# The putative effector-binding site of *Leishmania mexicana* pyruvate kinase studied by site-directed mutagenesis

Véronique Hannaert[a,*], Cédric Yernaux[a], Daniel J. Rigden[b], Linda A. Fothergill-Gilmore[c], Fred R. Opperdoes[a], Paul A.M. Michels[a]

[a] *Research Unit for Tropical Diseases, Christian de Duve Institute of Cellular Pathology and Laboratory of Biochemistry, Université Catholique de Louvain, ICP-TROP 74.39 Avenue Hippocrate, B-1200 Brussels, Belgium*
[b] *National Centre of Genetic Resources and Biotechnology, CENARGEN/EMBRAPA, Brasilia-DF, Brazil*
[c] *Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, UK*

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**Abstract The activity of pyruvate kinase of *Leishmania mexicana* is allosterically regulated by fructose 2,6-bisphosphate (F-2,6-$P_2$), contrary to the pyruvate kinases from other eukaryotes that are usually stimulated by fructose 1,6-bisphosphate (F-1,6-$P_2$). Based on the comparison of the three-dimensional structure of *Saccharomyces cerevisiae* pyruvate kinase crystallized with F-1,6-$P_2$ present at the effector site (R-state) and the *L. mexicana* enzyme crystallized in the T-state, two residues (Lys453 and His480) were proposed to bind the 2-phospho group of the effector. This hypothesis was tested by site-directed mutagenesis. The allosteric activation by F-2,6-$P_2$ appeared to be entirely abrogated in the mutated enzymes confirming our predictions.** 

*Key words:* Glycolysis; Pyruvate kinase; Allostery; Fructose-2,6-bisphosphate; *Leishmania mexicana*

## 1. Introduction

Pyruvate kinase (PYK; EC 2.7.1.40) catalyzes the final step of glycolysis, where the phospho group of phospho*enol*pyruvate (PEP) is transferred to ADP to form pyruvate and ATP. In most cells, this reaction is essentially irreversible and is one of the major control points of glycolysis, together with hexokinase and phosphofructokinase. In Trypanosomatidae, the situation is different due to the unique way in which their glycolytic pathway is organized. Trypanosomatids sequester the seven enzymes that convert glucose into 3-phosphoglycerate in a peroxisome-like organelle, termed the glycosome [1]. Only the enzymes catalyzing the last part of glycolysis, phosphoglycerate mutase, enolase and PYK are present in the cytosol. Glycolysis through the organelle seems unregulated: the activities of hexokinase and phosphofructokinase are not subject to regulation by the various effectors that are operational in many other cells [2,3]. In contrast, the activity of cytosolic PYK can be tightly controlled. Kinetic studies have demonstrated a positive cooperativity of the enzyme towards its substrate PEP, and an allosteric activation by micromolar concentrations of fructose 2,6-bisphosphate (F-2,6-$P_2$) [4–6]. Allosterically regulated PYKs from many other eukaryotes are fundamentally different because they respond to fructose 1,6-bisphosphate (F-1,6-$P_2$) and are essentially unresponsive to F-2,6-$P_2$. In most of these organisms, F-2,6-$P_2$ is instead a potent activator of phosphofructokinase. Trypanosomatid glycosomal phosphofructokinase, however, does not respond to F-2,6-$P_2$.

PYK has been extensively characterized in the two trypanosomatids *Trypanosoma brucei* and *Leishmania mexicana* and in the related organism *Trypanoplasma borelli* [5–10]. Like the enzyme of many other organisms, it is a homotetrameric enzyme with a subunit size of approximately 54 500 Da. The crystal structure of *L. mexicana* PYK in the absence of F-2,6-$P_2$ (T-state) has been recently reported [11]. *Leishmania* PYK shares the same overall fold as the enzymes from rabbit muscle, *Escherichia coli* and yeast [12–14]. Each subunit contains four domains: N-terminal, A, B and C. The active site lies in a pocket found between domains A and B; and in the allosterically regulated enzymes, domain C contains the effector site. A comparison between *Leishmania* T-state PYK and the yeast R-state enzyme reveals a possible structural basis for the different effector specificity. Two loops comprising residues 443–453 and 480–489 adopt very different conformations in the two enzymes, and Lys453 and His480 that are a unique feature of the trypanosomatid enzymes alone probably bind to the 2-phospho group of the effector molecule. To test this prediction, we have undertaken site-directed mutagenesis on the *L. mexicana* F-2,6-$P_2$-dependent PYK and assessed the effect of substitution of Lys453 and His480 on the allosteric regulation of the enzyme activity.

## 2. Materials and methods

### 2.1. Cloning of the L. mexicana PYK gene in an expression vector

The gene encoding PYK of *L. mexicana mexicana* (NHOM/B2/84/BEL46) was excised from the pLmPYK recombinant plasmid [6] and cloned into expression vector pET28a using the *Nde*I and *Bam*HI sites. The new recombinant plasmid named pET28a-LmPYK directs, under the control of the T7/lac promoter, the production of a fusion protein bearing an N-terminal extension of 20 residues including a $(His)_6$-tag.

*Corresponding author. Fax: (32)-2-762 68 53.
*E-mail address:* hannaert@trop.ucl.ac.be (V. Hannaert).

*Abbreviations:* F-1,6-$P_2$, fructose-1,6-bisphosphate; F-2,6-$P_2$, fructose-2,6-bisphosphate; PEP, phospho*enol*pyruvate; PYK, pyruvate kinase

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*2.2. Site-directed mutagenesis of L. mexicana PYK*

Site-directed mutagenesis of the *L. mexicana* PYK gene was performed on plasmid pET28a-LmPYK using PCR techniques as described by Mikaelian and Sergeant [15]. The *Leishmania* PYK Lys453 codon AAG was changed into either the Glu codon GAG or the Gln codon CAG, whereas the His codon CAT was changed into the Gln codon CAG or the Lys codon CTT. The proof-reading Vent DNA polymerase (New England Biolabs, USA) was used for amplification. The mutagenized PYK gene fragments were then excised from the plasmid by digestion with *Sac*I and used to replace the corresponding segment in the original plasmid containing the wild-type gene. Correctly mutagenized plasmids were then completely checked by sequencing before they were introduced into *E. coli* for protein expression.

*2.3. Overexpression and purification of wild-type and mutant L. mexicana PYK*

*L. mexicana* wild-type and mutated PYK were overexpressed in *E. coli* BL21(DE3) using the bacteriophage T7-RNA polymerase system [16]. *E. coli* cells containing the wild-type plasmid pET28-LmPYK or its mutant derivatives were grown in 50 ml LB medium supplemented with 30 μg/ml kanamycin. Expression was induced at an $OD_{600nm}$ of 0.5–0.8 by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside and growth was continued overnight at 30°C. Cells were collected by centrifugation (10 000 × *g*, 10 min at 4°C). The cell pellet was resuspended in 5 ml lysate buffer (50 mM triethanolamine/HCl, pH 7.2; 0.5 mM potassium phosphate; 150 mM KCl; 20% glycerol and the protease inhibitors leupeptin, pepstatin and E64, each at a concentration of 1 μM). Cells were lysed by two passages through an SML-Aminco French pressure cell at 800 psi. Nucleic acids were removed first by incubation with 100 units Benzonase (Merck, Germany) for 30 min at 37°C, and then with 5 mg of protamine sulfate for 15 min at room temperature. The lysate was centrifuged (10 000 × *g* 15 min at 4°C) and the supernatant used for purification of the recombinant enzyme by immobilized metal-affinity chromatography (TALON resin, Clontech, USA) exploiting the $(His)_6$-tag at the N-terminus of the PYK. The charged resin was first washed with lysate buffer plus 5 mM imidazole, then with lysate buffer plus 10 mM imidazole. The enzyme was subsequently eluted (1-ml fractions) with 100 mM imidazole in lysate buffer.

*2.4. Measurements of protein concentration and SDS/PAGE*

Protein concentrations were determined using the Bio-Rad protein assay, based on Coomassie brilliant blue [17], with bovine serum albumin (BSA) as standard. Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was done following standard techniques [18].

*2.5. Enzyme assays and kinetic studies*

The activity of PYK was measured spectrophotometrically by following the disappearance of NADH absorbance at 340 nm. The assay was performed at 25°C in a 1-ml reaction mixture containing 50 mM triethanolamine/HCl buffer, pH 7.2, 2.5 mM PEP, 2 mM ADP, 6 mM $Mg_2SO_4$, 50 mM KCl, 0.42 mM NADH and 1.6 units of beef heart lactate dehydrogenase (Roche, Germany). The reaction was initiated by the addition of 10 μl of diluted enzyme (dilution buffer: 25% glycerol; 0.5 mM potassium phosphate; 0.1 mg/ml BSA; 1 mM DTT; 0.2 mM EDTA in 50 mM triethanolamine/HCl buffer pH 7.2, containing 0.05 mM $NaN_3$). One activity unit is defined as the conversion of 1 μmol substrate/min under these standard conditions. Kinetic parameters for the substrates were determined under the above-mentioned reaction conditions with the following substrate concentrations: for PEP at a fixed concentration of 2 mM ADP; for ADP at 5 mM PEP. The modulation of the affinity of the enzymes for PEP by the allosteric effector F-2,6-$P_2$ was determined at 1 and 10 μM of this compound, and at 2 mM ADP, whereas the PEP concentration was varied between 0 and 18 mM. Activities were also measured at subsaturating concentrations of PEP and ADP, 0.7 mM and 0.3 mM, respectively, using varying concentrations of F-2,6-$P_2$ (0 to 11 mM). Kinetic parameters were calculated from Michaelis–Menten and Hill plots after optimal curve-fitting of experimentally determined data, using the SigmaPlot program.

## 3. Results and discussion

*3.1. Site-directed mutagenesis of L. mexicana PYK*

The crystal structure of *Leishmania* PYK has been determined in the T-state [11]. Attempts to soak the effector F-2,6-$P_2$ into crystals of the *Leishmania* enzyme showed no binding in the absence of divalent metal ions, or caused the crystals to crack when the metal ions were present. An understanding of the effector site is thus based on comparisons with the structure of *Saccharomyces cerevisiae* R-state PYK with F-1,6-$P_2$ present at the effector site. Both the two side chain hydroxyl groups and the main chain nitrogen that bind the 6-phospho group of F-1,6-$P_2$ in the yeast PYK are present in the *Leishmania* enzyme where they coordinate a sulfate ion to the protein in a similar manner. The 6-phospho-binding pocket is thus well conserved, but by contrast, the region of the effector site that binds the 1- or 2-phospho group is strikingly different (Fig. 1). Two loops in the effector site have different conformations and contribute to a radically changed pocket. The 2-phospho group probably interacts with either or both of Lys453 and His480, two residues located on these two loops. The altered structure of the Cβ3–Cβ4 loop in the *Leishmania* enzyme, compared to that of the *S. cerevisiae* protein, forces a change of orientation of the sugar ring in the predicted mode of F-2,6-$P_2$ binding. In this position the O1 and O3 hydroxyl groups could form hydrogen bonds with the Arg456 side chain (Fig. 1b) and the backbone oxygen of His483, respectively, while the fructose ring oxygen might hydrogen bond to the main chain nitrogen of Asn401. Despite these possible favorable interactions, it remains possible that an alteration in the Cβ3–Cβ4 loop conformation upon the transition to the R-state might result in the *Leishmania* enzyme acquiring a more 'open' structure, similar to that of the *S. cerevisiae* enzyme. The model also suggests, interestingly, that Arg456 might also bind to the 2-phospho group of F-2,6-$P_2$, in contrast to the role of the corresponding yeast residue Arg459 which binds the 1-phospho group of F-1,6-$P_2$ (Fig. 1).

Site-directed mutagenesis of *L. mexicana* PYK was used to confirm the importance of these residues for the effector binding. Lys453 and His480 were replaced by Gln (K453Q and H480Q) to eliminate the charge while retaining H-bonding ability, and by the negatively charged Glu (K453E) or Leu (H480L) with the intention to abolish the binding of the effector.

*3.2. Overexpression of the wild-type and mutant L. mexicana PYK in E. coli and purification of the enzymes*

*L. mexicana* wild-type and mutant PYKs were expressed in *E. coli* with a 20-residue N-terminal extension including six His residues. Optimal production of active, soluble proteins was obtained by inducing expression overnight at 30°C. Purification of the proteins was performed using immobilized metal-affinity chromatography exploiting the $(His)_6$-tag. Proteins were more than 95% pure as assessed by SDS/PAGE. A summary of the purification data is given in Table 1. Comparable amounts of pure proteins could be produced for the wild-type, K453E and K453Q PYK, but lower production levels were achieved for the two other mutants. In general, specific activities are lower for mutant proteins when compared with the wild-type PYK, except for K453Q.

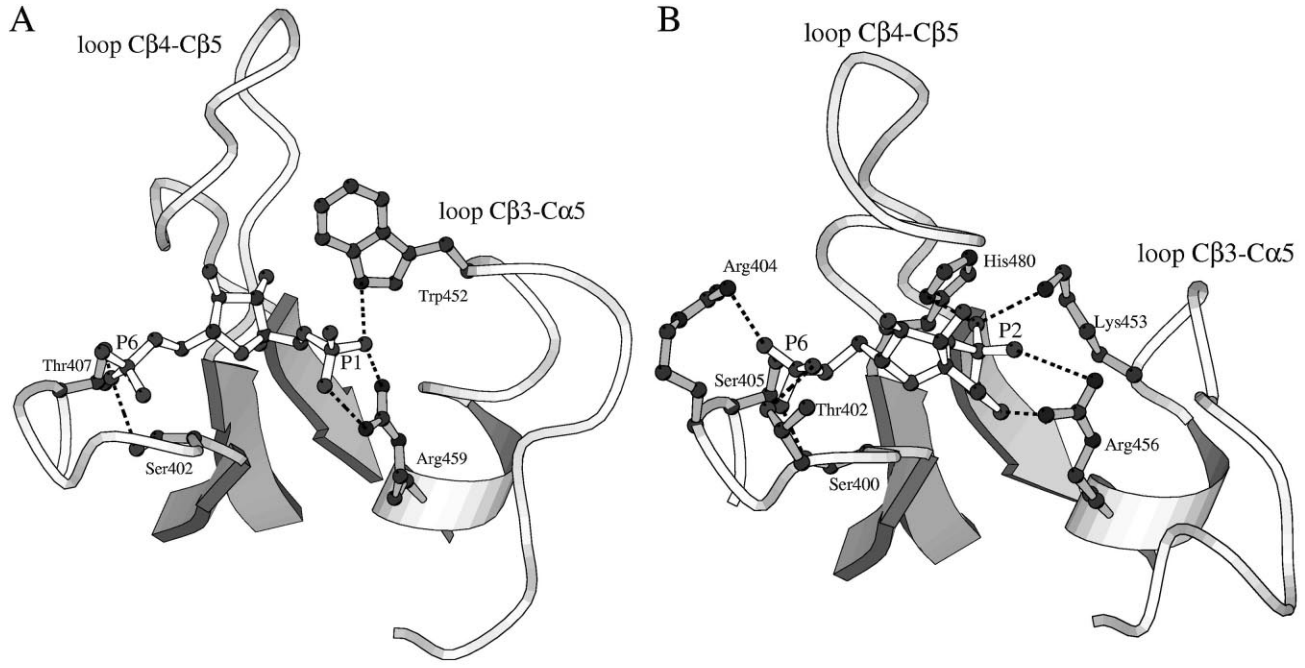


Fig. 1. Molscript [21] comparison of (a) the effector site of *S. cerevisiae* PYK with experimentally observed bound F-1,6-$P_2$ with (b) the corresponding region of *L. mexicana* PYK with a modelled conformation of bound F-2,6-$P_2$. The F-2,6-$P_2$ conformation was modelled by manual docking using O [22]. The side chains binding, or predicted to bind, the effector are labelled and drawn with shaded bonds in order to help distinguish them from the effector molecules. Possible hydrogen bonds are represented by dotted lines.

### 3.3. Kinetic analysis of the wild-type and mutant *L. mexicana* PYKs

Kinetic properties of wild-type and mutated PYKs have been determined with respect to the substrates PEP and ADP, and to the effector F-2,6-$P_2$ (Table 2). In the absence of F-2,6-$P_2$, substitution of Lys453 by Glu or Gln, and of His480 by Gln did not significantly alter the apparent affinity of PYK for its substrate PEP, with the concentrations of PEP giving half-maximal velocity values ($S_{0.5}$) and the Hill coefficients ($n$) all being comparable in wild-type and mutant enzymes. Addition of 10 μM of F-2,6-$P_2$ to wild-type PYK reduces the $S_{0.5}$ value of PEP from 2.67 to 0.57 mM without modification of the Hill coefficient. This concentration of F-2,6-$P_2$ was necessary to reach the maximal affinity for the substrate; only a lower level of activation was observed with 0.1 and 1 μM of the effector (data not shown). In contrast, none of the mutant enzymes is activated by F-2,6-$P_2$ at any of these concentrations. The kinetic profiles of the mutant enzymes resemble the low-affinity, unactivated wild-type PYK (Fig. 2).

The behavior of wild-type PYK and each mutant towards the second substrate (ADP) is similar: hyperbolic kinetics were observed with only marginal differences between the different forms of the enzyme.

Determination of the kinetics of wild-type PYK and its mutants with respect to the activator F-2,6-$P_2$ revealed important alterations (Table 2). All mutants showed a largely reduced apparent affinity for F-2,6-$P_2$. Modification of Lys453 into Glu almost completely abolished the binding of the activator, while the three other mutants showed a reduced apparent affinity: 234-, 42- and 22-fold in K453Q, H480L and H480Q, respectively. The Hill coefficient for F-2,6-$P_2$ binding decreases from a value of 1.95 in wild-type PYK to a value between 1.26 and 1.41 in the mutants.

All the substitutions made here result in a strongly decreased affinity for the activator in perfect agreement with the notion that Lys453 and His480 directly interact with the effector. The degree of reduction of F-2,6-$P_2$ affinity mirrors the degree of the change made in characteristics of the binding residues. Of the different mutants constructed, K453E appeared to be the most affected in the binding of F-2,6-$P_2$. This may be readily explained since the newly introduced negative charge would be positioned close to the F-2,6-$P_2$-binding site causing severe charge repulsion towards the in-

Table 1
Purification of recombinant wild-type and mutant *L. mexicana* PYKs from *E. coli* cells

| Type of enzyme | Total protein (μg) | Total activity (U) | Specific activity (U/mg) |
|---|---|---|---|
| Wild type | 742 | 59 | 79.5 |
| K453E | 726 | 24.7 | 34 |
| K453Q | 718 | 98.4 | 137 |
| H480L | 413 | 6.6 | 16 |
| H480Q | 303 | 10.7 | 35.3 |

The values given are for purifications from 50 ml of bacterial culture.

Table 2
Properties of recombinant wild-type *L. mexicana* PYK with respect to the substrates PEP and ADP, and to the effector F-2,6-$P_2$, compared with those of mutant PYKs

| Ligand | Modulator | Parameter | Wild type | K453E | K453Q | H480L | H480Q |
|---|---|---|---|---|---|---|---|
| PEP[a] | none | $S_{0.5}$ (mM) | 2.67 ± 0.03 | 2.37 ± 0.07 | 2.33 ± 0.09 | 3.56 ± 0.08 | 2.57 ± 0.10 |
| | | *n* | 1.59 | 1.70 | 1.54 | 1.85 | 1.61 |
| | F-2,6-$P_2$ | $S_{0.5}$ (mM) | 0.57 ± 0.02 | 1.96 ± 0.05 | 2.45 ± 0.08 | 3.24 ± 0.10 | 2.41 ± 0.06 |
| | | *n* | 1.26 | 1.88 | 1.50 | 1.74 | 1.59 |
| ADP[b] | | $K_m$ (mM) | 0.37 ± 0.02 | 0.49 ± 0.04 | 0.46 ± 0.02 | 0.69 ± 0.05 | 0.64 ± 0.04 |
| | | *n* | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| F-2,6-$P_2$[c] | | $S_{0.5}$ (μM) | 0.853 ± 0.034 | 35 420 ± 2 573 | 200 ± 9.63 | 35.98 ± 1.22 | 18.54 ± 0.61 |
| | | *n* | 1.95 | 1.41 | 1.34 | 1.30 | 1.26 |

The kinetic assay conditions for wild-type and mutant PYK were the following: the experiments were performed at 25°C in 50 mM triethanolamine/HCl, pH 7.2, containing 50 mM KCl and 6 mM $Mg_2SO_4$. The values given are means ± S.D. *n*, Hill coefficient.
[a]Buffer supplemented with 2 mM ADP and variable concentrations of PEP.
[b]Buffer supplemented with 5 mM PEP and variable concentrations of ADP.
[c]Buffer supplemented with 0.3 mM ADP and 0.7 mM PEP and variable concentrations of F-2,6-$P_2$.

coming ligand. The 234-fold affinity reduction of the K453Q shows that the simple hydrogen bond that could be made by the new Gln is a poor substitute for the original ionic interaction. The affinity reductions on substitution of His480 are less severe suggesting, perhaps, that His480 interacts with only one of the 2-phospho group oxygens while Lys453 makes multiple interactions, or that His480 is not fully ionized at the pH of the assay thereby diminishing the strength of its interaction with F-2,6-$P_2$. Again, the less drastic substitution of a Gln, retaining the hydrophilicity and hydrogen-bonding capability of the original His, leads to a lesser affinity reduction compared to the H480L mutation. The mutants' retention of pronounced sigmoidal kinetics in the presence of the allosteric activator suggests that Lys453 and His480 have a role, not just in effector binding, but also in transmission of the allosteric signal. It may be no coincidence that these two residues are positioned on the side of the effector-binding pocket closest to the subunit interface.

Because of its vital role in trypanosomatid metabolism and its unique kinetic characteristics, PYK has been proposed as a potential target for drugs against these highly pathogenic human parasites [5,9–11]. Notably, the effector-binding pocket is considered as a site for the binding of ligands that may cause alterations in the enzyme's activity and thus in the parasite's metabolism. Indeed, it has been shown that fructose-6-phosphate analogues such as 2,5-anhydro-D-mannitols inhibit specifically the activity of the trypanosomatid enzyme by binding at its regulatory site [19,20]. The mutant PYK forms here described provide further information about residues in the effector-binding pocket that confer the unique allosteric regulation to the trypanosomatid PYKs. Moreover, they may

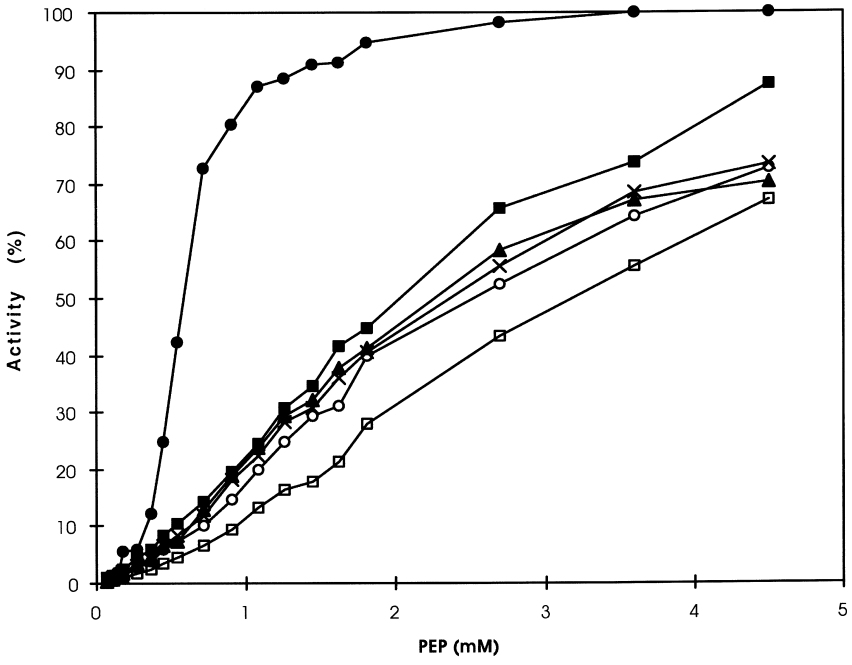


Fig. 2. PEP titration curves for wild-type and mutant PYKs in the presence or absence of 10 μM F-2,6-$P_2$. (○) wild-type−F-2,6-$P_2$, (●) wild-type+F-2,6-$P_2$, (■) K453E+F-2,6-$P_2$, (▲) K453Q+F-2,6-$P_2$, (□) H480L+F-2,6-$P_2$, (×) H480Q+F-2,6-$P_2$. Data were fit to Hill equation and the resulting kinetic parameters are given in Table 2.

assist in future screenings of selective enzyme inhibitors exploiting the unique regulatory site of the parasite's enzyme and in the analysis of the mechanism by which such inhibitors exert their inhibitory effect.

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