Structural basis for allosteric regulation of pyruvate kinase M2 by phosphorylation and acetylation

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Pyruvate kinase muscle isoform 2 (PKM2) is a key glycolytic enzyme and transcriptional coactivator and is critical for tumor metabolism. In cancer cells, native tetrameric PKM2 is phosphorylated or acetylated, which initiates a switch to a dimeric/monomeric form that translocates into the nucleus, causing oncogene transcription. However, it is not known how these post-translational modifications (PTMs) disrupt the oligomeric state of PKM2. We explored this question via crystallographic and biophysical analyses of PKM2 mutants containing residues that mimic phosphorylation and acetylation. We find that the PTMs elicit major structural reorganization of the fructose 1,6-bisphosphate (FBP), an allosteric activator, binding site, impacting the interaction with FBP and causing a disruption in oligomerization. To gain insight into how these modifications might cause unique outcomes in cancer cells, we examined the impact of increasing the intracellular pH (pHi) from ~7.1 (in normal cells) to ~7.5 (in cancer cells). Biochemical studies of WT PKM2 (wtPKM2) and the two mimetic variants demonstrated that the activity decreases as the pH is increased from 7.0 to 8.0, and wtPKM2 is optimally active and amenable to FBP-mediated allosteric regulation at pH 7.5. However, the PTM mimetics exist as a mixture of tetramer and dimer, indicating that physiologically dimeric fraction is important and might be necessary for the modified PKM2 to translocate into the nucleus. Thus, our findings provide insight into how PTMs and pH regulate PKM2 and offer a broader understanding of its intricate allosteric regulation mechanism by phosphorylation or acetylation.

The activity and the oligomeric state of PKM2 are modulated by post-translational modifications (PTMs) (such as phosphorylation (5–7), acetylation (8), etc.), many metabolites (9–12), and small molecules (13–16). In human cancer cells, it has been demonstrated that modification of PKM2 allows it to enter the nucleus in an inactive dimer state (8). Inside the nucleus, the dimeric/monomeric form of PKM2 up-regulates the transcription of several genes responsible for cell proliferation and tumorigenesis (5, 17). Specifically, it has been shown that phosphorylation of PKM2 at Ser37 results in peptidyl-prolyl cis-trans-isomerase NIMA-interacting 1 (PIN1)-mediated cis-trans-isomerization followed by its nuclear translocation as a dimer/monomer (5, 18–20). Furthermore, using a phosphoserine-mimicking variant of PKM2, hereafter referred to as PKM2 S37D, it has been reported that the recombinant enzyme exists in the tetrameric state at pH 7.0 (19). However, a complex of PKM2 S37D and PIN1 appeared as a mixture of tetramer and monomer, suggesting that PIN1 mediates a change in the oligomeric state of PKM2 S37D (19). Likewise, another phosphoserine-mimicking variant, PKM2 S37E, exists in the dimeric state, as observed on a nondenaturing gel using whole-cell lysates (18). It should be noted that the phosphomimetics (PKM2 S37E and PKM2 S37D) behave in a manner similar to the phosphorylated PKM2, as shown by previous studies (5, 18, 19).

Biochemical studies demonstrated that acetylation of PKM2 at Lys433 prevents it from binding fructose 1,6-bisphosphate (FBP), an allosteric activator known to stabilize PKM2 in the tetrameric state (8, 9). The crystal structure of wtPKM2-FBP (PDB entry 1T5A) (9) illustrates that Lys433 is present in the FBP-binding pocket of PKM2 and interacts with FBP. Therefore, acetylation (8) or mutation of PKM2 (21, 22) reduces its FBP-binding affinity. Using whole-cell lysate of PKM2 acetylated at Lys433 and a recombinant acetyl-lysine mimetic variant of PKM2 (hereafter referred to as PKM2 K433Q), gel filtration studies determined that the enzyme appeared in the dimeric and a mixture of monomeric/dimeric states, respectively, consequently resulting in its translocation to the nucleus (6, 8). The acetyl-lysine mimetic mutation, PKM2 K433Q, has been shown to exhibit similar characteristics as native acetylated PKM2 (8).

While previous studies observed the switch in tetramer-to-dimer/monomer state of PKM2 upon acetylation (8) or phosphorylation (18, 19), the underlying mechanism for disrupting the oligomerization is yet to be determined. An open question, which is mainly due to the lack of detailed structure-function studies, is how the PTMs impact active tetrameric PKM2 and convert it to the inactive dimer/monomer state. Given that the
Regulation of PKM2 by phosphorylation, acetylation, and pH

dimeric form of PKM2 benefits cancer cells through various non-glycolytic functions, such as oncogene transcription (23), there is a growing effort to develop selective PKM2 inhibitors and activators that can impair tumor growth (24). Drugs like TLN-232 that are in phase II clinical trials have been reported to elicit anti-cancer properties by suppressing glycolysis and inducing cell death (25). Using cell lysates, it was demonstrated that peptide aptamers decreased cell proliferation by converting PKM2 tetramers to dimers and inhibiting the enzyme (26, 27). Thus, understanding the mechanism of tetramer-to-dimer conversion by phosphorylation or acetylation and identification of critical residues will aid in the development of mechanism-based small-molecule inhibitors targeted toward specific allosteric sites to stabilize the dimeric form of PKM2.

As cell-based assays can be influenced by other interacting partners of phosphorylated or acetylated PKM2, to exclude the effect of any cellular partners, biochemical and structural studies were carried out using isolated PKM2 variants, S37E, S37D, and K433Q. In particular, to gain insight into the allosteric regulation mechanism of PKM2 by the PTMs, we aimed to identify the structural basis for the change in oligomerization by determining crystal structures of different variants. In general, our results demonstrate that phosphorylation or acetylation of PKM2 leads to structural changes in the FBP-binding pocket, consequently resulting in a decrease in the FBP-binding affinity and perturbing the tetramer to a mixture of tetramer and dimer states.

Another aspect of the present study is to understand the effect of pH on PKM2 and the two PTM mimetic variants. It has been proposed that in cancer cells, a relatively high intracellular pH (pHi) of 7.12–7.65 compared with pHi of 6.99–7.20 in normal cells results in the metabolic switch from oxidative phosphorylation to aerobic glycolysis, along with enhanced DNA synthesis and tumor growth (28–31). It has also been observed that there is a strong positive correlation between the level of aerobic glycolysis and GLUT-1 (glucose transporter 1) expression in cells (32). GLUT-1 enhances the influx of glucose in the cancer cells, thereby producing lactate, which is essential for tumor growth (33, 34). In tumors, the transcription of GLUT-1 is up-regulated following a cascade of events involving phosphorylated PKM2 (5). Interestingly, most of the enzymes in the glycolytic pathway, such as phosphofructokinase, fructose-1,6-bisphosphatase, triosephosphate isomerase, and aldolase, are known to be most active at alkaline pH (35).

As PKM2 is involved in both aerobic glycolysis and oncogene transcription and is amenable to FBP-mediated activation (in contrast to the M1 isoform), we hypothesized that pH might have an impact on the activity and oligomerization of wtPKM2 and the PTM mimetic variants. In addition, the acetyl-lysine mimetic, PKM2 K433Q, has been reported to be involved in the proliferation of cancer cells (8). Therefore, to test our hypothesis and to gain insight into the mechanism of PKM2 regulation at an elevated pH of ≤7.5, the activities and the oligomeric states of wtPKM2 and the two mimetic variants (S37E/S37D and K433Q) were examined at varying pH. As the pH in cancer cells ranges from 7.12 to 7.65, all assays in the present study were conducted within pH 7.0–8.0. Our results confirm that the activities of wtPKM2 and the mimetic variants decrease as pH increases, which is due to an increase in the dimeric form of the enzymes. Furthermore, at pH 7.5–8.0, acetyl-lysine and phosphoserine mimetics display significant dimer formation, and the dimer fraction is known to be essential for oncogene transcription. The results reveal that a shift in pH from neutral to alkaline (pH 8.0) alters the activity, allosteric regulation by FBP, and oligomeric state of wtPKM2 and the variants, thus providing insight into how cancer cells might be exploiting PKM2 to generate lactate for cell proliferation.

Results

Phosphoserine mimetics of PKM2 exhibit reduced activities, whereas the activity of the acetyl-lysine mimetic remains unchanged

To understand whether acetylation or phosphorylation of PKM2 influences its activity in vitro and to gain insight into how FBP influences the activities of the variants, pyruvate kinase assays were conducted at a pH of 7.5, in the absence and presence of FBP. Activity assays with the phosphoserine mimetics, PKM2 S37E and PKM2 S37D, show ∼4-fold reduction in turnover numbers ($k_{cat} = 37.8 ± 2.3$ and $33.3 ± 1.4$ s$^{-1}$ for PKM2 S37E and PKM2 S37D, respectively) compared with wtPKM2 ($k_{cat} = 141 ± 7$ s$^{-1}$) at pH 7.5 in the absence of FBP (Fig. 1A and Table S1). These results indicate that mutating Ser$^{37}$ is likely causing a conformational change, which might be responsible for the reduced activity. With the acetyl-lysine mimetic variant, kinetic studies indicate that PKM2 K433Q ($k_{cat} = 132 ± 10.6$ s$^{-1}$) and wtPKM2 ($k_{cat} = 141 ± 7$ s$^{-1}$) have comparable activities in the absence of FBP (Fig. 1A and Table S1).

To test how FBP influences the two mimetic variants, kinetic studies were performed with excess (0.5 mM) FBP, given that it binds tightly ($K_d$ of ∼10 nM) to wtPKM2 (14), with a cellular FBP concentration of ∼20–40 μM (36). FBP is undoubtedly able to activate both PKM2 S37E ($k_{cat} = 37.8 ± 2.3$ s$^{-1}$, −FBP versus $k_{cat} = 147.2 ± 1.6$ s$^{-1}$, +FBP) and PKM2 S37D ($k_{cat} = 33.3 ± 1.4$ s$^{-1}$, −FBP versus $k_{cat} = 189 ± 4.3$ s$^{-1}$, +FBP) at pH 7.5 (Fig. 1A and Table S1). In the presence of excess FBP, there is almost no activation of PKM2 K433Q ($k_{cat} = 132 ± 10.6$ s$^{-1}$, −FBP versus $k_{cat} = 217.5 ± 6.7$ s$^{-1}$, +FBP) compared with wtPKM2 (Fig. 1A and Table S1). This result is in agreement with previous studies suggesting that FBP fails to bind or activate PKM2 K433Q inside the cell (8).

FBP fails to activate other PKM2 variants, K433E and S37E/K433E

PKM1 is allosterically insensitive to FBP, and sequence gazing showed that the lysine residue at position 433 of PKM2 is replaced by glutamate in PKM1 (9). Therefore, we hypothesized that a Lys to Glu mutation (hereafter referred to as PKM2 K433E) should disrupt the electrostatic interaction between FBP and Lys$^{433}$, and consequently, FBP will not have any impact. Furthermore, to understand how FBP might influence the activity and oligomerization of PKM2 when both Lys$^{433}$ and Ser$^{37}$ were substituted, a double mutant PKM2 S37E/K433E was examined. Kinetic studies suggest that the turnover numbers of PKM2 K433E and PKM2 S37E/K433E decreased slightly ($k_{cat} = 58.6 ± 3.3$ s$^{-1}$ for PKM2 K433E and 84.7 ± 4.8 s$^{-1}$ for PKM2 S37E/K433E).
PKM2 variants exist in a mixture of oligomeric states

Previous in vitro studies have shown that acetyl-lysine (K305Q) and phosphotyrosine (Y105E) mimetics, including another mutation (K422R) of PKM2 regulate its activity by altering the oligomeric state (37). To gain insight into how the PKM2 variants in the present study influence the activity and to rule out the involvement of any other factors, gel filtration studies were performed in the absence and presence of FBP. At pH 7.5 in the absence of FBP, all PKM2 variants exist in a mixture of oligomeric states (Fig. 1A and Table S1). In the presence of 0.5 mM FBP at pH 7.5, there is little to no activation of PKM2 K433E (4.8 s⁻¹, −FBP versus 112.8 ± 3.7 s⁻¹, +FBP) as well as the double mutant (116.7 ± 5.4 s⁻¹, −FBP, 116.7 ± 5.4 s⁻¹, +FBP) (Fig. 1A and Table S1). The impact of FBP on the activities of these variants agrees well with a previous report where up to 0.25 mM FBP did not influence PKM2 K433E (12), and a recent study showed very little activation of this mutant (21).

Oligomerization of phosphoserine– and acetyl-lysine–mimetic variants depends on pH

To understand whether the FBP-mediated modulation of the oligomeric state of wtPKM2 and the variants is influenced by a pH change, gel filtration analyses were conducted in the physiological pH range of 7.0–8.0 (note that pH₇ in cancer cells is ~7.12–7.65) in the absence and presence of FBP (Fig. 2 and Fig. S1). At pH 7.0, without FBP, both wtPKM2 and the acetyl-lysine−/phosphoserine−mimetic variants were in the pure tetrameric state (Fig. 2). As the pH was shifted to 7.5, or to 8.0 in the absence of FBP, the two mimetic variants appeared as a mixture of tetramer and dimer. The elution profiles indicate that the dimeric form became more prominent at higher pH (Fig. 2, B–D). In the presence of 0.5 mM FBP, all enzymes were tetramers in the measured pH range (Fig. S1).

Activities of wtPKM2 and the phosphoserine– and acetyl-lysine–mimetic variants decrease with increasing pH

To understand whether the tetramer-to-dimer shift upon varying pH from near-physiological (pH 7.0–7.5) to alkaline (pH 8.0) is directly connected to the activity of the enzymes, and whether FBP has any impact, in vitro activity assays were performed at pH 7.0, 7.5, and 8.0 in the absence and presence of FBP (Fig. 3,Figs. S2, S7, and S8, and Table S1).

With wtPKM2 in the absence of FBP, the turnover number is inversely related to the pH. The activity decreased by 4-fold as the pH was increased from 7.0 to 8.0 (185 ± 16 s⁻¹ at pH 7.0 versus 43.3 ± 1.1 s⁻¹ at pH 8.0), which is due to the increase in dimer population (Figs. 2A and 3A and Table S1). In the presence of FBP, while the turnover number also decreased with increasing pH (251 ± 4.8 s⁻¹ at pH 7.0 versus 162.6 ± 6.3 s⁻¹ at pH 8.0), the overall activity is higher compared with when FBP is absent, considering that FBP induces tetramerization (Fig. 3A, Fig. S2A, and Table S1).

The phosphoserine mimetics, S37E and S37D, exhibit a similar trend as wtPKM2, but the decrease in their activities was more dramatic, with and without FBP, as the pH was increased from 7.0 to 8.0 (Fig. 3 (B and C), Fig. S2 (B and C), and Table S1). In particular, for PKM2 S37E and PKM2 S37D, the turnover numbers (k_cat) decreased from 123 ± 19.1 and 99.6 ± 3.1 s⁻¹ at pH 7.0 to 17.5 ± 1.4 and 18.4 ± 0.8 s⁻¹ at pH 8.0, respectively (Fig. 3 (B and C) and Table S1). In the presence of FBP, the k_cat decreased by ~2–3-fold for PKM2 S37E.
(226.3 ± 2.6 s⁻¹ at pH 7.0 versus 66.4 ± 2.8 s⁻¹ at pH 8.0) and PKM2 S37D (226 ± 2.6 s⁻¹ at pH 7.0 versus 89.3 ± 0.96 s⁻¹ at pH 8.0) (Fig. S2 (B and C) and Table S1).

The decrease in activity for the K433Q variant is quite similar in the presence and absence of FBP (Fig. 3 D, Fig. S2 D, and Table S1). In the absence of FBP, the activity decreased by ~7-fold as the pH was shifted from 7.0 to 8.0 (k_{cat} = 127.7 ± 9.6 s⁻¹ at pH 7.0 versus k_{cat} = 17.9 ± 0.6 s⁻¹ at pH 8.0) (Fig. 3 D and Table S1). In the presence of 0.5 mM FBP, the decrease in turnover number was ~4-fold (k_{cat} = 251.8 ± 4.4 s⁻¹ at pH 7.0 versus k_{cat} = 62.4 ± 0.8 s⁻¹ at pH 8.0) (Fig. S2 D and Table S1).

In general, the data demonstrate that the activities of wtPKM2 and the two mimetic variants decrease significantly when the pH is increased from near-optimal (7.0–7.5) (Fig. 3, Figs. S2, S7, and S8, and Table S1).

**FBP binds weakly to PKM2 variants compared with wtPKM2**

While the presence of excess FBP dramatically changes the oligomerization of wtPKM2 and all of the variants examined, its impact on the activity of the lysine (Lys⁴³³) variants is not significant (Figs. 1A, Fig. S1, and Table S1). It has been suggested that as PKM1 has a glutamate at position 433 instead of lysine; it does not bind FBP and is resistant to FBP-mediated activation (9). Likewise, with the dimeric PKM2 S437Y, we (14, 38) and others (13) have demonstrated that the variant is incapable of binding FBP and therefore is not activated. To understand how the PKM2 variants in the present study impact FBP binding, fluorescence studies were conducted, and the binding affinities (K_d) for FBP were determined at pH 7.5. All variants have a reduced FBP-binding affinity (K_d of ~0.6–7 μM) relative to wtPKM2 (K_d = 0.98 ± 0.1 nM) (Fig. 4, A–D), consistent with a recent report showing that the K433E variant has reduced FBP-binding affinity compared with wtPKM2 (21). These results imply that in addition to mutation of a residue in the FBP-binding pocket (Glu⁴³³) and Lys⁴³³ acetylation (Gln⁴³³), interestingly, phosphorylation at Ser⁴³, which is in the N terminus, also leads to a decrease in the FBP-binding affinity of PKM2.

**Phosphoserine and acetyl-lysine mimetics of PKM2 modulate substrate binding**

It has been shown that allosteric activators and inhibitors of wtPKM2 modulate the affinity for one of the substrates, PEP (K_{d}; 10–50 mM) and do not impact the ADP-binding affinity (K_{d}; 0.2–0.9 mM) (14, 16). Likewise, some PTMs of PKM2, such as K305Q (39) and Y105E (37), regulate its activity by altering the affinity for PEP. To gain insight into how the acetyl-lysine mimetics, phosphoserine mimetics, and other Lys⁴³³ variants of PKM2 alter substrate-binding affinities, each of the variant enzymes was titrated with increasing concentrations of PEP and ADP. The PEP binding affinities of S37E (K_{d} = 48.1 ± 1.7 μM) and K433Q (K_{d} = 30.5 ± 0.4 μM) were similar to wtPKM2 (K_{d} = 47.7 ± 1.7 μM), whereas PKM2 S37D (K_{d} = 151.7 ± 2.3 μM), PKM2 K433E (K_{d} = 117.7 ± 5.5 μM), and the double mutant (K_{d} = 313.4 ± 25.6 μM) have relatively weak binding.
affinities for PEP (Fig. 4, D–F). The ADP-binding affinities of most variants are quite similar to wtPKM2 ($K_d \approx 0.2-0.9$ mM) (Fig. S3) (14, 16), with the exception of PKM2 S37E, which has a significantly decreased affinity ($K_d = 9.8 \pm 0.3$ mM) (Fig. S3, inset). These results indicate that PKM2 S37E modulates the enzyme activity by decreasing its affinity for ADP, whereas the remaining variants (except PKM2 K433Q) exhibit decreased activity due to their low PEP-binding affinities relative to wtPKM2.

**Crystal structures of PKM2 variants unravel structural changes in the FBP-binding site**

Overall structure of PKM2 variants—To understand the molecular mechanism of tetramer-to-dimer switch of PKM2 by acetylation/phosphorylation and/or modification of the FBP-binding residue, crystal structures of most PKM2 variants discussed were determined (Figs. 5 and 6 and Figs. S4 and S5). The acetyl-lysine mimic, PKM2 K433Q, and the double mutant, PKM2 S37E/K433Q, were crystallized in the C2 ($P_1_2_1_2_1$) space group at 1.84 and 2.45 Å, respectively, whereas the phosphoserine mimetics (PKM2 S37E and PKM2 S37D) were crystallized in the $P_1_1_2_1$ space group at 1.90 and 2.17 Å, respectively. All attempts to determine the structure of PKM2 K433Q were unsuccessful. While the phosphoserine mimetics have four molecules in the asymmetric unit (asu), PKM2 K433Q and PKM2 S37E/K433E have two molecules each in the asu. However, both the acetyl-mimetic and the double mutant are symmetry-related tetramers, and PISA (40) predicts the structures to be tetrameric. Superimposition of the structures of all four PKM2 variants with wtPKM2-FBP (PDB entry 1T5A) showed that the overall structures are mostly similar, with no difference in the relative orientation of subunits (Fig. S4A). In general, each monomer in all four structures of PKM2 variants has one molecule of bound oxalate and Mg$^{2+}$ ion (Fig. S4B). Although FBP always co-purifies with wtPKM2 in our laboratory (14, 16) and as reported (12), it was absent in the C-domain of each monomer in the crystal structures of all PKM2 variants.

Structural alignment of a monomer of each variant over 396–440 C$_\alpha$ atoms with the monomer of wtPKM2-FBP (PDB entry 1T5A) resulted in root mean square deviation (rmsd) values of 0.288–0.367 Å, indicating that the monomers are identical (Fig. S4B). Some differences were observed in the B-domains of PKM2 S37E, PKM2 S37D, and S37E/K433E compared with wtPKM2-FBP, given that the B-domain is known to be highly mobile (9, 41). Specifically, rotation of the B-domains relative to the A-domain was observed in chains A and B of PKM2 S37E and PKM2 S37D, as well as chain A of PKM2 S37E/K433E, which is due to hinge-bending motion between residues 125 and 131, as seen previously with PKM2 S437Y (38) and wtPKM2 (9) (Fig. S4B). It is highly possible that the hinge-bending motion arises due to crystal packing effects. The active site of all the structures has one molecule of oxalate in each monomer (Fig. S4B).

Furthermore, the N-terminal region (residues 1–20 in PKM2 S37E or PKM2 S37D and residues 1–23 in PKM2 S37E/K433E) was unmodeled due to low electron density. One possible reason for such disorder might be the steric clashes caused by Glu$_{37}$ with Gin$_{14}$ and Thr$_{15}$, as well as Asp$_{37}$ with Gin$_{14}$ (Figs. 5 (A and B) and 6B). Minor structural changes were observed in the C-domains of PKM2 K433Q (chain A) and PKM2 S37D (chains B and D) ~20 Å away from the FBP-binding site, with residue Glu$_{396}$ swinging away from Arg$_{399}$, thereby breaking the H-bond that is present in wtPKM2 (Fig. S5). The side chain of Glu$_{396}$ was unmodeled in other monomers of PKM2 S37E and PKM2 S37E/K433E due to poor electron density.

**PKM2 variants disrupt the binding of FBP**—The crystal structures of all PKM2 variants were FBP-free, similar to our reported structure of the dimeric variant, PKM2 S437Y, which is incapable of binding FBP (38). Specifically, in PKM2 S37E, the FBP-binding pocket consists of one phosphate group tethered to Ser$_{434}$ in each chain, which is due to the presence of K$_2$HPO$_4$ in the crystal condition. In the FBP-binding pocket of PKM2 S37D, a phosphate molecule was also observed to be H-bonded to Ser$_{434}$ in chains A, B, and D, whereas, in chain C, the same position was occupied by a glycerol molecule. As the crystallization condition did not have any phosphate, the phosphate moiety is probably a portion of unbound FBP. The lack of FBP was verified as described under “Experimental procedures.” In PKM2 S37E/K433E, a nitrate, which is present in the crystal condition as NaNO$_3$, is H-bonded to Ser$_{434}$ of chain C, whereas the FBP-binding pocket of the remaining chains lacks any ligands. Likewise, the FBP-binding pocket in PKM2 K433Q does not contain any ligands.

Interestingly, significant movement of residues involved in interacting with the phosphate groups of FBP in wtPKM2-FBP (PDB entry 1T5A), such as Trp$_{482}$, Gin$_{432}$/Lys$_{433}$, and Arg$_{436}$, was observed in all of the variant structures (Figs. 5 and 6B).
and 6). In PKM2 K433Q, mutation of lysine at 433 to glutamine causes the side chain of Gln\textsuperscript{433} to flip away from the FBP-binding site, consequently breaking the contact between the −NH\textsubscript{3}\textsuperscript{+} of the lysine side chain and P1 of FBP that is otherwise present in wtPKM2-FBP (PDB entry 1T5A) (Fig. 6A). In addition, the side chain of Arg\textsuperscript{436} flips outward and away from the FBP-binding site. This movement breaks its contact with the -OH group of Ser\textsuperscript{434}, which is involved in H-bond interaction with P2 of FBP (Fig. 6A). Finally, Trp\textsuperscript{482} rotates ~180° about the C\textsubscript{\beta}–C\textsubscript{\gamma} bond, which breaks the H-bond between the indole ring nitrogen and P1 of FBP (Fig. S5A). The shift in residues was observed in all copies of the structure, except Gln\textsuperscript{433} in chain A and Trp\textsuperscript{482} in chain B, which could not be modeled.

In PKM2 S37E, S37D, and S37E/K433E, the side chains of Lys\textsuperscript{433} (or Glu\textsuperscript{433}), Arg\textsuperscript{436}, and Trp\textsuperscript{482} move away in a similar fashion to that of PKM2 K433Q (Figs. 5 (A and B) and 6B). Such movement resulted in the disruption of critical interactions between the side chains of Lys\textsuperscript{433}, Ser\textsuperscript{434}, and Trp\textsuperscript{482} and the phosphate oxygens of FBP otherwise observed in wtPKM2-FBP (PDB entry 1T5A). For PKM2 S37E, the shift in the side chain of Trp\textsuperscript{482} was observed in all chains. However, Lys\textsuperscript{433} was unmodeled in chains B, C, and D, and Arg\textsuperscript{436} could not be modeled in chains B and D. For PKM2 S37D, Lys\textsuperscript{433} and Arg\textsuperscript{436}...
were missing in chain D. For PKM2 K433Q, the shift in the side chains of Lys\(^{433}\) and Arg\(^{436}\) was observed in all chains. The side chain of Trp\(^{482}\) was unmodeled in chain B, but in chain A it was rotated in a manner described previously. For PKM2 S37E/K433E, Trp\(^{482}\) in chain A was oriented in a similar fashion compared with wtPKM2-FBP (PDB entry 1T5A). It is worth mentioning that the movements involving residues Arg\(^{436}\) and Lys\(^{433}\) were observed in previous FBP-free structures, such as apo-PKM2 S437Y (PDB entry 6B6U) (38) and S437Y in complex with serine (PDB entry 6NUB) (14) or cysteine (PDB entry 6NU5) (14) and PKM2 K305Q (PDB entry 4QG8) (37).

Additional structural alterations in the C-domain were confined to a loop (residues Gly\(^{518}\)–Phe\(^{521}\)), also known as the FBP activation motif (FAM), covering the FBP-binding site. With the exception of PKM2 S37E, the FAM displayed significant movement in the PKM2 variants relative to wtPKM2-FBP (PDB entry 1T5A) (Fig. S6). Such movement of the FAM was seen previously with PKM2 S437Y, which is incapable of binding FBP (38). In particular, the FAM in chain A of PKM2 K433Q shifted by 3.2 Å compared with the FAM in wtPKM2-FBP (PDB entry 1T5A) (Fig. S6A). In PKM2 S37D, the FAM of chain C moved 7 Å away from the FBP-binding pocket compared with that of wtPKM2-FBP (PDB entry 1T5A), thereby

Figure 5. Crystal structures of the phosphomimetic variants PKM2 S37E and PKM2 S37D. A, superimposition of PKM2 S37E (PDB entry 6WP4, magenta, chain A) and PKM2-FBP (PDB entry 1T5A, cyan) monomers, with a close-up view of the FBP-binding pocket (chain A) and N terminus (chain C). B, superimposition of chain A of PKM2 S37D (PDB entry 6WP5, deep salmon) and PKM2-FBP (cyan) monomers. Ser to Glu/Asp mutation causes Lys\(^{433}\) and Arg\(^{436}\) to move away from the FBP-binding site and rotates Trp\(^{482}\) by 180° about the C\(^{\beta}\)–C\(^{\gamma}\) bond. The mutation also results in a steric clash between Glu/Asp and the N terminus of the respective enzyme. C, superimposition of the FBP-binding pocket of PKM2 S37D (chain C, deep salmon) with PKM2-FBP (cyan) showing residues involved in binding FBP. In PKM2 S37D, in addition to Lys\(^{433}\) and Trp\(^{482}\), the FAM motif residues (Gly\(^{518}\)–Phe\(^{521}\)) are shifted compared with its counterpart in PKM2-FBP (shown as a loop). Dashed lines, H-bonds. Arrows represent the movement of specific residues. Oxygen, nitrogen, phosphorus, and carbon atoms are shown in red, blue, orange, and backbone colors, respectively. Composite omit 2\(F_o\) – \(F_c\) maps (blue mesh) contoured at 1.0 \(\sigma\) were generated for residues Lys\(^{433}\), Arg\(^{436}\), Trp\(^{482}\), Glu\(^{37}\), and Asp\(^{37}\).
opening up the pocket (Fig. S6B). The FAMs in chains A and B of PKM2 S37D aligned well with the FAM of wtPKM2-FBP (PDB entry 1T5A, cyan). Likewise, the rmsd between the FAM of the double mutant in chain A and wtPKM2-FBP (PDB entry 1T5A) was ~4.3 Å (Fig. S6C). The FAM in the three structures described above was observed to be in contact with a symmetry-related monomer in the adjacent asu, and therefore, the observed movement can be attributed to crystal-packing effects. The FAM of both wtPKM2-FBP and PKM2 S37E aligned well in all of the chains except in chain C, which could not be compared because of several unmodeled residues. Thus, the crystal structures of PKM2 variants confirm that movement of key residues in the FBP-binding site results in the breakage of critical interactions required for FBP binding, consequently promoting the dimerization of PKM2.

Discussion

The terminal step of glycolysis in cancer cells is catalyzed by PKM2, and not by PKM1 (8). The primary reason for the selective expression of PKM2 is its regulation by several small molecules (13–16), metabolites (10–12) including FBP (9), and PTMs, such as acetylation (8) and phosphorylation (5). The regulation of PKM2 for glycolysis and proto-oncogene expression is exploited by cancer cells for their own benefit to support growth and proliferation. The expression of proto-oncogenes by acetylation and phosphorylation of PKM2 has been proposed to require a change in the activity and the oligomeric state of the enzyme (5, 18, 19), although the molecular mechanism of the allosteric regulation is not clearly understood.

Phosphomimetic mutations at the N terminus alter key residues in the FBP-binding pocket, causing PKM2 dimerization and decreased activity

Gel filtration studies at pH 7.5 with the phosphoserine mimetics, PKM2 S37E and S37D, confirm that these variants exist in a mixture of tetramer and dimer (Fig. 1B). This result indicates that phosphorylation alone, without the involvement of any other factors, is sufficient to cause the oligomeric state
change, which is in agreement with a non-denaturing gel analysis of cancer cell lysates containing PKM2 S37E (18). In contrast, a previous gel filtration study with bacterially purified PKM2 S37D at pH 7.2 depicted it to be tetrameric (19), which is also consistent with our experiments performed at pH 7.0. As it is well-known that dimeric PKM2 has lower catalytic activity and substrate-binding affinity compared with tetrameric PKM2 (42), it is not surprising that the tetramer/dimer mixture of the phosphomimetic variants shows markedly reduced activities at pH 7.5 without FBP compared with tetrameric wtPKM2 (Fig. 1A and Table S1). Nevertheless, it is interesting to note that a phosphomimetic mutation of Ser37 at the N terminus of PKM2 can significantly influence the activity, although the residue lies at a distance of ~36 Å from the active site. The present data do not explain the mechanism of allosteric coupling between the active site and the N terminus of PKM2.

The FBP-binding affinities of the tetramer/dimer mixture of PKM2 S37E (Kd ~0.6 μM) and PKM2 S37D (Kd ~1 μM) were significantly lower compared with wtPKM2 (Kd ~1 nM) (Fig. 4, A–C). The low affinity for FBP is due to the structural changes occurring in the FBP-binding pocket, where the movement of Lys433 and Trp482 residues breaks the electrostatic/H-bond interaction between P1 of FBP and the side chains of these amino acids (Figs. 5 (A and B) and 7). The absence of FBP likely

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Figure 7. A proposed route for the regulation mechanism of PKM2 by acetylation and phosphorylation. I, PKM2 undergoes phosphorylation at Ser37 or acetylation at Lys433 in response to cellular stimuli, which guides tumors to enhanced proliferation. II, acetylation or phosphorylation results in structural changes in the FBP-binding pocket involving a shift of Lys433, Arg436, and Trp482 residues. III, movement of key residues results in disruption of electrostatic and H-bond interactions with FBP, which ultimately evicts FBP from the binding pocket. IV, FBP-free PKM2 undergoes an oligomeric state transition from tetramer to dimer. V, dimeric PKM2 translocates to the nucleus and subsequently facilitates transcription of oncogenes.
Regulation of PKM2 by phosphorylation, acetylation, and pH leads to a shift in Arg\(^{436}\) away from Ser\(^{434}\), and these two residues might be involved in a signal transduction network required for FBP-mediated activation of PKM2 (Figs. 5 and 7). In addition, the shift in the side chain of Glu\(^{396}\) breaks its H-bond interaction with Arg\(^{399}\), and both of these residues are located at the monomer interface (Fig. S5B). The association of these two residues along with other interactions involving Glu\(^{418}\) has been shown to play a part in the allosteric activation by FBP (43). However, these changes do not impede the allosteric activation of the phosphomimetics by FBP (Fig. 1A and Table S1). It is possible that the presence of excess FBP in the activity and gel filtration assays results in Lys\(^{433}\), Arg\(^{436}\), and Glu\(^{396}\) residues to occupy a position similar to that in wtPKM2. Consequently, the signal communication required for FBP-mediated activation of the phosphomimetics is turned “on.” Interestingly, Ser\(^{37}\) is located at the N terminus, and its mutation to Asp\(^{37}\) or Glu\(^{37}\) results in structural changes in the C-domain, including the FBP-binding site, which is 37.9 Å away. At present, we do not have an explanation for the mechanism of signal transfer from the N terminus to the FBP-binding pocket. However, the results with the phosphomimetics suggest that the coupling between the active site and the N terminus allosteric site might be occurring through an intramolecular signaling pathway involving the FBP-binding pocket.

Acetyl-lysine mimic of PKM2 reorganizes the FBP-binding pocket and disrupts FBP binding and FBP-mediated activation

On the basis of the gel filtration result at pH 7.5, it is evident that PKM2 K433Q undergoes a conformational change from a tetramer to a mixture of tetramer and dimer (Fig. 1B). However, the tetramer/dimer mixture of PKM2 K433Q shows comparable activity with predominantly tetrameric wtPKM2 (Fig. 1A and Table S1), consistent with that observed previously (8). Such similarity in the activities between the wtPKM2 and the acetyl-lysine mimic can be attributed to their similar PEP-binding affinities (47.7 ± 1.7 \(\mu\)M for wtPKM2 versus 30.5 ± 0.4 \(\mu\)M for PKM2 K433Q) (Fig. 4, D–F).

As expected, FBP is unable to activate PKM2 K433Q as it binds with an affinity (\(K_d \approx 1 \mu\)M), which is ~1000-fold lower than that of wtPKM2 (\(K_d \approx 1 \mu\)M) (Fig. 4, A–C). The weak binding affinity of FBP is due to the mutation of Lys\(^{433}\) and the rotation of Trp\(^{482}\), which breaks the interactions between the amino acid side chains and FBP, similar to that observed with the phosphomimetics (Fig. 6A). As expected, an excess of FBP can convert the dimeric fraction of PKM2 K433Q to tetramer, as seen in gel filtration studies (Fig. S1). The fact that in the presence of FBP, the tetrameric form of K433Q does not show an increased activity compared with the tetramer/dimer form (–FBP) (Fig. 1A and Table S1) indicates that there is a break in the signal transduction network required for FBP-mediated allosteric activation of PKM2, which is confirmed by our crystal structure.

On the basis of the crystal structure of PKM2 K433Q, one can imagine that the disruption of the signal communication process might be initiated by the outward movement of the Gln\(^{433}\) side chain away from the FBP-binding site (Figs. 6A and 7). The swinging-out motion is due to the steric collision between the P1 phosphate of FBP and the amide group of Gln\(^{433}\), which was also predicted by molecular modeling studies (8). Based on the position of FBP in the binding pocket of wtPKM2, it can be inferred that acetylation of Lys\(^{433}\) and concomitant movement of the side chain would disrupt the electrostatic interaction between the \(–NH_3^+\) of Lys\(^{433}\) and P1 of FBP (Figs. 6A and 7). Taken together, the steric collision–mediated movement of Gln\(^{433}\) side chain and the rotation of Trp\(^{482}\) (Fig. S5A) and the movement of the FAM motif (Fig. S6A) can be attributed to the decrease in the FBP-binding affinity of PKM2 K433Q and, consequently, the absence of FBP in the PKM2 K433Q structure. Although the molecular modeling studies of Lys\(^{433}\) acetylation did not show any movement of other residues except Lys\(^{433}\) in the FBP-binding pocket (8), our crystal structure shows that the H-bond interaction between Arg\(^{436}\) and Ser\(^{434}\) is disrupted due to the outward movement of Arg\(^{436}\) from the FBP-binding site. Interestingly, a previous study involving all-atom molecular dynamics simulation of PKM2 shows residues Lys\(^{433}\), Ser\(^{434}\), and Arg\(^{436}\) among others to be a part of the allosteric signal communication pathway (44). Finally, the shift in the side chain of Glu\(^{396}\) located at the monomer interface of PKM2 breaks the H-bond with Arg\(^{399}\) (Fig. S5A). Thus, our results confirm that mutation of Lys\(^{433}\) to Gln is associated with movements involving the side chains of Gln\(^{433}\), Arg\(^{436}\), and Glu\(^{396}\), thus breaking the signal relay network between these residues, including Ser\(^{434}\) and Arg\(^{399}\), responsible for disrupting FBP-mediated activation.

PKM2 variants K433E and S37E/K433E are unresponsive to FBP-mediated activation due to specific structural changes in the FBP-binding site

In the absence of FBP, PKM2 K433E and PKM2 S37E/K433E have relatively lower activities than wtPKM2, which is due to their low PEP-binding affinities (Figs. 1A and 4 (D–F) and Table S1). The results are in agreement with the gel filtration profiles of both variant enzymes showing significant dimer formation at pH 7.5 (Fig. 1B). There is negligible FBP-mediated activation of the K433E variant and almost no activation of the double mutant at pH 7.5, which can be attributed to their low FBP-binding affinities compared with wtPKM2 (Figs. 1A and 4 (A–C) and Table S1). These results are in agreement with those reported for PKM1, which has Gln\(^{433}\) instead of Lys\(^{433}\) and does not bind FBP (9). Furthermore, in yeast pyruvate kinase (YPK), mutation of threonine at 403 (analogous to Lys\(^{433}\) in PKM2) to glutamate prevents FBP binding (45). As expected, the gel filtration profile of both K433E and S37E/K433E in the presence of an excess of FBP (0.5 mM) at pH 7.5 is tetrameric, whereas the activities are slightly altered in the presence of FBP compared with their activities without FBP (Fig. 1A, Fig. S1, and Table S1). These data indicate a disruption in the relay of FBP-mediated activation signal from the allosteric site to the active site by a mechanism similar to PKM2 K433Q. In addition to the steric clash, similar to Gln\(^{433}\), Gln\(^{433}\) hinders FBP binding due to a charge repulsion between its negatively charged side chain (\(–COO^-\)) and phosphate of FBP. Also, the side chain of Gln\(^{433}\) moves away from the FBP-binding pocket along with...
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Physiological relevance of allosteric regulation of PKM2 with varying pH

Previous studies have shown that cancer cells maintain a slightly higher pH, ~7.5 compared with normal cells (pH ~7.1), which enhances aerobic glycolysis and oncogene transcription (30). As PKM2 is involved in both of the processes, to gain insight into the impact of pH perturbation on the activities and oligomerization, we examined wtPKM2 and the acetyl-lysine-/phosphoserine–mimetic variants at varying pH. Broadly, the results indicate that the turnover number of wtPKM2 and the two mimetic variants decrease as the pH is increased from 7.0 to 8.0, which is due to a change from tetramer to dimer at pH 7.0 to a mixture of tetramer and dimer at pH 8.0 (Figs. 2 and 3 and Table S1). These results are consistent with the fact that dimeric PKM2 has lower catalytic activity (42). Interestingly, even though wtPKM2 and the variants were tetrameric in the presence of FBP at all pH levels tested, the activities of the enzymes decreased as the pH was increased (Fig. 3 and Fig. S1). The decrease in the activity of pyruvate kinase with increasing pH has been observed in several studies (46, 47). In particular, with yPK using WT and an active site variant Lys240 (equivalent to Lys270 in PKM2), kinetic studies had demonstrated that the activities of the enzymes decreased as the pH was increased from 4.8 to 9.1 in the presence of FBP (47). Based on detailed kinetic isotopic studies, it was suggested that ionization of one or more groups in the active site in the pH range of 6–8 influences the catalytic activity. Specifically, γPK Lys246 (with a pK_a of 8.78) is involved in proton donation and phosphoryl group transfer in the catalytic cycle. Therefore, we can conclude that as the pH increases to ~8.0, ionization of Lys270 results in a loss of activity of wtPKM2 and PKM2 variants in the presence of FBP.

In particular, wtPKM2 exists in the tetrameric state with maximum catalytic activity at pH 7.0 in the absence of FBP, as seen in the activity assays and gel filtration studies (Figs. 2 and 3 and Table S1). Not surprisingly, at pH 7.0, wtPKM2 is unresponsive to FBP-mediated activation, as it is already tetrameric and in a state of maximum activation, and FBP can only activate wtPKM2 by converting dimer to tetramer (Fig. S1) (48). At pH 7.5 without FBP, wtPKM2 had a turnover number nearly identical to that at pH 7.0 (185 ± 16 s⁻¹ at pH 7.0, 141 ± 7 s⁻¹ at pH 7.5), but it can be allosterically activated by FBP (k_cat = 251 ± 4.8 s⁻¹ at pH 7.0 versus 295 ± 7.4 s⁻¹ at pH 7.5) (Table S1). One can speculate that cancer cells have adapted to function at a higher pH (above 7.5) by expressing the M2 isoform of PK over M1, which is amenable to allosteric regulation by FBP.

The acetyl-lysine-/phosphoserine–mimetic variants of PKM2 exist as a mixture of tetramer and dimer at pH 7.5, as seen in gel filtration studies, whereas they are predominantly tetrameric at pH 7.0 (Fig. 2). Thus, cancer cells maintaining a pH of 7.5 allows the dimeric fraction of acetylated and phosphorylated PKM2 to evolve into the nucleus, which agrees with the rationale for selective expression of PKM2 instead of PKM1 in cancer cells (6). The relatively low activity of PKM2 S37E (k_cat = 37.8 ± 2.3 s⁻¹) or PKM2 S37D (k_cat = 33.3 ± 1.4 s⁻¹) compared with wtPKM2 (k_cat = 141 ± 7 s⁻¹) at pH 7.5 is consistent with the fact that the phosphomimetics are required for the oncogene transcription function of PKM2 and not for glycolysis (Fig. 1A and Table S1). Moreover, one can imagine that FBP-mediated tetramerization and activation of PKM2 S37E and PKM2 S37D at pH 7.5 (Fig. S1, Fig. 1A, and Table S1) are important for cancer cells to maintain a fine balance between oncogene expression and aerobic glycolysis. At a relatively higher pH 8.0 in the absence of FBP, all of the enzymes under study had an increase in the dimer fraction, and consequently, their turnover numbers decreased significantly compared with that at 7.0 or 7.5 (Figs. 2 and 3 and Table S1).

Conclusions

Overall, the present study demonstrates that acetylation and phosphorylation cause a shift in the oligomeric state of PKM2 from tetramer to a tetramer/dimer mixture without the involvement of any additional factors. The PKM2 variants, K433E and S37E/K433E, can also cause a similar change in oligomerization. Acetylation, phosphorylation mimetics, and other mutations of PKM2 result in the evocation of FBP from the FBP-binding pocket, which leads to an increase in the dimeric state. Moreover, a mutation in Lys433 residue results in disruption of FBP-mediated activation, which confirms the importance of the residue in the activation process. The biophysical studies described here provide insight into how a shift in pH from ~7.1 to ~7.5 might allow cancer cells to keep PKM2 optimally active and responsive to FBP-mediated allosteric activation, consequently producing lactate and thus supporting its proliferation. However, a pH of 7.5 keeps the acetylated and phosphorylated PKM2 in a mixture of tetramer and dimer, indicating that the dimer fraction is necessary for transcription of oncogenes. Our findings provide a partial glimpse into the intricate molecular details of FBP-mediated PKM2 activation and demonstrate a pH-based regulation of PKM2. However, the mechanism by which phosphorylation at the N terminus of PKM2 significantly alters its activity and causes FBP to move...
out of the binding pocket located near the C terminus remains to be determined. It is also unclear what other residues are involved in the signal transduction network required for FBP-mediated activation of PKM2. Future studies can be directed to identifying potential residues in the pathway by mutational analysis.

**Experimental procedures**

**Expression and purification of wtPKM2 and PKM2 variants**

The wtPKM2 gene cloned in a pET28a vector was a gift from Matthew Vander Heiden. The plasmid was used as a template for the generation of the variants PKM2 K433Q, K433E, S37E, S37D, and S37E/K433E by site-directed mutagenesis using mutagenic oligonucleotides. All of the variant plasmids were transformed into Escherichia coli BL21 (DE3) (Life Technologies, Inc.). The culture and protein isolation procedures described below are applicable to wtPKM2 and PKM2 variants. Starter cultures were initiated with single colonies in Luria–Bertani medium. 2 liters of Terrific Broth medium was inoculated with 10 ml of the overnight grown starter culture. Post-inoculation, cells were grown further at 22 °C/170 rpm for 22 h and harvested by centrifugation at 4000 rpm for 30 min at 4 °C. The harvested cells (5 g) were resuspended in 25 ml of 20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM imidazole, and 5% glycerol (buffer A). The cells were lysed by sonication for 8 min (6-s ON/20-s OFF time) followed by centrifugation of the lysate at 30,000 rpm for 30 min at 4 °C. Each of the supernatant containing His6-wtPKM2 and PKM2 variants was purified by 10 ml of immobilized nickel-affinity resin pre-equilibrated with buffer A. The column was washed with 2 column volumes of buffer A, and the protein was eluted in the same buffer containing 450 mM imidazole. SDS-PAGE was used to visualize the protein and the protein was eluted in the same buffer containing 450 mM imidazole. The column was washed with 2 column volumes of buffer A, and the protein was eluted in the same buffer containing 450 and 1000 mM imidazole. SDS-PAGE was used to visualize the protein fractions, and the 450 mM fraction was dialyzed overnight in a buffer containing 20–50 mM Tris–HCl, pH 7.5, 100–150 mM KCl, 5% glycerol, 0.5 mM tris(3-hydroxypropyl) phosphine, and 0.5 mM EDTA (storage buffer). To remove any aggregated protein, the dialyzed and concentrated protein was passed through a HiLoad Superdex 200 16/600 (GE Healthcare) gel filtration column pre-equilibrated with storage buffer. A NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA) with the cuvette pathlength set at 1 mm was used to measure protein concentrations at 280 nm. The readout was in absorbance, and protein concentration was measured in mg/ml using the molecular mass of monomeric PKM2 (59.96 kDa) and molar extinction coefficient (ε280 nm) of 29.91 M⁻¹ cm⁻¹. Protein samples were stored at −80 °C until use. The protein concentration used in all experiments hereafter refers to monomeric PKM2.

**Pyruvate kinase activity assays**

To measure the activity of wtPKM2 and the variants in the presence and absence of FBP, a PKM2-lactate dehydrogenase coupled assay was performed using an Epoch microplate spectrophotometer (BioTek, Winooski, VT). The concentrations of all enzymes used were 25 and 13 nM in the absence and presence of FBP (0.5 mM), respectively. The enzymes were dissolved in 20 mM Tris–Cl, pH 7.5, 150 mM KCl, 5 mM MgCl₂, 4 units/ml lactate dehydrogenase, and 0.5 mM NADH. Reactions (100 μl) were initiated by varying the PEP concentration from 0.025 to 2 mM with a fixed ADP concentration of 0.8 mM. For the pH-based assays of wtPKM2, PKM2 K433Q, PKM2 S37E, and PKM2 S37D in the presence and absence of FBP, experiments were performed at pH 7.0, 7.5, and 8.0 separately. The assays for PKM2 K433E and PKM2 S37E/K433E were done only at pH 7.5. The concentrations of enzyme/FPB/ADP, PKM concentration, reaction volume, and buffer composition were identical to those described above. Reactions were monitored for a minute by recording the decrease in absorbance at 340 nm corresponding to the oxidation of NADH to NAD⁺. The rate of decrease in absorbance was used to calculate initial velocities in GraphPad Prism (GraphPad Software, Inc., San Diego, CA), which were plotted against PEP concentration. The resulting data from the activity assays (shown in Figs. S7 and S8) were fit using the Michaelis–Menten equation,

\[
Y = \frac{V_{max} X}{K_m + X}
\]  

(Eq. 1)

where \(Y\) is the initial velocity and \(X\) is the substrate (PEP) concentration.

**Ligand-binding assays**

The binding affinity of the substrates PEP/ADP and the allosteric activator FBP to the wtPKM2 and the variants was determined in a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA). For all binding assays, a 1.6 μM concentration of each enzyme was dissolved in 20 mM Tris–HCl, pH 7.5, 150 mM KCl, and 5 mM MgCl₂. The excitation and emission wavelengths were set at 295 and 340 nm with slit widths of 5 and 10 nm, respectively. The decrease in tryptophan fluorescence was monitored in titration experiments with varying concentrations of the appropriate ligand. Fractional saturation was calculated from the fluorescence intensity data, and the resulting data were plotted against the corresponding ligand concentration (Fig. 4 and Fig. S3). For PEP and ADP binding to wtPKM2 and most variants and for FBP binding to PKM2 S37E/K433E, the resulting curves were fit to one-site specific binding (Equation 2) using GraphPad Prism,

\[
Y = \frac{B_{max} X}{K_d + X}
\]  

(Eq. 2)

where \(Y\) is fractional saturation, \(B_{max}\) is maximum binding, and \(X\) is the ligand concentration.

The data for ADP binding to K433E (Fig. S3, blue trace) were fit to a one-site–specific binding equation incorporating the Hill coefficient, \(h\) (Equation 3)
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\[ Y = \frac{B_{max} \times X^h}{K^d + X^h} \]  
(Eq. 3)

As the enzyme concentration used was higher than the estimated \( K_d \), data for FBP binding to wtPKM2 and PKM2 K433E were fit using a binding equation (Equation 4), as reported by us elsewhere (49, 50) with Sigma Plot 12.5 (Systat Software Inc., Point Richmond, CA) (Fig. 4, A and B). Likewise, FBP-binding data for PKM2 K433Q, PKM2 S37E, and S37D (Fig. 4A) were fit using a variation of Equation 4 incorporating the Hill coefficient (Equation 5) using SigmaPlot,

\[ f = f_0 + \left( f_m - f_0 \right) \frac{(nP + x + K_d) - \sqrt{(nP + x + K_d)^2 - 4nPx}}{2nP} \]  
(Eq. 4)

\[ f = f_0 + \left( f_m - f_0 \right) \frac{(nP + x^b + K_d^b) - \sqrt{(nP + x^b + K_d^b)^2 - 4nPx^b}}{2nP} \]  
(Eq. 5)

where \( f \) is the fluorescence signal resulting from FBP binding to wtPKM2, \( f_m \) is the maximum fluorescence intensity, \( f_0 \) is the signal from wtPKM2 in buffer solution, \( P \) and \( x \) are total protein and added FBP concentrations, respectively, \( n \) is the number of binding sites, and \( h \) is the Hill coefficient.

**Gel filtration analysis of wtPKM2 and variants**

Solutions of wtPKM2 and PKM2 variants in storage buffer were injected separately onto a Superdex 200 10/300 GL gel filtration column (24 ml; GE healthcare) via a 0.2-ml loop at a rate of 0.5 ml/min using an AKTA Pure FPLC system (GE Healthcare Life Sciences). For wtPKM2, PKM2 K433Q, PKM2 S37E, PKM2 S37D, and PKM2 S37E/K433E, the experiments were conducted over a pH range of 7.0–8.0 at an enzyme concentration of 0.5 mg/ml (Fig. 2). Gel filtration experiments for PKM2 K433E and PKM2 S37E/K433E were conducted only at pH 7.5 (Fig. 1B). The assay at pH 7.5 was repeated in the presence of FBP with wtPKM2 and all of the variants (Fig. S1). Gel filtration molecular mass standards (Bio-Rad) containing vitamin B12 (1.35 kDa), horse myoglobin (17 kDa), chicken ovalbumin (44 kDa), bovine \( \gamma \)-globulin (158 kDa), and bovine thyroglobulin (670 kDa) were used to calibrate the column. UV absorbance at 280 nm was used to detect the eluted protein. The highest and lowest intensity values were set to 100 and 0%, respectively, to normalize the data, which were plotted against elution volume in GraphPad Prism.

**Crystallization of PKM2 variants**

To crystallize the PKM2 variants, ~10 mg/ml of variant enzymes in storage buffer were mixed with 2 mM oxalate and 5 mM MgCl₂, followed by incubation of the solution on ice at 4°C for 30 min. Crystal drops were set with 1 μl each of the protein and precipitant solutions using the sitting-drop vapor diffusion method at 22°C. Crystals of PKM2 S37E were obtained in a precipitant solution containing 0.2 M K₂HPO₄, 0.1 M HEPES, pH 7.0, and 20–24% PEG 3350. PKM2 S37D crystals were obtained in a precipitant solution with 0.2 M NaBr, 0.1 M bis-tris propane, pH 6.5, and 16–20% PEG 3350. PKM2 K433Q was crystallized in a precipitant solution containing 15% PEG 4000 and 0.25 M NaNO₃. Crystals of PKM2 S37E/K433E appeared in a precipitant solution containing 0.2 M NaNO₃ and 18% PEG 3350. For cryocooling, the crystals were looped and washed in a precipitant solution containing 25% (v/v) glycerol and mother liquor, followed by flash freezing in liquid nitrogen.

**Data collection and structure determination**

X-ray diffraction data for crystals of PKM2 variants were collected at 100 K at beamline 4.2.2 of the Advanced Light Source (Berkeley, CA) or beamline 19-ID-D of the Advanced Photon Source (Argonne, IL). For PKM2 S37E, data were collected using a 0.5-s exposure time, 0.1° oscillations, and 225-mm detector distance. Data for the PKM2 S37D crystal were collected using a 1-s exposure time, 0.25° oscillations, and 290-mm detector distance. For the K433Q variant, 0.2° oscillations, 0.2-s exposure time, and 220-mm detector distance were used to collect data. For the S37E/K433E variant, data were collected using 0.2° oscillations, 0.2-s exposure time, and 350-mm detector distance. The images obtained were processed and scaled using the XDS package (51) and SCALA (52), respectively. The structures were solved by molecular replacement using PHASER (53). Solutions for both PKM2 S37D and S37E structures were obtained using chain B of PKM2 S437Y (PDB entry 6BU6) as a starting model. For PKM2 K433Q, chain A of PKM2-serine (PDB entry 4B2D) was used as a model. The structure of PKM2 S37E/K433E was solved using chain C of the PKM2-cysteine structure (PDB entry 6NU1) as a starting model. Iterative rounds of refinement and model building were performed with PHENIX (54) and COOT (55), respectively.

For the PKM2 S37E structure (PDB entry 6WP4), poor electron density in the N terminus resulted in the absence of residues 1–20 in chains A and B and residues 1–21 in chains C and D along with the His tag. Residues 125–129 of the B-domain in chains C and D were unmodeled due to poor electron density. The FAM motifs comprising residues 514–522 were modeled in chains A and B, and D, but in chain C, residues 517 and 518 were absent.

In PKM2 S37D, the N-terminal His tag is absent in all four chains along with residues 1–20. The B-domain was disordered between residues 126 and 129 in chains A and B. In chain C, electron densities for these residues were completely missing. In chain D, residues 120–217 were also left unmodeled due to the absence of electron density. A portion of the FAM motif involving residues 515–519 was absent in chain D, and Mg²⁺ could not be modeled in the active site of chain D. To verify that the FBP-binding pocket lacks the complete FBP molecule, we fitted FBP with partial occupancy and did an occupancy refinement. However, with an occupancy of ~0.6, negative density was observed in the fitted region, except for the Ser³⁴³-bound phosphate, which confirmed that FBP is absent in the binding pocket.

In PKM2 K433Q, the N terminus is slightly disordered, and the His tag and first 13 residues were not modeled in both chains A and B. The B-domain was partially modeled in chain B with...
Regulation of PKM2 by phosphorylation, acetylation, and pH

Table 1
Data collection and refinement statistics

|                  | PKM2 S37E | PKM2 S37D | PKM2 K433Q | PKM2 S37E/K433E |
|------------------|-----------|-----------|-------------|-----------------|
| PDB code         | 6WP4      | 6WP5      | 6WP6        | 6WP6            |
| Data collection  |           |           |             |                 |
| Space group      | P1 2 1    | P1 2 1    | C1 2 1      | C1 2 1          |
| Unit cell dimensions |
| a, b, c (Å)      | 94.17, 132.63, 110.13 | 94.63, 117.57, 109.96 | 112.38, 94.23, 109.64 | 113.07, 92.92, 109.39 |
| α, β, γ (degrees) | 90, 112.74, 90 | 90, 112.63, 90 | 90, 94.84, 90 | 90, 94.27, 90 |
| Beamline         | APS 19-ID-D | APS 19-ID-D | APS 19-ID-D | APS 19-ID-D |
| Wavelength (Å)   | 1.000047  | 0.9786032 | 1.000029    | 0.976250        |
| Oscillation range (degrees) | 180 | 180 | 180 | 180 |
| Resolution range (Å) | 55.33–1.9 (1.968–1.9) | 19.84–2.17 (2.29–2.17) | 47.11–1.84 (1.968–1.84) | 61.27–2.45 (2.538–2.45) |
| No. of observations (no. of reflections) | 677,560 (81,775) | 440,348 (63,318) | 356,710 (52,319) | 146,222 (18,024) |
| No. of unique reflections | 192,145 (26,293) | 116,911 (17,088) | 96,634 (14,320) | 41,013 (5839) |
| Reduction (%)    | 3.5 (3.1)  | 3.8 (3.8)  | 3.7 (3.7)   | 3.6 (3.1)       |
| Rmerge (%)       | 98.2 (92.1) | 99.7 (99.9) | 98.0 (99.9) | 98.7 (98.6) |
| Completeness (%) | 77.8 (77.8) | 67.8 (67.8) | 57.8 (57.8) | 47.8 (47.8) |
| Wilson B-factor (Å²) | 24.54 | 34.02 | 25.91 | 42.10 |
| Refinement       |           |           |             |                 |
| Rwork (%)        | 18.8 (29.7) | 21.6 (30.0) | 20.3 (26.3) | 21.5 (26.8) |
| Rfree (%)        | 22.1 (33.7) | 25.9 (35.6) | 23.0 (28.2) | 25.4 (29.5) |
| No. of reflections | 191,611 | 116,845 | 96,273 | 39,992 |
| No. of molecules per asu | 4 | 4 | 2 | 2 |
| No. of atoms     | 15,205 | 14,184 | 6991 | 7258 |
| Proteins         | 186 | 100 | 66 | 30 |
| Ligands          | 563 | 209 | 385 | 63 |
| Water            |             |             |             |                 |
| Average B-factor (Å) |
| Macromolecules   | 29.02 | 41.86 | 30.23 | 49.7 |
| Ligands          | 36.96 | 45.68 | 32.48 | 45.9 |
| Water            | 30.55 | 37.65 | 32.48 | 45.9 |
| rmsd for bonds lengths (Å) | 0.0088 | 0.0088 | 0.007 | 0.004 |
| rmsd for bond angles (degrees) | 0.77 | 0.94 | 0.96 | 0.80 |
| Ramachandran (%) | 98.12 | 97.6 | 97.84 | 97.6 |
| Favored          | 1.68 | 2.19 | 1.74 | 2.3 |
| Allowed          | 0.20 | 0.16 | 0.43 | 0.1 |
| Outliers         |             |             |             |                 |

residues 122–206 untraced owing to the absence of density in that region. In chain A, residues 128–130 of the B-domain were unmodeled. The FAM motif was somewhat disordered in chain A, whereas in chain B, residues 515–519 of the FAM motif were absent. In chain A of K433Q, the position of the P1 phosphate group of FBP seen in wtPKM2-FBP (PDB entry 1T5A) is occupied by Glu223 from the neighboring asu. No phosphate or other ligands were observed in the FBP-binding pocket.

Likewise, in the double mutant S37E/K433E, a significant portion of the N terminus (residues 1–23) was absent along with the His tag. The B-domain and FAM motif were well-modeled in chain A. However, in chain C, residues 126–128 and 518–520 were missing in the B-domain and FAM motif, respectively, due to poor electron density. Mg2+ could not be modeled in chain A.

Refinement strategies used in the initial rounds of refinement for PKM2 K433Q and PKM2 S37E were rigid-body, TLS, restrained coordinate refinement, individual isotropic ADP, and simulated annealing. However, in the later rounds, restrained coordinate, occupancy, and individual isotropic ADP refinements were used. For PKM2 S37D and PKM2 S37E/K433E, restrained coordinate refinement, rigid-body, simulated annealing, individual ADP, target weights, and occupancy refinement were used in the initial rounds of refinement, and in the later rounds simulated annealing was switched off. Rigid-body refinement was also turned off for PKM2 S37D.

Composite omit maps were generated to verify the final models. For validating the structures, the wwPDB validation server was used (56). Figures related to the crystal structures were created using PyMOL (57). The coordinates of the structures have been deposited in the Protein Data Bank (PDB entries 6WP3, 6WP4, 6WP5, and 6WP6). Data-processing, refinement, and validation statistics are summarized in Table 1.

Data availability

Atomic coordinates and structure factors have been deposited in the Protein Data Bank as entries 6WP3, 6WP4, 6WP5, and 6WP6. All other data are contained within the article. The amino acid sequence of this protein can be accessed through the UniProt KB Protein Database under UniProt ID P14618.

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Abbreviations—The abbreviations used are: PKM2, pyruvate kinase muscle isoform 2; PK, pyruvate kinase; yPK, yeast PK; PEP, phosphoenolpyruvate; FBP, fructose 1,6-bisphosphate; rmsd, root mean square deviation; asu, asymmetric unit; pH, intracellular pH; GLUT-1, glucose transporter-1; PIN1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; PTM, post-translational modification; PDB, Protein Data Bank; FAM, FBP activation motif.

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