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Succinyl-5-aminoimidazole-4-carboxamide-1-ribose 5′-Phosphate (SAICAR) Activates Pyruvate Kinase Isoform M2 (PKM2) in Its Dimeric Form

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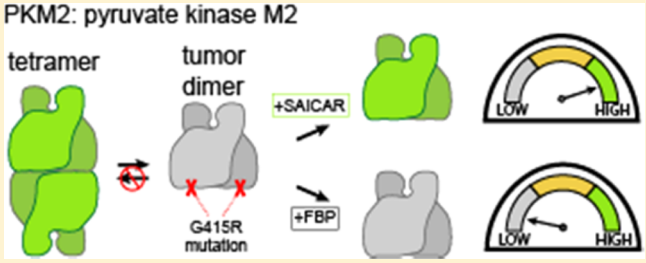
Supporting Information

ABSTRACT: Human pyruvate kinase isoform M2 (PKM2) is a glycolytic enzyme isoform implicated in cancer. Malignant cancer cells have higher levels of dimeric PKM2, which is regarded as an inactive form of tetrameric pyruvate kinase. This perceived inactivity has fueled controversy about how the dimeric form of pyruvate kinase might contribute to cancer. Here we investigate enzymatic properties of PKM2^{G415R}, a variant derived from a cancer patient, which we show by size-exclusion chromatography and small-angle X-ray scattering to be a dimer that cannot form a tetramer in solution. Although PKM2^{G415R} binds to fructose 1,6-bisphosphate (FBP), unlike the wild type this PKM2 variant shows no activation by FBP. In contrast, PKM2^{G415R} is activated by succinyl-5-aminoimidazole-4-carboxamide-1-ribose 5′-phosphate (SAICAR), an endogenous metabolite that we previously showed correlates with an increased level of cell proliferation and promotes protein kinase activity of PKM2. Our results demonstrate an important and unexpected enzymatic activity of the PKM2 dimer that likely has a key role in cancer progression.



Pyruvate kinase isoform M2 (PKM2) is one of four human pyruvate kinase isoforms[1,2] that, unlike other pyruvate kinases, is prevalent in many types of cancer.[2] Replacement of PKM2 with other pyruvate kinase isoforms hinders cancer cell survival and growth, highlighting its importance in cell proliferation.[3] Interestingly, PKM2 has long been used as a marker for cancer. In cancerous cells, PKM2 is abundantly found as a dimer, with the level of PKM2 dimer correlated with malignancy, and thus has been named tumor PKM2.[4] How PKM2 contributes to malignancy is still controversial, but its contribution is believed to arise from a combination of metabolic[5] and cell signaling roles.[6]

Although found to exist in different oligomeric forms in cells, like other pyruvate kinases, PKM2 is considered to have maximal pyruvate kinase activity as a tetramer.[7] Earlier biochemical studies found the PKM2 dimer to be nearly inactive.[7] The low activity of the dimer was caused by a K_M for phosphoenolpyruvate (PEP) that is much higher than physiological PEP concentrations, while the tetrameric PKM2 has a low-micromolar K_M for PEP.[7] This led to the belief that the dimeric PKM2 is a nearly inactive enzyme at physiological PEP concentrations. How a nearly inactive form of pyruvate kinase might contribute to cell proliferation has been unclear. Initially, it was thought that the nearly inactive pyruvate kinase allows cancer cells to divert more glycolytic intermediates to anabolic processes.[5] However, recent evidence shows that the dimeric PKM2 may have an additional active role in cell growth.[8−14]

In addition to its classical role in generating ATP from ADP and the phosphate donor PEP, PKM2 also has been found to phosphorylate protein substrates.[9,10,12−16] A significant fraction of PKM2 in cancer cells is located in the nucleus,[17] and this proportion increases when cancer cell proliferation is stimulated.[14] Purification of this nuclear PKM2 showed that it can phosphorylate various nuclear proteins using PEP as a phosphate source.[9−11,16] Mutational studies such as those using the variant PKM2^{R399E} showed that this dimer-prone mutant promotes PKM2's unusual protein kinase activity.[13−15] This led to the belief that dimeric PKM2 may be responsible for the unusual signaling activity and contribute to cancer. However, a challenging aspect of this model is how dimeric PKM2 could maintain significant activity given its high K_M for PEP that,

Received: June 29, 2016
Revised: July 30, 2016
Published: August 2, 2016

DOI: 10.1021/acs.biochem.6b00658
Biochemistry 2016, 55, 4731−4736

under physiological conditions, would severely limit its ability to phosphorylate protein substrates.

Both the oligomeric state and activity of PKM2 are influenced by bound ligands. For decades, PKM2 and several other pyruvate kinase isoforms like PKL and PKR have been known to be induced by fructose 1,6-bisphosphate (FBP).[18,19] The binding of FBP shifts PKM2 to a tetrameric oligomeric state and lowers the K_M for PEP.[7] More recently, we reported that SAICAR, an intermediate of *de novo* purine nucleotide biosynthesis, activates PKM2 in an isozyme-specific manner.[12] SAICAR levels are enriched when cancer cells are under stress conditions, and binding of SAICAR to PKM2 promotes cancer cell survival.[12] In addition, the binding of SAICAR induces the protein kinase activity of PKM2 *in vitro* and in cultured cancer cells.[16]

Given the apparent switch in substrate specificity upon binding to SAICAR and the prevalence of dimeric PKM2 in cancer, we sought to determine whether there was a connection between SAICAR binding and the oligomeric state of PKM2. Here we report that SAICAR stimulates the PKM2 dimer without inducing formation of a PKM2 tetramer. We focused on a PKM2 variant identified in patient-derived tumors, $PKM2^{G415R}$,[20] and show that this variant can bind to FBP but does not form a tetramer. FBP cannot activate $PKM2^{G415R}$, suggesting that FBP-mediated PKM2 activation requires the tetramerization of PKM2. SAICAR, on the other hand, still activates $PKM2^{G415R}$ and in fact binds better to the dimeric form of PKM2 and improves substrate binding. Our results support an active role for the PKM2 dimer in cancer and suggest that the allosteric activation of PKM2 by SAICAR may in fact preferentially act via the dimeric form.

EXPERIMENTAL PROCEDURES

Materials. The plasmid encoding GST-PKM2 in the pGEX-6P-1 vector (GE Life Sciences) was custom synthesized by GenScript. Oligonucleotides were synthesized by Operon. Site-directed mutagenesis was performed using QuikChange XL II kits (Stratagene) and confirmed by sequencing from the T7 promoter and from the T7 terminator sites. Lactate dehydrogenase (LDH) was purchased from Roche. SAICAR was purchased from Toronto Research Chemicals. All other chemicals were from Sigma-Aldrich unless specifically noted otherwise.

Protein Expression and Purification. Wild-type PKM2 and its mutants were expressed and purified from *Escherichia coli* strain Rosetta (DE3) pLysS (Novagen). Cells were grown in Terrific Broth at 37 °C, and protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG, 0.5 mM) followed by incubation for 18 h at 16 °C. The following steps were conducted at 4 °C or on ice unless noted otherwise. Cells were collected by centrifugation (4000*g* for 20 min), and the pellet was resuspended in buffer A [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, and 1 mM DTT]. Cells were lysed by sonication, and debris was removed by centrifugation (45000*g* for 45 min) and then filtration through a 1.1 μm glass fiber filter (Thermo Scientific). The supernatant was loaded onto a 5 mL GSTrap column (GE Life Sciences) pre-equilibrated in buffer A. The resin was washed with buffer A, and the bound protein was eluted with a linear gradient of buffer B [20 mM Tris-HCl (pH 8), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10 mM reduced glutathione] over 20 column volumes. Fractions containing target protein were combined and digested with PreScission protease[21] (GE Life Sciences) in buffer C [50 mM Tris-HCl (pH 6.9), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT] overnight. Cleaved proteins were purified by gel filtration chromatography on a HiLoad 16/600 Superdex 200 column (GE Healthcare) with buffer D [10 mM HEPES (pH 7), 150 mM NaCl, 5 mM $MgCl_2$, and 0.25 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP)]. The protein concentrations were determined by measuring the absorbance at 280 nm under denaturing conditions, using a calculated extinction coefficient of 29910 M^{-1} cm^{-1}.[22,23] Protein contents were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and Coomassie Blue staining.

Pyruvate Kinase Activity Assay. Enzyme activity was measured in vitro with a lactate dehydrogenase (LDH) coupled assay as previously described.[12] PKM2 samples were diluted to the desired concentration in the assay buffer [50 mM Tris-HCl (pH 7.6), 100 mM KCl, 6.2 mM $MgCl_2$, and 1 mM DTT] and incubated with ligands at 37 °C for 30 min in a UV-transparent 96-well plate. After incubation, ADP (final concentration of 2 mM), NADH (final concentration of 220 μM), and LDH (final concentration of 5 units/mL) were added to the solution. Reactions were initiated by the addition of PEP (final concentration of 150 μM for typical experiments). The rate of NADH oxidation was spectroscopically measured at 340 nm using a Tecan Infinite M2 microplate reader.

Size-Exclusion Chromatography. Size-exclusion chromatography was performed using a Superdex S200 10/300 GL column (GE Healthcare). Purified $PKM2^{WT}$ or $PKM2^{G415R}$ (0.20 mL in buffer D) was loaded onto the column and eluted with buffer D (0.3 mL/min at 4 °C). Samples with FBP or SAICAR were incubated for 1 h at 4 or 18 °C before being loaded.

Small-Angle X-ray Scattering. Size-exclusion chromatography−small-angle X-ray scattering (SEC−SAXS) experiments were performed at BioCAT (beamline 18-ID, Advanced Photon Source at Argonne National Laboratory[24]). The instrument used for the experiment used a 12 keV (1.03 Å) X-ray beam and a Pilatus 3 1M detector (Dectris). Samples were passed through a Superdex 200 Increase 10/300 GL column (GE Life Sciences), and the eluted solution was directed to a 1.5 mm quartz capillary sample cell at a rate of 0.75 mL/min. The sample-to-detector distance was ∼3.5 m, which resulted in a q range of 0.004−0.38 $Å^{-1}$ (0.5 s exposure every 2 s). Data were corrected for background scattering by subtracting the buffer signal. Radius of gyration (R_g) values were calculated from Guinier approximations and $P(R)$ curves using PRIMUS.[25]

Isothermal Titration Calorimetry (ITC). ITC measurements were performed by using a Microcal ITC_{200} calorimeter at 25.0 °C. Protein solutions were dialyzed against buffer D. Ligands were dissolved in buffer D. Both protein and ligand solutions were degassed in vacuum before use. A $PKM2^{WT}$ or $PKM2^{G415R}$ solution (300 μM, 0.22 mL) was placed in the sample cell. A FBP or SAICAR solution (3 mM, 20 injections at 2 μL/injection) was injected at 180 s intervals. The baseline-corrected data were analyzed with Microcal Origin software.

RESULTS

The $PKM2^{G415R}$ Mutant Does Not Form a Tetramer in Solution. To isolate the potential activities for a dimeric form of PKM2, we sought to find a PKM2 variant that was unable to form a tetramer. Given that PKM2 has long been known to preferentially form a dimer in cancers, known as a "tumor

DOI: 10.1021/acs.biochem.6b00658
Biochemistry 2016, 55, 4731−4736

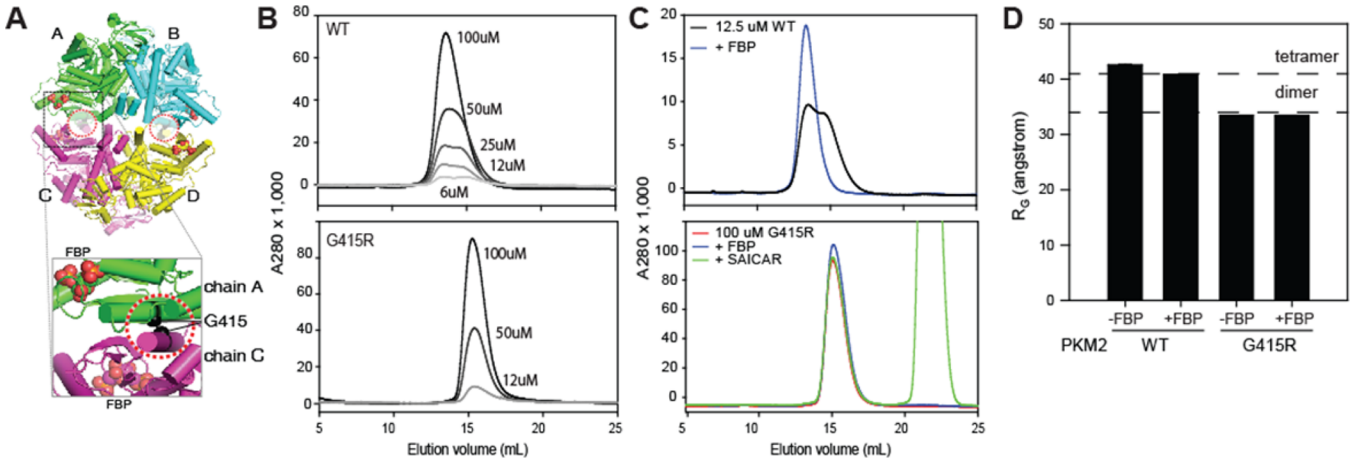


Figure 1. PKM2^{G415R} is constitutively a dimer. (A) Structure of the PKM2 tetramer (Protein Data Bank entry 3ME3[29]). The Cα atom of residue G415 is shown as a black sphere in each of the four chains. (B) PKM2^{WT} (top) but not PKM2^{G415R} (bottom) displays concentration-dependent gel filtration elution changes. (C) FBP does not alter the oligomeric state of PKM2^{G415R}. Gel filtration experiments show PKM2^{WT} to have an FBP-dependent shift, whereas PKM2^{G415R} was unaffected. (D) Small-angle X-ray scattering experiments suggest that PKM2^{G415R} is constitutively a dimer. Values for the radius of gyration (R_g) of PKM2^{WT} and PKM2^{G415R} were calculated from Guiner plots, using scattering data taken with PKM2 in the presence and absence of FBP. For reference, the R_g values calculated from a PKM2 tetramer crystallographic structure (Protein Data Bank entry 3ME3, chains A–D) or a hypothetical dimer (chains A and B only) are shown as dashed lines.

dimer", we analyzed the locations of mutations in PKM2 isolated from patient-derived tumors.[20] One amino acid change, a glycine-to-arginine mutation at position 415 (G415R), is located at the dimer–dimer interface of the PKM2 tetramer (Figure 1A). On the basis of its location at the interface and the introduction of two opposing positive charges, we speculated that this change might disrupt tetramer formation.

To test this hypothesis, human PKM2^{G415R} was recombinantly expressed and purified from *E. coli* and compared to human PKM2^{WT} by gel filtration chromatography (Figure 1B,C). At low-micromolar protein concentrations, PKM2^{WT} eluted at two major peak positions, which we interpret as tetrameric and dimeric species. We observed that either increasing concentrations of PKM2^{WT} or addition of FBP predominantly shifted the elution profile to the larger species, consistent with the larger species corresponding to a tetramer and smaller species to a dimer. In contrast to the wild type, PKM2^{G415R} eluted in a single peak corresponding to the smaller dimeric species and failed to shift to the larger tetrameric profile at a high (100 μM) protein concentration or upon the addition of FBP.

To independently verify that these gel filtration elution profiles reflect differences in oligomeric states, radius of gyration (R_g) values of wild-type and variant PKM2 were determined using SAXS[26] (Figure 1D). To minimize aggregation, SAXS measurements were recorded following an in-line size-exclusion column. To maximize the signal-to-noise ratio, PKM2 variants were applied to the column at concentrations of 100 μM. PKM2^{WT} had an R_g value of 43 Å (Figure 1D and Figure S1), consistent with the R_g value calculated from crystallographic structures of tetrameric PKM2 (41 Å). The binding of FBP did not significantly alter the R_g of PKM2^{WT} (41 Å), which is expected at the high concentrations used in these experiments. On the other hand, PKM2^{G415R} showed a much smaller R_g value [33 Å (Figure 1D and Figure S1)]. Guinier plots of these SAXS data showed that the molecular weight of PKM2^{G415R} in solution is approximately one-half of that of the PKM2^{WT} tetramer in solution (Figure S1). The R_g was not affected by FBP and was nearly identical to the R_g value expected from one-half of tetrameric PKM2 (34 Å). These SAXS results showed that PKM2^{G415R} indeed remains a dimer even at a high protein concentration or in the presence of FBP.

The PKM2^{G415R} Dimer Is in Its Tense Form and Cannot Be Activated by FBP. To assess the activation and properties of the PKM2 dimer, we measured the pyruvate kinase activity of PKM2^{G415R} with varying ADP and PEP concentrations (Figure 2). Previous work found the dimeric form of PKM2^{WT}

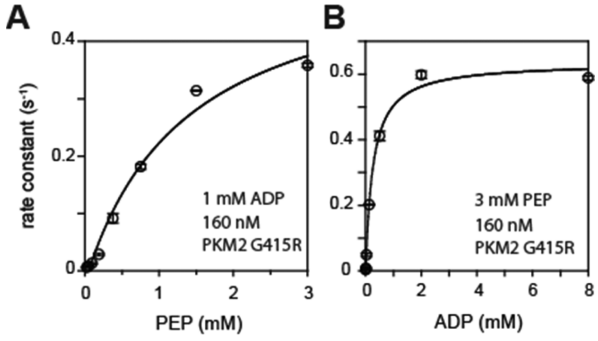


Figure 2. Pyruvate kinase activity of PKM2^{G415R}. (A) With titration of PEP, the PKM2^{G415R} variant shows a K_M of 1.2 mM PEP and a k_{cat} of 0.57 s^{-1}. (B) With titration of ADP, PKM2^{G415R} shows a K_M of 0.25 mM ADP.

to have relatively low activity and to be considered to exist in a tense form.[7] Here, we found PKM2^{G415R} to have a weak affinity for PEP (K_M = 1.2 mM PEP; k_{cat} = 0.57 s^{-1}) and a low-millimolar K_M for ADP (0.25 mM ADP). These values are comparable to those of the tense form reported for the wild-type PKM2 dimer.[7,12]

FBP is well-known for promoting the tetramerization and coincident activation of PKM2.[6,27] Our SAXS and size-exclusion experiments showed that FBP is unable to convert PKM2^{G415R} to a tetrameric form (Figure 1). To see the extent that activity might be impacted, we compared pyruvate kinase activities of PKM2^{WT} and PKM2^{G415R} at varying FBP

DOI: 10.1021/acs.biochem.6b00658
Biochemistry 2016, 55, 4731–4736

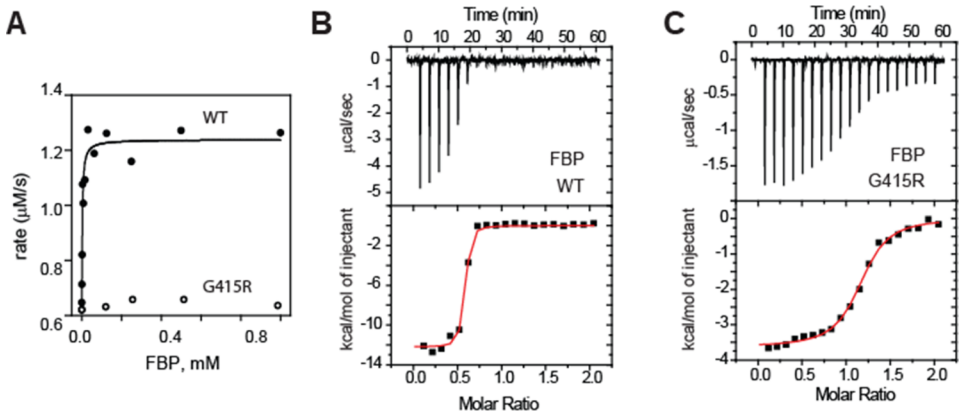


Figure 3. PKM2^{G415R} binds to FBP but is not activated. (A) Pyruvate kinase activity of PKM2^{WT} (●) and PKM2^{G415R} (○) in the presence of FBP. (B and C) Isothermal titration calorimetric analysis of binding of FBP to (B) PKM2^{WT} (0.54 site; K_d = 0.21 μM; ΔH = −12.2 kcal/mol) and (C) PKM2^{G415R} (1.0 site; K_d = 1.0 μM; ΔH = −4.7 kcal/mol).

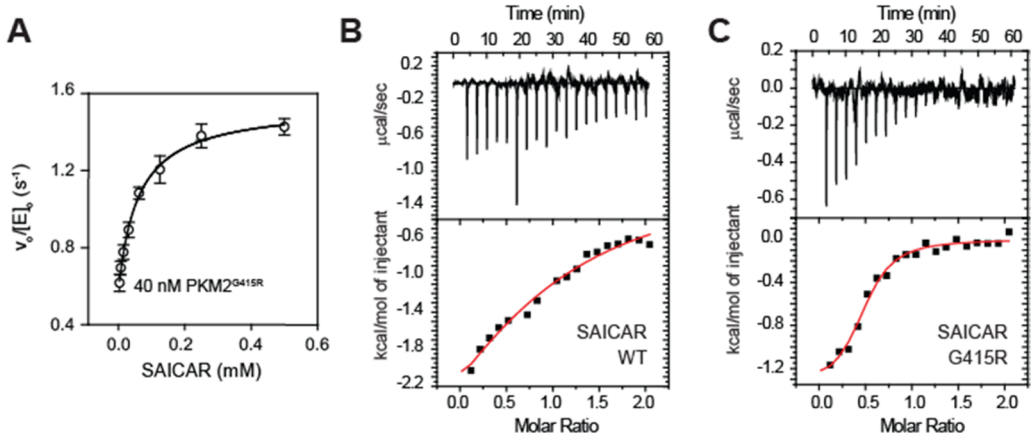


Figure 4. SAICAR still binds to PKM2^{G415R} and activates it. (A) Normalized pyruvate kinase activity of PKM2^{G415R} in the presence of SAICAR. (B and C) Isothermal titration calorimetric analysis of binding of SAICAR to (B) PKM2^{WT} (1.0 site per PKM2^{WT} monomer; K_d = 300 μM; ΔH = −5 kcal/mol) and (C) PKM2^{G415R} (0.48 site per PKM2^{G415R} monomer; K_d = 12 μM; ΔH = −1.4 kcal/mol).

concentrations (Figure 3A). In contrast to the clear activation of PKM2^{WT}, PKM2^{G415R} appeared to be unaffected by the presence of FPB and remain in the tense form.

A simple possibility for explaining these results could be that the G415R substitution interferes with FBP binding. To determine whether FBP still binds to PKM2^{G415R}, the interaction of FBP with PKM2 was analyzed by isothermal titration calorimetry (Figure 3B,C). For PKM2^{WT}, we observed an apparent binding isotherm consistent with a submicromolar K_D (Figure 3B). The contribution of enthalpy to binding suggests that FBP induced formation of the PKM2 tetramer (calculated buried surface area of 2400 Å^2). Interestingly, for PKM2^{G415R}, titration of FBP promoted a clear change in the measured heat released, with a K_D approximately 5-fold higher than that of PKM2^{WT} (Figure 3C). Compared to that of the FBP−PKM2^{WT} interaction, the enthalpy contribution was much smaller and consistent with FBP binding (buried surface area of 326 Å^2) without drastic changes in PKM2 quaternary structure. Taken together, these results demonstrate that PKM2^{G415R} can bind to FBP but fails to exhibit activation in concert with conversion to a tetrameric form.

The PKM2^{G415R} Dimer Can Be Activated by SAICAR without Forming a Tetramer. Our previous work showed that PKM2^{WT} can be activated by SAICAR.[12] To determine if activation might be intrinsically coupled to formation of the PKM2 tetramer, we monitored the distribution of size-exclusion peaks after incubation with SAICAR. The addition of SAICAR had no measurable effects on oligomeric state for either PKM2^{WT} or PKM2^{G415R} (Figure 1). This raised the possibility that SAICAR primarily exerts its influence on PKM2 without altering its oligomerization state. Given the inability of PKM2^{G415R} to tetramerize, we therefore used this cancer variant to investigate whether SAICAR could stimulate the dimeric form of PKM2. Remarkably, pyruvate kinase assays performed showed a distinct 2-fold activation by SAICAR [EC_{50} = 60 ± 20 μM; 1.0 ± 0.4 SAICAR per PKM2 monomer (Figure 4A)]. Importantly, the EC_{50} is approximately 5-fold lower than the EC_{50} (0.3 mM) observed with PKM2^{WT} activation by SAICAR,[12] suggesting that SAICAR binds to PKM2^{G415R} better than it binds to PKM2^{WT}.

Tighter binding of SAICAR to PKM2^{G415R} compared with that of PKM2^{WT} was further supported by ITC experiments (Figure 4B,C). Compared with PKM2^{WT}, which bound to SAICAR with an affinity of 300 ± 90 μM, PKM2^{G415R} bound to SAICAR significantly better, with a K_D of 12 ± 3 μM. Taken together, these results demonstrate that SAICAR binds to the PKM2 dimer and activates it without inducing a tetramer.

Taken together, these results show that there are two different modes of activating inactive dimeric PKM2 (Figure 5). First, dimeric PKM2 can be activated by becoming tetrameric

DOI: 10.1021/acs.biochem.6b00658
Biochemistry 2016, 55, 4731−4736

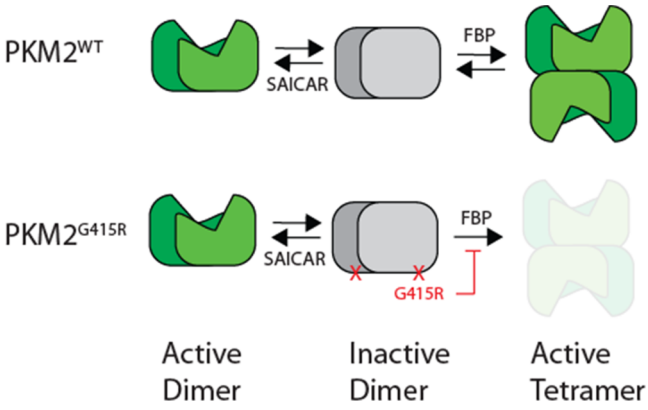


Figure 5. Different modes of PKM2 activation by FBP and SAICAR.

PKM2. This oligomerization state change is induced by FBP. Alternatively, the inactive PKM2 dimer can be activated by forming an active PKM2 dimer. SAICAR binding is an example in which the inactive PKM2 dimer becomes an active one without having its oligomerization state altered. The $PKM2^{G415R}$ variant from a patient-derived tumor shows that tetramer formation is dispensable and that it can be activated by an alternative mechanism.

DISCUSSION AND CONCLUSIONS

The relevance of PKM2 in cancer has been demonstrated in multiple systems.[6,27,28] Interestingly, quickly proliferating cells have higher levels of dimeric PKM2, which was widely considered to be an inactive form of pyruvate kinase. Many different models have been proposed to explain how nearly inactive pyruvate kinase can contribute to malignant cell growth. The results presented here show that dimeric PKM2 should not be considered a constitutively inactive form, but one that can be specifically activated by ligands such as SAICAR.

Our findings open new possibilities for regulation and activation of PKM2. Whereas tetramerization is one route to PKM2 activation, the PKM2 dimer is not merely an inactive pool but represents a physiologically important target for specific activation. Our findings that SAICAR bound more tightly to the dimeric $PKM2^{G415R}$ variant suggest that the dimeric form can be targeted by unique regulatory ligands that allow for richer responses to the metabolic state of the cell. We speculate that the pressure for fast proliferative growth in cancer cells selects for variants of PKM2 such as G415R that stabilize the dimeric state, which in turn favor ligand binding and biochemical properties of the PKM2 dimer. With dual substrate specificity, the ability of PKM2 to phosphorylate protein targets has been linked to cellular proliferation.[6,10,13,16,28] We previously reported that SAICAR-bound PKM2 can phosphorylate proteins much more efficiently than the FBP-bound form.[16] The higher activity we report here for dimeric PKM2 in the presence of SAICAR is consistent with the previous suggestion that protein kinase activity is accomplished by the dimeric form.[6] We anticipate that other means of controlling the dimer−tetramer equilibrium, such as through post-translational modifications, will also be uncovered, and that proper allosteric regulation of the PKM2 dimer will prove to be an important contributor to cellular adaptation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00658.

Figure S1 showing additional SAXS results (PDF)

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Author Contributions

M.Y. prepared all reagents and performed biochemical experiments. S.C., J.M.T., and L.P. performed SAXS experiments and analysis. M.Y., Y.-S.L., and G.D.B. designed experiments and analyzed data. Y.-S.L. and G.D.B. conceived the project. M.Y., Y.-S.L., and G.D.B. wrote the manuscript.

Funding

This work was supported by National Institutes of Health Grants R21CA181751 to G.D.B. and Y.-S.L. and R01CA168658 to Y.-S.L. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract DE-AC02-06CH11357. The SAXS experiment was supported by Grant 9 P41 GM103622 from the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health. Use of the Pilatus 3 1M detector was provided by Grant 1S10OD018090-01 from NIGMS.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Shelby Winans for the preparation of the $PKM2^{G415R}$ plasmid, and Cynthia Wolberger and Beverly Wendland for allowing us to use their equipment.

ABBREVIATIONS

PKM2, pyruvate kinase isoform M2; FBP, fructose-1,6-bisphosphate; SAICAR, succinyl-5-aminoimidazole-4-carboxamide-1-ribose-5′-phosphate; ITC, isothermal titration calorimetry; SAXS, small-angle X-ray scattering.

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DOI: 10.1021/acs.biochem.6b00658
Biochemistry 2016, 55, 4731−4736