# Supporting Information

## Morgan et al. 10.1073/pnas.1217157110

### SI Materials and Methods

**Cloning.** cDNA corresponding to human M1PYK (M1 isoform of pyruvate kinase) and M2PYK isoforms were synthesized by GENSCRIPT. An *NdeI* restriction site was added to the 5′ end of each gene, which also incorporated the ATG start codon. An *XhoI* restriction site was added downstream of the stop codon. All genes were codon optimized for *Escherichia coli* expression. Each gene was cloned by GENSCRIPT into a pUC57 vector. Inserts were digested from the pUC57 using *NdeI* and *XhoI* restriction enzymes and then purified using the Qiagen gel purification kit. The digested inserts were ligated into an *NdeI-XhoI*-digested pET28a vector (Novagen) and verified by DNA sequencing. A Quikchange site-directed mutagenesis kit (Stratagene) was used to generate all mutants as per the manufacturer's instructions.

**Expression of Human Pyruvate Kinases.** Chemically competent BL21(DE3) *E. coli* cells (Novagen) were transformed with pET28a_M2PK or pET28a_M1PK. Single colonies of transformed *E. coli* harboring the *M2PYK* gene construct were picked from lysogeny broth kanamycin plates (50 μg/mL) and used to inoculate 50 mL of 2× tryptone yeast-extract (TY) medium (containing 50 μg/mL of kanamycin). Cultures were grown overnight at 37 °C with agitation. Twenty milliliters of the overnight culture was used to inoculate 1 L of 2× TY medium containing kanamycin (50 μg/mL). One-liter cultures were grown to an $OD_{600}$ of 0.8–1.0, and expression was induced at 20 °C by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. The incubation was then continued for 18 h, and the cells were harvested at 15,500 × *g* in a JLA-9.1000 rotor for 12 min at 10 °C. One-liter cell pellets were then frozen in liquid nitrogen and stored at −80 °C.

**Purification of Human Pyruvate Kinases.** All purification steps were performed at 4 °C unless stated otherwise. Cell pellets were resuspended in lysis buffer [50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole (pH 8.0), and 1 tablet EDTA free protease inhibitors (Roche)] to a final volume of 30 mL/5 g (~1 L of cell culture) of cells and lysed using a constant cell disruption system (pressure set to 25 MPa). The lysate was centrifuged at 23,000 rpm at 10 °C for 45 min, and the supernatant was filtered through a 0.2-μm syringe filter. The supernatant was loaded onto a 5-mL immobilized metal affinity Hitrap HP Sepharose column (precharged with cobalt) at 2 mL $min^{-1}$. The column was maintained at a constant flow rate (2 mL $min^{-1}$) throughout the purification process. The column was washed with 10 column volumes (CV) of buffer A (50 mM $NaH_2PO_4$, 300 mM NaCl, and 20 mM imidazole, pH 8.0). An additional wash step (15 CVs) of 20% (vol/vol) buffer B (50 mM $NaH_2PO_4$, 300 mM NaCl, and 250 mM imidazole, pH 8.0) was also included, which minimized contaminating proteins. The $His_6$M1/2PYK protein was eluted over a five-CV gradient using 100% buffer B. Eluted fractions were pooled and concentrated using a Vivaspin column (GE Healthcare) (molecular mass cutoff, 30 kDa). Samples containing $His_6$M1/2PYK were pooled, concentrated (10–20 mg/mL) and loaded onto a Superdex 200 10/300 GL column, pre-equilibrated with Dulbecco's PBS without calcium and magnesium [PBS-CM; Sigma catalog no. D5652 (8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 2.7 mM KCl, and 137 mM NaCl, pH 7.4)]. $His_6$M1/2PYK was concentrated using a Vivaspin column (molecular mass cutoff, 30 kDa) and quantified using the theoretical extinction coefficient, ε280 nm = 28,590 $M^{-1}{\cdot}cm^{-1}$.

**Measurement of M2PYK Activity.** A 25-mL solution of 2× assay buffer (PBS-CM supplemented with 4 mM ADP, 40 U/mL LDH, 20 mM $MgCl_2$, 200 mM KCl, 1 mM DTT, and 1 mM NADH) was prepared fresh on the day of experiments. Five milliliters of assay was removed, and phosphoenolpyruvate (PEP) as added to a final concentration of 10 mM (PEP solution). Twofold serial dilutions of PEP were prepared in a 96-well master block (Greiner Bio-one catalog no. 780270) using 2× assay buffer for dilution; PEP concentrations typically spanned 10 mM to 78 μM. Using a multichannel pipette, 50 μL of each PEP concentration was transferred to a column on a 96-well plate.

M2/1PYK samples were diluted from 10 mg/mL to 0.002 mg/mL in PBS-CM containing 1 mM DTT. For fructose-16-bisphosphate (F16BP) experiments, 1 mM F16BP (100 mM stock was prepared in PBS-CM buffer) was included in the 0.002-mg/mL protein solution. All protein solutions were incubated for 30 min at 37 °C before the assay. To start the reaction 50 μL of M2/1PYK (using a multichannel pipette) was added to a column of wells containing the PEP titration (plates were pre incubated for 10 min at 37 °C in the SpectraMax M5 microplate reader). Final reagent concentrations were as followsL 0.001 mg/mL M2/1PYK, 2 mM ADP, 0.5 mM NADH, 100 mM KCl, 10 mM $MgCl_2$, 20 U/mL LDH, ±500 μM F16BP, 1 mM DTT, and 1× PBS. The PEP solution was serially diluted from 5 mM to 39 μM.

M2PYK modulator assays were performed as follows. Two solutions were prepared to generate serial dilutions for modulator titration experiments. Solution 1 was PBS-CM supplemented with 1 mM DTT and 0.0025 mg $mL^{-1}$ of M2/1PYK. Solution 2 was PBS-CM supplemented with 0.0025 mg $mL^{-1}$ of M2/1PYK and modulator, either 20 μM triiodo-L-thyronine (25 mM stock solution prepared in 100 mM NaOH), or 1 mM F16BP (100 mM stock solution prepared in PBS-CM) or, 10 mM Phe (80 mM stock). When required, the pH values of all ligand stocks were adjusted to 7.4. Twofold serial dilutions were prepared in a 96-well master block using solutions 1 and 2 and incubated for 30 min at 37 °C before the assay. To start the reaction 50 μL of M2/1PYK plus modulator (using a multichannel pipette) was added to a column of wells containing 2× assay buffer supplemented with 1 mM PEP (plates were preincubated for 10 min at 37 °C in the SpectraMax M5 microplate reader). Final reagent concentrations were as follows: 0.00125 mg/mL M2PYK, 2mM ADP, 0.5 mM PEP, 0.5 mM NADH, 100 mM KCl, 10 mM $MgCl_2$, 20 U/mL LDH, and 1× PBS. The modulator solutions were serially diluted over the range required for $IC_{50}$ determination.

Plates were agitated for 10 s, and the decrease in absorbance at 340 nm was measured for 2 min using a plate reader. Initial reaction rates were determined using SoftMax Pro software (Molecular Devices). Substrate–velocity curves were plotted using KaleidaGraph graph-plotting software (Synergy Software).

The kinetic parameters ($V_{max}$, $S_{0.5(PEP)}$, and $n_H$) were determined by fitting the data to Eq. **1** (allosteric equation), and $IC_{50}$ values were determined by fitting the data to Eq. **2** (a four-parameter logistic model) using KaleidaGraph v3.6 software (Synergy Software).

$$(a * x \wedge b)/(c + x \wedge b) \quad \textbf{[1]}$$

$$a + (d\text{-}a)/(1 + 10 \wedge ((x\text{-}e) * f)). \quad \textbf{[2]}$$

The terms are as follows: *a* is the maximum response ($V_{max}$); *b* is a measure of cooperativity ($n_H$); *c* is the substrate concen-

tration that provokes a response halfway between baseline and the maximum response [$S_{0.5(PEP)}$]; $d$ is the baseline response; $e$ is the ligand concentration that provokes a response halfway between baseline and maximum; and $f$ is the slope. In Eq. **1** when $b = 1$ this equation is the same as the Michaelis-Menten equation.

**Crystallization and Data Collection.** Single crystals of M1PYK and M2PYK were obtained at 17 °C by vapor diffusion using the hanging drop technique. The drops were formed by mixing 1.5 μL of protein (10 mg/mL) solution with or without ligands [Phe was added to a final concentration of 25 mM; ATP, oxalic acid (OX), and F16BP were added to a final concentration of 1 mM] with 1.5 μL of a well solution, composed of 10–16% PEG 8,000 or 3,350, 100 mM sodium cacodylate (pH 7.0), 50 mM $MgCl_2$, and 100 mM KCl. The drops were equilibrated against a reservoir filled with 0.5 mL of well solution. Crystals grew to maximum dimensions (0.5 × 0.2 × 0.1 mm) after 2 wk. Before data collection crystals were dipped in a freezing solution consisting of well solution supplemented with 25% (vol/vol) glycerol, which eliminated the appearance of ice rings. Diffraction data were collected using both a rotating copper anode generator (φ scans were 1° over 360°) and at the Diamond synchrotron radiation facility in Oxfordshire, United Kingdom on beamline IO3 to a resolution of 2.55 Å (M2PYK-ATP/OX/F16BP), 2.9 Å (M2PYK-R489A-Phe), and 2.85 Å (M1PYK). All datasets were obtained from a single crystal flash-frozen in liquid nitrogen at 100 K.

**Structure Determination.** All M1 and M2 structures were solved by molecular replacement using the program PHASER (1). A monomer from the previously determined tetrameric structure of M2PYK (3BJF, accession no. NM_002654) was divided into two search regions. Region 1 (residues 117–219, which is equivalent to a complete B-domain) and region 2 (residues 1–116, 220–532) served as search models. There was a clear molecular replacement solution for all structures. The resulting model was then subjected to 10 cycles of rigid body refinement using the program REFMAC (2). For M1PYK, residues were mutated to correspond to the published M1PYK amino acid sequence (accession no. NM_182470) using the program COOT (3). Further manual changes were made to models to adjust side chain conformations and add additional residues to the N terminus. The model was then subjected to several rounds of restrained refinement, and ligands molecules were added where clear unbiased $F_o$-$F_c$ electron density was observed. Water molecules were added to the model using COOT, and after several rounds of restrained refinement $R/R_{free}$ values converged (Table S2). For M2PYK-ATP/OX/F16BP and M2PYK-R489A-Phe, translation libration screw (TLS) refinement was used. TLS groups were residues 26–36, 37–223, 224–388, and 389–531 for the M2PYK-ATP/OX/F16BP structure and 28–116, 117–214, 215–390, and 391–531 for the M2PYK-R489A-Phe structure.

**ACKNOWLEDGMENTS.** We thank Dr. J. Dornan for excellent advice and practical help, and the staff at the Synchrotron facilities at European Synchrotron Radiation Facility, Grenoble, France and Diamond, United Kingdom. This project has been funded by the Medical Research Council. The Centre for Translational and Chemical Biology and the Edinburgh Protein Production Facility were funded by the Wellcome Trust and the Biotechnology and Biological Sciences Research Council.

1. McCoy AJ, et al. (2007) Phaser crystallographic software. *J Appl Cryst* 40(Pt 4):658–674.
2. Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 53(Pt 3):240–255.
3. Emsley P, Cowtan K (2004) Coot: Model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60(Pt 12 Pt 1):2126–2132.

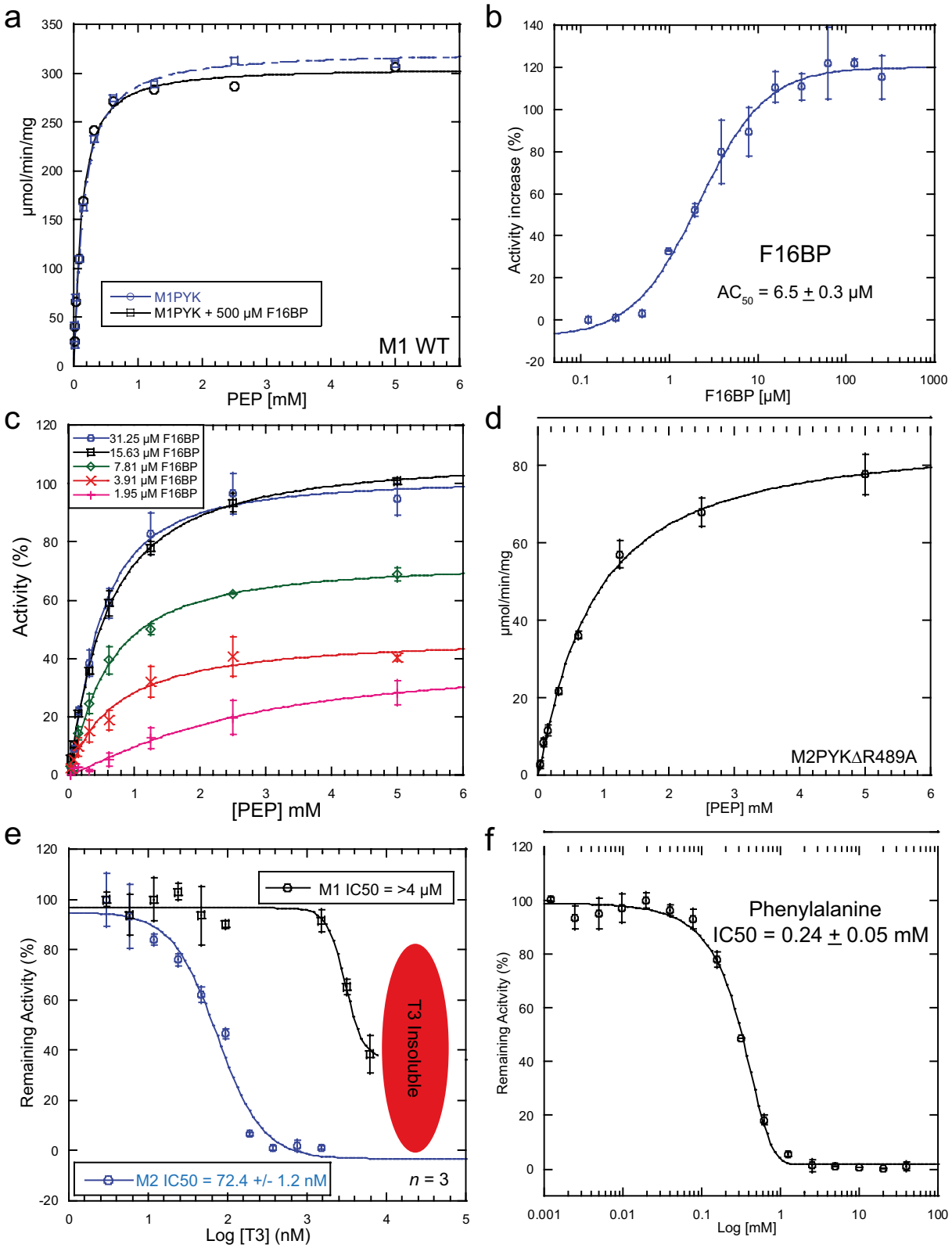



**Fig. S1.** PYK kinetics. Kinetic measurements were performed at 37 °C in the absence (black line) or presence of saturated F16BP (blue dashed line). Concentration response curves observed for the titration of PEP against (*A*) M1PYK and (*D*) M2PYKΔR489A. Concentration–response curves observed for titration of (*B*) F16BP, (*E*) T3, and (*F*) Phe against M2PYK activity. (*C*) Kinetic behavior of M2PYK shows characteristics of V-type allosteric regulation. Changes were observed in $V_{max}$ and $S_{0.5(PEP)}$ in the presence of increasing concentrations (1.95–31.25 µM) of the allosteric effector F16BP.

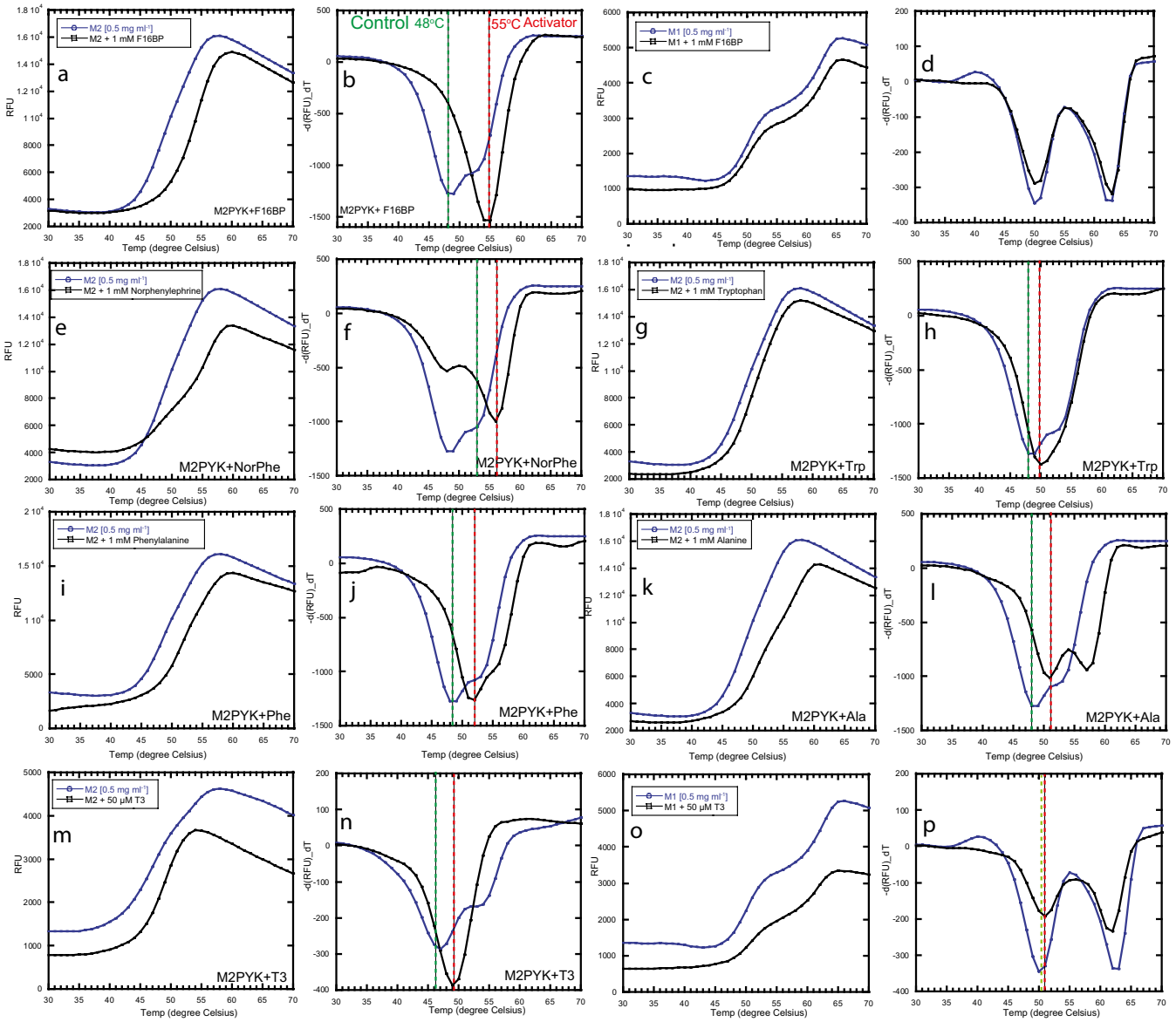



**Fig. S2.** Stabilization of M2PYK by binding modulators. Thermal shift assay results for 0.5 mg/mL M2PYK and M1PYK in the absence (blue line) and the presence (black line) of modulators; (*A* and *B*) M2PYK plus 1 mM F16BP; (*C* and *D*) M1PYK plus 1 mM F16BP; (*E* and *F*) M2PYK plus 1 mM norphenylephrine; (*G* and *H*) M2PYK plus 1 mM tryptophan; (*I* and *J*) M2PYK plus 1 mM phenylalanine; (*K* and *L*) M2PYK plus 1 mM alanine; (*M* and *N*) M2PYK plus 1 µM T3; (*O* and *P*) M1PYK plus 1 µM T3. The unfolding of M1PYK in PBS buffer gives $T_m$ values of 50 °C and 62 °C. The unfolding of M2PYK in PBS buffer gives $T_m$ values of 48 °C and 54–55 °C, which shift to 49 °C in the presence of saturating T3. No significant shift is observed for M1PYK in the presence of saturating T3. A green dashed line indicates the control $T_m$, and a red dashed line indicates the $T_m$ in the presence of ligand.

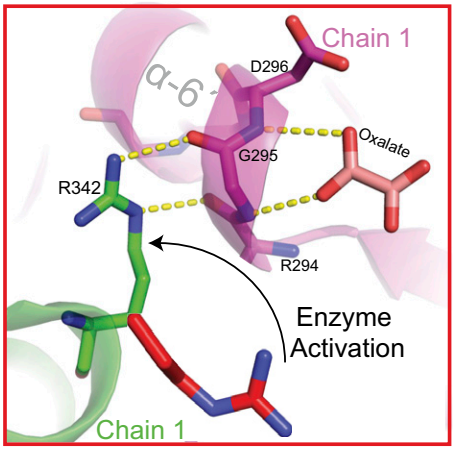



**Fig. S3.** Active site formation. A section of the A-A interface for the F16BP-bound M2PYK structure (chain 1 is shown in purple, and chain 2 is shown in green). The active site is formed when Arg342 from chain 1 hydrogen bonds to the Aα6′ helix (chain 2). The hydrogen bond interactions between Arg342 (NE)...Arg294 (O) and Arg342 (NH2)...Gly295 (O) stabilize the Aα6′ helix, allowing the substrate PEP (or oxalic acid) to bind. Without the interchain Arg342 bridge, monomeric M2PYK is unable to form the active site, therefore rendering it inactive.

**Table S1. Identification of metabolites as PYK modulators**

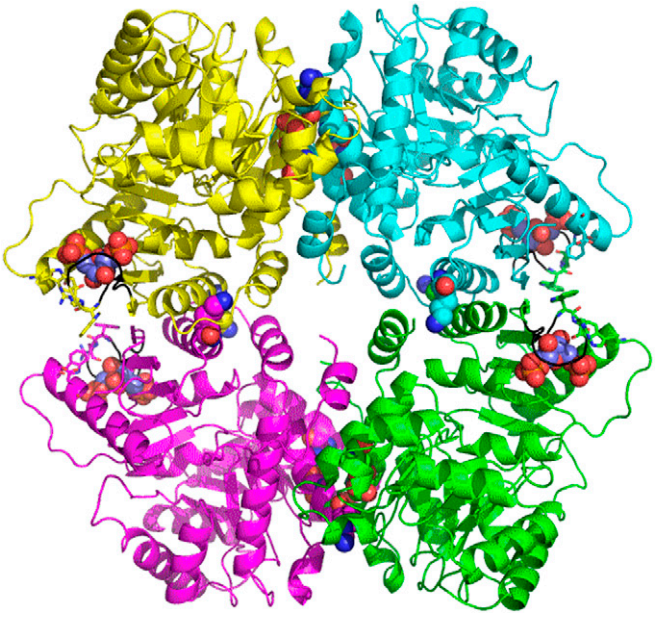
| Compound | M2PYK activity (%) | SD | M1PYK activity (%) | SD |
|---|---|---|---|---|
| Phenylalanine | 0 | 0 | 75 | 4 |
| Alanine | 10 | 4 | 109 | 12 |
| 1,3-Dihydroxyacetone dimer | 22 | 3 | 95 | 10 |
| Tryptophan | 24 | 2 | 92 | 8 |
| Oxalic acid | 42 | 13 | 42 | 7 |
| Ribose-5-phosphate | 51 | 3 | 102 | 8 |
| Thymidine-5-monophosphate | 52 | 7 | 79 | 12 |
| GMP | 52 | 2 | 92 | 14 |
| Methionine | 53 | 13 | 98 | 9 |
| 2,3-Diphosphoglyceric acid | 54 | 8 | 88 | 22 |
| 3-Methyl histidine | 62 | 5 | 94 | 15 |
| Proline | 62 | 3 | 95 | 7 |
| Deoxyguanosinediphosphate | 63 | 0 | 95 | 4 |
| Fructose-6-phosphate | 69 | 4 | 94 | 6 |
| Valine | 70 | 5 | 91 | 17 |
| ATP | 71 | 1 | 103 | 6 |
| AMP | 75 | 5 | 93 | 13 |
| Leucine | 79 | 10 | 95 | 8 |
| *N*-Acetyl-L-alanine | 83 | 5 | 103 | 5 |
| Hydroxyproline | 85 | 7 | 96 | 8 |
| D-Fructose-6-phosphate | 86 | 6 | 90 | 14 |
| Glutamine | 86 | 5 | 99 | 12 |
| Threonine | 91 | 26 | 109 | 7 |
| Glycine | 91 | 4 | 95 | 15 |
| Isoleucine | 91 | 32 | 92 | 9 |
| ADP | 95 | 10 | 108 | 18 |
| Taurine | 97 | 10 | 95 | 10 |
| Uridine | 97 | 5 | 92 | 15 |
| Succinic acid | 99 | 5 | 99 | 6 |
| Lysine | 99 | 30 | 91 | 16 |
| D-Glucose-6-phosphate | 99 | 4 | 102 | 7 |
| D-Glucose | 100 | 5 | 97 | 9 |
| Arginine | 100 | 3 | 100 | 5 |
| D/L-Isocitrate | 102 | 2 | 100 | 6 |
| Malonic acid | 103 | 18 | 97 | 14 |
| Fumarate | 106 | 11 | 96 | 15 |
| PEP | 108 | 7 | 98 | 14 |
| Thymidine | 110 | 7 | 84 | 19 |
| Glutamic acid | 111 | 15 | 94 | 7 |
| Citric acid | 112 | 8 | 133 | 17 |
| D-Fructose | 114 | 10 | 94 | 10 |
| Galactose | 117 | 13 | 99 | 5 |
| Glucoheptonic acid | 119 | 15 | 92 | 9 |
| Methyl-malonic acid | 122 | 4 | 95 | 8 |
| Glycylglycine | 133 | 4 | 102 | 7 |
| Glucuronic acid | 142 | 18 | 96 | 8 |
| Thiamine | 144 | 18 | 95 | 12 |
| α-Ketoglutarate | 154 | 20 | 98 | 8 |
| Cys-HCl | 177 | 14 | 132 | 9 |
| Succinic acid | 187 | 31 | 97 | 9 |
| *N*-Acetyl glutamic acid | 230 | 62 | 95 | 5 |
| Cis-aconitic acid | 240 | 9 | 96 | 13 |
| Malic acid | 262 | 33 | 101 | 5 |
| Tartaric acid | 276 | 68 | 96 | 9 |
| Maleic acid | 262 | 33 | 101 | 8 |
| Histidine | 303 | 8 | 112 | 13 |
| Serine | 391 | 15 | 130 | 7 |
| Fructose-1,6-bisphosphate | 624 | 28 | 95 | 1 |

Activity is expressed as a percentage deviation from that of the WT enzyme in the absence of ligand, which equals 100%. Assay conditions: PBS (8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 2.7 mM KCl, and 137 mM NaCl, pH 7.4) buffer with saturating [ADP] = 2 mM, [KCl] = 100 mM, and [$MgCl_2$] = 10 mM and subsaturating [PEP] = 0.5 mM in the presence of 1 mM ligand. Red, activity ≤75% to that of WT in the absence of modulator; green, activity ≥125% to that of WT in the absence of modulator. Cys-HCl, cysteine hydrochloride.

**Table S2. Data collection and refinement statistics**

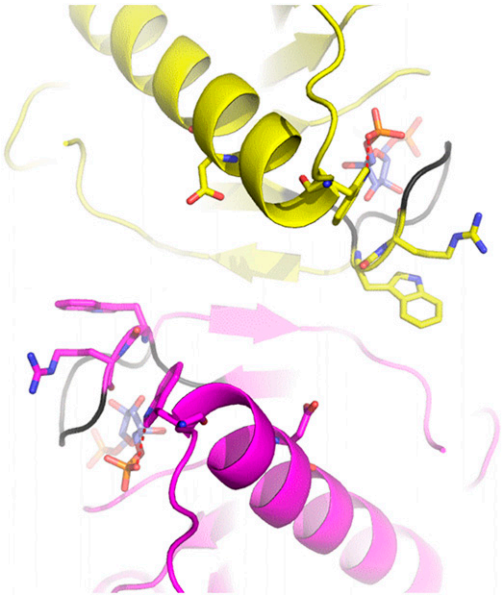
| Parameter | M2PYK-R489A (T-State) | M2PYK-ATP/OX/F16BP (R-State) | M1PYK |
|---|---|---|---|
| Data collection | | | |
| Space group | $P2_1$ | $P2_1$ | C2 |
| Cell dimensions | | | |
| *a*, *b*, *c* (Å) | 97.6, 70.7, 168.3 | 81.6, 139.4, 111.3 | 238.0, 192.2, 109.1 |
| α, β, γ(°) | 90.0, 105.92, 90 | 90.0, 90.5, 90.0 | 90, 90, 90 |
| Solvent content (%) | | 48 | 53 |
| Wavelength (Å) | 0.98 | 0.98 | 0.98 |
| Resolution | 80.94–2.91 | 66.1–2.55 | 40.0–2.85 |
| Rmerge (%) | 7.9 (79.5) | 8.5 (63.3) | 9.8 (34.6) |
| I/σI | 12.6 (2.0) | 15.6 (2.6) | 6.1 (2.3) |
| % completeness | 99.6 (99.9) | 98.2 (93.6) | 93.8 (95.5) |
| Multiplicity | 3.7 (3.8) | 6.9 (6.5) | 2.1 (2.1) |
| Refinement | | | |
| Monomers in ASU | 4 | 4 | 8 |
| No. reflections | 46,146 | 75,250 | 101,863 |
| $R_{work}/R_{free}$ | 25.8/26.5 | 21.5/25.4 | 22.3/28.0 |
| Average B-factor (Å$^2$) | 31.4 | 41.6 | 41.4 |
| No. residues | 1,995 | 2,457 | 4,098 |
| rmsd | | | |
| Bond lengths (Å) | 0.005 | 0.006 | 0.006 |
| Bond angles (°) | 0.75 | 0.88 | 0.918 |

Values in the parentheses are for the outer shell. Five percent of reflections were used as a test set for the calculation of $R_{free}$.



**Movie S1.** The R- to T-state transition. Phe (black carbon spheres) binding induces T-state conformational change, whereas F16BP (navy carbon spheres) induces R-state. Formation (and destruction) of major interactions bridging either the C-C or A-A interface are shown by spheres. Individual chains are highlighted (cyan, yellow, pink, and green), and effector loops are colored in black.

Movie S1



**Movie S2.** A view of part of the C-C interface (orthogonal to that shown in movie 1). An enlargement of the C-C interface highlights conformational changes and side chain movements that occur as the protomers rotate from R- (effector bound) to T- (Phe bound) state. Effector loops colored black and hydrogen bonds shown as dashed red lines. The effector loop curls round F16BP to stabilize the R-state.

Movie S2