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# Pyruvate kinase type-II isozyme in *Plasmodium falciparum* localizes to the apicoplast

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## Abstract

Bioinformatics research on *Plasmodium falciparum* revealed two isoforms of pyruvate kinase: type-I and type-II enzymes. The type-I enzyme shows typical glycolytic properties, while type-II enzyme is involved in fatty acid type-II biosynthesis and has been predicted to localize to the apicoplast with the targeting signal in its N-terminus. The type-I and type-II isoforms have the same evolutionary origin as *Toxoplasma gondii* isozymes, *Tg*PyKI and *Tg*PyKII, respectively; however, *Tg*PyKII localizes to both the mitochondrion and the apicoplast. Accordingly, we made a recombinant full length of *P. falciparum* pyruvate kinase type-II protein using a wheat germ cell-free expression system and obtained a specific antibody against the type-II protein. Fluorescent microscopic analysis revealed that *P. falciparum* type-II enzyme was localized only to the apicoplast, not to the mitochondrion. The data suggest differences in localization and metabolic pathways between *P. falciparum* and *T. gondii* pyruvate kinase isoforms.

### Keywords

*Plasmodium falciparum*; Pyruvate kinase II; Apicoplast; Mitochondria; Cell-free expression

Pyruvate kinase (EC 2.7.1.40) catalyzes the essentially irreversible transphosphorylation of phosphoenolpyruvate (PEP) to ADP. The activities of most mammalian and bacterial pyruvate kinases are allosterically regulated by fructose 1,6-bisphosphate, and pyruvate kinase is known to play a regulatory role in glycolysis. The glycolytic end product, pyruvate, feeds into various metabolic pathways, and hence pyruvate kinase is important in several primary metabolic reactions.

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Many organisms have pyruvate kinase isozymes with different kinetic properties, and most pyruvate kinases in eukaryotes are reported to be located in the cytosol. Two types of pyruvate kinase were characterized in *Toxoplasma gondii* [1,2]. Pyruvate kinase type-II isozyme (*Tg*PyKII) was localized in both the mitochondrion and the apicoplast, whereas pyruvate kinase type-I (*Tg*PyKI) was located in the cytosol. *Tg*PyKII exhibited only 18% overall amino acid identity with *Tg*PyKI and showed novel properties of exhibiting high pH optima and GDP dependency [2].

The malaria bioinformatics website (http://sites.huji.ac.il/malaria/), compiled and maintained by Hagai Ginsburg, reports two isoforms of pyruvate kinase in *Plasmodium falciparum*. The type-I enzyme (*Pf*PyKI) has been characterized enzymologically in detail [3]. The type-II enzyme (*Pf*PyKII) was predicted to have an apicoplast targeting signal in the N-terminus; however, experimental localization has not been confirmed. Phylogenetic analysis indicated that *Pf*PyKI and *Pf*PyKII have the same evolutionary origin as *Tg*PyKI and *Tg*PyKII, respectively, suggesting that type-II has a proteobacterial origin [2]. Thus, we questioned whether both *Pf*PyKII and *Tg*PyKII are localized in both the apicoplasts and the mitochondria.

In this study, we made recombinant *Pf*PyKII protein in a wheat germ cell-free expression system, purified the recombinant protein, created an antibody, and localized *Pf*PyKII by immunofluorescent microscopy.

The *Pf*PyKII gene was amplified from *P. falciparum* genomic DNA. The two primers were 5′-ACTGGATCCCCATATTGCCTATGAT-3′ and 5′-TCGGGATCC CTAATTTGTTAGACATGG-3′ (*Bam*HI site is underlined). The first denaturation at 95 °C was for 10 min and each of 30 reaction cycles consisted of 94 °C for 30 s, 47 °C for 30 s, and 65 °C for 2 min, and a final elongation cycle step at 65 °C for 5 min using KOD-plus DNA polymerase (Toyobo Co. Ltd, Osaka, Japan). Thereafter, the amplified DNA products were treated with *Bam*HI and inserted into a plasmid pEU-EO1G-N2 (Cell-Free Science and Technology Research Center, Ehime University, Ehime, Japan) using the LigaFast ligation kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The plasmid was electroporated into the *Escherichia coli* DH10B (Takara Bio, Kyoto, Japan) and the bacteria were grown in a plate. The right directional clones were detected by DNA sequencing using the ABI PRISM BigDye terminator cycle sequencing kit (Applied Biosystems, Foster, CA, USA) and loaded onto an ABI PRISM 310 DNA sequencer. After plasmid purification using Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany), the plasmid was further purified by $C_SCl_2$ ultra-centrifugation at 391,000 ×*g* for 16 h at 25 °C to avoid endotoxin contamination. The purified plasmid containing a glutathione S-transferase (GST) coding region and an SP6 promoter upstream of the DNA inserted region was treated with SP6 RNA polymerase (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The method of mRNA production and translation in wheat germ was described previously [4].

The GST-pyruvate kinase isozyme fusion protein in the wheat germ extract was purified using an affinity column of glutathione sepharose 4B (GE Healthcare). The pyruvate kinase isozyme was cut from the fusion protein by PreScission Protease (GE Healthcare) according to the manufacturer's recommendations. Pyruvate kinase isozyme purity was analyzed by

SDS–PAGE on 8% polyacrylamide gel as described by Laemmli [5] (Data not shown). The recombinant protein concentration was determined by Bradford assay [6] using bovine serum albumin (BSA) as a standard.

Anti-recombinant *Pf*PyKII antibody was produced through a commercial company (Immuno-Biological Laboratories, Takasaki, Japan). Briefly, purified recombinant *Pf*PyKII from wheat germ extracts was used to immunize a BALB/c mouse. Following six injections of pyruvate kinase isozyme (5 μg each) at 1-week intervals, the whole IgG was isolated from peritoneal fluid with a HiTrap rProtein A FF column (GE Healthcare). The whole cell lysate of $1\times10^8$ erythrocytic stage *P. falciparum* parasites (FCR-3 strain) was separated on 8% acrylamide gel and blotted onto a nitrocellulose Hybond-C Extra membrane (GE Healthcare). The membrane was blocked for 20 min with 2% skimmed milk in Tris-buffered saline containing 0.2% Tween 20, incubated for 1 h with primary antibodies (1:3000), probed with alkaline phosphatase-conjugated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) (1:5000), and detected with a BCIP-NBT system (Roche, Basel, Switzerland). Molecular sizes of the protein bands were determined with reference to pre-stained Rainbow molecular weight markers (GE Healthcare).

Cells were fixed and stained using the procedures described by Tonkin et al. [7]. Cells were briefly fixed with 4% EM grade para-formaldehyde (ProSciTech, Thuringowa, Queensland, Australia) and 0.0075% EM grade glutaraldehyde (ProSciTech) in phosphate-buffered saline (PBS) for 30 min. Fixed cells were washed once in PBS and permeated with 0.1% Triton X-100/PBS for 10 min. Cells were washed again and treated with 0.1 mg/ml of sodium borohydride ($NaBH_4$)/PBS for 10 min. Following another wash, cells were blocked in 3% BSA/PBS for 1 h. For staining anti-*Pf*PyKII antibody-binding structure and apicoplast, anti-*Pf*PyKII mouse antibody (diluted 1/1000) and anti-acyl carrier protein (ACP) rabbit antibody (diluted 1/500; gifted by Geoff McFadden, University of Melbourne, Australia) were added and allowed to bind for a minimum of 1 h in 3% BSA/PBS. AlexaFluor goat anti-mouse 594 (red) and anti-rabbit 488 (green) secondary antibodies (Invitrogen, Carlsbad, CA, USA) were added at 1:1000 dilution (in 3% BSA/PBS) and allowed to bind for 1 h, while cells settled onto a previously flamed cover slip coated with 1% polyethylenimine (PEI; Sigma, St Louis, MO, USA). For staining the anti-*Pf*PyKII antibody-binding structure and mitochondrion, citrate synthase-GFP construct (gifted by Geoff McFadden) transformed *P. falciparum* was used. Anti-*Pf*PyKII mouse antibody (diluted 1/1000) was added and allowed to bind for 1 h, followed by addition of AlexaFluor goat anti-mouse 488 (green) antibody and Cy5-conjugated anti-GFP rabbit (red) antibody (diluted 1/1000; Sigma) and allowed to bind for 1 h. Cells were mounted in 50% glycerol with 0.1 mg/ml of 1,4-diazabicyclo[2,2,2]octane (DABCO, Sigma). The microscopic system was a DeltaVision restoration system (Applied Precision, Washington, USA) on an Olympus IX70 inverted microscope equipped with a mercury vapor lamp (100 W) and appropriate barrier emission filters. Images were taken 0.2 μm apart and deconvolved using softWoRx Explorer Suite (Applied Precision).

The deduced amino acid sequence of *Pf*PyKII (NCBI Accession# NP_700836), exhibiting low overall identity (21%) to that of *Pf*PyKI (NCBI Accession# CAG25081), contained a pyruvate kinase signature (PROSITE; PS00110) as did other species and other consensus

regions, such as multiple binding sites of ADP, PEP, and divalent cations (Fig. 1). Based on protein alignment, *Pf*PyKII was predicted to be a monovalent cation-independent enzyme. Most of the monovalent cation-binding sites were conserved; however, two binding sites, $Thr^{113}$ and $Glu^{117}$ (in *Felis catus* pyruvate kinase), were substituted by Ile and Lys, respectively. These substitutions are a common characteristic of monovalent cation-independent pyruvate kinases. We found three-specific long insertions in the middle of domain B, A2, and C of *Pf*PyKII, as in *Tg*PyKII. These insertions were different in length, but the insert positions were the same as in *Tg*PyKII.

Following six injections of pyruvate kinase isozyme at 1-week intervals, the whole IgG was isolated from mouse peritoneal fluid. Western blot analysis showed a single band (~80 kDa) in the *P. falciparum* lysate (Fig. 2), which was different from the mass in the type-I enzyme (55.6 kDa), indicating no cross-reaction with the type-I enzyme. Preimmune serum detected no bands in the $1\times10^8$ *P. falciparum* lysate (data not shown). The antibody was used in immunofluorescence microscopy.

The stained structure from the anti-*Pf*PyKII antibody in *P. falciparum* merged into the apicoplast stained pattern (Fig. 3A), suggesting that *Pf*PyKII localizes to the apicoplast. To determine if *Pf*PyKII localizes to the mitochondria, we analysed the immunolocalization of *Pf*PyKII in a *P. falciparum* cell line expressing the citrate synthase fused to GFP, which targets to the mitochondria [7] (Fig. 3B). The merged image showed that anti-*Pf*PyKII stain is adjacent to, but not associated with, the mitochondria. The two stains were distinguishable in all the stages (data not shown). Thus, we concluded that *Pf*PyKII localizes to the apicoplast, not to the mitochondrion. The data indicate a different localization of type-II pyruvate kinase in *P. falciparum* from that in *T. gondii*.

A recombinant protein of *P. falciparum* pyruvate kinase type-II isozyme (*Pf*PyKII) was created using a wheat germ cell-free system. All our previous attempts for production of this recombinant protein in *E. coli* systems have failed. Probably, it was due to its biased codon usage. The efficiency of production of the protein in our study was not high; nevertheless, the wheat germ cell-free system is useful for creation of the recombinant protein.

In addition, we showed localization of *Pf*PyKII in the apicoplast by immunofluorescent assay. Despite its proteobacterial origin, *Pf*PyKII was localized only to the apicoplast, not to the mitochondrion as in *Tg*PyKII, which is localized to both the mitochondrion and the apicoplast. The difference in metabolic pathways in the organelles between *T. gondii* and *P. falciparum* might reflect differences in their internal environment and in the metabolic relationships between those organelles in the two parasites. Further investigation to reveal these potential differences will contribute to understanding survival of *T. gondii* and *P. falciparum* in the host.

As suggested by Ralph et al. [8], pyruvate kinase in the apicoplast might dephosphorylate PEP imported into the apicoplast via PEP transporter on the apicoplast membrane and supply pyruvate for fatty acid synthesis and the non-mevalonate 1-deoxy-D-xylulose-5-phophate (DOXP) pathway in the organelle. Fleige et al. [9], reporting on carbohydrate metabolism in the *T. gondii* apicoplast, indicated that *Tg*PykII was localized in the

apicoplast. These findings suggest differences between *T. gondii* and *P. falciparum* in the mitochondrion and apicoplast metabolic pathways, even though *T. gondii* and *P. falciparum* have comparable organelle components, and were thought to have similar enzyme components in both the apicoplast and the mitochondrion. The pathway differences might reflect differences in intracellular environments or different abilities to import metabolites into those organelles. We expected that the difference in enzymatic properties between *Tg*PyKII and *Pf*PyKII would help in understanding their roles in the two parasites, but several attempts to express the active recombinant enzyme have failed. As pyruvate kinase has been thought to play a role only in glycolysis in the cytosol, pyruvate kinases localized with cell organelles are unique. Non-glycolytic pyruvate kinases have been found only in the apicomplexan parasites, such as *Plasmodium* sp, *Theileria* sp, and *T. gondii*. Characterization of non-glycolytic pyruvate kinases would increase the understanding of the unique metabolic pathways in protozoan parasites.

In addition to uncertainty about metabolic pathways, we are also uncertain about the origin of *Pf*PyKII. Although *Pf*PyKII exhibits a typical bipartite signal in the N-terminus, *Pf*PyKII has a proteobacterial origin, which is indicative of the apicoplast protein, and not a cyanobacterial or plastidic origin [2]. We suggest that *Pf*PyKII might have been obtained from endosymbiotic bacteria. Originally *Pf*PyKII may have localized in both the mitochondrion and the apicoplast, as in *T. gondii*; subsequently *P. falciparum* may have lost the mitochondrial location during evolutionary development.

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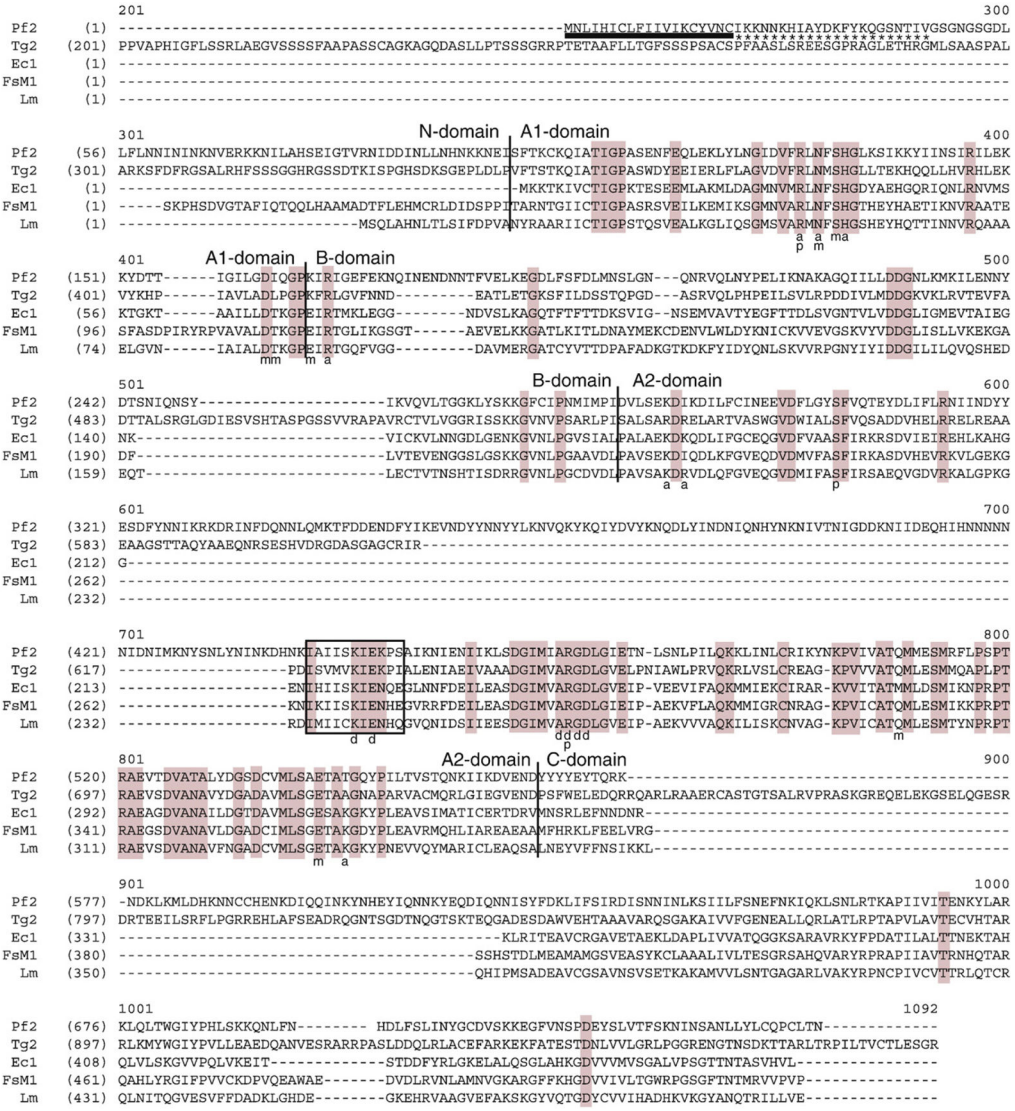


**Fig. 1.**

Amino acid sequence alignment of *P. falciparum* pyruvate kinase type-II isozyme (*Pf*PyKII) with four pyruvate kinases from other species. Sequence data accession numbers are: Pf2, *Pf*PyKII (this study; PF10_0363); Tg2, *Toxoplasma gondii* II (AB118155); Ec1, *Escherichia coli* isozyme I (1PKY_A); FsM1, *Felis catus* isozyme M1 (P11979); Lm, *Leishmania mexicana* (CAA52898). *Vertical lines* indicate divisions between four three-dimensional domains (N, A, B, and C) as described previously [10]. An open black *box* indicates the pyruvate kinase signature sequence (PROSITE, PS00110); *p* indicates PEP binding sites; *a*, ADP binding sites; *d*, divalent cation binding sites; *m*, monovalent cation binding sites; *dashes*, gaps in the alignment. DNA sequences analyses were performed using the VectorNTI suite (InforMax, Executive Way Frederick, MD, USA). The thick underline in the N-terminal of *P. falciparum* sequence is signal sequence, and following asterisks indicates probable plastid transit peptide. It is conceivable that these sequences compose apicoplast

targeting signal. Targeting signals in the N-terminal were analyzed by SignalP [11] and PlasmoAP [12].

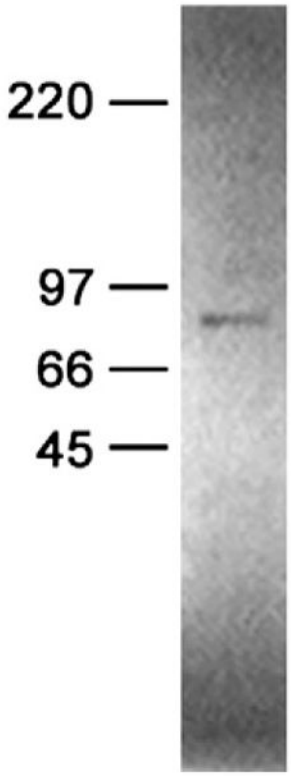


**Fig. 2.**

Specificity of anti-*Pf*PyKII IgG shown by Western blot analysis. The purified recombinant *Pf*PyKII was detected by Western blot analysis with the antibody against the recombinant *Pf*PyKII. Rainbow molecular weight markers (kDa) are indicated on the left.

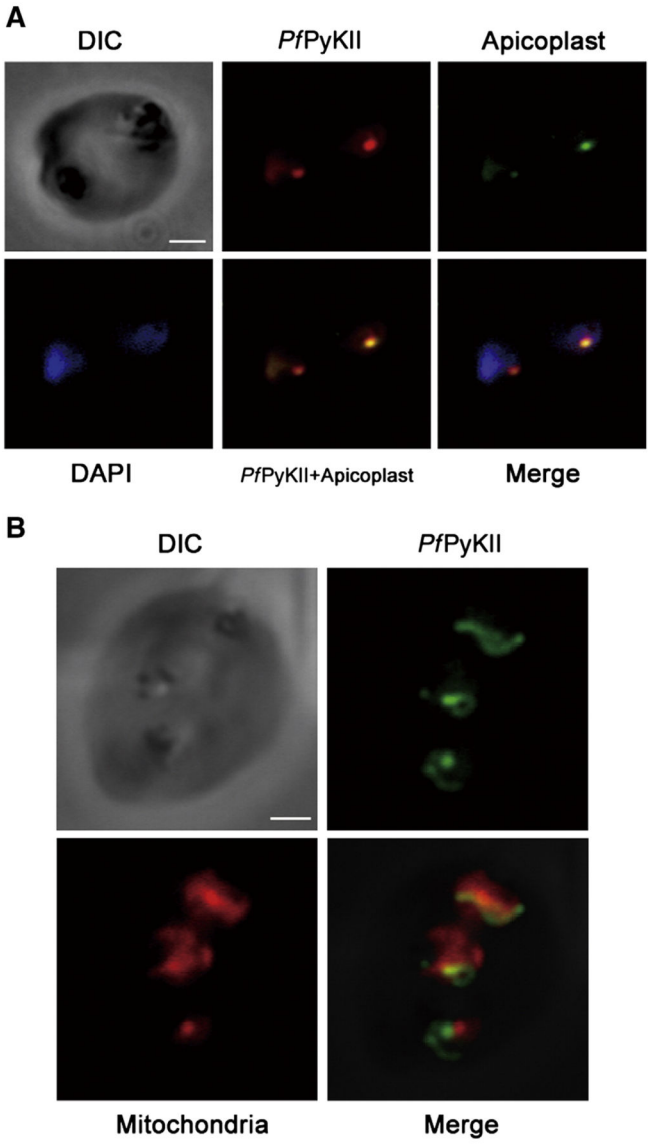


**Fig. 3.**

Immunofluorescent microscopic analysis of co-localization of *Pf*PyKII with the apicoplast, but not with the mitochondrion in red blood cells infected with *P. falciparum*. A: Anti-*Pf*PyKII and anti-*P. falciparum* ACP antibodies detected by AlexaFlour goat anti-mouse 594 (red) and goat anti-rabbit 488 (green) secondary antibodies, respectively. Immunofluoresecnce of *P. falciparum* ACP antibody shows the apicoplast. Merged images indicate co-localization of *Pf*PyKII and *P. falciparum* ACP. Nucleus stained by DAPI (blue). B: Red blood cells infected with parasites expressing citrate synthase fused to GFP targeting the mitochondrion (Tonkin et al. 2004). GFP detected by Cy5-conjugated goat anti-GFP (red). Anti-*Pf*PyKII antibody detected by AlexaFluor goat anti-mouse 488 (green) IgG. Merged image shows that *Pf*PyKII does not co-locate with the mitochondrion. White scale bars are 2 μm.