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A critical review of the role of M_2PYK in the Warburg effect

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Abstract

It is becoming generally accepted in recent literature that the Warburg effect in cancer depends on inhibition of M_2PYK, the pyruvate kinase isozyme most commonly expressed in tumors. We remain skeptical. There continues to be a general lack of solid experimental evidence for the underlying idea that a bottle neck in aerobic glycolysis at the level of M_2PYK results in an expanded pool of glycolytic intermediates (which are thought to serve as building blocks necessary for proliferation and growth of cancer cells). If a bottle neck at M_2PYK exists, then the remarkable increase in lactate production by cancer cells is a paradox, particularly since a high percentage of the carbons of lactate originate from glucose. The finding that pyruvate kinase activity is invariantly increased rather than decreased in cancer undermines the logic of the M_2PYK bottle neck, but is consistent with high lactate production. The "inactive" state of M_2PYK in cancer is often described as a dimer (with reduced substrate affinity) that has dissociated from an active tetramer of M_2PYK. Although M_2PYK clearly dissociates easier than other isozymes of pyruvate kinase, it is not clear that dissociation of the tetramer occurs *in vivo* when ligands are present that promote tetramer formation. Furthermore, it is also not clear whether the dissociated dimer retains any activity at all. A number of non-canonical functions for M_2PYK have been proposed, all of which can be challenged by the finding that not all cancer cell types are dependent on M_2PYK expression. Additional in-depth studies of the Warburg effect and specifically of the possible regulatory role of M_2PYK in the Warburg effect are needed.

Introduction

Many researchers have concluded that the pyruvate kinase (PYK) reaction, the last reaction in glycolysis, plays a pivotal role in controlling metabolism in cancer tissues. In fact, even in 1975 it was known that tumor cell respiration can be stimulated by the use of PYK inhibitors [46, 47]. More recent discussions on the controllers of Warburg metabolism often reference Christofk, *et al.* to indicate PYK's role in tumor growth [48]. Given the appeal of targeting Warburg metabolism to treat cancer and the growing acknowledgment of the role of M_2PYK

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in controlling that metabolism, it is not surprising that there has now been considerable effort to directly target M_2PYK activity for drug design [49–68].

Unfortunately, the race to identify a M_2PYK-targeting cancer drug has led to several poorly supported explanations for how M_2PYK functions in cancer and many of those explanations are based on data that have not been fully scrutinized (or speculated ideas that were never supported by data!). Therefore, the goal of this review is to critically evaluate M_2PYK functions in the context of Warburg metabolism. However, rather than simply dismiss M_2PYK as an important enzyme in Warburg metabolism, we conclude with a speculation about the role of increased glycolysis in cancer metabolism.

A quick overview of PYK

Historically, the unique properties of the pyruvate kinase protein in cancers were identified simultaneously with isozyme expression patterns in normal tissues [75–84]. There are four isozymes expressed in mammals [75, 87, 88]. LPYK (found in liver and pancreas) and PYK from erythrocytes (R-PYK) are products of one gene as a result of alternative start sites [89–93]. M_1PYK (found in heart, muscle and brain and characterized by a hyperbolic response of activity over a concentration range of PEP) and M_2PYK (also referred to as KPYK due to its presence in kidney and characterized by a sigmoidal response of activity over a concentration range of PEP), originate from a second gene *via* alternative RNA splicing [75, 87, 88, 96, 97], and differ by only 22 amino acids. In early fetal tissue, M_2-PYK is the only isozyme detected. Near the time of birth, expression of M_2PYK is displaced by tissue specific isozymes in many tissues [75, 88, 100, 101]. Despite this general trend for a change in isozyme expression, several adult tissue types continue to express M_2-PYK, including adult lung, kidney and many smooth muscle organs [75, 88, 100, 104–106]. Re-expression of M_2PYK is an early event in transformation of normal tissue into cancer [112]; therefore, the serum level of M_2PYK has been evaluated as a marker for many types of cancers [113–115]. These well-established facts about M_2PYK set a background for the discussion of a role of M_2PYK in Warburg metabolism.

M_2PYK in cancer tissue/cells is NOT an "inhibited" isozyme of pyruvate kinase

The often-repeated assumption that the M_2PYK present in cancer cells is an inhibited form of pyruvate kinase seems to have been derived from a comparison of the enzymatic properties of M_2PYK to those of M_1PYK [96, 97]. Indeed, in the absence of post-translational modifications, protein:protein interacting partners, or allosteric effectors, M_1PYK has a lower higher apparent affinity (*i.e.,* We use apparent affinity to refer to the concentration of substrate at ½ V_{max} activity. For a sigmoidal response fit to the Hill equation, this parameter is sometimes referred to as $S_{0.5}$ or $K_{0.5}$. When data are hyperbolic, the parameter is a K_M. Our use of apparent affinity is intended to collectively refer to both.) for the substrate, phosphoenolpyruvate (PEP), compared to M_2PYK (Table 1) [88, 123]. In addition, M_1PYK is often used in comparative studies of an enzyme that increases flux through the PYK reaction [48]. However, when considered in the context of all the various types of regulatory mechanisms that can alter substrate affinity, it is better to state that

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M_2PYK has a larger range of substrate affinities that can be accessed *via* various regulatory mechanisms. In contrast, inhibition by hydrophobic amino acids is the only regulatory mechanism for M_1PYK [124] and given the low affinities for these ligands, it is unclear if the collective concentration of all hydrophobic amino acids provides a mechanism for control of M_1PYK that is physiologically meaningful. (M_1PYK often is reported as not being allosterically regulated.) Unfortunately, in this comparison to M_1PYK, the "tunability" of M_2PYK's affinity for PEP seems to have been completely overlooked in the assignment of M_2PYK as "inhibited."

In fact, a decrease in the apparent affinity of M_2PYK does not in itself imply reduced PYK activity in the cell. A few different scenarios (or combinations of these) could result in the same or increased activity in the cell, despite a reduced substrate affinity for one isozyme. For simplicity in these hypothetical considerations, we will consider a true K_M instead of an $S_{0.5}$. 1) The activity in a cell depends upon the cellular concentration of substrate. If PEP concentrations are sufficiently high relative to K_M values for both isozymes, both the M_1PYK and M_2PYK enzymes could have the same level of catalytic turnover, despite differences in K_M (Figure 1). 2) If an isozyme with lower substrate affinity also has a higher V_{max}, then the cell could have equal or higher activity compared an enzyme with tighter substrate affinity, but lower V_{max} activity (Figure 2). 3) When an enzyme with lower substrate affinity (*e.g.,* M_2PYK) is expressed at higher concentrations in the cell, the cell can have equal or higher total activity compared to when an isozyme with higher substrate affinity (*e.g.,* M_1PYK) is expressed at a lower concentration. Therefore, an isozyme switch to an enzyme with a reduced substrate affinity does not imply reduced enzyme activity and metabolic flux in the cell (Figure 2). Therefore, the reduced affinity of M_2PYK for PEP, when compared to M_1PYK does not imply reduced PYK activity in the cell.

Despite the often-quoted idea that M_2PYK is inhibited, the similar kinetic/enzymatic properties of M_2PYK, LPYK, and RPYK (in the absence of any other regulatory mechanism) give little reason to consider M_2PYK to be "inhibited" [88, 128]. LPYK and M_2PYK have similar apparent affinities for PEP and similar specific activity values in the absence of regulatory effectors. Despite the M_2PYK *vs.* LPYK similarities, the LPYK expressed in hepatocytes is displaced by M_2PYK expression in heptocarcinomas, much like M_2PYK expression is favored in most other cancer tissues. Therefore, pointing to the apparent affinity of M_2PYK for PEP (in the absence of effectors) as the primary property that makes M_2PYK important for cancer survival seems unwarranted. Furthermore, if comparisons between isozymes can be used to define one isozyme as inhibited or not inhibited and given that LPYK is not commonly referenced as an "inhibited" form, then the LPYK *vs.* M_2PYK comparison would support that M_2PYK is also not inhibited.

Many past reviews of the role of M_2PYK in cancer biology have focused primarily on the isozyme expression switch that results in M_2PYK being expressed in cancer tissues. However, that focus overlooks the fact that M_2PYK is expressed in several normal adult tissues (*e.g.,* lung, kidney, many smooth muscles) [75, 100, 104–106]. An isolated focus on the PYK isozyme expression switch also ignores the evidence that the "cancer" form of M_2PYK has properties that differ from those of M_2PYK expressed in normal adult tissues [82, 128–140]. A search of the primary literature for direct comparisons of the kinetic/

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enzyme properties of M_2PYK isolated from normal *vs.* from cancer tissue/cells reveals relatively few studies. Nonetheless, those reports that are available indicate that the M_2PYK isolated from cancer has higher (NOT lower) affinity for PEP when compared to M_2PYK isolated from normal tissue [128, 138, 141]. In total, there is very little evidence to support the often-repeated idea that M_2PYK isolated from cancer cells is inhibited.

Focusing on functional outcomes of regulatory mechanism is more important than focusing on tetrameric M_2PYK dissociating into dimer/monomer

At the protein level *in vitro*, the M_2PYK subunits exist in an equilibrium between a tetramer and dimers/monomers. The observations of a quaternary structure change *in vitro*, with knowledge that dissociated dimers of other PYK isozymes lack activity, have led to the acceptance that M_2PYK can easily be inhibited. In fact, that idea can be extended as a mechanism of control if the percentage of the protein that exists in each quaternary state can be modulated by the various regulatory mechanisms: post translational modifications (PTMs), protein:protein interactions, or small molecule allosteric effectors [6, 34, 39, 44, 142–151].

It is often assumed that any reference to "inhibition" of M_2PYK implies a dimeric state of the enzyme. However, it is important to note that there are many examples of enzymes/binding proteins that are inhibited (with altered substrate affinity or altered k_{cat}) without multimeric dissociation. Therefore, the focus in the literature on a potential M_2PYK dimer-tetramer equilibrium may be overemphasized. What is more relevant to metabolism are the maximal enzymatic capacity in the cell (which intrinsically includes enzyme expression level), the specifics of whether the regulatory mechanism alters substrate affinity and/or specific activity of M_2PYK and how those two ideas combine to determine the metabolic flux through the PYK reaction in the cell.

In fact, the ability of enzyme expression levels to influence the metabolism flux through the PYK reaction in the cell depends on whether a given regulatory mechanism alters the enzymes K_M for substrate or prevents catalytic turnover. To exemplify this concept, consider the potential of M_2PYK becoming inhibited in cancer (the common consideration in much of the literature). More specifically, if the "inhibited" form of M_2PYK only has reduced substrate affinity, then upon a switch from M_1PYK expression to M_2PYK in cancer, the reduced affinity would initially cause reduced flux through the PYK reaction and a build-up of up-stream glycolytic intermediates (Figure 3). However, once concentrations of PEP increase sufficiently (due to reduced activity at low concentrations of PEP), that increased substrate availability would drive flux through the pyruvate kinase reaction. Therefore, the new steady-state would include high concentrations of metabolites upstream of the PYK reaction, but would also include flux through the PYK reaction to result in the observed increased lactate production. In this scenario, the concentration of the M_2PYK enzyme would also influence the rate of flux through the PYK reaction. In turn, it would be the build-up of glycolytic intermediates that would provide us with the most reasonable explanation for why a lack of a Pasteur effect and a high rate of glycolysis even under

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aerobic conditions may be beneficial to dividing cells. Due to increased substrate availability (*i.e.,* glucose-6-phosphate), flux through the pentose phosphate pathway would be increased. The pentose phosphate pathway is largely responsible for producing the NADPH needed to regenerate the antioxidant glutathione and permit cancer cells to live with the increased oxidative stress known to exist in cancer. It follows in this logic that the rate of flux through the PYK reaction at the bottom of glycolysis influences NADPH production in the pentose phosphate pathway. This scenario emphasizes a new steady-state condition to explain a high rate of glycolysis and that new steady-state would be associated with an increase in concentrations of glycolytic intermediates, but would also allow lactate production. That new steady-state could include cancer-specific rates of glycogen turnover [152–154]. As noted, overexpression of M_2PYK in this scenario could also result in increased glycolytic flux. Importantly, the scenario presented in Figure 3 is used to explain common ideas about Warburg metabolism rather than what is actually supported by data, which is that the activity of PYK is increased rather than decreased in cancer cells [128, 138, 141, 155].

As a further contrast, a comparison of tumor tissue with non-tumor esophageal tissue failed to identify the increase in glycolytic intermediates [156] that would be anticipated by the scenario in Figure 3. Although it is generally thought that most lactate is derived from glycolysis [109, 157, 158], Figure 3 also does not consider that lactate can be derived from the metabolism of glutamine or serine [158–164]. Therefore, although Figure 3 offers an explanation that circumvents the apparent paradox introduced by M_2PYK "inhibition" *vs.* increased lactic acid production, the entire assumption of the M_2PYK inhibition is what should be questioned. Despite the lack of support in cancer metabolism, the description in Figure 3 is not completely useless in that it is consistent with the increased levels of glycolytic intermediates identified for metabolism in other tissues that result in high levels of lactate production (e.g., ischemia in the brain and electroshock-induced seizures in the brain.) [165, 166]. Furthermore, there are indications for variable metabolism used by different types of cancer [167–169], leaving the possibility open that there may be a unique type of cancer that uses the scenario described in Figure 3.

In contrast to a K-type regulation represented in Figure 3, if "inhibition" of M_2PYK means that the enzyme lacks catalytic turn over, then a regulatory mechanism that shifts M_2PYK towards the inhibited form would reduce flux through the PYK reaction step, even when intermediate metabolite concentrations are increased. If indeed, the M_2PYK in cancer lacks or has greatly reduced catalysis (Figure 4), then the only way to gain flux through the PYK reaction is to activate the enzyme. Even higher levels of enzyme expression would have no relevance if that enzyme has a complete lack of activity. If indeed Warburg metabolism includes both increased levels of glycolytic intermediates and increased lactate production as commonly reported, then the dynamic balance between k_{cat}-activated and k_{cat}-inhibited forms of M_2PYK would be challenging to explain.

Despite this thought exercise that can provide an explanation for how a reduced substrate affinity for M_2PYK in cancer (a commonly repeated theme in the literature) could provide a description of why M_2PYK could dictate Warburg metabolism, it is contrary to data that indicates increased PYK activity in cancer [128, 138, 141, 155]. Fructose-1,6-bisphosphate (Fru-1,6-BP) causes an approximately 8-fold increase in the apparent affinity for PEP [148,

170]. This change is also associated with a doubling of the V_{max}. This change in catalytic activity does not imply a complete lack of catalysis in the non-activated or inhibited form(s), but does suggest that changes in both specific activity and substrate affinity contribute to activation/inhibition.

M_2PYK tetramer-dimer equilibrium may not have physiological relevance

Unfortunately, in the studies of M_2PYK, there has been considerably more focus on the tetramer dissociation upon "inhibition" than providing the clear characterizations of the functional/enzymatic outcomes associated with the form of M_2PYK found in cancer. Nonetheless knowing what causes the tetramer to dissociation has the potential to indirectly contribute to our knowledge of functional outcomes if the functional properties of the dissociated dimer are well characterized. This conversation can be initiated by evaluating if there is convincing data that the M_2PYK dimer retains activity, and even that conversation should be preceded with a cautionary note derived from previous studies of M_1PYK.

Many more protein denaturation and refolding studies have been completed with M_1PYK than with M_2PYK. For the M_1PYK protein, denaturation progresses from the stable tetramer to an expanded tetramer, to an inactive dimer before further unfolding steps are observed. The observation of the stable dimer is relevant to our current consideration [171–174], given the focus on the tetramer to dimer transition in studies of M_2PYK in cancer. However, indications that the dimeric form of M_1PYK is active are thought to be artifacts because PEP stabilizes the tetrameric form of that enzyme [173, 175, 176]. Therefore, even when isolated dimers of the M_1PYK protein are added to an assay, the presence of PEP in the assay causes dimers to rapid assemble into active tetramers. Thus, the study of M_1PYK exemplifies that an inactive dimeric form of a protein can easily be misinterpreted to have enzymatic activity. The conclusion of studies of M_1PYK is that the dimeric form of the enzyme is not catalytically active.

Keeping in mind the lesson learned from studies of M_1PYK, it is important to note that both substrates of the enzyme, ADP and PEP, stabilize the M_2PYK tetramer [145, 177]. However, given that M_2PYK isolated from cancer cells/tissues has properties that are different from M_2PYK isolated from normal tissue [82, 128–140], a more specific consideration of M_2PYK isolated from cancer may be warranted. Like the M_1PYK form, there is a rapid re-association of dimers of the cancer form of M_2PYK when PEP is present [149]. For all conditions studied, Hofmann *et al.* and co-workers found evidence for activity for the dimer, leading them to consider the dimeric form of cancer-M_2PYK to be "suboptimal" (without specifying if that implied reduced k_{cat} or reduced substrate affinity) [145, 149, 150]. Assays of the isolated dimer produced activity in the Mazurek laboratory [178], but for the reason identified in the M_1PYK example, without evidence that the protein maintains a dimer form in all assay conditions, data collected from that design does not actually support that the dimer maintains activity.

Several studies probe M_2PYK structure function questions with mutations, but they do not always define if the mutations cause the protein to behave like the M_2PYK from normal tissues *vs.* from cancer tissues. For example, the G415R mutant dimer fails to tetramerize in

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the presence of Fru-1,6-BP but shows activity: again, the influence of PEP in the assay condition on tetramerization was not evaluated [179]. In contrast to any indication that the dimer retains activity, a K305Q mutation that prevent tetramer formation is reported to “abolish activity” [170].

More direct data indicate that detection of dimer forms of M_2PYK do not correlate with cell proliferation [180]. Finally, the argument has recently been made that cellular concentrations of Fru-1,6-BP, an allosteric activator that promotes both high PEP affinity and stabilization of the tetrameric state of M_2PYK, are sufficiently high to predict that unmodified M_2PYK (presumably equal to that found in normal tissue) is always a tetramer in the cell [147]. Therefore, despite what is often reported, there is little to no evidence that establishes that the M_2PYK dimer does or does not retain activity

Also relevant to this topic, several studies have reported the use of antibodies to detect specific oligomeric states of M_2PYK (*e.g.,* [181]). However, antibody binding to a given multimeric form of an antigen protein that is in equilibrium with some other multimeric form will of necessity influence the equilibrium of the subunit assemblies. Even in tissues that have been crosslinked (i.e., samples for histological analysis), tetramer dissociation to dimer may be possible. The idea that antibody binding causes dissociation might best be supported by considering that an antibody that specifically recognizes the monomers of M_2PYK promotes dissociation into monomers [51, 144] in the same way that antibodies to dimers identifies dissociation into dimers. Furthermore, antibody staining is not quantitative: if the percentage of M_2PYK in the dimer form of M_2PYK changes from 0.0001% of the total M_2PYK protein to 0.0003%, then stated in isolation, a 3-fold change would sound important. However, the percentage of the total protein that is under consideration is so small as to make the observation seem negligible. For these reasons, antibodies detection of dimeric M_2PYK should be used with extreme caution.

Overall, the M_2PYK tetramer dissociates *in vitro* into dimers and monomers much easier than other isozymes of PYK [144, 145, 148, 149, 170]. It follows that it is challenging to prove that M_2PYK dimers exist in cells, especially given the influence of various ligands and molecular crowding to promote subunit association. At least in normal tissue, and possibly in cancer tissue, dissociation may not have a physiological relevance due to ligand concentrations [147].

Level of M_2PYK protein/mRNA expression and maximal enzyme capacity of the PYK reaction

Up to now, the distinction between responses to regulatory mechanisms has been emphasized. In particular, if response does not completely eliminate catalysis, then total activity levels can influence metabolic flux. It follows that a modification in the level of enzyme expression is one conceivable way to control total PYK activity. The level of M_2PYK protein is upregulated in many cancer cell types, which is consistent with upregulation of the mRNA encoding the protein [182–186]. However, that upregulation is often offset by the loss of expression of some other tissue specific isozyme [75, 187]; therefore, protein expression of M_2PYK alone may not correlate with an increase in total

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PYK activity in cancer. Furthermore, expression levels of M_2PYK are not likely to correlate with cancer progression for those cancer types that are derived from normal tissues, which already express M_2PYK [188, 189]. If a given range of PYK activity is what is needed for cancer metabolism, then it follows that excess PYK activity provided by some other PYK isozyme may also support rapid cell proliferation, which, in turn, may be the bases of several observations that suggest the M_2PYK isozyme is not required in all types of cancers [190–195].

Despite that the level of M_2PYK contribution to total PYK activity may depend on cell type, the “maximum enzyme capacity” for the PYK activity is increased in cancer cells [128, 138, 141]. Furthermore, increased pyruvate kinase activity (and other glycolytic enzyme activities) has been correlated with the growth rate of tumors/malignancy [141, 155]. This correlation again indicates more PYK activity in rapidly growing tumors, not inhibition of PYK.

Post translational modifications of M_2PYK in cancer

Although we can clearly question the representation that M_2PYK is “inhibited” and the “evidence” that a tetramer/reduced-activity-dimer equilibrium is central to a regulatory mechanism of M_2PYK *in vivo*, we accept that PYK is a control site in glycolysis [10, 11]. (Although this too has been questioned in the context of cancer *via* flux analysis [196, 197].) It also remains that forced expression of M_1PYK in place of M_2PYK limits xenograph tumor growth [15] and activation of M_2PYK suppresses tumor growth [26]. Therefore, M_2PYK continues to be central in cancer biology and it is worth exploring what properties may be unique in cancer to cause this cancer-central role.

Again, M_2PYK in cancer cells is modified compared to M_2PYK in normal tissues [82, 128–140]. The early techniques used to identify these differences included isoelectric focusing, making it reasonable that the observed alterations at the protein level could be due to PTMs. Unique substrate affinities for the M_2PYK protein purified from normal *vs.* cancer tissue could also be interpreted as consistent with PTMs that are unique in cancer [128]. As exemplified by the observation that phosphorylation promotes the dimer form of M_2PYK [39, 142], modification by PTMs may work in concert with other regulatory mechanisms. Recent literature record a large number of post-translational modifications on M_2PYK [198] and many more are included in the various data banks (Table 2).

Unfortunately, the studies of many of these PTMs fall well short of satisfying what, if any, role they play in cancer metabolism. 1) The influence on the metabolic activity of M_2PYK or their influence on cancer biology has not been detailed for most PTMs that have been identified. There are, however, some exceptions with more information available [9, 15, 23, 34, 35, 39, 44, 45, 98]. 2) Most, if not all, of these PTMs were identified from cancer cell lines. Because only a small fraction of cells from a cancer tumor are capable of being developed into a cell line, it remains unknown if regulatory mechanisms for M_2PYK identified in cell lines represent those that are the most abundant in tumors. 3) Many of these PTMs were originally identified on fragments of the M_2PYK protein as detected by mass spectrometry. This identification approach does not distinguish if the PTMs can be added to

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the whole protein or if they are added to peptide fragments that result from protein degradation. Due to the high sequence similarity, this approach also fails to distinguish if the modification was present on only M_2PYK, only M_1PYK, or both. If modifications can be added to both isozymes, then a stream of questions follow (e.g., Can the PTM be added to both M_1PYK and M_2PYK? Does the PTM regulate the two enzymes the same way? etc.). 4) Finally, the mass spectrometry techniques used to identify modifications on peptide fragments are very sensitive, leaving the possibility that modifications are only added to a negligibly small fraction of the total M_2PYK present in the cell. If only a few molecules of M_2PYK include a given PTM, it would be hard to imagine a regulatory role for that PTM. Even though the field seldom acknowledges that many of the identified PTMs may not have physiological roles, it remains plausible that PTMs may contribute to the unique properties of M_2PYK found in tumors.

Allosteric Regulation of M_2PYK by small molecule effectors

It is well established that the response of M_2PYK to allosteric effectors differs from that of other mammalian PYK isozymes [88, 89, 101, 106, 136, 138, 139, 199–202]. This includes the allosteric activation by Fru-1,6-BP that results in an increased affinity of M_2PYK for PEP, and alanine and phenylalanine, both allosteric inhibitors that decrease affinity of the enzyme for PEP. Although these responses are qualitatively shared with LPYK, the concentrations and the resulting substrate affinities with effector bound are unique for the two enzymes.

Yuan *et al.* recently extended the characterizations of amino acid regulators of M_2PYK [143]. They found that in addition to phenylalanine and alanine, tryptophan, methionine, valine, and proline acted as allosteric inhibitors. The ability of hydrophobic amino acids to act as inhibitors parallels the response from M_1PYK [124]. However, these inhibitory effects on M_2PYK appear to be within concentration ranges that are more relevant to physiological conditions. Consistent with earlier studies [148, 170], inhibitions by amino acids were reported to include change in both V_{max} and substrate affinity for PEP. Histidine and serine acted as activators in the Yuan *et al.* study. The activation by serine only increased V_{max} without influencing substrate affinity. Importantly, these influences of allosteric amino acid effectors were measured with an M_2PYK protein expressed in *E. coli* that would lack any cancer-specific PTMs.

Regulation by L-cysteine and L-serine can be further highlighted. An inhibition by L-cysteine [139, 140, 203] appears to be unique to the M_2PYK purified out of cancer cells [128]. L-cysteine reduces both V_{max} and the apparent affinity for PEP. [138]. L-serine has been known to be an activator of M_2PYK for some time [135, 148, 204, 205], with some evidence for a differential response between the normal and cancer enzymes from chicken.

ATP is often listed as a common allosteric inhibitor of mammalian PYK isozymes. However, without a binding site identified *via* co-crystallography, it has been challenging to establish whether the inhibition is allosteric or *via* competitive binding with ADP in the active site. Nonetheless, the cancer form of M_2PYK has been reported to lack sensitivity to ATP, in contrast to the ATP inhibition that is observed for M_2PYK isolated from normal tissue [206].

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Succinyl-5-amino imidazole-4-carboxamide-1-ribose-5'-phosphate (SAICAR), an intermediate of the *de novo* purine nucleotide biosynthesis pathway activates M_2PYK, but not M_1PYK [207]. SAICAR concentrations are reported higher in cancer cells than normal cells that express M_2PYK (*i.e.,* lung fibroblasts). It remains undefined if SAICAR causes a change in specific activity or modifies substrate affinity.

M_2PYK metabolic functions regulated by protein:protein interactions

A number of proteins that interact directly with the M_2PYK protein have been identified in recent years (Table 3). In those studies, it is not always clear if the proposed protein:protein interactions are thought to alter metabolic functions *via* enzyme activity in the cytosol or *via* nuclear functions (see below). However, some studies have directly tested the influence of protein:protein interactions on the metabolic activity of M_2PYK. Interactions of M_2PYK with tyrosine-phosphorylated peptides, SOCS3, PRL, JMJD8 causes reduced PYK activity [74, 110, 117, 119]. In contrast, interaction of M_2PYK with DAPk results in increased PYK activity [11]. Although thought to occur in the cytoplasm, the influence of a direct interaction with PanK4 and TRIM35 on the activity of M_2PYK was not determined [108, 126]. Also, whether altered activities of M_2PYK are a result of changes in specific activity or substrate affinity have not typically been determined for these protein interactions.

Much like PTMs, quantitation is lacking in these protein:protein interaction studies. This begs the question of whether there is a sufficient percentage of M_2PYK in the protein:protein interaction to warrant consideration of that interaction as a regulatory mechanism. It should also be kept in mind that the percentage of each interacting protein that participates in a protein:protein interaction is dependent on the concentrations of both proteins. Therefore, there is a note of caution for all reports of proteins that directly interact with M_2PYK when interactions are detected in systems that overexpress M_2PYK or the other protein, thus artificially driving protein:protin interactions that may not occur at a meaningful level under normal expression levels. Nonetheless, given the examples of protein:protein interactions that can alter M_2PYK metabolic activity, protein:protein interactions may work in concert with PTMs and/or small molecule allosteric ligands to control M_2PYK activity.

The role of the affinity of M_2PYK for ADP

Control of metabolic flux via M_2PYK may not depend solely on changes in the enzyme's affinity for PEP. Removal of competition between PYK and mitochondrial oxidative phosphorylation for ADP has been offered as an explanation for why tumor cell respiration can be stimulated by inhibitors of PYK [46, 47].

This idea would require M_2PYK to have a higher affinity for ADP than the mitochondrial electrogenic adenine nucleotide translocator (ANT). In non-proliferating cells, the apparent affinity for ADP reported for PYK isozymes are much lower (200–400 μM) [88, 208] than values reported for ANT in non-proliferating cells (1 to 100 μM) [209]. In non-proliferating cells respiration creates a membrane potential and pH gradient across the mitochondrial inner membrane that drives release of ATP in exchange for cytosolic ADP via the

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electrogenic ANT located in the mitochondrial inner membrane. In turn, this leads to a high cytosolic ATP/ADP ratio and a free ADP concentration (in the range of 25 μM). That ADP range is considerably below the K_M of PYK, a scenario that restricts PYK flux. Therefore, inhibition of PYK in non-proliferating cells has no effect on the respiration rate.

The conditions are much different in rapidly proliferating cancer cells. In these cells, ATP/ADP exchange is not driven by the electrogenic ANT [210], due inhibition of the mitochondrial outer membrane voltage-dependent anion channel (VDAC) [211]. This results in a low cytosolic ATP/ADP ratio and therefore a higher free cytosolic ADP concentration that promotes PYK activity, aerobic glycolysis, and the Warburg effect. Since PYK is a major user of ADP in proliferating cells, inhibition of its activity greatly impacts the free ADP concentration which overcomes inhibition of the ANT and thereby stimulates respiration. These details are consistent with tumor cell respiration being stimulated by inhibitors of PYK.

The influence of M_2PYK on glycolytic flux *in vivo*

Throughout the discussion of M_2PYK as a glycolytic enzyme, it becomes clear that many of the measurements that are easily obtained (maximal enzyme activity in a tissue, enzyme concentrations, mRNA concentrations, responses to substrates, responses to effectors and metabolite concentrations) do not easily translate into knowledge of flux through a pathway *in vivo*. This concept is the bases of flux analysis [212–214], which also includes increasing concentrations of other enzymes in a pathway [215]. Within flux analysis, it is recognized that a change in enzyme activity will both reduce substrate concentration and increase product for that enzymatic step. However, those changes in substrate and product concentrations will alter activity of other enzymatic steps. Thus, the ability of each enzyme to control flux is measured as a "control strength" value instead of attempting to assign a single enzyme as a "rate limiting step" in the pathway. Surprisingly, there are few flux analysis evaluations of the metabolism found in cancer cells and little evidence that M_2PYK is a control point in the flux analysis studies that have been completed [196, 197].

Consistent with this lack of evidence for M_2PYK as a control point and inconsistent with the highly popular speculation for inhibition of M_2PYK in cancer, there is little evidence for increased concentrations of glycolytic intermediates in cancer [165, 216]. Even in a theoretical consideration, a potential increase in glycolytic intermediates, all of which are phosphorylated, must be limited to prevent depletion of inorganic phosphate: fructose intolerance is caused by phosphate depletion due to the accumulation of fructose 1-phosphate. Of special note is that most flux analysis *via* isotope labeling [217] can identify increased labeling of glycolytic intermediates without evaluating for increases in the concentrations of those glycolytic intermediates.

M_2PYK in non-canonical role: nuclear translocation of M_2PYK to act as a protein kinase

Given the many questions that have often challenged a metabolic role for M_2PYK in cancer, it is not surprising that there have been many attempts to identify some function for M_2PYK

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in cancer that is outside of the traditional metabolic function. These proposed non-canonical/ non-metabolic/ moonlighting functions reported for M_2PYK parallel proposals for a non-canonical functions for a large number of other enzymes [218].

Both a PEP-dependent protein kinase activity of M_2-PYK [24, 134, 151, 207, 219–229] and a translocation of dimeric M_2PYK into the nucleus to act as a transcription factor [34, 73, 120, 125, 230–235] have been proposed for M_2PYK. The protein kinase activity appears to primarily be detected in the nucleus using nuclear proteins as substrates. However, it is less defined if all roles prescribed to the nuclear M_2PYK dimer rely on that proposed protein kinase activity. The nuclear functions of a M_2PYK dimer have been proposed to regulate gene transcription of both glycolytic functions and other cancer-specific properties.

A few considerations introduce a strong caution for the proposed PEP-dependent protein kinase activity. Presumably, the substrate peptide must bind in the nucleotide portion of the active site. However, that nucleotide binding site is similar in all pyruvate kinase isozymes, begging the question of why the other mammalian isozymes do not also have a protein kinase activity. (A protein kinase activity is also reported for yeast PYK [236].) A second caution is derived from the PYK control included in efforts to identify a protein kinase that uses PEP as a phosphate donor [237, 238]. In those studies, even in a dialyzed protein extract, there is a probability that assays include contaminating concentrations of both PYK and ADP, resulting in a transfer of a phosphate from PEP to ADP (*via* the PYK reaction) and, in turn, ATP being the true phosphate donor. It follows for studies of M_2PYK as a protein kinase that even undetectable contamination levels of ADP and an ATP-dependent protein kinase could result in substrate protein phosphorylation and be interpreted as a PEP-dependent protein kinase activity for PYK. This becomes increasingly relevant given that cellular concentrations of ADP are largely bound to proteins [208], hence tight binding and a high possibility of proteinaceous extracts including contamination by ADP. It has also been shown that “enzyme cycling” systems, such as the one possible with PYK, ADP and a protein kinase, amplifies signal [239], an outcome that may be relevant if the protein kinase activity for PYK is an artifact due to contamination. Therefore, absolute proof of a protein kinase activity for M_2PYK is challenging. Co-crystallization of a substrate peptide in the active site of M_2PYK would greatly strengthen an argument for M_2PYK acting as a protein kinase. Despite these considerations, there continues to be large numbers of studies reporting detection of protein kinase activity for M_2PYK. However, there has also been a challenge that these protein kinase detections are not reproducible [240].

M_2PYK in non-canonical roles: M_2PYK translocation into the mitochondria and circulating in the blood

Several non-canonical functions have been proposed that do not have obvious dependencies on nuclear translocation. It has been proposed that M_2PYK alters mitochondrial fusion *via* a protein:protein interaction with p53 [85, 241]. Translocation of M_2PYK into the mitochondria, followed by a protein-protein interaction with and phosphorylation of Bcl2 to regulate apoptosis has also been proposed [229].

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M_2PYK circulating in the blood may also have a role in cancer [242, 243]. M_2PYK in blood promotes angiogenesis in tumors. Much like other non-canonical functions, this activity is reported to depend on a dimer form of the enzyme. Interestingly, the caveats regarding cellular concentrations of metabolites that were used to question the dissociation of tetramer in the cytoplasm are not likely to apply to dissociation of the M_2PYK tetramer in circulating blood or in other cellular compartments beyond the cytoplasm. Rather than or in addition to circulating M_2PYK, M_2PYK released by neutrophils at a wound site may facilitate angiogenesis [244].

Studies of M_2PYK are plagued with a lack of quantitative studies

All non-canonical functions of M_2PYK and many proposed PTM and protein:protein interactions can be collectively questioned by the observation that M_2PYK is not required in all cancer types [190–195]. The recent report that M_1PYK can promote tumor cell growth [195] may offer an even more specific challenge to these proposed non-metabolic/non-canonical roles for M_2PYK in cancer (as well as many of the identified regulatory mechanisms).

A second reason that all of the various PTM, protein:protein interactions and non-canonical functions of M_2PYK can collectively be questioned is the lack of quantitation and good techniques to probe only regulatory features of the M_2PYK protein. Most of these non-canonical functions are heavily influenced by antibody/western blot detection techniques, which will give a result when combined with sensitive chemiluminescent detection (even if they are non-meaningful physiologically). Observations from those non-quantitative antibody-based approaches are often paired with knock-out, knock-down and overexpression strategies, which seldom neatly probe regulatory features, a third reason to question many reported observations. To fully appreciate why changing expression levels is not a good probe of function, consider the role of M_2PYK in controlling glycolytic flux: Would knock-out, knock-down, or overexpression of the enzyme identify if a protein phosphorylation subtly alters glycolytic flux? The answer no because the entire glycolytic pathway would be perturbed and alterations in many downstream metabolite would be expected to act as further signals for change. It follows that knockout, knock-down and overexpression strategies may not be good tools to evaluate the potential roles of non-canonical roles of M_2PYK. Cell culture and *in vivo* studies that include M_2PYK proteins with point mutations that mimic or prevent individual types of the regulatory mechanisms starts to address the need for better probing approaches. However, even that design is challenged: mutant M_2PYK introduced *in vivo* might mimic chronic changes in enzyme function, but they don't recapitulate the reversible acute responses that would be possible with PTMs, allostery, and protein:protein interactions *in vivo*. Therefore, inducible regulatory mechanisms may have an important role in the future [245].

What can we speculate about Warburg metabolism based on primary literature

The Warburg effect is the incomplete switch from glycolytic production of lactic acid to complete glucose oxidation when O_2 is present. In other words, the Warburg effect is an

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incomplete Pasteur Effect, or perhaps even better characterized as rapid glycolysis on top of oxidative phosphorylation [246]. Rather than increasing the concentrations of glycolytic intermediates to force flux (by mass action) into side pathways that are important for cell proliferation and growth, we visualize that increased expression of enzymes at the branch points of these side pathways, namely glucose-6-phosphate dehydrogenase [247] and 3-phosphoglycerate dehydrogenase [248], readily explains increased synthesis of NADPH, ribose 5-phosphate, serine, and glycine. Increased expression of all of the glycolytic enzymes [249], including M_2PYK [216], assures that generation of intermediates of the glycolytic pathway is not rate-limiting for glycolysis as well as the side reactions. We believe the Warburg effect as it relates to glucose metabolism is due to upregulation of the enzymes of the glycolytic pathway coupled with increased expression of pyruvate dehydrogenase kinases [250] to partially inhibit the pyruvate dehydrogenase complex. In turn, this partial inhibition of the pyruvate dehydrogenase complex restricts the levels of pyruvate (a result of increased glycolytic flux) that can enter the citric acid cycle and, instead, conserves sufficient quantities of pyruvate for the recycling of NADH produced by glycolysis back to NAD^+ (although citric acid cycle continues to function [217, 251]). The Warburg effect further depends upon reduced mitochondrial uptake of ADP due to suppression of the activity of the mitochondrial ANT [210] which decreases the cytosolic ATP/ADP ratio and sets a higher steady state concentration of free ADP required for M_2PYK activity as discussed in greater detail above.

An explanation for outcomes in studies with M2PYK

Unfortunately, the summary of M_2PYK is that there are so many functions and regulatory mechanisms reported for M_2PYK (beyond catalysis in glycolysis), that it is easy to question if any of these observations have valid physiological relevance. In particular, the finding that M_2PYK is not required for all cancers [190–195] seems to refocus studies on the role of M_2PYK back to its metabolic function.

We propose that the many types of potential regulatory mechanisms of M_2PYK allows a fine tuning of activity to a level that supports the metabolic flux levels needed for cancer survival. Therefore, drug activation of M_2PYK [49–56, 61–64] or expression of M_1PYK [48] that lacks sensitivity to regulatory mechanisms may introduce too much PYK activity, an outcome that would prevent some glucose-6-phosphate from entering the pentose phosphate pathway to produce the required NADPH. This scenario would be toxic to the cell. As a contrast, overexpression of M_2PYK can be accommodated because of cellular mechanisms that reduce activity to the cancer-specific activity range. On the other hand, any knock-down, knock-out, or drug designs that inhibit M_2PYK activity [59, 60, 63, 65–68] has the potential to reduce the total PYK activity below a threshold needed for cell survival.

In Warburg metabolism, increased flux through glycolysis results from additional PYK activity, not reduced PYK activity. Although we can conceive how that increased flux could be accompanied with increased concentrations of glycolytic intermediates when there is a switch from expression of M_1PYK to M_2PYK (Figure 3), we anticipate that many cancer types do not have this phenotypic change (as compared to comparable normal tissue) [156].

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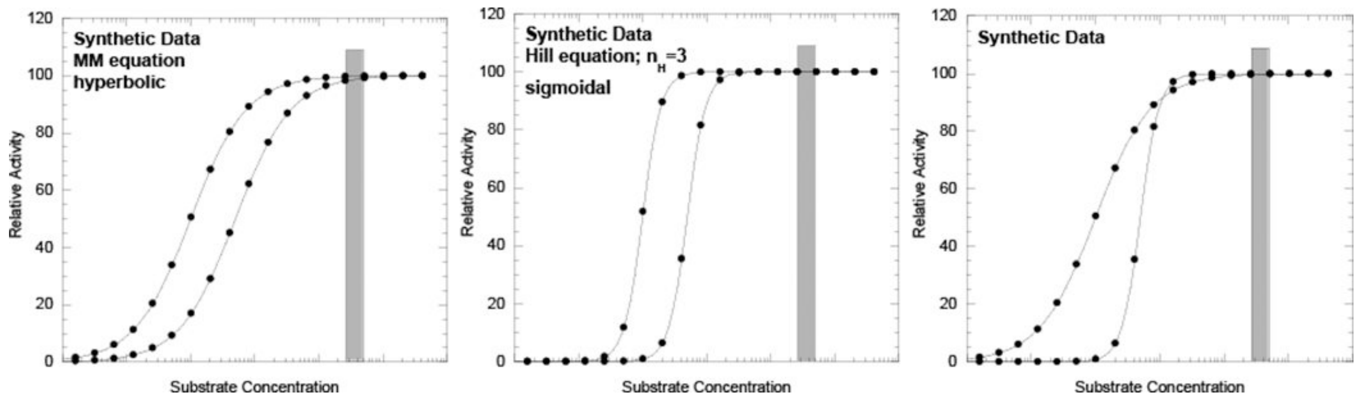

Figure 1.

Synthetic data for two isozymes with different K_M values as a demonstration that reduced substrate affinity of an isozyme alone is not sufficient to predict activity in the cell. If substrate concentrations are sufficiently high in the cell (indicated by gray column) two isozymes with different affinity for substrate can give rise to the same level of activity. The Michaelis Menten equation was used to generate synthetic data in the left panel here and in Figures 2, and 4. Data in the left panel are hyperbolic (consistent with M_1PYK data) on a linear x-axis, but appear sigmoidal in this figure due to the logarithmic scale of the x-axis. Data in the middle panel were derived from the Hill equation (with a n_H value equal to 3). The resulting sigmoidal response is more representative of that found for M_2PYK. The panel on the right compares a hyperbolic response with a higher substrate affinity (representing M_1PYK) to a sigmoidal response with a lower substrate affinity (representing M_2PYK). As exemplified in this figure, the ideas discussed in the text can be represented by the simpler comparison of hyperbolic data. Therefore, all other figures in this review include only data generated from the simpler Michaelis Menten equation. Nonetheless, the reader should keep in mind that M_2PYK has a sigmoidal response.

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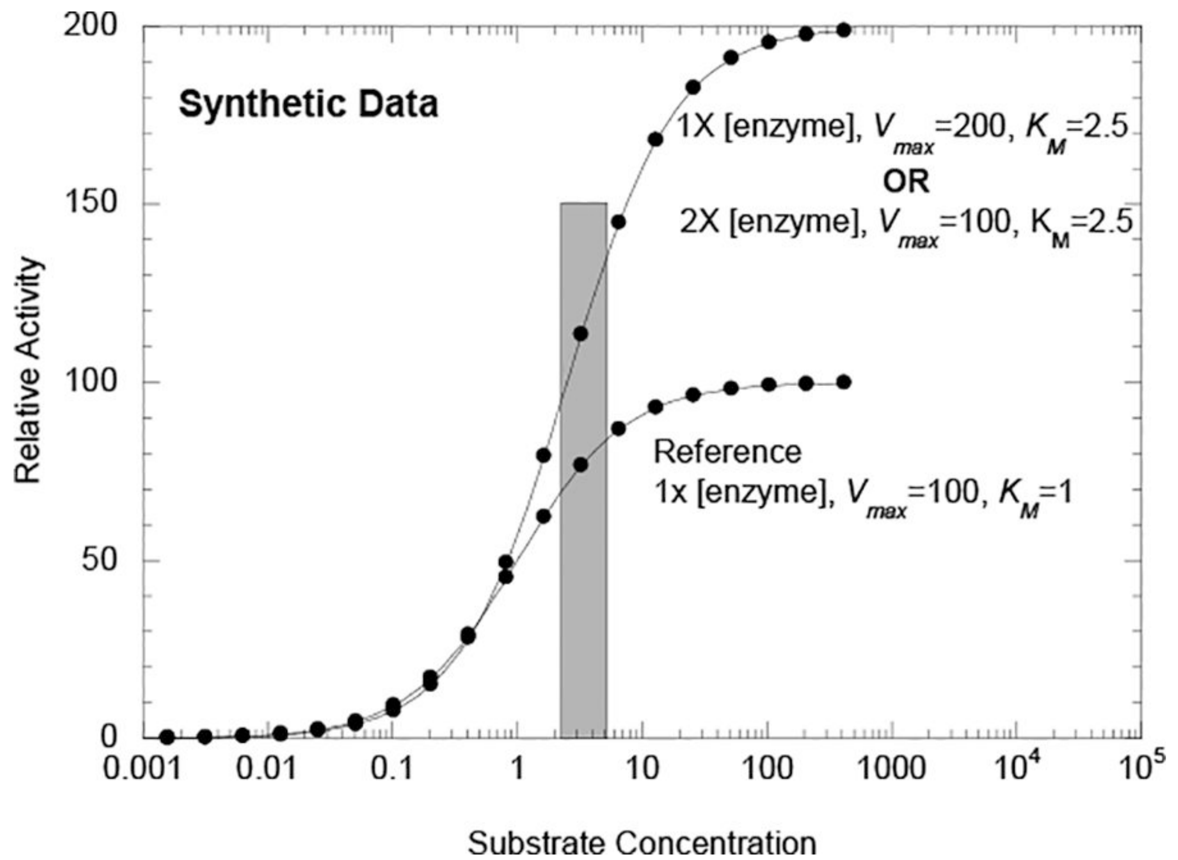

Figure 2.

Synthetic Data for two isozymes with altered K_M and enzyme increased enzyme concentration and/or increased V_{max} activity to demonstrate the possibility that an isozyme with lower substrate affinity can give rise to higher activity even at sub-saturating substrate concentrations, if it also has either higher specific activity or if it is expressed at higher concentrations.

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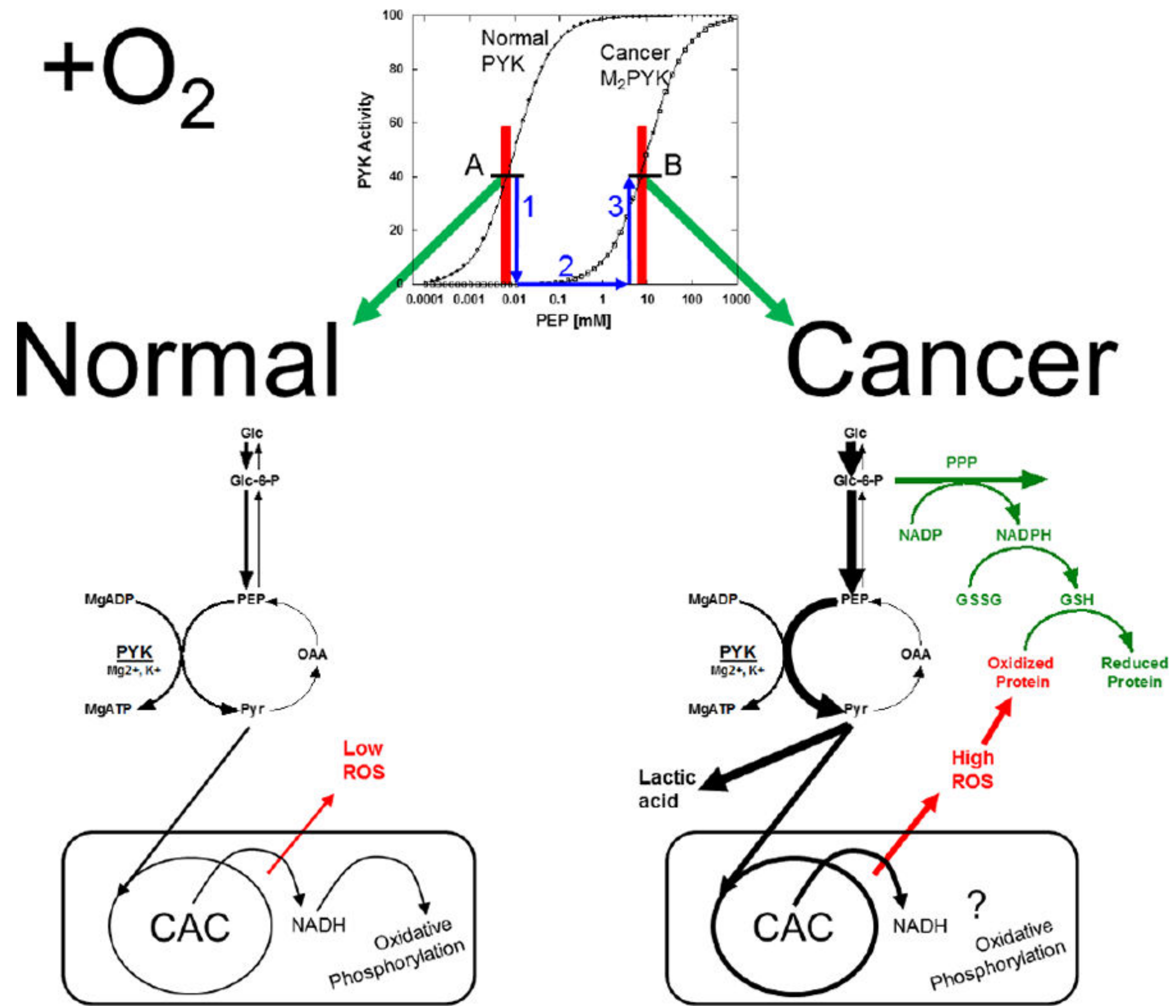

Figure 3.

A role for altered affinity of cancer M_2PYK for PEP in creating a new steady-state in Warburg metabolism. The top panel represents the activity response curves of either normal PYK or cancer M_2PYK. “Normal” PYK can be M_1PYK, LPYK, RPYK, or M_2PYK isolated from normal tissue. Concentrations of PEP in the cell are represented by red boxes and the respective PYK activity at that PEP concentration is represented by black dashes intersecting the red box (also marked with letters A and B at the beginning and the two steady-state PYK activities) and the response curve. The blue arrows trace changes related to the progression to cancer: 1) The PYK isozyme changes to cancer M_2PYK, which has little activity at the PEP concentration present in normal tissue. 2) PEP concentrations build up due to reduced PYK activity. 3) Due to increased substrate availability caused by PEP build up, the cancer form of M_2PYK has activity at high PEP. This condition maintains high levels of glycolytic intermediates and allows for high flux to lactic acid production. Green arrows connect the two steady-state PYK activities (A and B) with panels representing the respective metabolism. In these two lower panels, the thickness of arrows is intended to represent the relative concentrations of intermediates in the pathway indicated by the respective arrow. Left) When normal PYK is expressed, normal metabolism is at a steady-state and in the presence of O_2, that steady-state flux of carbon from sugar into the mitochondria for

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oxidative phosphorylation. Right) When cancer M_2PYK is expressed, a new steady-state flux is established that includes higher concentrations of glycolytic intermediates. This includes high rates of flux through the cancer PYK reaction and increased production of lactic acid. We anticipate higher flux through the mitochondria as well, thus generating higher levels of reactive oxygen species (ROS) and oxidative stress. However, the increased concentration of glucose 6-phosphate in the new steady-state condition drives increased flux through the pentose phosphate pathway due to substrate availability. This change produces increased NADPH. NADPH is needed to regenerate glutathione, which counteracts the increased oxidative stress, thus allowing cancer cells to live with higher intracellular ROS concentrations.

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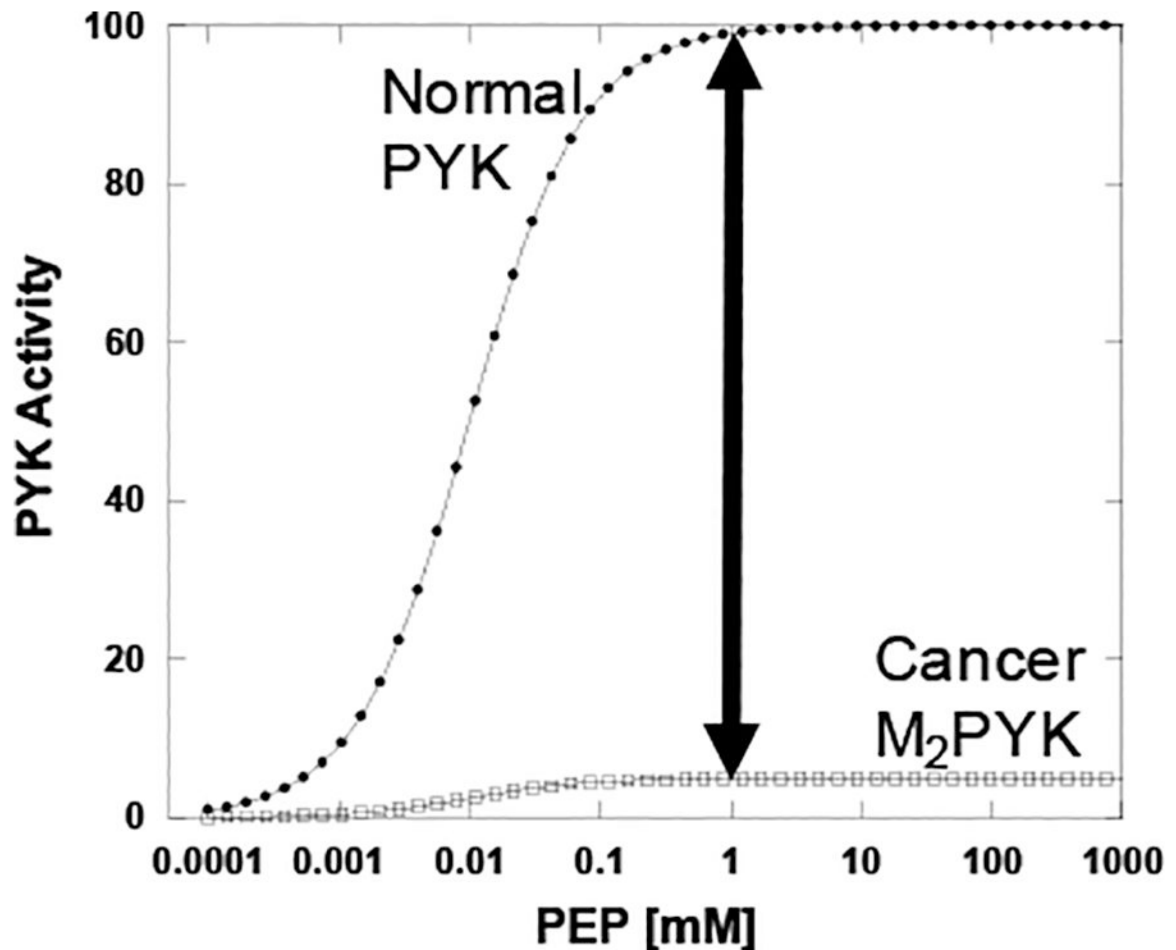

Figure 4.

A theoretical response curve of normal PYK and cancer M_2PYK, if "inhibited" cancer M_2PYK involves reduced k_{cat}. Here 5% activity compared to Normal PYK is used as an example, although the points in the text are more relevant if inhibition removes all catalytic activity. "Normal" PYK can be M_1PYK, LPYK, RPYK, or M_2PYK isolated from normal tissue. In the absence of changes in M_2PYK expression, the only way to increase flux through the PYK reaction would be to activate the M_2PYK protein, thus requiring a dynamic control of M_2PYK to achieve both inhibition for build-up of glycolytic intermediates and to allow sufficient flux through the PYK reaction to generate high levels of lactic acid. The need for that dynamic regulation might predict oscillating flux of high glycolytic intermediate dispersed with periods of high lactic acid production.

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Table 1:

Properties of pyruvate kinase isozymes in the absence of regulators

	M_1PYK	LPYK	RPYK	M_2PYK (normal tissue)	M_2PYK (cancer tissue or cell)
In the absence of regulators[a]					
Specific Activity[b]	230–780	60–560	60–300	130–500	770
K_M or $S_{0.5}$ PEP (mM)	0.04–0.09	0.3–1.0	0.5–0.6	0.09–0.4[c]	0.4
K_M ADP(mM)	0.3–0.4	0.1–0.6	0.4–0.6	0.2–0.4	??
Full ranges when including outcomes of regulations					
Specific Activity[b]	Unregulated	Unregulated	Unregulated	34–500[f]	??
K_M or $S_{0.5}$ PEP (mM)	Unregulated[d]	0.02–10[e]	??	0.078–3.0[f]	??
Allosteric regulations					
Allosteric activator (lowest effective conc.)	None	Fru-1,6-BP (0.00006 mM)[n]	Fru-1,6-BP (0.003mM)[o]	Fru-1,6-BP (0.00005 mM)[l]	Fru-1,6-BP (0.00005 mM)[h]
				Serine (0.8mM)[m]	Serine??
				SAICAR (0.1mM)[p]	SAICAR??
Allosteric inhibitor (lowest effective conc.)	Phenylalanine[d] (5mM)	Alanine (0.5mM)[n]	Alanine	Phenylalanine (0.4mM)[m]	Phenylalanine (4.8mM)[h]
				Alanine (0.8mM)[m]	Alanine??
					Cystine (0.05mM)[g]

Physiological range[s]		
	Normal tissues	Cancer tissues
Intracellular pH	7.0–7.2[i]	7.12–7.65
phosphoenolpyruvate	0.0035–0.397 μmole/g fresh wt[j]	1.02 × normal[q]
Fru-1,6-BP	0.009–0.120[j] μmole/g fresh wt[j,k]	0.99 × normal[q]
Serine	0.19–1.27 μmole/g fresh wt[j]	1.26 × normal[q]
Alanine	0.38–1.64 μmole/g fresh wt[j]	1.01 × normal[q]
Phenylalanine	7.87 nmol/10^6 cells[r]	1.82 × normal[q]

[a] Data in this section are from [88, 89, 252] and represents a summary of findings from many studies and from multiple mammalian species.

[b] Specific activity is the number of μmoles of pyruvate formed per min per mg of enzyme.

[c] Range expanded based from 0.2–0.4 reported in [88] down to 0.09–0.4 based [123]

[d] The affinity of M_1PYK for the allosteric inhibitor, phenylalanine, is low at pH values below 7.5 [124]. Therefore, under normal physiological conditions, this enzyme may not be regulated and values are the same as in the absence of regulators. Phenylalanine concentration that produces an effect [124, 253].

[e]The highest value is in the presence of alanine and the lowest is in the presence of Fru-1,6-BP, however, these values are dependent on pH (7.5 used as the upper limit for considerations here), monovalent cation type, divalent cation type, and ion concentrations [254, 255].

[f]A phenylalanine-dependent decrease in specific activity was reported in [143] but not in [177].

[g]Although inhibition is shown, estimation of PEP affinity in the presence of Cys was not included. [130]

[h]Like control of M_1PYK activity by phenylalanine, the concentration required to inhibit M_2PYK is likely above the physiological range. [82]

[i][256–258]

[j]Data are from [259] and represent a summary of findings from many studies and from many mammalian tissues.

[k]Excludes the very low concentration of 0.0009 (μmole/g fresh wt.) found in adipose.

[l][116]

[m][143][253]

[n][89, 254]

[o][260, 261]

[p][262]

[q][156]

[r][263]

[s]Given that the cell is 70% water, μmole/g fresh wt ÷ 0.7 approximates the mM concentration.

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Table 2:

PTMs identified on M_2PYK (or a MPYK isozyme without distinguishing between M_1PYK and M_2PYK)

PTM type	Amino Acid Residue Location if known	Ref
Glycosylation		[1, 2]
O-GlcNAc (Serine/threonine)	403 and 405	[3–6]
O-methylation (glutamic acid/aspartic acid)		[7]
Oxidation	358	[8]
Methylation (arginine)	445, 447 and 455	[9]
MARlation		[10]
N-acetylserine	2	Uniprot HPRD
N6-acetyllysine	3, 62, 89, 135, 162, 166, 206, 207, 230,266,305, 433 and 498	Uniprot HPRD PhosphoSitePlus [13–18]
Nitrosylation (cysteine)	49	HPRD
Nitrosylation (tryptophan)		[19]
Nitrosylation (tyrosine)		[20, 21]
Phosphorylation (serine/threonine)	37, 41, 45, 50, 57, 60, 77, 80, 87, 93, 95, 97, 100, 127, 129, 172, 195, 202, 222, 243, 249, 287, 328, 437 and 454	Uniprot HPRD PhosphoSitePlus [22–38]
Phosphorylation (tyrosine)	83, 105, 148, 149, 175, 370, 390 and 466	Uniprot HPRD PhosphoSitePlus [30, 39–45]
Sumolation		[94]
Succinylation (lysine)	62, 66, 115, 135, 247, 311, and 498	[95]
Ubiquitination (lysine)	66, 115, 125, 136, 141, 151, 166, 186, 188, 206 224, 261, 277, 311, 322, 336, 367, 475	PhosphoSitePlus [98, 99]
4-hydroxylation (prolines	403 and 408	Uniprot and [70]
Prolyl isomerization		[102, 103]

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Table 3:

Proteins reported to interact directly with M_2PYK

DAPk	[11]
DDB2	[12]
HERC1	[69]
HIF-1α	[70]
HPV-16 E7	[71]
HSP40	[72]
HSP90	[24]
JMJD5	[73]
JMJD8	[74]
MDM2	[85]
MST1	[86]
Oct-4	[107]
PanK4	[108]
PHD3	[70, 109]
Phosphotyrosine peptide	[110, 111]
PML	[116]
PRLr	[117]
p53	[118]
SOCS3	[119]
SREBP	[120–122]
SUMO-E3 Ligase PIAS3	[94]
TGIF2	[125]
TRIM35	[126]
TSC22DS	[127]