Characterization of a Glucose-Repressed Pyruvate Kinase (Pyk2p) in Saccharomyces cerevisiae That Is Catalytically Insensitive to Fructose-1,6-Bisphosphate

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We have characterized the gene YOR347c of Saccharomyces cerevisiae and shown that it encodes a second functional pyruvate kinase isoenzyme, Pyk2p. Overexpression of the YOR347c/PYK2 gene on a multicopy vector restored growth on glucose of a yeast pyruvate kinase 1 (pyk1) mutant strain and could completely substitute for the PYK1-encoded enzymatic activity. PYK2 gene expression is subject to glucose repression. A pyk2 deletion mutant had no obvious growth phenotypes under various conditions, but the growth defects of a pyk1 pyk2 double-deletion strain were even more pronounced than those of a pyk1 single-mutation strain. Pyk2p is active without fructose-1,6-bisphosphate. However, overexpression of PYK2 during growth on ethanol did not cause any of the deleterious effects expected from a futile cycling between pyruvate and phosphoenolpyruvate. The results indicate that the PYK2-encoded pyruvate kinase may be used under conditions of very low glycolytic flux.

Pyruvate kinase is the last enzyme in the glycolytic pathway of sugar catabolism. It catalyzes the irreversible conversion of phosphoenolpyruvate (PEP) into pyruvate by the addition of a proton and the loss of a phosphate group, which is transferred to ADP. Pyruvate kinases from a wide range of organisms have been extensively studied, and much is known about their physical and catalytic properties (8, 35, 38, 39). Nearly all characterized eukaryotic pyruvate kinases are tightly regulated and are activated by fructose-1,6-bisphosphate (FBP). The mammalian muscle isozyme M1 is the only known pyruvate kinase that displays hyperbolic kinetics and lacks allosteric control (40). On the other hand, pyruvate kinases from prokaryotes can be activated by either FBP or other sugar phosphates (e.g., ribose phosphate), and those from trypanosomes can be activated by fructose-2,6-bisphosphate (35).

In the yeast Saccharomyces cerevisiae, pyruvate kinase has been thought to be encoded solely by the PYK1 gene. The gene was cloned and sequenced by Burke et al. (16), and part of the nucleotide sequence was revised by McNally et al. (36). PYK1 codes for a 54.5-kDa protein (Pyk1p) consisting of 500 amino acids. Mutants defective in the PYK1 gene fail to grow on fermentable carbon sources and are even inhibited by them (17, 18, 30). However, they grow normally on ethanol or other gluconeogenic carbon sources. Under those conditions, hexose phosphates are provided by the gluconeogenic pathway, which uses the enzymes FBP and PEP carboxykinase to bypass the 6-phosphofructo-1-isomerase and pyruvate kinase reactions, respectively. The concentrations of glycolytic metabolites in pyk1 deletion mutants after growth on an ethanol-containing medium are similar to the wild-type levels, but after addition of glucose, a large increase in the amounts of PEP and phosphoglycerates can be observed (12, 17). These results suggested that Pyk1p is the main enzyme which catalyzes the conversion of PEP into pyruvate in S. cerevisiae.

The biochemical properties of yeast Pyk1p suggest that it plays a central regulatory role in carbon metabolism, undergoing changes in activity during the transitions between fermentation, when sugars are abundant, and gluconeogenesis, when ethanol is consumed. The other tightly regulated reaction is catalyzed by the hetero-octameric phosphofructokinase enzyme (see reference 27 and references cited therein). Yeast cells, like other organisms or cells which are able to carry out glycolysis and gluconeogenesis, have developed multiple regulatory mechanisms to avoid the simultaneous operation of these antagonistic metabolic pathways (7, 22, 42). One of these mechanisms is thought to be the activation of pyruvate kinase by FBP, which is the most potent and important activator of yeast Pyk1p. This glycolytic metabolite shifts the sigmoidal characteristics of a PEP saturation curve to a hyperbolic curvature without affecting the maximum velocity of the enzyme but drastically lowers the $K_m$ values for PEP and, to a lesser degree, ADP (2, 29, 56). Under conditions of glucose excess, the activity of pyruvate kinase should be high enough to provide sufficient amounts of ATP and pyruvate, which is a central metabolite involved in a variety of metabolic pathways. On the other hand, under gluconeogenic conditions, down-regulation of the Pyk1p-catalyzed reaction is necessary in order to avoid a futile cycling between pyruvate and PEP via the ATP-consuming reactions catalyzed by pyruvate carboxylase and PEP carboxykinase (15, 51, 53). Accordingly, the concentration of FBP is high in cells growing on fermentable carbon sources. During gluconeogenic growth, Pyk1p remains nearly inactive due to the low concentration of its allosteric activator, FBP (2, 3, 10, 23, 34). Thus, it is sometimes assumed that the positive control of Pyk1p by FBP provides the mechanism for switching between glycolysis and gluconeogenesis. Additionally, although the basal level of expression of the PYK1 gene is already very high, the amount of enzyme is still increased 4- to 20-fold in cells grown on fermentable sugars compared to those grown on gluconeogenic carbon sources (10, 33).

Unexpectedly, after completion of the yeast genome sequencing project, a new open reading frame (ORF) of S. cerevisiae, on chromosome XV, was identified which has a high
degree of similarity to the *PYK1* sequence (44). Here, we report the characterization of this gene, whose expression appears to be subject to glucose repression and which encodes a functional pyruvate kinase that is catalytically almost insensitive to FBP.

**MATERIALS AND METHODS**

**Yeast strains and growth conditions.** Unless otherwise stated, all yeast strains used in this work were derived from strain CEN.PK2-1C (*MATa leu2-3,112 ura3-52 trpl-289 his3-D12 MAL2-8 SUC2*) or CEN.PK2-1D (*MATa; otherwise identical to CEN.PK2-1C*) or the corresponding diploid strain, CEN.PK2. These strains harbor the *HIS3*/*MATa*, *URA3*, and *VW18* (for repression of *PYK2* expression in *Saccharomyces cerevisiae*), respectively, in their genomes (27). The *reg1* (*leu2*) deletion strain in the CEN.PK genetic background was obtained by S. Müller (Darmstadt, Germany). The *CAT8* (MSP9) gene was previously cloned as a multicopy suppressor of a *reg1* mutant for growth on glucose (11, 31). In strain EBY152 (*MATa can2-D1139 HIS3*), derived from the wild-type strain ENY.WA1A (K.-D. Eintian, Frankfurt, Germany), the *CAT8* construct is completely replaced by the *HIS3* gene (31). Strains WAY.pyl1 (MATS *ura3 his3-D12 his3-D2-LLE2*) and WAY.7-D2/A (MATS *ura3 his3-D12 his3-D2-LLE2*) are isogenic to ENY.WA1A and were obtained from M. Rose and K.-D. Eintian. FY1679 has been described by Winstone et al. (54). Yeast cells were grown at 30°C in YEP medium (1% yeast extract, 2% Bacto Peptone), in synthetic minimal (SM) medium [0.17% Difco yeast-nitrogen base (YNB), 10 mM KCl, pH 6.0 supplemented per the manufacturer's demands], or in synthetic complete (SC) medium supplemented with different carbon sources.

**Molecular biology techniques.** DNA and RNA were purified and manipulated according to procedures previously described (47, 49). Plasmid transformations of yeast cells were carried out by the freeze method (19). *Escherichia coli* JM101, DH5α, and SURE (Stratagene GmbH) were transformed by electroporation. pUC7, pUC18, pUC19, pUC21, and the yeast-*E. coli* shuttle vectors from the series of Gietz and Sugeio (25) served as vectors. All other experiments were performed as described previously (9).

**Construction of plasmids.** The *PYK2* gene was cloned by PCR with a pair of primers designed to amplify a DNA fragment enclosing the complete gene as well as its 5′ and 3′ regulatory regions. One oligonucleotide (5′-CTTGAACAATGGTGTTGcCCTCGGTGTC-3′) is located from −574 to −552 bp in front of the ATG start codon, and it contains an XhoI restriction site at its 5′ end. The other oligonucleotide (5′-ATCGGcGGTATTTACcAATA-3′) is located from 745 to 765 bp behind the *PYK2* ORF, just behind a Xcel restriction site (at 741 bp). PCR with the Expand High Fidelity PCR System (Boehringer, Mannheim, Germany), with this oligonucleotide pair as primers and whole cells of strain FY1679 as the template (45 s at 95°C, 45 s at 50°C, 3.5 min at 68°C; 30 cycles), yielded a 2.8-kb DNA fragment. The fragment was cleaved with XhoI and SacI and cloned into plasmids YEplac195 and pUC19, resulting in plasmids YEplac195-PYK2 and pUC-PYK2, respectively. To delete most parts of the ORF of *PYK2*, the strategy described in reference 20 was employed. This strategy takes advantage of the ability of PCR primers to amplify the upstream and downstream sequences of the region to be deleted along with the whole cloning vector, thereby replacing the ORF with a unique restriction site. One oligonucleotide (5′-CGGATCCGCTTCACTCCTTGTG-3′) was complementary to the sequence at position −16 to −3 bp, next to the start codon of the *PYK2* gene, with a BamHI restriction site at its 5′ end. The other oligonucleotide (5′-CGGATCCGGTTGATTTGCGGACcGAAGCTGATCATA-3′) was located at positions +1422 to +1438 bp, at the end of the *PYK2* ORF, and also contains a BamHI restriction site at its 5′ end. PCR with the Expand High Fidelity PCR System, with this oligonucleotide pair as primers and pUC-PYK2 as the template (45 s at 95°C, 30 s at 50°C, 6 min at 68°C; 25 cycles), yielded a 4.1-kb DNA fragment which was isolated, cleaved with *BamHI*, and self-ligated, resulting in pDPYK2. In this plasmid, the DNA sequence encoding amino acids 2 to 474 of *PYK2* and the *PYK2* gene, was recloned into YEplac195. This construct, called pJJH85. In parallel, the *PYK2* gene was amplified by PCR with the Expand High Fidelity PCR System, with this oligonucleotide pair and strain FY1679 as the template (45 s at 95°C, 30 s at 50°C, 6 min at 68°C; 25 cycles), yielded a 2.8-kb DNA fragment. The fragment was cleaved with *BamHI* and *SacI* and cloned into plasmids YEplac195 and pUC19, resulting in plasmids YEplac195-pPYK2 and pUC-pPYK2, respectively. To delete most parts of the ORF of *PYK2*, the strategy described in reference 20 was employed. This strategy takes advantage of the ability of PCR primers to amplify the up-
The **PYK2** gene, including the complete promoter region (574 bp of the 5’ region in front of the ATG codon) and 741 bp of the 3’ noncoding region, was amplified by PCR using chromosomal DNA from wild-type strain FY1679. The 2.8-kb DNA fragment was cloned into the 2μm-based multicopy plasmid YEplac195 and transformed into the wild-type yeast strain VW1A and three different **pyk1** deletion mutant strains (VWH3A [see Materials and Methods], EBY55 [12], and EBY56 [13]). The transformants were plated on a uracil-free medium (to select for plasmid uptake) which was supplemented with 3% ethanol and 0.1% galactose (to allow all transformants to grow). After 3 days, the colonies were replica plated onto the same basic medium containing 1% glucose as the sole carbon source. Not only the wild-type cells but also the **pyk1** deletion mutants containing the **PYK2** gene on the multicopy vector grew very well on the glucose medium, with similar growth rates. Transformants of the **pyk1** deletion mutant strains containing only the cloning vector YEplac195 without an insert did not grow on the glucose medium within 1 week.

In order to avoid false conclusions due to error-prone PCR amplification of the **PYK2** gene, the PCR-generated **PYK2** fragment was replaced with a chromosomal copy of **PYK2** by the gap repair method. Again, **pyk1** mutant cells transformed

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**FIG. 1.** Amino acid sequence alignment of (from top) yeast Pyk2p and Pyk1p and the rat muscle M1, rat kidney M2, and rat liver L pyruvate kinase isoenzymes. The region purportedly involved in the allosteric properties of pyruvate kinases is indicated by the thick line below the sequences. Asterisks indicate amino acids identical in all five proteins, and dots indicate amino acids conserved in at least three proteins.
with a multicopy plasmid containing this genome-derived copy of *PYK2* (YPEpPYK2) grew very well on glucose media. The plasmid YEpPYK2 was reisolated from cells of the *pyk1* mutant strain growing on glucose and was shown by restriction enzyme analysis to be identical to the originally transformed plasmid. After prolonged growth on nonselective YEP medium with 3% ethanol as the sole carbon source, several cells had lost the plasmid, as judged by their uracil auxotrophy, and concomitantly the ability to grow on a medium with glucose. Thus, overexpression of the *PYK2* gene on a multicopy plasmid restores the ability of *pyk1* deletion mutants to grow on fermentable carbon sources. This stands in contrast to a similar situation reported for isocitrate lyase, in which a homolog, *ICL2*, present in the yeast genome is under no circumstances able to complement an *icl1* defect (28). The results reported above suggest that *PYK2* encodes a functional pyruvate kinase.

A *pyk2* deletion mutant showed growth properties identical to those of the wild-type strain under all conditions tested (see below). After crossing it with a *pyk1* deletion mutant, the diploid strain was sporulated, and spores containing *pyk1* as well as *pyk2* deletion alleles were obtained. Cells of the *pyk1* *pyk2* double-deletion mutant were able to grow on YEP medium with 3% ethanol, like wild-type cells. However, like cells of a *pyk1* single-deletion strain, they were not able to grow on a medium with 2% glucose. Growth of the *pyk1* mutant strain (VWH3A), the *pyk2* mutant strain (EBY118A), the *pyk1* *pyk2* double-mutant strain (EBY121), the *pyk1* mutant strain containing plasmid YEpPYK2 (EBY120A), and a corresponding wild-type strain (CEN.PK2-1C) was tested on various media with different carbon sources and under various conditions.

The cells were plated on SM, SC, and YEP media containing either 2, 0.5, or 0.1% glucose, maltose, or galactose or 2% raffinose or sucrose, with or without 0.7 M NaCl or 1 M sorbitol and with either ammonium or proline as a nitrogen source, or on SC medium containing 3% ethanol, with or without 2, 1, or 0.5% glucose, and were incubated at 30 or 37°C. No significant growth differences among the wild-type strain, the *pyk2* mutant strain, and the *pyk1* mutant strain containing plasmid YEpPYK2 were found. The growth properties of the *pyk1* deletion mutant and the *pyk1* *pyk2* double mutant were similar in that neither strain grew on SM or SC medium with 0.1 to 2% glucose or 0.1 to 2% galactose under any conditions. However, there were also distinct differences. For instance, the *pyk1* mutant strain grew very slowly on SC medium with small amounts of maltose or with 2% raffinose, and the *pyk1* *pyk2* double mutant did not. Also, the growth-inhibiting effect of increasing concentrations of glucose added to an ethanol-based SC growth medium was more pronounced in the *pyk1* *pyk2* double-mutant strain, which, unlike the *pyk1* mutant strain, did not grow at all on a mixture of 3% ethanol and >0.5% glucose.

Pyruvate kinase activities were determined in the different strains after growth on YEP medium with 3% glycero–ethanol or 2% glucose (Table 1). As already shown previously (10), pyruvate kinase activity in wild-type cells is induced about fourfold on a glucose medium compared to an ethanol medium. The same was true for a *pyk2* deletion strain, which did not differ from the wild-type strain. Only a very low specific activity close to the limit of detection could be determined in a *pyk1* deletion strain, and the level was even lower in the *pyk1* *pyk2* double-mutant strain. Interestingly, pyruvate kinase activity in the *pyk1* deletion strain containing plasmid YEpPYK2 on a glycero–ethanol medium was much higher than in the wild-type strain and was decreased to wild-type levels on a glucose medium (Table 1).

The concentrations of some of the glycolytic metabolites were determined in the different strains during growth on YEP medium with 3% ethanol and 1 h after addition of 2% glucose to the cultures (Table 2). No significant differences among the wild-type strain, the *pyk2* deletion strain, and the *pyk1* deletion strain containing YEpPYK2 were observed in the concentrations of any of the various metabolites, including ATP, either during growth on ethanol or after addition of glucose. Moreover, no significant differences in metabolite concentrations were found between the *pyk1* and the *pyk1* *pyk2* deletion strains. Both strains accumulated large amounts of glycolytic metabolites after addition of glucose, and their ATP levels were reduced, characteristic for a block in glucose catabolism. However, during growth on ethanol, the concentrations of metabolites in both strains were similar to those in the wild-type strain. The physiological data provide in vivo evidence that a single chromosomal copy of the *PYK2* gene provides only very low levels of pyruvate kinase activity under these conditions but that overexpression from a multicopy vector can completely complement the *pyk1* defects.

**Biochemical characterization of Pyk2p.** The kinetic properties of partially purified Pyk1p and Pyk2p enzymes were compared (Table 3). Pyruvate kinase activities from the wild type and the *pyk2* deletion strain were strongly dependent on the addition of FBP to the assay. Under standard conditions, FBP activated Pyk1p enzyme activity about 8-fold and lowered the *Kₘ* value for PEP about 30-fold. In contrast, the *PYK2*-encoded activity and its *Kₘ* value for PEP were only marginally affected by FBP, and the *Kₘ* value of Pyk2p for PEP, with or

### TABLE 1. Pyruvate kinase activities in different yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Pyruvate kinase activity (µmol min⁻¹ mg of protein⁻¹) in</th>
<th>YEP + 3% ethanol–3% glycerol</th>
<th>YEP + 2% glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEN.PK2-1C Wild type</td>
<td></td>
<td>1.212</td>
<td>4.914</td>
<td></td>
</tr>
<tr>
<td>VWH3A</td>
<td><em>pyk1Δ</em></td>
<td>0.038</td>
<td>NG</td>
<td></td>
</tr>
<tr>
<td>EBY118A</td>
<td><em>pyk2Δ</em></td>
<td>1.280</td>
<td>4.704</td>
<td></td>
</tr>
<tr>
<td>EBY121A</td>
<td><em>pyk1Δ</em> <em>pyk2Δ</em></td>
<td>0.017</td>
<td>NG</td>
<td></td>
</tr>
<tr>
<td>EBY120A</td>
<td><em>pyk1Δ</em> YEpPYK2</td>
<td>7.763</td>
<td>4.928</td>
<td></td>
</tr>
</tbody>
</table>

*Activities are mean values of data from at least two independent experiments. Cells were grown overnight in the indicated medium to the mid-exponential growth phase.

TABLE 2. Concentrations of internal metabolites in various yeast strains

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CEN.PK2-1C (wild type)</th>
<th>VWH3A (pyk1Δ)</th>
<th>EBY118A (pyk2Δ)</th>
<th>EBY121A (pyk1Δ pyk2Δ)</th>
<th>EBY120A (pyk1Δ YEpPYK2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc-6-P</td>
<td>2.6/3.0</td>
<td>2.0/2.2</td>
<td>1.7/2.7</td>
<td>2.7/2.2</td>
<td>2.0/3.1</td>
</tr>
<tr>
<td>Fru-1,6-bP</td>
<td>0.5/0.9</td>
<td>0.9/1.6</td>
<td>0.8/0.8</td>
<td>0.9/2.0</td>
<td>0.7/0.9</td>
</tr>
<tr>
<td>3-P-glycerate</td>
<td>0.3/2.3</td>
<td>1.1/3.0</td>
<td>0.7/3.2</td>
<td>1.3/9.8</td>
<td>0.5/4.2</td>
</tr>
<tr>
<td>PEP</td>
<td>0.6/1.5</td>
<td>0.8/7.0</td>
<td>0.8/1.6</td>
<td>0.8/7.8</td>
<td>0.4/2.2</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.2/1.5</td>
<td>0.1/0.4</td>
<td>0.3/1.5</td>
<td>0.1/0.6</td>
<td>0.4/1.8</td>
</tr>
<tr>
<td>ATP</td>
<td>4.4/4.0</td>
<td>3.3/1.9</td>
<td>4.1/4.2</td>
<td>4.6/2.0</td>
<td>4.9/5.4</td>
</tr>
</tbody>
</table>

*Activities were determined in cells grown overnight in YEP medium supplemented with 3% ethanol (first value) and 1 h after the addition of 2% glucose (second value). Mean values of data from three independent experiments are given.

**Abbreviations:** Glc-6-P, glucose-6-phosphate; Fru-1,6-bP, fructose-1,6-bisphosphate; 3-P-glycerate, 3-phosphoglycerate.
without the addition of FBP, was similar to the \( K_m \) value of the FBP-activated Pyk1p (Table 3). Also, after extensive dialysis of the Pyk2p preparation, activation of pyruvate kinase activity by FBP was less than 2-fold (data not shown). Moreover, activation did not differ for Pyk2p enzymes from ethanol-grown cells and those from glucose-grown cells. Only at very low concentrations of PEP, below 0.1 mM, did activation by FBP reach about 10-fold, in contrast to Pyk1p activity, which was activated by FBP more than 1,000-fold under these conditions. Fructose-2,6-bisphosphate did not activate Pyk2p activity at all (data not shown). In contrast to Pyk1p, the kinetic properties of Pyk2p rather resemble the mammalian muscle isoenzyme M1 in being unaffected by FBP. Interestingly, the rat M1 and M2 Pyk isoenzymes, which differ significantly with respect to their regulatory properties, are encoded by the same gene and are produced by alternative splicing of the same RNA (43). The two isoenzymes differ only in the sequence from amino acids 389 to 433, which is hydrolyzed outside the cells, producing only small amounts of free sugars. Moreover, Pyk2p expression was even induced by ethanol. \( \beta \)-Galactosidase activities were slightly higher during growth on 2% glucose when proline was used as a (poor) nitrogen source instead of ammonium sulfate. No change in promoter activity was seen after a heat shock. In contrast to the PYK2::lacZ fusion, which was repressed by glucose about 40-fold compared to ethanol (Table 4), Pyk2p enzyme activities were repressed no more than 2-fold (Table 1). This may be explained by the high selective pressure for the PYK2-containing plasmid in the pyk1 deletion mutant during growth on glucose but not on ethanol, which may lead to an increase in the copy number of the plasmid and a deregulation of PYK2 expression during growth on glucose. A similar observation has been made with overexpression of GDH2, encoding glutamate dehydrogenase, in a pgil phosphoglucone isomerase mutant during growth on glucose (11). Interestingly, the Pyk2p enzyme activity adjusted exactly to wild-type pyruvate kinase levels under these conditions. In contrast, there is no selective pressure for the PYK2::lacZ fusion construct.

Transcriptional repression by glucose is a common mechanism in yeast cells (22, 24, 45). The glucose repression pathway triggers repression of genes that are dispensable or unimportant to cells growing on glucose. Phosphorylation of glucose by one of the two yeast hexokinases, Hxk1p and Hxk2p, has been proposed to be involved in the triggering reactions for glucose repression of the GAL, MAL, and SUC genes (21, 32, 46). However, the ability of the PYK2 promoter to respond to glucose in hsk1 hsk2 double mutants containing only the GLK1-
encoded glucokinase remained intact (Table 4), similar to the glucose-repressed gluconeogenic genes FBP1 and PCK1 (55). Ethanol induction of the PYK2 promoter was not affected in a cat8 deletion mutant. The CAT8 (MSP8) gene encodes a transcription factor required for derepression of gluconeogenic enzymes during growth on ethanol (26). Another central component of the glucose repression pathway is the GLC7-encoded protein phosphatase 1, which is targeted by the Reg1p (Hex2p) protein to other proteins in the glucose repression regulatory pathway (52). Interestingly, glucose repression of the PYK2 promoter was relieved in a reg1 deletion mutant (Table 4). Altogether, our results suggest that the PYK2 gene is subject to the general mechanisms of glucose repression.

### DISCUSSION

We have shown that the previously discovered ORF designated YOR347c/O6342 (44), which has a high level of sequence similarity to the PYK1 gene, encodes a second functional yeast pyruvate kinase isoenzyme. The unusual properties of PYK2 gene expression and of the encoded enzyme, namely, the very low enzyme activity concomitant with a relatively high transcript level during growth on an ethanol medium, the strong repression of the gene by high concentrations of glucose, the lack of an obvious phenotype in a pyk2 deletion mutant, and the catalytic insensitivity to FBP, suggest that Pyk2p is used by the cells only under very specific conditions. Our results are compatible with the notion that Pyk2p activity may be used by the cells under conditions in which the level of glycolytic flux is very low. This is the case, for example, under starvation conditions, i.e., when the cells are consuming internal glucose derived from storage carbohydrates or when the external glucose supply is low. Under such conditions, the PYK2 gene is derepressed and the low levels of intracellular FBP may not be sufficient to activate the Pyk1p enzyme.

On the other hand, overexpression of the unregulated Pyk2p enzyme activity during growth on ethanol did not cause any deleterious effects on the cells, as might be expected if regulation of pyruvate kinase activity by FBP provides the mechanism for switching between glycolysis and gluconeogenesis. A similar observation was made by Maitra and Lobo (34), who isolated a yeast mutant with a Pyk1p enzyme which did not require FBP for activity. In contrast to our results, this mutant could grow on an ethanol medium even faster than the wild-type strain, but only after a considerable lag period of 3 to 4 days without growth. Altogether, the results suggest that control parameters other than FBP-dependent regulation of pyruvate kinase, capable of maintaining a level of PEP sufficient for gluconeogenesis and of avoiding the energy-wasting cycling by the pyruvate kinase-pyruvate carboxylase-PEP carboxykinase enzyme reactions, must operate in the cells. They could be as-yet-unidentified allosteric effectors of pyruvate kinase activity. On the other hand, it is tempting to speculate that the gluconeogenic enzymes form specific enzyme complexes (1, 50) that allow channeling of intermediate metabolites, e.g., from oxaloacetate to glyceraldehyde-2-phosphate. In this case, PEP would not be accessible to pyruvate kinase during growth on ethanol.

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