The dimorphic yeast *Yarrowia lipolytica* possesses an atypical phosphofructokinase: characterization of the enzyme and its encoding gene

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The phosphofructokinase from the non-conventional yeast *Yarrowia lipolytica* (YlPfk) was purified to homogeneity, and its encoding gene isolated. YlPfk is an octamer of 869 kDa composed of a single type of subunit, and shows atypical kinetic characteristics. It did not exhibit cooperative kinetics for fructose 6-phosphate (Hill coefficient, $h = 1.1$; $S_{0.5} = 52 \mu M$), it was inhibited moderately by MgATP ($K_i = 3.5 \text{ mM}$), and it was strongly inhibited by phosphoenolpyruvate ($K_i = 61 \mu M$). Fructose 2,6-bisphosphate did not activate the enzyme, and AMP and ADP were also without effect. The gene *YIPFK1* has no introns, and encodes a putative protein of 953 aa, with a molecular mass consistent with the subunit size found after purification. Disruption of the gene abolished growth in glucose and Pfk activity, while reintroduction of the gene restored both properties. This indicates that *Y. lipolytica* has only one gene encoding Pfk, and supports the finding that the enzyme consists of identical subunits. Glucose did not interfere with growth of the *Yipfk1* disruptant in permissive carbon sources. The unusual kinetic characteristics of YlPfk, and the intracellular concentrations of glycolytic intermediates during growth in glucose, suggest that YlPfk may play an important role in the regulation of glucose metabolism in *Y. lipolytica*, different from the role played by the enzyme in *Saccharomyces cerevisiae*.

INTRODUCTION

*Yarrowia lipolytica* is an obligate aerobic non-conventional yeast that has attracted much attention because of some important characteristics. Among them are its unusual ability to grow on hydrocarbons, its ability to excrete important organic acids, its potential as a host for the expression of heterologous proteins (for reviews see Barth & Gaillardin, 1996), and its ability to shift between a yeast and a hyphal form (Domínguez et al., 2000). In spite of its basic and industrial interest, biochemical studies on its central metabolic pathways are scarce (Flores et al., 2000). Regarding the regulatory steps of glycolysis, a pathway nearly ubiquitous in all life forms, it is known only that the hexokinase activity of *Y. lipolytica* is low, about 1/10 of that of *Saccharomyces cerevisiae* (Petit & Gancedo, 1999), and that it is strongly inhibited by trehalose 6-phosphate (Petit & Gancedo, 1999), and that pyruvate kinase exhibits kinetic and regulatory properties significantly different from those of the enzyme from *S. cerevisiae* (Hirai et al., 1975). Nothing is known about the characteristics of Pfk (phosphofructokinase; EC 2.7.1.11), the enzyme that catalyses the physiologically irreversible phosphorylation of Fru-6-P (fructose 6-phosphate) to Fru-1,6-P2 (fructose 1,6-bisphosphate). The activity of this enzyme in different organisms is affected by a large variety of effectors, thus making Pfk an example of a ‘multimodulated enzyme’ (for reviews, see Uyeda, 1979; Sols, 1981; Kemp & Foe, 1983). The diversity of effectors that influence the activity of Pfk in different organisms has made it a qualified candidate to play a critical role in the regulation of glycolysis. In fact, in the yeast *S. cerevisiae*, it was thought to be the ‘bottleneck’ of this pathway; however, this view has been shaken by the fact that overproduction of the two subunits of *S. cerevisiae* Pfk did not alter the fermentation rate of this yeast (Davies & Brindle, 1992). Although the regulatory hierarchy of Pfk as controller of the yeast glycolytic pathway is still unsettled, its regulability, i.e. its capacity to be affected by different effectors (Hofmeyr & Cornish-Bowden, 1991), in a variety of organisms is undisputed, and the importance of its
regulatory properties is shown by other results (Tornheim, 1988; Ogushi et al., 1990; González-Mateos et al., 1993). In addition to its role as a glycolytic enzyme, other functions have recently been described for Pfk in some organisms, such as its ability to modulate glucose-induced microautophagy in *Pichia pastoris* (Yuan et al., 1997), and to affect tubulin dynamics in the slime mould *Dictyostelium discoideum* (Orosz et al., 1999).

The metabolic peculiarities of *Y. lipolytica*, a yeast which diverged early from other yeasts (Souciet et al., 2000), make it interesting to study the kinetic and regulatory characteristics of the Pfk from this organism, as well as the physiological effects of the disruption of its encoding gene(s). In the work described here, we purified to homogeneity Pfk from *Y. lipolytica*, and characterized its physical and regulatory properties, which are discussed with regard to the particular metabolic regulation of this organism. We also demonstrate that there is only one gene encoding this protein, and show that it is essential for growth in glucose.

**METHODS**

**Materials.** All chemical reagents and enzymes used in genetic assays and protein purification were obtained from Roche Molecular Biochemicals, Biotools, Amersham Biosciences or Sigma. The auxiliary enzymes and biochemicals for the Pfk assay were from Sigma.

**Organisms and growth conditions.** The following strains of *Y. lipolytica* were used in this study, as indicated where appropriate: CJM244 (MATa lys1-23 ura3-302 leu2-270) provided as INAG 35667 by A. Domínguez (Instituto de Microbiología Bioquímica, Salamanca, Spain), and P01a (MATa leu2-270 ura3-302). The following *S. cerevisiae* strains were used: VW1a (MATa ura3-52 leu2-3,112 his3A1 trpl-289 MAL2-8' SUC2 GAL) and the pfk1 pfk2 mutant HD152-1D (MATa pfk1::HIS3 pfk2::HIS3 ura3-52, his3A1 leu2-3,112 trpl-289 MAL2-8' SUC2 GAL) provided by J. Heinisch (Universität Osnabrück, Germany). Yeasts were grown at 30°C in 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose (YPD), or in minimal medium YNB (Difco), with the carbon sources indicated in each case at 2% (w/v). Liquid cultures were shaken at 200 r.p.m. in a New Brunswick orbital shaker. Solid media were identical to liquid, but with 2% (w/v) agar added. Auxotrophic requirements were added when necessary at 10-5 U (mg protein)⁻¹. This preparation was subjected to SDS-PAGE analysis on 10% gels, stained with Coomassie blue as previously described (Martínez-Costa et al., 1994), and judged to be homogeneous by this criterion (see Results). For comparative kinetic studies between YIPfk and ScPfk, the cell extracts were dialysed against 10 mM HEPES, 0.5 mM PMSF, 2.5 μg leupeptin ml⁻¹ and 20% (v/v) glycerol, pH 7.0.

**Enzyme activity assay.** Total Pfk activity was measured in an assay mixture containing 50 mM HEPES, 100 mM KCl, 5 mM MgCl₂, pH 7.0, 2 mM P₃, 0.4 mM NH₄Cl, 40 μM cAMP, 0.15 mM NADH, 1 mM MgATP, 1:2 U aldolase, 1 U triosephosphate isomerase, 1 U glycerol-3-phosphate dehydrogenase, 5–10 μl enzyme preparation and 2 mM Fru-6-P, in a final volume of 1 ml. Assays for kinetic studies were carried out under the same conditions, omitting P₃, NH₄Cl and cAMP unless otherwise stated, with 5–10 μl purified enzyme, and the indicated concentrations of MgATP, the corresponding effector and Fru-6-P. When the effect of citrate was studied, the concentration of MgCl₂ was 20 mM. In all cases, the reaction mixture without Fru-6-P was preincubated for 5 min. The reaction was started by the addition of Fru-6-P, and followed by measuring the rate of the absorbance change at 340 nm at 25°C. When Pfk activity was assayed during purification, glucose-6-P was added to Fru-6-P in a 3:1 molar proportion. Auxiliary enzymes were desalted as described previously (González-Mateos et al., 1993; Martínez-Costa et al., 1994). One unit (U) of activity is defined as the amount of enzyme that catalyses the conversion of 1 μmol substrate min⁻¹ under the conditions described above.

**Size-exclusion FPLC.** Isocratic size-exclusion chromatography was performed at 20°C on a Superdex 200 HR 10/30 column (Amersham Biosciences). The column was equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl. Samples of 0.2 ml were applied to the column, and eluted at a flow rate of 0.5 ml min⁻¹ with the same buffer. Fractions of 0.1 ml were collected, and tested for Pfk activity. When indicated, the elution buffer contained 10 mM Fru-6-P. For calibration, standard proteins ranging from 13-7 to 880 kDa were used.

**Isolation of the YIPFK1 gene.** Degenerate oligonucleotides were designed against two regions of Pfk in which conserved amino acid sequences were found in diverse organisms. The design took into account the high G+C content of the *Y. lipolytica* genome (http://www.kazusa.or.jp/codon/). The oligonucleotides were GTCCGGT- CYATYGACAACACAGATG for the 5’ region, and GTACTCGG- TRCCGGGSGATRTT for the 3’ region (the standard abbreviations to represent ambiguity are used; Nomenclature Committee of the International Union of Biochemistry, 1985), corresponding to VGSIDNDM and NVPGT, respectively. A PCR reaction was
performed using these oligonucleotides, and \textit{Y. lipolytica} genomic DNA as a template. The conditions were: 30 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. A DNA fragment of about 1 kb was isolated, cloned into pGEMT-easy (Promega), and sequenced. The translated sequence of this fragment was highly similar to that of other Pfk's. To obtain the rest of the coding region, 5' and 3' RACE reactions were performed using the RLM-RACE kit (Ambion; catalogue no. 1700) and the following oligonucleotides: for the 5' RACE, inner primer TCACAGCAAAAGCTCGC-GAGTGC, and outer primer CCCACTGAGTAGCATCGGGACGAC, and for the 3' RACE, CAACGTTTGGGCTTCAATCGTC. For the 5' RACE reaction, the conditions were as follows: three cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C; followed by a cycle of 1 min at 94°C, 1 min at 60°C, and 5 min at 72°C. For the 3' RACE, the following conditions were used: 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1-2 min at 72°C; followed by one cycle of 1 min at 94°C, 1 min at 50°C, and 5 min at 72°C. The products of the reaction were cloned into pGEMT-easy, and sequenced. All the products were sequenced at least twice in each direction. The sequence corresponding to the coding region of the \textit{YIPFK1} gene encoding the \textit{Y. lipolytica} Pfk has been deposited in the GenBank database under the accession number \textit{AY142710}.

To obtain a DNA fragment comprising the whole coding region of \textit{YIPFK1}, the following oligonucleotides were used: for the 5' region, CGCGCTTCAAAACACCGCATT, and for the 3' region, AATTTCACACAACCACCCCCTTA. A PCR reaction was done using genomic \textit{Y. lipolytica} DNA and the following conditions: three cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; followed by a cycle of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C, followed by one cycle of 1 min at 94°C, 1 min at 48°C, and 5 min at 72°C. The product was cloned into pGEMT-easy to yield plasmid pCL79, and sequenced.

**Expression of \textit{YIPFK1} in \textit{Y. lipolytica} and \textit{S. cerevisiae}**

\textit{YIPFK1} was expressed in \textit{Y. lipolytica} using the plasmid pCL49, which carries the \textit{YITEF1} promoter and the \textit{YIPXR2} terminator (Flores & Gancedo, 2005). The 3 kb \textit{YIPFK1} ORF was excised from pCL79 with NotI, and cloned in the same site in pCL49. To express \textit{YIPFK1} in \textit{S. cerevisiae}, a monocopy plasmid, pCL80, and a multicopy plasmid, pCL81, were constructed. Both plasmids carried the coding region of the \textit{YIPFK1} gene under the control of the \textit{S. cerevisiae} \textit{ADH1} promoter; and they were constructed as follows. Plasmid pCL80 was obtained by inserting in the BamHI site of pRS316 (Sikorski & Hieter, 1989) a cassette carrying the promoter and terminator of \textit{ADH1} excised from plasmid pAAH5 (Ammerer, 1983), and then introducing in the blunt-ended HindIII site the PCR fragment carrying \textit{YIPFK1} (obtained using Pwo DNA Polymerase; Roche). Plasmid pCL81 was created by cutting plasmid pDB20 (Becker \textit{et al.}, 1991) with NotI, and inserting the 3 kb NotI–NotI fragment from pCL79 carrying \textit{YIPFK1}.

**Disruption of the chromosomal \textit{YIPFK1} copy.** To disrupt the \textit{YIPFK1} gene, plasmid pCL79 was cut with NotI, and the fragment containing the \textit{YIPFK1} gene was inserted in the NotI site of a pKS plasmid (Stratagene) lacking the piece between \textit{Xhol} and \textit{KpnI} in the polylinker. The resulting construction was digested with NotI, and the internal 1-66 kb NotI–NotI fragment from \textit{YIPFK1} was replaced by a DNA fragment of 2-1 kb containing the \textit{YIEU2} gene obtained by NotI digestion of plasmid \	extit{pTA62} (Gaillardin & Ribet, 1987) to give plasmid pCL83. The 3-4 kb NotI–NotI fragment from pCL83 was used to transform \textit{Y. lipolytica}. Transformants were selected by growth in the absence of leucine on minimal medium agar containing 3% glycerol and 2% ethanol, and replica plated to minimal medium glucose agar. Those transformants unable to grow on glucose were selected for further study.

**Nucleic acid manipulations.** Recombinant DNA techniques were done according to established protocols. DNA sequencing was performed by the dideoxy chain-termination method. Sampling of the yeast to extract RNA was done by rapid filtration and flash freezing in liquid nitrogen, as described by Belinchón \textit{et al.} (2004). Total RNA for RACE reactions was extracted from \textit{Y. lipolytica} using the TRIzol LS reagent (Invitrogen). Southern analysis was performed using established protocols. Probes were labelled with \textit{32P} using the Rediprime II Random Prime labelling system. Genomic DNA was obtained as described by Hoffman & Winston (1987).

**Other methods.** Protein concentration was determined by Bradford’s dye-binding method (Bradford, 1976), using bovine \textit{gamma-globulin} as the standard. Yeasts were sampled during the exponential phase of growth, and metabolite extraction was done as described previously (Gamo \textit{et al.}, 1993). Metabolites were determined by standard enzymic techniques (Bergmeyer \textit{et al.}, 1987). Multialignments of Pfk sequences were conducted by means of the program \textit{CLUSTAL W} (Higgins \textit{et al.}, 1994).

**RESULTS**

**Purification and molecular properties of \textit{Y. lipolytica} Pfk**

Cell-free extracts of glucose-grown \textit{Y. lipolytica} CJM 244 had a Pfk activity of 63 ± 8 mU (mg protein)\textsuperscript{−1}, corresponding to a total activity of 6 ± 1 U (g wet weight)\textsuperscript{−1}. This value was about threefold lower than that shown by extracts of glucose-grown \textit{S. cerevisiae} done in parallel [18-5 ± 1-8 U (g wet weight)\textsuperscript{−1}]. \textit{YIP FK} was purified by the procedure summarized in Table 1. As shown, two effective steps, namely ion-exchange chromatography on DE52, and affinity chromatography on Blue Sepharose, led to a pure enzyme preparation with a specific activity of 10-5 U (mg protein)\textsuperscript{−1}. The final preparation showed only one band in SDS-PAGE (Fig. 1a), indicating that it was homogeneous, and that \textit{YIP FK} is likely to be composed of a single type of subunit of molecular mass 109 ± 2 kDa.

When the purified enzyme was subjected to FPLC size-exclusion chromatography on Superdex 200HS 10/30, Pfk activity eluted as a single peak (Fig. 1b) with a molecular mass of 869 ± 26 kDa, suggesting that the native enzyme is an octamer of identical subunits.

**Kinetic and regulatory properties of \textit{Y. lipolytica} Pfk**

We studied different kinetic properties of \textit{YIPFK} using the purified enzyme, obtained as described above, and dialysed cell extracts from \textit{Y. lipolytica} and \textit{S. cerevisiae} to compare some parameters of the enzymes from both origins. As shown in Fig. 2(a), \textit{YIP FK} exhibited a high affinity for Fru-6-P (\textit{S}\textsubscript{0.5} = 52 μM), and no evidence of cooperativity for this substrate was found \textit{K} (Hill coefficient) 1-1. This contrasts with the sigmoidal curve and \textit{S}\textsubscript{0.5} values within the mM range shown by Pfk's of most organisms; compare, for example, the \textit{h} and \textit{S}\textsubscript{0.5} values of ScPfk with those of \textit{YIP FK} (2-8 and 1-48 mM versus 1-04 and 90 μM, respectively). Also, \textit{YIP FK} was only slightly inhibited by MgATP (Fig. 2b), showing a \textit{Ki} value of 3-5 mM, about 10-fold higher than
that of the *S. cerevisiae* Pfk (Fig. 2b, inset). An increase in the MgATP concentration from 1 to 5 mM brought about an increase of the \( k_{\text{cat}} \) value of YlPfk for Fru-6-P to 0.51 mM, as expected, but the saturation curve for this substrate remained hyperbolic (\( h 1:1 \)) (results not shown). The calculated \( k_{\text{cat}} \) value (152 s\(^{-1}\)) is about one order of magnitude lower than those known for Pfks from other yeast species, such as *S. cerevisiae* (Hofmann & Kopper-schläger, 1982), *Schizosaccharomyces pombe* (Reuter et al., 2000), *P. pastoris* (Kirchberger et al., 2002) and *Kluyveromyces lactis* (Bär et al., 1997).

The most remarkable difference in the behaviour of the YlPfk was its insensitivity to Fru-2,6-P\(_2\) (Fig. 3a), a strong activator of eukaryotic Pfks (Van Schaftingen, 1987). As can be seen in Fig. 3(a), the enzyme from *S. cerevisiae* was activated about 100-fold by saturating concentrations of Fru-2,6-P\(_2\) with a \( K_{\text{act}} \) (activation constant) value of 1.3 \( \mu \)M, while the enzyme from *Y. lipolytica* remained basically unaffected.

Phosphoenolpyruvate markedly inhibited YlPfk, with a \( K_i \) value of 61 \( \mu \)M, two orders of magnitude lower than the value found for ScPfk (Fig. 3b). With respect to other typical allosteric effectors of Pfks from other sources, the activity of YlPfk was affected as follows compared with the control [\( 0-63 \pm 0.08 \) mU (mg protein)]\(^{-1}\): it was inhibited 80% by 5 mM citrate [\( 0.12 \pm 0.02 \) mU (mg protein)]\(^{-1}\), activated three- to fourfold by 5 mM P\(_i\) [\( 2.1 \pm 0.2 \) mU (mg protein)]\(^{-1}\) and 5 mM NH\(_4\)\(^+\) [\( 2.4 \pm 0.1 \) mU (mg protein)]\(^{-1}\), and not significantly affected by 5 mM ADP [\( 0.43 \pm 0.04 \) mU (mg protein)]\(^{-1}\] and 1 mM AMP [\( 0.63 \pm 0.08 \) mU (mg protein)]\(^{-1}\] (enzyme activities are reported as means \( \pm \) SEM of three to five determinations). The saturation curve for citrate was sigmoidal (\( h 2:1 \)), with a \( K_i \) value of 3.3 mM; the curves for P\(_i\) and NH\(_4\)\(^+\) were hyperbolic, with \( K_{\text{act}} \) values of 0.62 and 0.66 mM, respectively (results not shown).

Table 2 shows the concentrations of glycolytic metabolites related to Pfk function that we measured in *Y. lipolytica*, as compared with those determined in *S. cerevisiae*. The most striking result concerned the reaction product of Pfk, Fru-1,6-P\(_2\), whose intracellular concentration (80 \( \mu \)M) actively growing in YPD was 2.5 times the control [\( 0.12 \pm 0.08 \) mU (mg protein)]\(^{-1}\].

Table 1. Purification of *Y. lipolytica* Pfk

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity [U (mg protein)(^{-1})]</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>31</td>
<td>232.5</td>
<td>15.4</td>
<td>0.066</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Polyethylene glycol 0–5%</td>
<td>30</td>
<td>79.8</td>
<td>13.5</td>
<td>0.17</td>
<td>2.6</td>
<td>88</td>
</tr>
<tr>
<td>DE52 chromatography</td>
<td>10</td>
<td>6.8</td>
<td>5.9</td>
<td>0.87</td>
<td>13.2</td>
<td>38</td>
</tr>
<tr>
<td>Blue Sepharose chromatography</td>
<td>1.8</td>
<td>0.076</td>
<td>0.8</td>
<td>10.5</td>
<td>159.5</td>
<td>5</td>
</tr>
</tbody>
</table>

![Fig. 1.](image-url) (a) SDS-PAGE and (b) size-exclusion FPLC of purified *Y. lipolytica* Pfk. (a) A 1 \( \mu \)g quantity of purified enzyme was loaded on the gel. The positions and molecular masses of marker enzymes are indicated on the right. (b) Elution was carried out in the presence of 10 mM Fru-6-P. Enzyme activity was measured at 2 mM Fru-6-P, 1 mM MgATP. Arrows indicate the theoretical elution volumes of YlPfk forms corresponding to octamer, tetramer, dimer and monomer, and their calculated \( M_r \). Inset, native molecular mass determination of YlPfk. Molecular size standards: a, the 48-subunit form of ferritin, 880 kDa; b, thyroglobulin, 669 kDa; c, the 24-subunit form of ferritin, 440 kDa; d, catalase, 232 kDa; e, aldolase, 158 kDa; f, albumin, 67 kDa; g, ovalbumin, 43 kDa; h, chymotrypsigen A, 25 kDa; i, ribonuclease A, 13.7 kDa. Addition of Fru-6-P to the chromatography buffer was necessary to elute the enzyme in an active form.
Isolation of the \textit{YlPFK1} gene, and comparison of the encoded protein with other Pfks

The gene encoding \textit{YlPfk} was isolated as described in detail in Methods. Basically, degenerate oligonucleotides corresponding to two regions of conserved amino acid sequences in Pfks of diverse organisms were designed taking into account the characteristics of the codon usage of \textit{Y. lipolytica}. A PCR reaction using genomic \textit{Y. lipolytica} DNA as the template allowed the isolation of a piece of DNA of about 1 kb whose translated sequence was highly similar to that of other Pfks. Using 5' and 3' RLM-RACE reactions, the sequence corresponding to the entire coding region of the \textit{YlPFK1} gene was obtained. The structural gene does not exhibit introns, and its G+C content was close to 57.5 mol\%, a result not surprising in view of the high G+C content of the \textit{Y. lipolytica} genome (Dujon et al., 2004). The \textit{YIPFK1} gene encodes a putative protein of 953 aa, a value in agreement with the subunit size found in the purification. This protein presents a high sequence similarity with Pfks of other origins, showing around 50% identity with Pfks from diverse organisms (e.g. 54% with the protein of \textit{Giberella zeae}, 53% with Pfk2 from \textit{Candida albicans}, 52% with the enzyme from \textit{Sch. pombe}, 48 and 49% with the $\alpha$ and $\beta$ subunits, respectively, from \textit{S. cerevisiae}, and as much as 43–44% with Pfk isoenzymes.

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{fig2}
\caption{Dependence of \textit{Y. lipolytica} Pfk activity on the substrate concentration. Assays were carried out as described in Methods. (a) MgATP concentration was 1 mM. (b) Fru-6-P concentration was 25 $\mu$M. Insets, comparison of the kinetic behaviour of YlPfk (●) and ScPfk (□) assayed in dialysed cell extracts. Conditions were as described above, except that the MgATP dependence of ScPfk was assayed at 1-5 mM Fru-6-P. Activity is expressed relative to the maximal activity obtained for each preparation.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{fig3}
\caption{Effect of (a) fructose-2,6-P$_2$ and (b) phosphoenolpyruvate on \textit{Y. lipolytica} Pfk. Assays were carried out as described in Methods. Fructose-2,6-P$_2$ activation was assayed at 50 $\mu$M Fru-6-P and 5 mM MgATP. Phosphoenolpyruvate (P-enolpyruvate) inhibition was assayed at 50 $\mu$M Fru-6-P and 3-5 mM MgATP. Insets, results with dialysed cell extracts from \textit{Y. lipolytica} (●) and \textit{S. cerevisiae} (□). Conditions for YlPfk were as described above. The effect of Fru-2,6-P$_2$ and phosphoenolpyruvate on ScPfk activity was studied in the presence of 5 mM and 0-5 mM MgATP, respectively, and at 1 mM Fru-6-P. Activity is expressed relative to the maximal activity obtained for each enzyme in the presence of Fru-2,6-P$_2$ (a, inset) and in the absence of phosphoenolpyruvate (b, inset).}
\end{figure}
Table 2. Intracellular concentrations of selected metabolites in Y. lipolytica and S. cerevisiae grown in YPD

Yeasts were sampled as described in Methods. Concentrations (mM) of metabolites were obtained by multiplying the values in μmol (g wet weight)\(^{-1}\) by 2.5; this assumes that the intracellular water is 50%, and that the wet weight/dry weight ratio is 5. Results represent the means (± SEM) of between three and five determinations of five independent cultures.

<table>
<thead>
<tr>
<th>Metabolite (mM)*</th>
<th>Y. lipolytica</th>
<th>S. cerevisiae</th>
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<tbody>
<tr>
<td>Glucose-6-P</td>
<td>0.44 ± 0.03</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>Fruuctose-6-P</td>
<td>0.19 ± 0.01</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>Fruuctose-1,6-P</td>
<td>0.08 ± 0.01</td>
<td>1.53 ± 0.44</td>
</tr>
<tr>
<td>Phospho-ribose</td>
<td>0.06 ± 0.01</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Phospho-ribose-2-carboxylic</td>
<td>0.27 ± 0.03</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.35 ± 0.04</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>ATP</td>
<td>0.61 ± 0.09</td>
<td>2.24 ± 0.18</td>
</tr>
</tbody>
</table>

*Citrate concentration was also examined in Y. lipolytica, but it was below the detection limit of the assay (25 μM).

from mammals. An alignment of the deduced amino acid sequence of Ylpfk with those of Pfks from different organisms (Fig. 4) revealed that all functional domains containing amino acid residues assigned to binding sites for substrates and allosteric effectors (Poorman et al., 1984; Shirakihara & Evans, 1988; Martínez-Costa et al., 2004) are conserved in this Pfk.

When expressed in S. cerevisiae under the control of the S. cerevisiae ADH1 promoter, either in mono- or multicopy plasmids, the Ylpfk1 gene complemented the lack of growth in glucose of a S. cerevisiae pfk1 pfk2 mutant (results not shown).

Physiological effects of the disruption of the YLPFK1 gene

The chromosomal copy of YLPFK1 was disrupted by deletion of an internal 1.66 kb fragment and substitution by the YILEU2 gene, as described in Methods. A Southern analysis showed the correctness of the disruption (Fig. 5a). Disruption of the YLPFK1 gene resulted in glucose-negative growth (Fig. 5b), and abolished detectable Pfk activity. The findings on protein structure, which indicated a protein composed of identical subunits, are in agreement with the findings obtained in the gene disruption. Reintroduction of the YLPFK1 gene restored growth on glucose (Fig. 5b), thus confirming that the inability to grow in glucose of the disruptant was caused by the lack of Pfk. Although glucose did not support growth of the Ylpfk1 disruptant, it did not interfere with growth on permissive carbon sources (Fig. 5b), which is in contrast with the situation observed in S. cerevisiae (C.-L. Flores & C. Gancedo, unpublished results).

DISCUSSION

In this study, we isolated and characterized the Pfks from Y. lipolytica and its encoding gene. Ylpfk1 was purified to homogeneity, and was found to consist of a single type of polypeptide chain of 109 kDa, the native enzyme being a homo-octamer of 869 kDa. The identical structure of the subunits was further supported by the lack of growth in glucose after disruption of the unique YLPFK1 gene, as well as by the functional complementation of a S. cerevisiae pfk1 pfk2 mutant. Additionally, the recent availability of the Y. lipolytica genome (http://cbi.labri.fr/Genolevures) shows that there is no other ORF with significant similarity to the one described in this paper. In contrast to Pfk from other organisms, which are tetrameric proteins, the enzymes from yeasts are octameric, except for the Rhodotorula glutinis Pfk, which was suggested to be a tetramer (Schröter & Kopperschläger, 1996). However, the subunit composition of yeast Pfks varies between species: while the enzymes from S. cerevisiae, K. lactis, P. pastoris and C. albicans are hetero-octameric proteins (Kopperschläger et al., 1977; Heinisch et al., 1993; Bär et al., 1997; Lorberg et al., 1999; Kirchberger et al., 2002), those of the fission yeasts Sch. pombe and Y. lipolytica are homo-octameric (Reuter et al., 2000; this work). Thus, the acquisition of an octameric structure does not necessarily result from the interaction between two different types of subunits. It is usually assumed that Pfk subunits from eukaryotes arose by duplication, fusion and mutation of an ancestral prokaryotic gene (Poorman et al., 1984), which, in the case of several yeast species, was followed by a second duplication event leading to two different genes (Heinisch et al., 1989). Phylogenetic analyses indicate that Y. lipolytica separated early from other yeast genera, after the branching of Sch. pombe (Kurtzman & Blaž, 1998; Souciet et al., 2000). Interestingly, the Pfks of Sch. pombe and Y. lipolytica are composed of a single type of subunit, thus making attractive the hypothesis that the appearance of a second gene encoding a different subunit occurred after the separation of these species. The possible physiological advantages, if any, of the gene duplication have not been addressed.

The kinetic study of the purified Pfks from Y. lipolytica, and the isolation of its encoding gene, have revealed important differences between this organism and other yeasts. Unlike the enzyme from S. cerevisiae and most other cells, Ylpfk has a high affinity for Fru-6-P, and lacks co-operative interactions in the presence of this substrate, suggesting that the enzyme is primarily in the more active R conformation, as assumed by the model of Monod et al. (1965). Additionally, Ylpfk shows little inhibition by MgATP, and is insensitive to the allosteric activators Fru-2,6-P2, AMP and ADP, whereas it is highly sensitive to inhibition by phosphoenolpyruvate. In bacteria, phosphoenolpyruvate is the main allosteric inhibitor of Pfks (Blangy et al., 1968), but in S. cerevisiae and animal cells, this compound is of little physiological relevance because of its intracellular concentration, which is usually several orders of magnitude lower than the K_i values of Pfk isozymes (inset of Fig. 3b,
Table 2; Martinez-Costa et al., 2004). In contrast, our data suggest that phosphoenolpyruvate could play a physiological role in the regulation of YlPfk, as its intracellular concentration during growth in YPD (0-27 mM) is about fourfold higher than its $K_i$ value for YlPfk (61 $\mu$M). Under these conditions, the inhibition by phosphoenolpyruvate would not allow the enzyme to work near $V_{\text{max}}$, even if the concentration of Fru-6-P (0-19 mM), much higher than the $S_0$ value (52 $\mu$M), would permit it. Therefore, phosphoenolpyruvate, probably in conjunction with the activators $P_i$ and $NH_4^+$, may contribute to maintaining YlPfk within a range of in vivo activity able to respond efficiently to variations of Fru-6-P concentration, even in the absence of co-operative kinetics. To our knowledge, no data are available on the effect of this metabolite on Pfks from other yeast species, except S. cerevisiae. It has been reported (Habison et al., 1983) that partially purified Pfk from Aspergillus niger is inhibited by phosphoenolpyruvate with a $K_i$ value of 0-15 mM, and also by citrate ($K_i$ 0-1-0-4 mM), the latter inhibition being released by Fru-2,6-P$_2$ activation (Arts et al., 1987). Inhibition by citrate is a common feature of different eukaryotic Pfks; however, in Y. lipolytica, at least during growth in YPD, the role of this inhibitory effect is uncertain because of the high $K_i$ value of 3-3 mM, and the undetectable intracellular concentration of citrate under these conditions.

Fig. 4. Alignments between amino acid sequences of Pfks from different organisms. The sequences shown are from Y. lipolytica (Yl), Sch. pombe (Sp), S. cerevisiae (Sc), human muscle (Hm) and E. coli (Ec). Amino acids identical to YlPfk are shaded. Putative residues assigned to the binding of substrates and allosteric effectors (Poorman et al., 1984; Shirakihara & Evans, 1988; Martinez-Costa et al., 2004) are indicated by black triangles.
The atypical kinetic properties of YlPfk, and the levels of key glycolytic intermediates determined in *Y. lipolytica*, point to a role of this enzyme in the regulation of glycolysis that is different from the role played in *S. cerevisiae*. The high concentration of Fru-1,6-\(P_2\) found in *S. cerevisiae* during growth in glucose, greatly exceeding the concentration of Fru-6-\(P\) (Table 2; Bañuelos et al., 1977; Breitenbach-Schmitt et al., 1984), suggests that in this yeast, a rate-limiting step of the glycolytic pathway is located after the reaction catalysed by Pfk. However, in *Y. lipolytica*, the situation appears to be different since the concentration of Fru-1,6-\(P_2\) in cultures growing in YPD (over 50-fold lower than that in *S. cerevisiae*) was about half that of Fru-6-\(P\), thus suggesting that in this organism, Pfk may have an important role on the control of glucose utilization.

All the amino acid residues assigned to the binding of Fru-2,6-\(P_2\) and AMP/ADP are conserved in YlPfk (Fig. 4). Therefore, the lack of activation by these effectors could be related to other unidentified residues important for either the binding to their allosteric sites or the signal transduction route from these sites to the catalytic site. It is also possible that the insensitivity of YlPfk towards Fru-2,6-\(P_2\) may be due to an evolutionary adaptation to the lack of this compound; however, this seems unlikely. Although no data on Fru-2,6-\(P_2\) concentration in *Y. lipolytica* are available, two ORFs, YAL10E17635g and YAL10F27885g, have been found in the available *Y. lipolytica* genome (http://cbi.labri.fr/Genolevures) with similarity to *S. cerevisiae* 6-phosphofructose-2-kinase and fructose-2,6-bisphosphatase, respectively, the enzymes responsible for the metabolism of this compound.

Among eukaryotic Pfks with abnormal allosteric properties, the *Sch. pombe* enzyme was reported to have little sensitivity to ATP inhibition (Reuter et al., 2000), and that from *K. lactis* shows no Fru-6-\(P\) co-operativity (Bår et al., 1997); however, both enzymes are sensitive to usual Pfk effectors, including Fru-2,6-\(P_2\) and AMP. A nonallosteric Pfk has been described in the slime mould *D. discoideum* (Martínez-Costa et al., 1994).

*S. cerevisiae* mutants lacking Pfk activity do not grow in glucose because of limited functionality of the pentose phosphate pathway, and because of the fermentative sugar metabolism in this yeast. In contrast, in *K. lactis*, a yeast with a more oxidative metabolism than *S. cerevisiae*, pfk mutants grow in glucose at a rate similar to that of the wild-type (Heinisch et al., 1993). However, an additional block of respiration, or mutations that affect the function of the pentose phosphate pathway, precluded growth of *K. lactis* pfk mutants in glucose (Jacoby et al., 1993). In this context, the lack of growth in glucose of the *Y. lipolytica* pfk mutant is unexpected, since this yeast has an obligate oxidative metabolism, and even possesses an active complex I in the respiratory chain (Djafarzadeh et al., 2000). The most likely explanation of this growth phenotype would be a problem with the functionality of the pentose phosphate pathway, perhaps related to NADP\(^+\) regeneration. However, direct measurements of the glucose flux going through this pathway in *Y. lipolytica* are not yet available.

An interesting difference between the behaviour of *Y. lipolytica* pfk1 and *S. cerevisiae* pfk1 pfk2 mutants is their response to glucose. This sugar inhibits growth in media with gluconeogenic carbon sources not only of the S.

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**Fig. 5.** Disruption of *YIPFK1* gene in *Y. lipolytica*, and phenotypic analysis of the disruption. (a) Scheme of the *YIPFK1* gene (white box), its disruption with the *YILEU2* gene (grey box), and Southern blot analysis of the disruption. Genomic DNA was digested with EcoRV, and probed with the indicated 439 bp *Ncol–Nof* fragment obtained from plasmid pCL79 (for details, see Methods). PO1a, wild-type; CLP5, strain with disrupted *YIPFK1*. (b) Phenotypic analysis of the disruptant. The disruptant strain CLP5 was transformed with plasmids carrying either the *YIPFK1* gene with the correct orientation relative to the promoter, or with the reversed gene. Two independent transformants with a reintroduced *YIPFK1* gene in the correct orientation, and one with the reversed orientation, are shown. The carbon sources of the corresponding agar plates are indicated; and the media were supplemented with leucine and uracil. The relevant characteristics of the strains are indicated in the diagram.
cerevisiae pkf1 pkf2 mutant (C.-L. Flores & C. Gancedo, unpublished results), but also of other glycolytic mutants (Ciriacy & Breitenbach, 1979). In contrast, the growth of the Y. lipolytica pkf1 mutant under similar conditions remained unaffected by the sugar. This difference might be due to a variation in the magnitude of catabolite repression among the two yeasts. While glucose exerts a strong repression on pathways that allow the use of gluconeogenic carbon sources in S. cerevisiae (Gancedo, 1998; Carlson, 1999), this repression is less marked in Y. lipolytica (Barth & Scheuber, 1993; R. Jardón, C.-L. Flores & C. Gancedo, unpublished results). This fact, and the function of an active respiration, may allow the Y. lipolytica pkf1 mutant to withstand the presence of glucose.

The peculiar regulatory characteristics of the YIPfk, together with the differences in kinetic properties of pyruvate kinase (Hirai et al., 1975), and the low hexokinase activity found in this organism (Petit & Gancedo, 1999), indicate that the glycolytic flux in this organism is regulated in a different way from that described in the conventional yeast S. cerevisiae.

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Y. lipolytica Phk and disruption of its gene.


