Evidence That Does Not Support Pyruvate Kinase M2 (PKM2)-catalyzed Reaction as a Rate-limiting Step in Cancer Cell Glycolysis*

Received for publication, November 18, 2015, and in revised form, February 23, 2016 Published, JBC Papers in Press, February 25, 2016 DOI 10.1074/jbc.M115.704825

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It has been recognized that the rate-limiting function of pyruvate kinase M2 (PKM2) in glycolysis plays an important role in distributing glycolytic intermediates for anabolic and catabolic purposes in cancer cells. However, after analysis of the catalytic capacity of PKM2 relative to other glycolytic enzymes, the regulation range of PKM2 activity, metabolic flux control, and thermodynamics, we suggest that the PKM2-catalyzed reaction is not a rate-limiting step in cancer cell glycolysis. Hexokinase and phosphofructokinase 1 (PFK1), the first and third enzyme along the pathway, are rate-limiting enzymes that limit the overall glycolytic rate, whereas PKM2 and lactate dehydrogenase, the last two enzymes in the pathway, are for the fast removal of upstream intermediates to prevent the obstruction of the pathway. The argument is in accordance with the catalytic capacity of glycolytic enzymes, regulation range of enzyme activities, metabolic flux control, and thermodynamics.

During tumorigenesis, pyruvate kinase (PK) isotype in cells switches from pyruvate kinase M1 (PKM1) or pyruvate kinase L (PKL) to PKM2 (1, 2), suggesting that PKM2 plays a part in tumor initiation and development. PK catalyzes the last step of glycolysis and is commonly regarded as a rate-limiting enzyme. In cancer cells, PKM2 is considered as a rate-limiting enzyme, because its enzyme activity is sensitive to allosteric regulation (3, 4) and it catalyzes a thermodynamically favorable reaction. It is proposed that when it is allosterically inhibited, its catalytic rate is lower than the upstream glycolytic rate; hence, phosphoenolpyruvate (PEP)4 and upstream glycolytic intermediate may accumulate and flow to anabolic pathways; when it is allosterically activated, its activity is higher than the upstream glycolytic rate, and the upstream glycolytic intermediate would flow to pyruvate, which is then converted to lactate or enters mitochondria for complete oxidation. There are several lines of evidence that support this hypothesis (1, 4–11).

However, the evidence to support PKM2 as a rate-limiting enzyme is not sufficient. If PKM2 is a rate-limiting enzyme, the following lines of evidence are required: (a) PKM2 catalyzes an irreversible reaction; (b) its enzyme activity relative to other glycolytic enzymes is low; (c) its activity is sensitive to regulation; and (d) when its activity is allosterically activated, its catalytic rate should be higher than the upstream glycolytic rate and when its activity is allosterically inhibited, its catalytic rate should be lower than upstream glycolytic rate. So far, there is evidence of points a and c but no b and d.

Experimental Procedures

Cell Lines

Murine breast cancer cell line 4T1, human breast cancer cell line Bcap37, human cervical cancer cell line HeLa, human gastric cancer cell line SGC7901, human colon cancer cell line RKO, human liver cancer cell lines HepG2 and SMMC7721 were used in this study. Cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin, and 2 mM L-glutamine (complete RPMI 1640 medium).

Reagents and Enzymes

Glucose, Glc-6-P, fructose 6-phosphate (Fru-6-P), fructose 1,6-bisphosphate (FBP), glyceraldehyde 3-phosphate (GAP3), 3-phosphoglycerate (3PG), 2-phosphoglycerate (2PG), PEP, pyruvate, ADP, ATP, AMP, NAD(P), NAD(P)H, hexokinase 2 (HK2), phosphohexose isomerase (PGI), aldolase, triose-phosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), enolase, PK, and LDH were all from Sigma. PFK1 was from XIMEI (China).

Preparation of Recombinant PKM2, PKM1, and PKL

Recombinant PKM2, PKM1, and PKL was cloned and purified as the method described by us previously (12). The cDNA of PKM2, PKM1, and PKL was amplified from the cDNAs of the human breast cancer cell line MCF-7, human muscle, and human liver, respectively. Specimens of human muscle and human liver were obtained from the Tissue Bank of the Second

* This work was supported in part by China National 973 Project 2013CB911303, China Natural Sciences Foundation Projects 81272456 and 81470126, and the Fundamental Research Funds for the Central Universities, National Ministry of Education, China (to X. H.). The authors declare that they have no conflicts of interest with the contents of this article.

1 Supported by China Natural Sciences Foundation Project 81302398.

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4 The abbreviations used are: PEP, phosphoenolpyruvate; HK, hexokinase; LDH, lactate dehydrogenase; Fru-6-P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; GAP3, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; HK2, hexokinase 2; PGI, phosphohexose isomerase; TPI, triose-phosphate isomerase; LDHA, lactate dehydrogenase A; α-GPDH, α-glycerophosphate dehydrogenase.
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Affiliated Hospital, Zhejiang University. The use of human tissue was approved by the hospital's Institutional Review Board. The cDNA of PKM2, PKM1, and PKL was cloned into pQE-30 (Qiagen, Hamburg, Germany) with an N-terminal His6 tag and expressed in *Escherichia coli* strain XL-1 blue (Qiagen). Each clone was confirmed by sequencing by Invitrogen. When the *E. coli* culture attained an absorbance (600 nm) of 0.7, expression was induced by 1 mM isopropyl-

Briefly, a reaction was initiated by addition of the cell lysate (2–20 μg of protein) into the reaction buffer, reactant, and when appropriate the cofactor in a total volume of 1 ml. The amounts of the lysates used and the reaction times were carefully tested for each enzyme to maintain the linearity for each reaction. The absorbance at a wavelength of 260 nm (phosphoglycerate mutase and enolase) or 340 nm (HK, PGI, PKF1, aldolase, TPI, GAPDH, PGK, PKM2, and LDH) were monitored at 37 °C with a spectrophotometer (DU® Series 700, Beckman Coulter, Inc.). To get the linearity of each reaction, we added different amounts of cell lysate into the reaction mixture for different enzyme assays. The reaction mixture for each enzyme measurement was as described below.

**HK**—Reaction mixture contained 50 mM Hepes, 0.1 mM glucose, 0.5 mM ATP, 0.2 mM NADP, 1 unit of glucose-6-phosphate dehydrogenase, and the cell lysate was added into the reaction mixture to a final concentration of 20 μg of protein/ml.

**PGI**—50 mM Hepes, 5 mM MgCl2, 2 mM Fru-6-P and cell lysate was added into the reaction mixture to a final concentration of 5 μg of protein/ml.

**PKF1**—50 mM Hepes, 100 mM KCl, 5 mM MgCl2, 5 mM Na2HPO4, 1 mM NH4Cl, 5 mM Fru-6-P, 1.5 mM ATP, 0.2 mM NADH, 0.1 mM AMP, 1 unit of α-glycerophosphate dehydrogenase (α-GPDH), 1 unit of TPI, 1 unit of aldolase, and cell lysate was added into the reaction mixture to a final concentration of 20 μg of protein/ml.

**TPI**—50 mM Hepes, 5 mM EDTA, 1 mM GA3P, 0.2 mM NADH, 1 unit of α-GPDH and cell lysate was added into the reaction mixture to a final concentration of 15 μg of protein/ml.

**GAPDH**—50 mM Hepes, 5 mM Na2HPO4, 0.2 mM EDTA, 1 mM GA3P, 1 mM NAD, and cell lysate was added into the reaction mixture to a final concentration of 4 μg of protein/ml.

**PGK**—50 mM Hepes, 10 mM 3PG, 4 mM ATP, 6 mM MgSO4, 0.2 mM EDTA, 0.2 mM NADH, 1 unit of GAPDH, and cell lysate was added into the reaction mixture to a final concentration of 4 μg/ml.

**Phosphoglycerate Mutase**—50 mM Hepes, 5 mM MgCl2, 2 mM 3PG, 1 unit of enolase, and cell lysate was added into the reaction mixture to a final concentration of 20 μg of protein/ml.

**Enolase**—50 mM Hepes, 1 mM MgCl2, 50 mM KCl, 1 mM EDTA, 1 mM 2PG, and cell lysate was added into the reaction mixture to a final concentration of 20 μg of protein/ml.

**PKM2**—50 mM Hepes, 100 mM KCl, 5 mM MgCl2, 0.5 mM EDTA, 0.2 mM NADH, 1.5 mM ADP, 5 mM PEP, 1 unit of LDH, and cell lysate was added into the reaction mixture to a final concentration of 2 μg of protein/ml.

**LDH**—50 mM Hepes, 0.05% BSA, 2 mM pyruvate, 0.2 mM NADH, and cell lysate was added into the reaction mixture to a final concentration of 2 μg of protein/ml.

**Measurement of Enzyme Activity at Physiological Substrate Concentration**

The condition is the same as above, except for the concentration of substrates. The PEP and ADP for PKM2 was 0.25 mM.
and 50 μM, respectively; the Fru-6-P for PFK1 was 0.1 mM, and the glucose for HK was 2 mM.

**Measurement of Glycolytic Intermediates**

A million (1 × 10⁶) cells per well in complete RPMI 1640 medium were seeded into 6-well plates and allowed to attach overnight. The culture media were then replaced with 2 ml of fresh complete RPMI 1640 medium plus 6 mM glucose and cultured for 2 h. After removal of the medium, cells were quickly washed twice with ice-cold PBS. Prechilled 400 μl of 0.6 M HClO₄ was added, shaken on ice for 10 min, and aspirated. The solution was neutralized with 40 μl of 3 M K₂CO₃ and kept on ice for about 30 min. After centrifugation, the supernatant was used for spectrophotometric determination of glycolytic intermediates (24).

**Assay of Glucose, Glc-6-P, and Fru-6-P**—100 μl of supernatant and 10 μl of 20 mM NADP were added to the reaction buffer (200 mM Hepes, 5 mM MgCl₂, pH 7.3) for a total volume of 1 ml. The reaction was started with 1 unit of G6PDH to measure Glc-6-P. After reaction termination, 1 unit of PGI was added to measure Fru-6-P. 10 μl of 50 mM ATP and 1 unit of HK2 were added to measure glucose.

**Assay of FBP, Dihydroxyacetone Phosphate, and GA3P**—100 μl of supernatant and 10 μl of 20 mM NADH were added to the reaction buffer (200 mM Hepes, 5 mM EDTA, pH 7.3) for a total volume of 1 ml. The reaction was started with 1 unit of α-GPDH to measure dihydroxyacetone phosphate. After reaction termination, 1 unit of TPI was added to measure GA3P. 1 unit of aldolase was added to measure FBP.

**Assay of 3PG**—100 μl of supernatant, 10 μl of 20 mM NADH, and 1 unit of PGK were added to the reaction buffer (200 mM Hepes, 5 mM MgCl₂, pH 7.3) for a total volume of 1 ml. The reaction was started with 1 unit of GAPDH to measure 3PG.

**Assay of 2PG, PEP, and Pyruvate**—100 μl of supernatant and 10 μl of 20 mM NADH were added to the reaction buffer (200 mM Hepes, 5 mM MgCl₂, pH 7.3) for a total volume of 1 ml. The reaction was started with 1 unit of PEP to measure pyruvate. After reaction termination, 10 μl of 50 mM ADP and 1 unit of PK were added to measure PEP. 1 unit of enolase was added to measure 2PG.

**In Vitro Glycolytic System**

4T1 cells cultured in complete RPMI 1640 medium with 6 mM glucose, and at 70% confluence the cells were washed twice with ice-cold PBS and lysed with M-PER™ mammalian protein extraction reagent supplemented with Halt™ protease inhibitor mixture. The resultant crude 4T1 cell lysates when reconstituted with a buffer (200 mM Hepes, pH 7.3, 5 mM MgCl₂, 0.5 mM EDTA, 5 mM Na₂HPO₄, 50 mM KCl, 10 mM glucose, 1.5 mM ATP, 1.5 mM ADP, 2 mM NAD, and 0.2 mM NADH), empirically determined to be suitable for all 11 relevant enzymes, were able to fulfill an entire conversion of glucose to lactate in the cell-free system. Unless otherwise specified, each reaction was initiated by adding 4T1 cell lysates to the above buffer in a total volume of 600 μl; because the volumes of added cell lysates were small as compared with total reaction volumes, the levels of endogenous glycolytic metabolites and cofactors were negligible. The action was terminated by adding 600 μl 1 M HClO₄, then neutralized with 100 μl 3 M K₂CO₃. After keeping on ice for about 30 min and centrifugation, the supernatant obtained was analyzed for the lactate generated. When PKM2 was assayed, the reaction mixture was the same as above, except ADP was 50 μM. The amounts of generated lactate were determined by HPLC as described by us previously (25).

**Metabolic Flux Control Assay**

Metabolic flux control assay is aimed at describing the control ability of each glycolytic enzyme to glycolysis. The control is described as the infinitesimal fractional change in the activity of the enzyme that causes the fractional change of flux (26). Using the in vitro glycolytic system as described above, we monitored the flux change by adding extra glycolytic enzyme to the system, so we could get a serial glycolysis flux with different enzyme activity, e.g. when we tried to determine the contribution of HK to glycolytic rate, we added a serial amount of pure HK2 into the in vitro glycolytic system. The fluxes were reflected by the amount of generated lactate. The amounts of generated lactate were determined by HPLC as described by us previously (25). For PKM2, assay, the condition was the same as above except that ADP concentration in the in vitro glycolytic system was 50 μM.

To plot the graph about flux and enzyme activity, flux values were normalized with respect to the value of basal flux (without adding any extra enzyme), and enzyme activity values were divided by their respective $K_m$ values as described before (27).

**Calculation of the Gibbs Free Energy Change ($\Delta G$) of the Glycolytic Reactions**

$\Delta G$ can be calculated according to the following equation: $\Delta G = \Delta G_m^{o} + RT\ln Q$, where $\Delta G_m^{o}$ denotes the standard transformed Gibbs free energy at 37 °C, and Q can be calculated from the glycolytic intermediates data (Table 2), and ATP/ADP and NAD/NADH were set as 10 and 78.8, respectively. (We determined the ATP, ADP, NAD, and NADH concentrations in 4T1, HeLa, and Bcap37 cells, which were relatively stable and so
the average value was used for Q value calculation.) For example, the Q value of the reaction catalyzed by HK was calculated as $\text{[Glc-6-P]}/\text{[ADP]}/\text{[glucose]}/\text{[ATP]}$.

The question is that $G_310$ is not available. We calculated $G_310$ from $G_{298}$ according to Equation 1,

$$
\Delta G = \Delta H - T\Delta S 
$$

(Eq. 1)

Because the change of $\Delta H$ and $\Delta S$ is negligible between $298 \text{ K}$ (25 °C) and $310 \text{ K}$ (37 °C) (28, 29), the equation can be expressed as shown in Equation 2,

$$
\Delta G^0_{310} = \frac{310}{298} \Delta G^0_{298} + \left( 1 - \frac{310}{298} \right) \Delta H^0_{298} 
$$

(Eq. 2)

where $\Delta G^0_{298}$ and $\Delta G^0_{310}$ is from Refs. 30, 31.

siRNA Knockdown Experiment

$1 \times 10^5$ 4T1 cells per well in complete RPMI 1640 medium were seeded into 6-well plates, then transfected with either negative control siRNA (siR-Ribo<sup>TM</sup> negative control, Ribobio, China) or HK2, PFK1, PKM2, and LDHA siRNA, and cultured
for 72 h. The sequences of deployed siRNAs were as follows: HK2 sense, CCAAGAUGUCUCGAUAU-dTdT; PFK1 sense, GCACAGCUUGAGCCAUAG-dTdT; and antisense, AUAUCGAGACACUUCUUGG-dTdT; PKM2 sense, CCAUAUCGUGUCACCAACCAAA-dTdT; and antisense, UUGAGGAGCAAGGAUAAAUGG-dTdT; and LDHA sense, GGAUGACGUUCGCCCUUGUUU-dTdT, and antisense, ACAAAGGCGAACGCUAACC-dTdT. The final concentrations for negative control of HK2, PFK1, and PKM2 siRNA were 50 nM and for LDHA siRNA was 20 nM. The culture media were then replaced with 2 ml of fresh complete RPMI 1640 medium plus 6 mM glucose and cultured for a further 5 h. The culture media were collected for glucose and lactate determination. Cells were counted and collected for Western blot detection and enzyme activity assays, and the glucose consumption and lactate generation by intact cells and intracellular glycolytic intermediates were measured as described above.

**PFK1 mRNA Detection**

As the antibody against PFK1 is not commercially available, we used real time PCR to determine the transcriptional level of this enzyme. 1 × 10^6 4T1 cells per well in complete RPMI 1640 medium were seeded into 6-well plates, then transfected with either negative control siRNA or PFK1 siRNA (final concentration 50 nM), and cultured for 48 h. RNA was extracted and used to synthesize the first-strand cDNA using the Superscript system (Life Technologies, Inc.) according to the manufacturer’s instructions. Real time PCR was performed using the Power SYBR Green PCR Master Mix protocol (Applied Biosystems). Sequences of the primers for real time PCR was as follows: forward 5’-CCCCCAGTCTCTAAGGGTGG-3’ and reverse 5’-ATCATGTACGACCAGCACCC-3’. The first question is as follows. In cancer cells is PKM2 activity relatively low in comparison with other glycolytic enzymes?

**Results and Discussion**

The first question is as follows. In cancer cells is PKM2 activity relatively low in comparison with other glycolytic enzymes?
We measured the glycolytic enzymes in seven randomly picked cancer cell lines. These cancer cells express PKM2 (Fig. 1). The pattern of the enzyme activities in these cancer cells was remarkably similar (Fig. 2). The activities of PKM2 in seven cancer cells lines were all the highest in glycolytic enzymes (Fig. 2). By contrast, the activities of HK and PFK1 were lower than those of other glycolytic enzymes, except that PFK1 activity was higher than that of aldolase in 4T1 cells. The relative activities of PKM2 in the tested cancer cells were 34.8–58.1-fold higher than HK, and 23.4–92.1-fold higher than PFK1 (Fig. 2). Similarly, PKM2 activities in clinical tumors were also far higher than those of HK (Table 1).

Nevertheless, the above measurement of enzyme activities was carried out under the condition of saturating substrate concentration. It was necessary to measure the actual enzyme activities under physiological substrate concentrations. We then measured the concentration of glucose, Fru-6-P, PEP, and pyruvate in the tested cancer cells (Table 2), and the enzyme activities according to the substrate concentration in the cancer cells (Fig. 3). PKM2 activities even under physiological PEP concentrations were 3.5–6.6-fold higher than HK and 2.7–24.1-fold higher than PFK1 (Fig. 3).

The catalytic rate of HK may represent the maximal glycolytic rate, but the actual glycolytic rates of cancer cells were

### Table 1

| Cells    | HK (nmol min⁻¹ mg⁻¹ protein) | PFK1 (nmol min⁻¹ mg⁻¹ protein) | PKM2 (nmol min⁻¹ mg⁻¹ protein) |
|----------|------------------------------|--------------------------------|--------------------------------|
| 4T1      | 142 ± 6                      | 258 ± 7                        | 702 ± 35                       |
| HepG2    | 151 ± 13                     | 40 ± 8                         | 690 ± 31                       |
| SGC7901  | 113 ± 8                      | 32 ± 9                         | 420 ± 24                       |
| SMMC7721 | 130 ± 23                     | 34 ± 8                         | 470 ± 27                       |
| Hela     | 120 ± 12                     | 39 ± 5                         | 631 ± 75                       |
| RKO      | 122 ± 21                     | 34 ± 14                        | 737 ± 65                       |
| Bcap37   | 128 ± 4                      | 36 ± 3                         | 867 ± 26                       |
lower than the HK rate (Fig. 4). As a result, the activities of PKM2 in cancer cells at physiological concentration of PEP are many fold higher than the actual glycolytic rates (Fig. 4).

The second key question is as follows. If PKM2-catalyzed reaction is a rate-limiting step in glycolysis, the activity of PKM2 should be jump between $x$ and $y$ ($x$ denotes the actual glycolytic rates in cancer cells, and $y$ denotes the actual activities of PKM2, e.g. for 4T1, $x$ and $y$ are 21.5 and 702 nmol min$^{-1}$ mg$^{-1}$ protein, respectively, Fig. 4A). According to the general principle of metabolic flux control, this change magnitude of enzyme activity seems far too large for an enzyme to function as a rate-limiting enzyme.

The activity of PKM2 is sensitive to regulation (32). The most potent allosteric regulator is FBP, which tetramerizes four subunits of PKM2 and the tetramer is the form with high activity; without FBP, PKM2 molecules dissociate to a dimer, which is a form with low activity. The $V_{\text{max}}$ of PKM2 dimer and tetramer is comparable, but the $K_m$ value of the dimer and tetramer toward PEP is 0.5 and 0.03 mM, respectively (33). Under physiological PEP concentrations, in the tested cancer cells, if all the molecules of PKM2 were tetramer, its catalytic rate would be
2.7–3.6-fold higher than if all the enzyme molecules were dimer (Fig. 5A). The subsequent questions then are as follows. Are the catalytic rates of PKM2 dimer and tetramer lower and higher, respectively, than the overall glycolytic rate? Could this regulation possibly limit the rate at this step?

In fact, when we measured PKM2 activities, we did not add FBP into the assay system. Under such conditions, most PKM2 molecules should be dimers. Indeed, the $K_m$ value of PKM2 in the tested cancer cells, except 4T1 PKM2, were 0.46–0.61 mM (Fig. 6), which were similar to the reported value of the PKM2 dimer (33). The $K_m$ value of 4T1 PKM2 was 0.13 mM, although significantly lower than that of enzymes from other cell lines, and about 4-fold higher than that of dimeric $K_m$. We do not know why PKM2 from 4T1 has a lower $K_m$ value, but probably it is because 4T1 is a mouse cancer cell line and others are all human cancer cell lines. To objectively assess whether the fluctuation of PKM2 activity can possibly play a part in rate-limiting, we compared the actual glycolytic rate of the tested cancer cells with the calculated rate of PKM2 in tested cells. The comparison led to the conclusion that even if all molecules of PKM2 were dimers, the activities were 50–128-fold higher than the actual glycolytic rate in the tested cancer cells (Fig. 5B); if all molecules of PKM2 were tetramers, the activities were 175–342-fold higher than those of the actual glycolytic rates in the tested cancer cells, indicating that, no matter whether tetramer or dimer, the activities of PKM2 were far higher than the overall glycolytic rate, i.e., the regulation range of PKM2 activity by FBP unlikely makes the enzyme-catalyzed reaction a rate-limiting step in cancer cell glycolysis. Although the activity of PKM2 can be subjected to many regulations (1, 3–5, 7–9, 32, 34, 35), these...
regulations are less potent than FBP, and hence are less likely to make this reaction a rate-limiting step.

We then did metabolic flux analysis. The results were in our expectations (Fig. 7). HK was the major rate-limiting enzyme, followed by PFK1. Aldolase and PGI exerted a minor rate-limiting effect on glycolysis. Other glycolytic enzymes, including PKM2, have no detectable rate-limiting effect on glycolysis. The results of metabolic flux assay excellently match the results of the relative activities of enzymes.

It would be worthwhile to discuss the change of Gibbs free energy accompanied by the reaction catalyzed by PKM2. The standard change of Gibbs free energy of this reaction is $-32.87 \text{ kJ/mol}$, and the actual changes of Gibbs free energy of this reaction in tested cancer cells are $-25.28$ to $-24.38 \text{ kJ/mol}$ (Fig. 8A). A reaction with such a large change in the Gibbs free energy in a metabolic pathway is commonly considered as a rate-limiting step, but the prerequisite condition is that the catalytic capacity of the corresponding enzyme that catalyzes this reaction should be low in comparison with other enzymes along the metabolic pathway, and the regulation range of its activity should make its catalytic rate fluctuate around the overall rate of the pathway. As the PKM2-catalyzed rate is far higher than the actual glycolytic rate of cancer cells, the regulation of PKM2 activity would hardly exert a significant impact on the overall rate of glycolysis. As such, the large change of the Gibbs free energy of the reaction catalyzed by the quite excessive catalytic capacity of PKM2 (both low and high catalytic form) drives PEP from the upstream glycolytic flux unconditionally, rapidly, and effectively to pyruvate, i.e. we cannot perceive a biochemical basis that the PKM2-catalyzed reaction can be lower than the upstream rate so that PEP and its upstream intermediates can accumulate. Therefore, PKM2 is unlikely a rate-limiting step.

**FIGURE 8.** Gibbs free energy change in cancer cells and the relative activities of PFK1, PKM2, and LDH to HK at physiological substrate concentrations. A, standard and actual change of Gibbs free energy in cancer cells. The standard change of Gibbs free energy $\Delta G_{\text{std}}$ is from Ref. 30; the actual change of Gibbs free energy $\Delta G$ is calculated as described as “Experimental Procedures.” B, activities of enzymes relative to HK, which is defined as 1. Data are mean ± S.D., $n = 3$. 

| Glycolytic reactions | $\Delta G^\circ$ (kJ/mol) | $\Delta G$ (kJ/mol) |
|----------------------|--------------------------|---------------------|
| Glc + ATP $\rightarrow$ G6P + ADP | $-16.41$ | $-30.42$ |
| F6P + ATP $\rightarrow$ F1,6BP + ADP | $-14.39$ | $-19.58$ |
| PEP + ADP $\rightarrow$ Pyruvate + ATP | $-32.87$ | $-24.38$ |
| Pyruvate + NADH $\rightarrow$ Lactate + NAD | $-23.62$ | $-3.22$ |

regulations are less potent than FBP, and hence are less likely to make this reaction a rate-limiting step.

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Regulation of PKM2 activity does not significantly affect the rates of glucose consumption and lactate generation by intact 4T1 cells. A, PKM2 siRNA transfection significantly reduced the amount and activity of PKM2. B, PKM2 siRNA transfection did not significantly decrease glucose consumption and lactate generation. C, PKM2 activator TEPP-46 significantly enhanced PKM2 activity. D, TEPP-46 did not significantly increase glucose consumption and lactate generation.

Down-regulation of LDH does not significantly affect the rates of glucose consumption and lactate generation by intact 4T1 cells. A, LDHA siRNA transfection significantly reduced the amount and activity of LDH. B, LDHA siRNA transfection did not significantly decrease glucose consumption and lactate generation.
enzyme, and hence its catalyzed reaction is unlikely a rate-limiting step in cancer cell glycolysis. By contrast, the large change of the Gibbs free energy of the reactions catalyzed by HK and PFK1 (Fig. 8A), the low activities of HK and PFK1 relative to other glycolytic enzymes (Figs. 2 and 3), and their sensitivity to allosteric regulation (36, 37) make them perfect rate-limiting enzymes.

We then carried out additional experiments to provide additional proof. After knocking down PKM2, the amount and activity of this enzyme reduced significantly, but glucose consumption and lactate generation in cells did not decrease significantly (Fig. 9, A and B). Conversely, after the cells were treated with the PKM2 activator, despite a significant enhanced activity of PK, glucose consumption and lactate generation remained nearly unchanged in comparison with control cells (Fig. 9, C and D). LDHA knockdown did not significantly affect glucose consumption and lactate generation (Fig. 10).

In contrast, knocking down HK2 and PFK1 significantly reduced glucose consumption and lactate generation (Fig. 11). Nevertheless, given the large change of HK activity (reduced by 50%) and PFK1 activity (reduced by 75%), the reduction of glucose consumption and lactate generation remained nearly unchanged in comparison with control cells (Fig. 9, C and D). LDHA knockdown did not significantly affect glucose consumption and lactate generation (Fig. 10).

In addition, we determined the concentrations of FBP, PEP, and pyruvate in intact 4T1 cells with or without HK2, PFK1, PKM2, or LDHA siRNA transfection or with or without TEPP-46 treatment. The conditions of cell culture, transfection, or treatment with TEPP-46 were the same as described in Figs. 9–11. Cells were washed twice with ice-cold PBS and lysed with 0.6 M HClO4 and neutralized with 3 M K2CO3, and then the intermediates were measured as described under “Experimental Procedures.” Data are mean ± S.D., n = 3.

FIGURE 11. Down-regulation of HK and PFK1 significantly decreases glucose consumption and lactate generation by 4T1 cells. A, HK2 siRNA transfection significantly reduced the amount and activity of HK. 4T1 cells were transiently transfected with HK2 siRNA or negative control siRNA for 72 h; cells were collected and lysed; and the cell lysates were subjected for Western blot and enzyme assay for HK. B, HK2 siRNA transfection significantly decreased glucose consumption and lactate generation. Cells were incubated in culture medium for a further 5 h; the concentrations of glucose and lactate in culture medium were determined, and then glucose consumption and lactate generation were calculated. C, PFK1 siRNA transfection significantly reduced the amount and activity of PFK1. 4T1 cells were transiently transfected with PFK1 siRNA or negative control siRNA for 48 h; RNA was extracted and subjected to real time PCR determination of PFK1; enzyme assay for PFK1 activity was carried 72 h after siRNA transfection. D, PFK1 siRNA transfection significantly decreased glucose consumption and lactate generation. Cells were incubated in fresh culture medium for further 5 h; the concentrations of glucose and lactate in culture medium were determined, and then glucose consumption and lactate generation were calculated. The detailed procedure of siRNA knockdown, Western blot, enzyme activity assay, and determination of glucose and lactate is described under “Experimental Procedures.” Data are mean ± S.D., n = 4.

FIGURE 12. Concentrations of FBP, PEP, and pyruvate in intact 4T1 cells with or without HK2, PFK1, PKM2, or LDHA siRNA transfection or with or without TEPP-46 treatment. The conditions of cell culture, transfection, or treatment with TEPP-46 were the same as described in Figs. 9–11. Cells were washed twice with ice-cold PBS and lysed with 0.6 M HClO4, and then the intermediates were measured as described under “Experimental Procedures.” Data are mean ± S.D., n = 3.
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seems that the concentration change of these intermediates is not necessarily associated with the rate change of glycolysis.

The intermediate determination raises several biochemical issues, e.g. it is understandable that LDHA knockdown elevated the concentration of pyruvate, but it is beyond our understanding that LDHA knockdown also significantly elevated the concentration of PEP. Does it imply that PKM2-catalyzed reaction is at near equilibrium state, so that elevation of pyruvate caused elevation of PEP? Such an interpretation is of course incorrect, because the $K_{eq}$ of this reaction is $2 \times 10^4$, and if it is approaching an equilibrium state, the concentration of pyruvate would reach the molar range, wreaking osmotic havoc. Hence, this is a puzzle to be resolved.

Alteration of enzyme activities of HK or PFK1 altered overall glycolytic rates in intact cells and also lowered the concentration of FBP. However, alteration of enzyme activities of PKM2 and LDH did not alter the overall glycolytic rates in intact cells, but it could alter the concentrations of FBP, PEP, or pyruvate. As the overall glycolytic rate is not changed, the alteration of PKM2 and LDH activities should not change the overall metabolic fate of glucose. Nevertheless, there should be some meaning of these changes, but so far, based on the available data, we are not able to plausibly interpret what the concentration fluctuation of these intermediates associated with the activity change of PKM2 and LDH implies.

Then, what is the possible role of PKM2 in glycolysis? Because no matter whether a tetramer or dimer, the catalytic capacity of PKM2 in cancer cells is far higher than the actual glycolytic rate; and a reasonable interpretation is that it is for the rapid removal of PEP to prevent accumulation of upstream glycolytic intermediates. To avoid the accumulation of pyruvate, cancer cells also express excessive LDH (Figs. 2 and 8B), which rapidly converts pyruvate to lactate. It should be noted that the standard change of Gibbs free energy of the LDH-catalyzed reaction is $-23.62$ kJ/mol, and in cancer cells, as the mass action ratio is smaller than the equilibrium constant, the actual change of Gibbs free energy is $-7.29$ to $-3.22$ kJ/mol (Fig. 8A), which is sufficient to drive this reaction from pyruvate to lactate. Conversely, most cancer cells produce a quantity of lactate, converting 80–90% of incoming glucose to lactate, even with ample oxygen.

Taken together, the results suggest that PKM2 and LDH, the last two enzymes in the pathway, are probably for the fast removal of upstream intermediates to prevent the obstruction of the pathway. In addition, the pattern of activities of glycolytic enzymes in cancer cells and the thermodynamics of the reactions laid the fundamental biochemical basis for the Warburg effect.

Author Contributions—X. H. conceived the concept and designed the study; J. X. and C. D. performed the experiments; X. H., J. X., and C. D. analyzed the data; J. X. and C. D. drew the graphs; and X. H. wrote the paper.

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