2SO_4 . Protein spectra were recorded between 250 nm and 200 nm wavelengths at 22 °C in mdeg on Jasco J810 spectropolarimeter in 0.1 cm cell. A scanning with buffer without protein was recorded under identical conditions to determine the background spectra. CD spectra was analyzed using the programme CDsstr, part of CDPro [29].

3. Results

3.1. Identification of a putative *P. falciparum* transketolase (PfTk)

A putative transketolase gene was predicted and annotated on chromosome 6 (Gene ID PFF0530w, <http://www.PlasmoDB.org>) of the *P. falciparum* genome. The PfTk gene was predicted to have a single exon which was not interrupted by intron. The exon has a complete open reading frame of 1.9 Kb capable of encoding a protein consisting of 633 amino acids with a calculated molecular weight of 70 kDa. The alignment of the derived protein sequence of PfTk with transketolases from yeast, kinetoplastids, plants and *E. coli* revealed the presence of highly conserved residues throughout the sequences along with two notable motifs (Fig. 1). The first motif, located in N-terminal domain is common to all ThDP-dependent enzymes. The consensus sequence for this motif in transketolase, GDGXXXXLXXLVXXXDXN, begins with the highly conserved residues Gly-Asp-Gly (GDG) which is followed by 21 residues of less homology [30,31]. A second motif (THDSIGLGEDGPTHQPIE) was found to be specific for transketolases, and is denoted as ‘Tk motif’ (Fig. 1). This motif is similar to the nucleotide-binding motif of NADPH-dependent dehydrogenase [32].

3.2. Over-expression and purification of PfTk

According to the sequence information available at www.PlasmoDB.org for PfTk, specific forward and reverse primers were designed and used for the amplification of complete ORF of PfTk by PCR using genomic DNA of *P. falciparum* as template. PCR specifically amplified a fragment of 1.9 Kb, the predicted size of PfTk, which was subsequently cloned into an expression vector pCR[®]T7/NT-TOPO[®]. Transformation of the construct TOPO-T7-PfTk into *E. coli* strain BL21(DE3)Rosetta expressed a soluble protein of expected size which was confirmed by Western immunoblotting using anti-His antibodies (results not shown). Since the temperature for expression of the intact PfTk was found to be critical (as the expressed protein was significantly degraded at 37 and 25 °C), therefore the expression of PfTk was carried out at 18 °C which significantly protected the recombinant protein. The protein was purified to homogeneity by Ni-NTA affinity chromatography (Fig. 2A) and ammonium sulfate precipitation (Fig. 2B). The subunit molecular weight of PFTK was ~70 kDa on SDS-PAGE which was also confirmed by Western blotting using anti-His antibodies. Gel filtration of the native recombinant protein on the Superdex-200 column, calibrated with standard molecular weight markers, showed a single peak with retention volume of 10.5 ml (Fig. 3B). When elution volumes of marker proteins were plotted as a function of log molecular mass, PfTk was found to have a molecular mass of about 418 kDa, suggesting the hexameric nature of the protein (Fig. 3A). SDS-PAGE analysis of glutaraldehyde cross-linked PfTk also confirmed the above results (Fig. 3B, inset, lane 3). The subunit configuration of PfTk at low concentrations of GdmCl was studied by size exclusion chromatography (SEC) and glutaraldehyde cross-linking (Fig. 3B). In size exclusion experiments, for 0.5 M GdmCl-treated enzyme, two peaks with retention volumes of 10.5 and 13.05 ml were obtained. For 1.0 M GdmCl-treated PfTk only one peak with retention volume of 13.05 ml was obtained. These results indicated that at 0.5 M GdmCl, hexamer and dimer

species existed, whereas at 1.0 M GdmCl only dimer species were present. SDS-PAGE analysis of glutaraldehyde cross-linked GdmCl-treated PfTk showed similar results (Fig. 3B, inset, lanes 4&5).

3.3. Kinetic studies

Kinetic studies were performed to validate the transketolase activity of the recombinant protein. Recombinant PfTk displayed standard Michaelis-Menten kinetics (Fig. 4A) with K_m values of 2.25 ± 0.5 mM for F6P (Fig. 4A, inset) and 4.78 ± 0.2 mM for hydroxypyruvate (HP). PfTk catalyzed oxidation of F6P on a broad range of pH, but optimally at pH 8.9 (Fig. 4B). The effect of p-hydroxyphenylpyruvate (HPP) on the enzyme activity was determined by Lineweaver-Burk double reciprocal plot. HPP competitively inhibited the enzyme activity in presence of HP as a substrate (Fig. 4C) with a K_i value of 305 μ M (Fig. 4D). However, the inhibition was noncompetitive with F6P (data not shown). pCMB (5 mM) and NEM (10 mM) significantly inhibited enzyme activity (77 and 72%, respectively). EDTA and O-phenanthroline were effective at 10 mM showing enzyme inhibition by 79 and 65%, respectively.

3.4. Localization of PfTk in the parasite

To localize PfTk in the parasite, anti-PfTk antibodies were raised using the recombinant PfTk. The antibody titer was found to be 1:102400. The antibodies specifically recognized the purified recombinant PfTk as well as Tk from *P. falciparum* lysate on a Western blot (Fig. 5A). Confocal microscopy results showed the cytosolic and nuclear localization of PfTk in the ring, trophozoite and schizont stages of the parasite (Fig 5B). These results suggested that PfTk is distributed both in the cytoplasm and nucleus of the parasite.

3.5. Molecular modeling and CD analysis

A homology model for PfTk was built based on the pair wise sequence alignment of *P. falciparum* sequence with the homologue transketolases of *S. cerevisiae* (ID: 1TRK, 2.0 Å resolution) [26], *L. mexicana* (ID: 1R9J, 2.22 Å resolution) [27] and *E. coli* (ID: 1QGD, 1.90 Å resolution) (Fig. 6, inset). The average sequence similarity of PfTk with *S. cerevisiae*, *L. mexicana* and *E. coli* homologue was 50, 43 and 41%, respectively. The structural model of PfTk, was constructed using the Modeller program [24], which generated the structure by the satisfaction of spatial restraints. Refinement of the homology model removed the steric constraints and obtained stable conformation by energy minimization of 1000 iterations. After the refinement process, the model was validated using PROCHECK program [28]. The Ramachandran plot [33] showed normal distribution of points with phi (ϕ) values and psi (ψ) values clustered in a few distinct regions with 87.5 and 11.7% of residues occupying core and allowed regions, respectively. Only a few residues (0.2%) were in the disallowed regions (Table 1). The root mean square (RMS) deviation of the residue C- α atoms between PfTk and 1TRK was 1.30 Å, which is indicative of a good quality model and suggested similarity between the two models. Superposition of the PfTk homology model on the template also revealed a close structural resemblance of modeled PfTk with template (data not shown). The α and β contents of the model protein were found to be 42 and 25%, respectively as predicted by the program Prostat available with Insight 2000.1 [25]. CD analysis of PfTk was performed to validate experimentally the helix content as predicted by molecular modeling. Analysis of the CD spectra suggested that PfTk is composed of 39% α -helices, 26% β -sheets and the remaining part assumed to be randomly coiled structure (Fig. 6). These results are in close agreement with PfTk homology model prediction for helix contents.

4. Discussion

The availability of *P. falciparum* genome sequence has provided an opportunity to identify novel drug targets. Selection of parasite proteins as drug targets is usually based on its functional importance, conservation across different species and having least or no homology with the host. The *P. falciparum* transketolase is one such candidate which shows low homology with the host. Transketolase plays a key role in tumor cell proliferation, neurodegenerative diseases, diabetes and cancer [34,35,14]. The inhibitors of transketolase are also used in the treatment of cancer [15].

We have successfully cloned, expressed and biochemically characterized transketolase from *P. falciparum*. The alignment of the protein sequence of PfTk with transketolases from different sources showed its high sequence identity with yeast, plants and kinetoplastids. Similar to other known transketolases, PfTk contains the conserved 'ThDP' and 'Tk' motifs. The conserved 'ThDP' motif is found to be crucial both for the binding of ThDP to transketolase and for the activation of the enzyme [36]. The 'Tk' motif, known to form a part of the active site of enzyme, was also identified in PfTk (Fig. 1). The conserved residues of the 'Tk' motif are directly involved in the cofactor and donor (aldehyde) substrate binding [37].

The occurrence of transketolase activity has been described in a wide range of prokaryotic and eukaryotic organisms [38–42]. Initial attempts to express PfTk in BL21(DE3) cells were not fruitful probably due to differences in the codon usage between *E. coli* and *P. falciparum* [43,44]. Therefore, the expression was carried out in BL21(DE3)Rosetta, which alleviated the problem of codon bias, thereby resulting in successful expression of PfTk. The analysis of native mass (~ 418 kDa) of PfTk indicated the hexameric nature of the protein whereas transketolases from other sources are either dimeric or tetrameric in nature [38,41,45,46]. SEC and glutaraldehyde cross-linking experiments suggested that treatment of PfTk with low concentration of GdmCl (1.0 M) resulted in dissociation of native hexameric enzyme into functionally active dimers. The stabilization of any other intermediate was not observed. This suggested the possibility of the stabilized hexameric enzyme existing as trimer of dimers.

PfTk resembled the transketolases from *S. cerevisiae* and spinach [42] in utilizing F6P and HP as substrate. The recombinant PfTk has specificity for both the substrates, whereas the host (human) enzyme is reported to utilize F6P only [47]. The K_m value for F6P (2.25 ± 0.5 mM) in case of PfTk is nearly one third of the host enzyme (7 mM). The higher affinity of PfTk for fructose-6-phosphate as compared to human transketolase is suggestive of greater utilization of F6P by the parasite to meet its requirement of nucleotides for rapid multiplication. PfTk resembled spinach transketolase in utilizing hydroxypyruvate and ribose-5-phosphate for the production of heptulose sugars [39]. However, it is different from host transketolase which does not utilize hydroxypyruvate [47]. PfTk was strongly inhibited by p-hydroxyphenylpyruvate (HPP) at very low concentrations. The inhibition was noncompetitive with F6P and was competitive with hydroxypyruvate. However, in *S. cerevisiae*, HPP competitively inhibited the transketolase activity with both the substrates [48]. This led investigators to suggest that inhibition by p-hydroxyphenylpyruvate might be one of the possible mechanisms for the regulation of tyrosine biosynthesis. The inhibition of PfTk by pCMB and NEM indicated the presence of functional -SH groups in the enzyme. Significant inhibition of the enzyme activity by metal chelators, EDTA and O-phenanthroline, indicated that divalent metal ions have a role in enzyme function. The necessity of divalent cations for enzyme activity has been also reported for mammalian Tk [49].

We also examined the localization of transketolase in the parasite. Western blot analysis indicated the presence of PfTk in the parasite lysate and the results of the confocal microscopy demonstrated its cytosolic and nuclear localization in the parasite. The presence of PfTk both in the cytoplasm and nucleus is surprising because the established enzymatic function of transketolase is related to cytoplasm only. However, evidence for the dual localization of TkTL1 is also available [50]. The TkTL1 gene representing one of the three transketolase genes in human genome (TkT, TkTL1 and TkTL2) is activated during malignancies, particularly, when there is a strong need of pentose for DNA synthesis during cell cycle [51]. Although the exact function of transketolase in the nucleus is not yet known, but its presence might be associated with its role in apoptosis or some other function yet to be investigated.

The three dimensional model of PfTk was found to be similar to the 3D-structure of *S. cerevisiae* transketolase [26]. The PfTk model is an average structure based on restraints derived from the coordinate sets of the template. Similar to *S. cerevisiae* transketolase, the PfTk model can also be divided into N-terminal, middle and C-terminal domains. A ThDP-binding pocket as reported for *S. cerevisiae* transketolase was also identified in the PfTk model. The proposed ThDP binding pocket in *S. cerevisiae* was formed mainly by His69, Leu118, Ile191, His263 and Ile416, whereas in PfTk, isoleucine was replaced by valine. A hydrophobic pocket constituted by Phe438, Phe441 and Tyr444 was also identified in the PfTk model. The analysis of CD spectra confirmed that PfTk contained 39% α -helices, 26% β -sheets and the rest was randomly coiled structures, which is in close agreement with the PfTk homology model calculations.

In conclusion, we have expressed and characterized PfTk, a putative drug target in *P. falciparum* having low homology with its human host. Evidence has also been presented that PfTk, a hexameric protein, can efficiently utilize both fructose-6-phosphate and hydroxypyruvate as substrates and the enzyme is localized both in the cytoplasm and nucleus. The absence of transaldolase in the genome of *P. falciparum* raises the question whether the erythrose-4-phosphate, needed for the shikimate pathway, is produced by transketolase alone or in association with some other enzyme. So far the regulatory role of transketolase in the malarial parasite has not been reported. It is likely that this enzyme may regulate a reversal step of PPP for the production of erythrose-4-phosphate and thereby ribose-5-phosphate which is needed for rapid multiplication of the parasite [3, 11]. The information obtained in the present study on the structure and kinetics of PfTk may be exploited in the design of parasite specific transketolase inhibitors.

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Legends to figures:

Fig. 1. Amino acid sequence alignment of transketolases from *L. mexicana* (LmTk, CAD-20572), *Trypanosoma brucei* (TbTk, XP-823559), *Arabidopsis thaliana* (AtTk, NP-567103), *Oryza sativa* (OsTk, NP-001056711), *E. coli* (EcTk, NP-754872), *P. falciparum* (PfTk, XP-966097), *P. yoelii* (PyTk, XP-731078) and *S. cerevisiae* (ScTk, NP-015399). Black and grey backgrounds represent identical and homologous residues respectively. The two conserved regions in the transketolase family [ThDP box i.e., GDGXXXLXXLVXXXDXN and Tk motif i.e., THDSIGLGEDGPTHQPIE] are indicated in box.

Fig. 2. 10% SDS-PAGE of purified recombinant PfTk. (A) *E. coli* lysate expressing PfTk was purified by Ni-NTA affinity chromatography, Lane 1: Molecular weight standard marker; lane 2: Soluble fraction of induced cells loaded on Ni-NTA column; lane 3: Flow through; lane 4: 20 mM imidazole wash; lanes 5-7: protein eluted with 250 mM imidazole (B) The eluted fractions containing PfTk were pooled and further purified by ammonium sulfate precipitation. Lane 1: Molecular weight standard marker; lane 2: Purified PfTk obtained after ammonium sulphate precipitation. Arrow indicates the molecular mass of PfTk.

Fig. 3. Determination of native molecular mass and oligomeric forms of PfTk. (A) The native molecular mass of the protein was determined by FPLC using Tricorn Superdex-200 gel filtration column. The column was calibrated with molecular weight standard markers- (a) albumin (67 kDa) (b) aldolase (158 kDa), (c) catalase (232 kDa), (e) Ferritin (440 kDa). Arrow indicates (d) the molecular mass of PfTk. Data are the representative of three independent experiments. (B) SEC profiles for GdmCl-treated PfTk on Superdex-200 column. The numbers in the figure represent concentrations of GdmCl at which the samples were incubated and run on the column. Inset: 6% SDS-PAGE profile of glutaraldehyde cross-linked samples. Lane 1: Molecular weight standard markers; lanes 2 and 3: uncross-linked and glutaraldehyde cross-linked PfTk respectively; lanes 4 and 5: cross-linked 0.5 M and 1.0 M GdmCl-treated PfTk samples.

Fig. 4. Kinetics of PfTk and its inhibition by HPP. (A) Michaelis-Menten plot at different concentrations of F6P. PfTk activity was measured as described in materials and methods and expressed as $\mu\text{molemin}^{-1}$. K_m value was calculated from Lineweaver-Burk plot (inset). (B) Effect of pH on the PfTk activity was determined as described in section 2.7. (C) Double reciprocal plot showing competitive inhibition when HP was used as a substrate. The different reactions were run first in the absence of HPP and then in the presence of different concentrations of HPP. (D) The slopes of the double reciprocal graph are plotted against the HPP concentration.

Fig. 5. Identification and immunolocalization of PfTk in *P. falciparum*. (A) Western Blot experiment on parasite lysate with antiserum raised to recombinant PfTk. Lane 1:

Prestained Protein Ladder; lane 2: Recombinant PfTk; lane 3: *P. falciparum* lysate. Arrow indicates transketolase position in gel. (B) Localization of PfTk in *P. falciparum* by confocal microscopy. Parasites at ring (panel I), trophozoite (panel II) and schizont (panel III) stages were fixed and immunostained with anti-PfTk (green) antibody followed by FITC-labelled secondary antibody. The parasites were stained with DAPI (blue) and slides were visualized by confocal microscopy. In each panel (a) represents phase contrast of infected RBCs, (b) and (c) indicate the location of nucleus and PfTk respectively in *P. falciparum*, (d) Merged picture of b & c.

Fig. 6. Far-UV CD spectrum and Molecular modeling of PfTk. Protein spectra were recorded between 250 and 200 nm wavelength at 22 °C in mdeg on Jasco J810 spectropolarimeter in 0.1 cm cell. The spectrum was analyzed using the programme CDsstr part of CDPro. Inset: The model structure of PfTk was constructed by homology modeling using the Modeller program interfaced with Insight II 2000.1 [24,25]. Helices are colored red, strands as green and coils as blue.

Fig. 1.

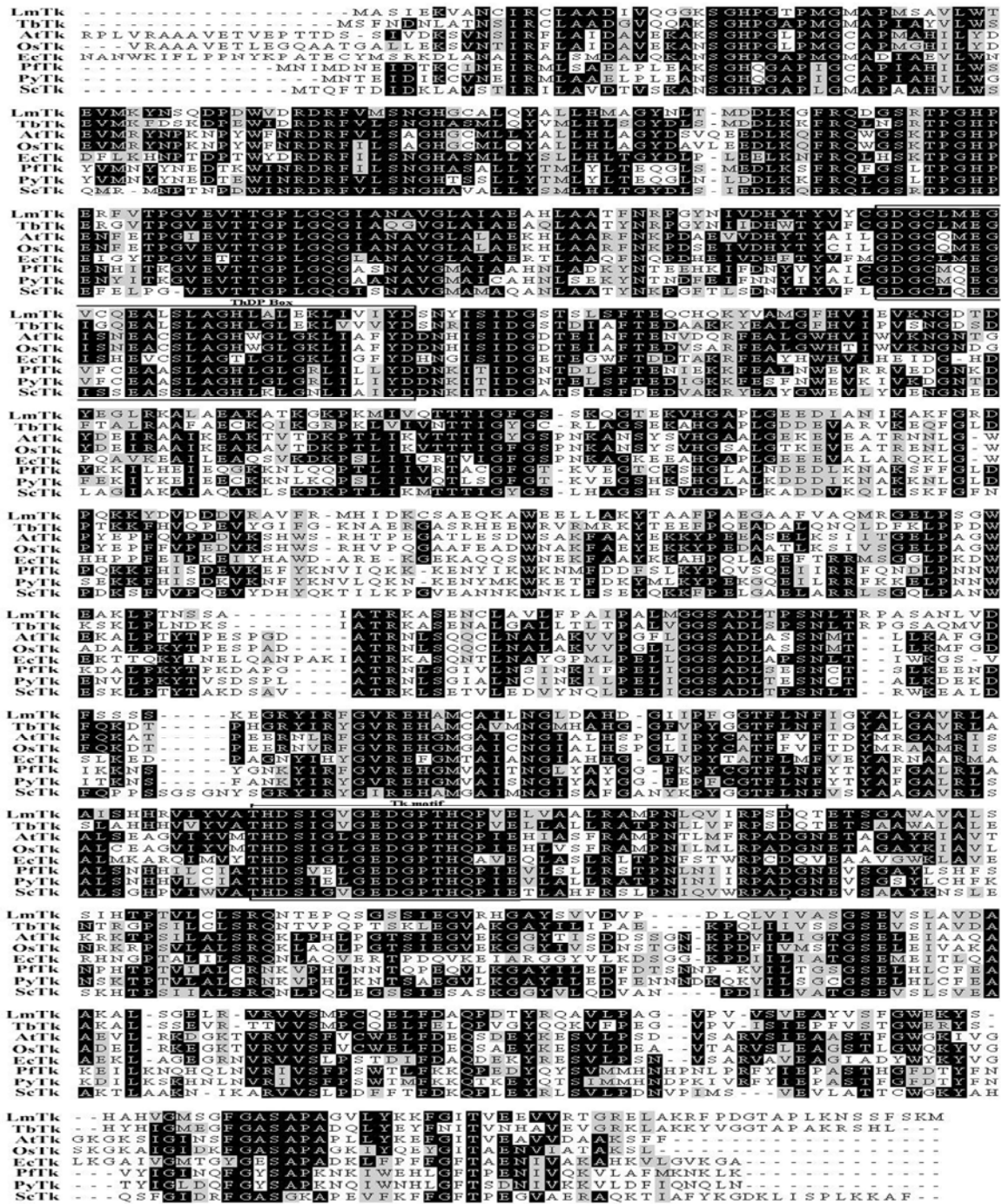


Fig. 2A

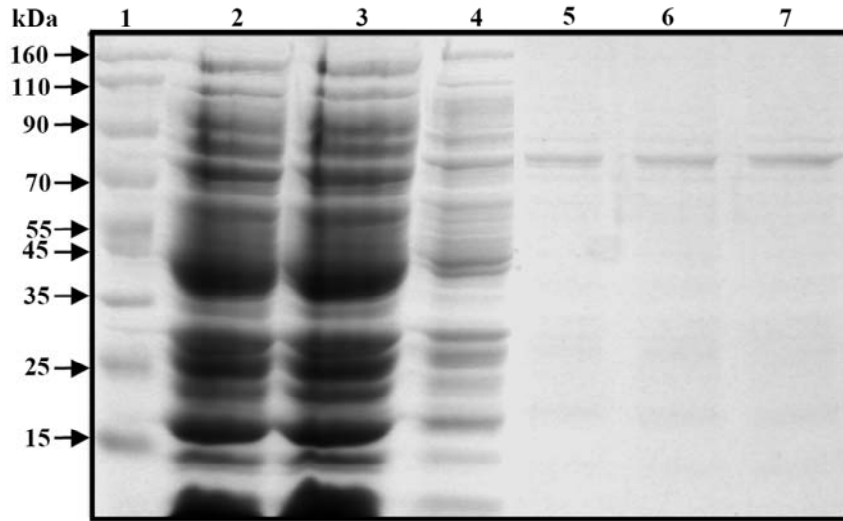


Fig. 2B

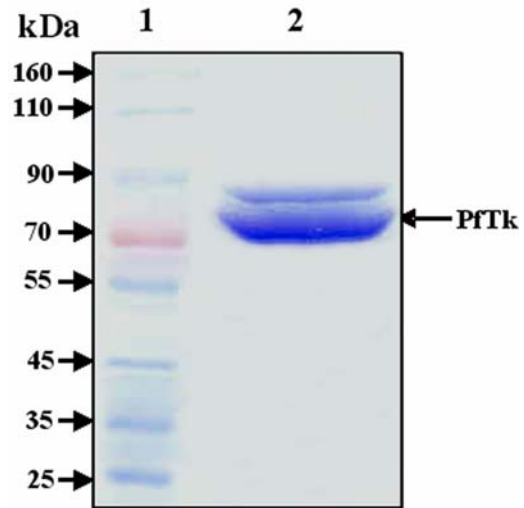


Fig. 3 A

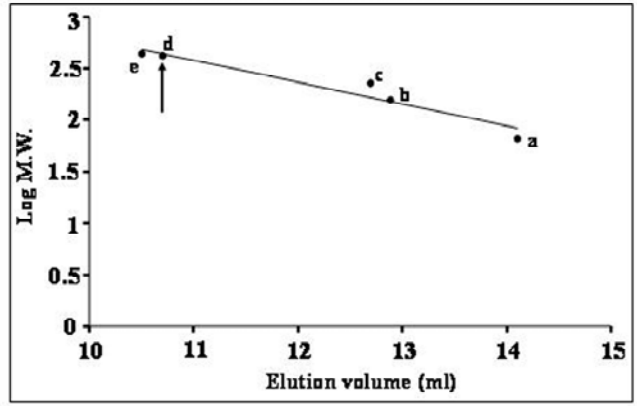


Fig. 3 B

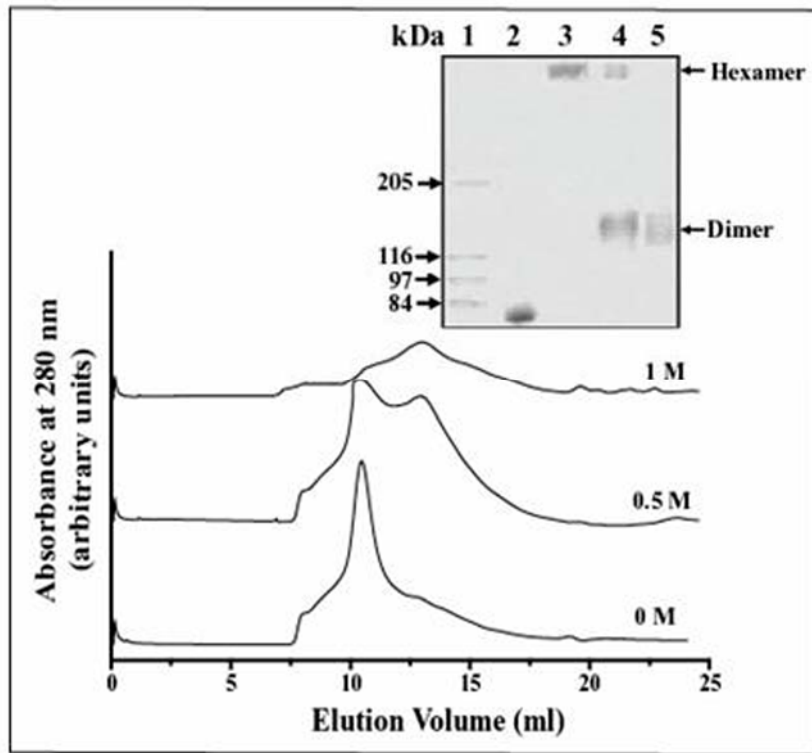


Fig. 4 A

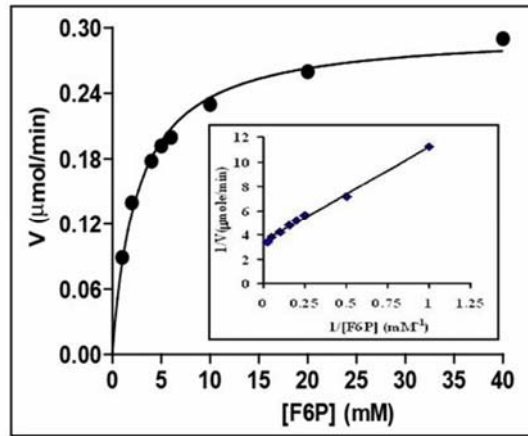


Fig. 4 B

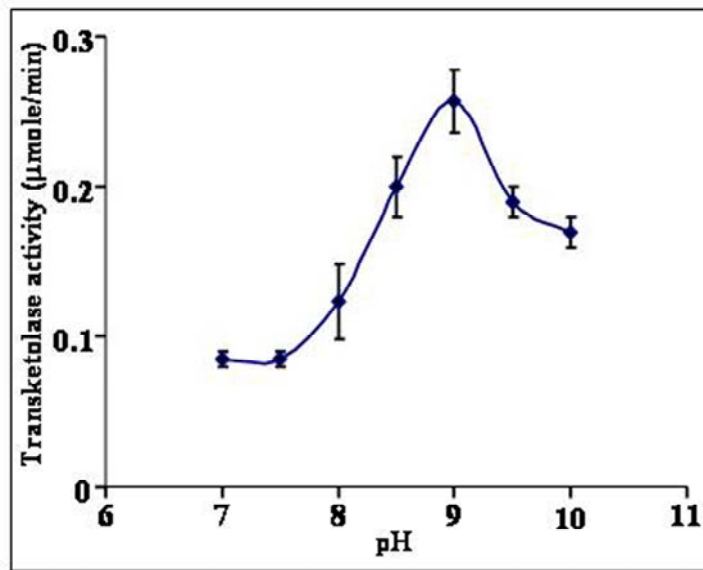


Fig. 4 C

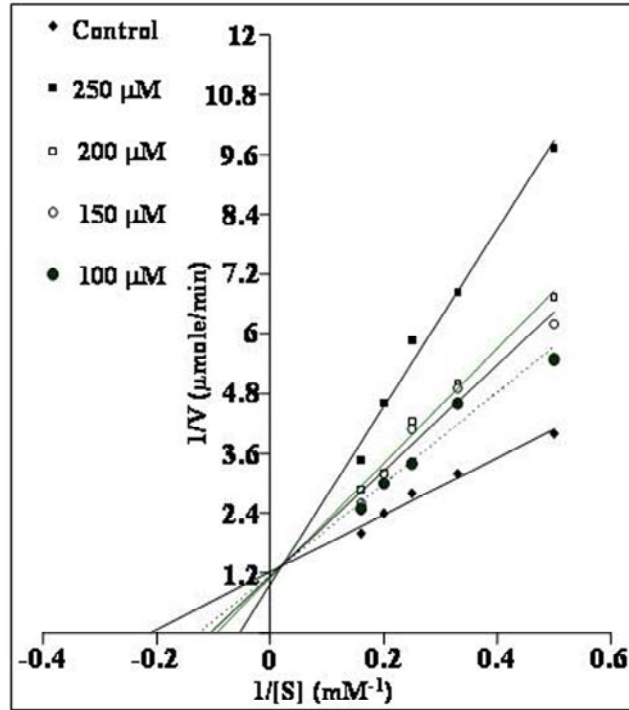


Fig. 4 D

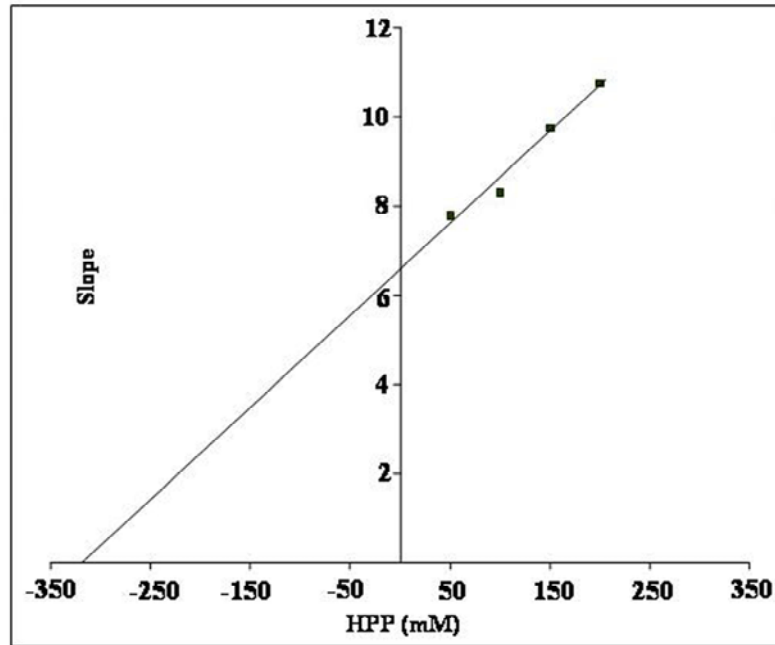


Fig. 5 A

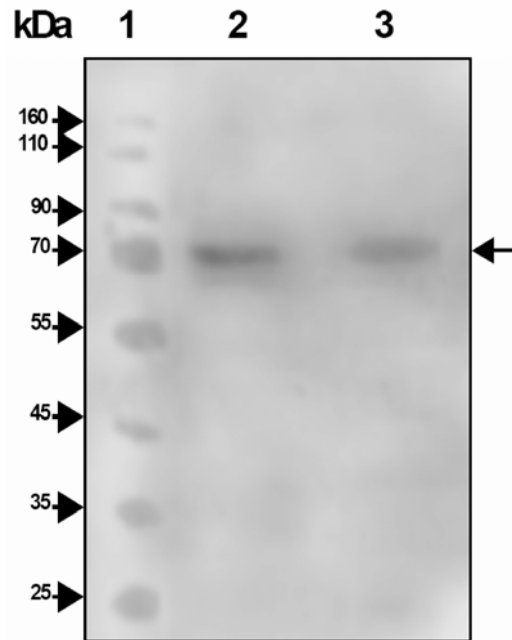


Fig. 5 B

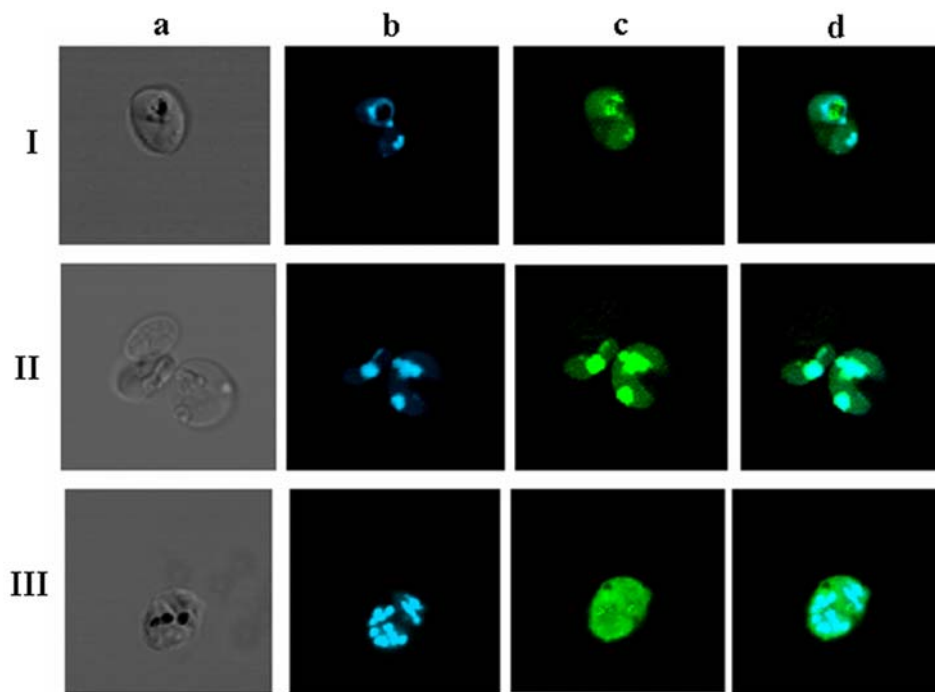


Fig. 6

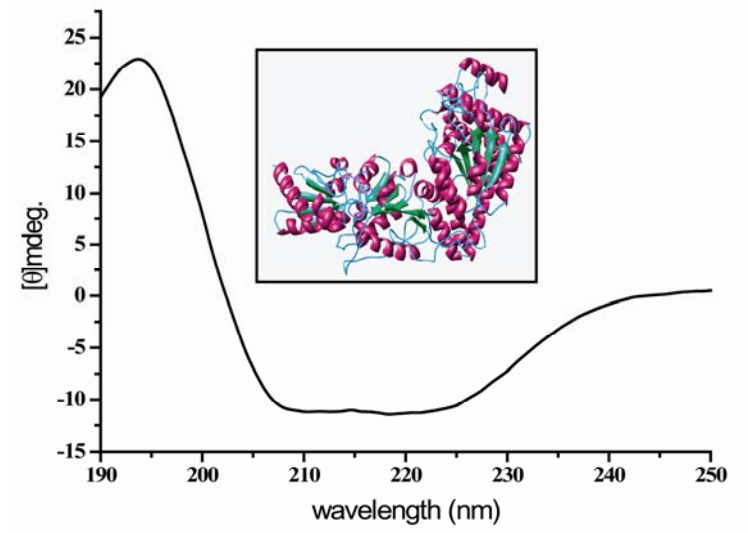


Table 1.

PROCHECK Ramachandran scores for the *P. falciparum* putative transketolase structure

Ramachandran Plot (%)	<i>P. falciparum</i> transketolase (PFTk)	<i>S. cerevisiae</i> transketolase
Core	87.5	89.2
Allowed	11.7	10.8
Generous	0.7	0.0
Disallowed	0.2	0.0