Single Point Mutations in Either Gene Encoding the Subunits of the Heterooctameric Yeast Phosphofructokinase Abolish Allosteric Inhibition by ATP*§

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Yeast phosphofructokinase is a heterooctameric enzyme subject to a complex allosteric regulation. A mutation in the PFK1 gene, encoding the larger α-subunits, rendering the enzyme insensitive to allosteric inhibition by ATP was found to be caused by an exchange of proline 728 for a leucine residue. By in vitro mutagenesis, we introduced this mutation in either PFK1 or PFK2 and found that the exchange in either subunit drastically reduced the sensitivity of the holoenzyme to ATP inhibition. This was accompanied by a lack of allosteric activation by AMP, fructose 2,6-bisphosphate, or ammonium and an increased resistance to heat inactivation. Yeast cells carrying either one mutation or both in conjunction did not display a strong phenotype when grown on fermentable carbon sources and did not show any significant changes in intermediary metabolites. Growth on non-fermentable carbon sources was clearly impaired. The strain carrying both mutant alleles was more sensitive to Congo Red than the wild-type strain or the single mutants indicating differences in cell wall composition. In addition, we found single pfk null mutants to be less viable than wild type at different storage temperatures and a pfk2 null mutant to be temperature-sensitive for growth at 37 °C. The latter mutant was shown to be respiration-dependent for growth on glucose.

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Phosphofructokinase (Pfk1; E.C. 2.7.1.11) is a central enzyme of glycolysis found in almost all organisms from bacteria to higher eukaryotes. It catalyzes the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate under ATP consumption, i.e. the second irreversible glycolytic reaction. Because of its allosteric properties, Pfk has been postulated to play a major role in the control of the glycolytic flux (reviewed in Ref. 1). However, this role has been questioned by metabolic control calculations (2, 3). In view of such models, the sensitivity of Pfk to allosteric regulation by a vast number of small molecules, especially in eukaryotes, remains a puzzling observation (reviewed in Ref. 4). Thus, ATP not only functions as a substrate, but also as an allosteric inhibitor at millimolar concentrations (5). Under physiological conditions, AMP (also in millimolar concentrations) and fructose 2,6-bisphosphate (effective in the micromolar range) are thought to be the most potent allosteric activators (6, 7). This is also true for Pfk from bakers’ yeast, Saccharomyces cerevisiae, which is a heterooctameric enzyme composed of 4α- and 4β-subunits (8), encoded by PFK1 and PFK2, respectively (note that this nomenclature is used by German and American groups (i.e. Ref. 9), whereas Maitra and co-workers use the reverse designations, as in Ref. 10). Deletion mutants in either one of the genes still ferment glucose and are capable of growth on this carbon source, whereas pfk1 pfk2 double deletions are glucose-negative (11). A number of mutant alleles have been isolated for both PFK genes, and their phenotypic consequences have been investigated in detail (Refs. 12–14, to name just a few studies). The genes have been cloned and sequenced (15), providing the basis for targeted amino acid exchanges by site-directed mutagenesis. In addition to sequence comparisons between Pfk’s from a variety of organisms, this approach was aided by the determination of the x-ray structure of eubacterial Pfk (16–18). Based on these works, we could show that critical amino acid residues in the active center are functionally conserved in the yeast and human muscle Pfk (11, 19). A hypothesis originally brought up by Poorman et al. (20) attributes the origin of eukaryotic Pfk subunits to a gene duplication event, with the N-terminal half of each subunit retaining catalytic function and the C-terminal half evolving allosteric binding sites. This assumption was substantiated by our findings, when we abolished activation of yeast Pfk by fructose 2,6-bisphosphate using site-directed in vitro mutagenesis in sequences encoding the C-terminal halves of each subunit (21).

As stated above, the importance of allosteric control of Pfk for yeast metabolism is an ongoing controversy. Since inhibition by ATP seems to be the principle regulatory mechanism (AMP and fructose 2,6-bisphosphate function by reversing this inhibition (see Refs. 6 and 7)), we started here to investigate this control at the level of enzyme structure and physiological consequences of altering ATP inhibition. For this purpose, we took advantage of a previous work, in which mutants insensitive to ATP inhibition were obtained in a genetic screen and shown to be caused by a defect in the α-subunits encoded by PFK1 (Ref. 22; note again that these authors designate this gene encoding the larger Pfk subunits as PFK2). After identifying the altered base, we introduced the equivalent exchange by in vitro mutagenesis in the yeast PFK1 and PFK2 genes. Our data provide first clues to the localization of the regulatory ATP binding...
domain in eukaryotic Pfk subunits, for which x-ray structure analyses are not yet available.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Growth Conditions—**Yeast strain MCH-7B (derived from HD56–5A (Ref. 21) by deletion of the TRP1 coding region) and its isogenic pfk deletion derivatives (Table I (available as supplemental material in on-line version)) were used as recipients for transformations with plasmids carrying in vitro mutated PPK alleles, for enzyme purification and determination of kinetic parameters.

For amplification of plasmid DNA, *E. coli* strain DH5αF' (Life Technologies, Inc.) was used throughout.

Standard media and growth conditions were employed (23). Rich media were based on 1% yeast extract and 2% bacto-peptone (Difco) and supplemented with 2% glucose (YPD) or 1% glycerol and 3% ethanol (YEPE) for growth of mutants unable to utilize glucose. Yeast transformations were performed by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA) (24). Transformed yeast cells were recovered, amplified in E. coli (25). Transformation of yeast was carried out by a freeze method according to Ref. 26.

Deletions in the PFK genes were obtained by inverse PCR according to a method described by (27). For this purpose, we first constructed plasmids carrying either the PFK1 or the PFK2 gene in the pUK1921-derivative vector pUK19ea (obtained by digestion of pUK1921 (Ref. 28) with EcoRI/Xcl, fill-in with Klenow, and religation to delete the BamHI site from the polylinker). PFK1 was subcloned as a 4-kg SphI fragment (= pJJH424; from -960 to +3006 bp according to the published sequence reading the A from the translation start codon as +1) and PFK2 as a 4-kg PstI fragment (= pJJH422; from -899 to +3001 bp). For deletion of the open reading frames, the oligonucleotide pairs Prom1.1/pP1–60 and pProm/ZF2–60, each carrying a BamHI recognition sequence (Table II (available as supplemental material in on-line version)), were used as DNA templates. The products were then digested with BamHI and religated to yield pJJH428 (pK1A) and pJJH429 (pK2A).

To the latter construct more 3'-non-coding sequences of PFK2 were added as an EcoRI/BamHI fragment (now enclosing sequences 830 bp downstream of the translational stop codon; pJJH463). pJJH428 and pJJH463 were linearized at the single BamHI sites to introduce a set of marker genes (HIS3, URA3, TRP1, and LEU2) from the YEp vector series (29) as additional fragments into each. The resulting plasmids were used to construct a series of otherwise isogenic single deletion mutants by taking advantage of homologous recombination using the one-step gene replacement method (30).

For localization of the PFK1 mutation in strain GT4-AC (22) the coding sequence was amplified from whole cells by PCR using the oligonucleotide pairs PFK1FusX/PFK1endX (Table II (supplemental material)). From the product, the 2-kg BgII fragment (comprising about two thirds of the 5' end of PFK1) and the 2.2-kg PstI/XhoI fragment (containing about two thirds of the 3' end of PFK1, with the XhoI site introduced by one of the primers) were used to substitute either the BgII or the PstI/SalI fragment of *Yp1* (Fig. 1, based on the 2-μm vector Yep352 carrying PFK1 and its flanking regions as a 4-kg SphI fragment (Ref. 11)).

As recipient plasmids for *in vivo* recombination, we constructed the yeast multicopy plasmids pJH684 (pLB12196–2929, URA3) and pJH685 (pLB2177–2830, LEU2) again by inverse PCR (27) using the oligonucleotide pairs PFK1delXho-5'/PFK1delXho-3' and PFK2delXho-5'/PFK2delXho-3' (Table II (supplemental material)), respectively, to introduce a single XhoI site. Plasmids pJH686 (PFK1, bp +1988 to +3006) and pJH687 (PFK2, bp +1991 to +2892) were then constructed as templates for *in vitro* mutagenesis by subcloning the 3' end of each PFK gene as a BglII/BamHI fragment into pUK1912 linearized with BamHI, taking advantage of the internal BglII restriction site in the *PFK2* gene (Ref. 31).

Enzyme Purification, Determination of Specific Activities, and Metabolite Determinations—Pfk was purified for determination of kinetic parameters using a modification of the method of (32), based on precipitation with polyethylene glycol and affinity purification on Affi-Gel Blue columns, as described (11). Activation by fructose 2,6-bisphosphate was determined in 50 mM sodium phosphate buffer at pH 7.0, containing 1 mM magnesium chloride, with 3 mM ATP and 0.5 mM fructose 6-phosphate in a standard assay mixture containing 0.2 mM NADH, 0.3 units of aldolase, 1.5 units of triosephosphate isomerase, and 0.5 units of glyceral-3-phosphate dehydrogenase without other effectors. The ancillary enzymes were purchased from Roche Biochemicals (Mannheim, Germany) in ammonium sulfate suspension. To test the effect of ammonium activation, the ancillary enzymes were purchased from Sigma (Munich, Germany) as dried powder, resuspended in 1 ml of phosphate buffer, and added to a final concentration of 1 unit/ml each. For enzymatic determinations in crude extracts, 50 mM potassium phosphate buffer, pH 7.0, containing 20 mM magnesium chloride was used with the same ancillary enzymes as described above, but with substrate concentrations of 2.0 mM fructose 6-phosphate and 1 mM ATP, unless indicated otherwise. Allosteric activators like 0.5 mM AMP, 0.5 mM ADP, and 10 μM fructose 2,6-bisphosphate were added as indicated.

To determine glucose consumption and ethanol production rates, cells were grown overnight to late logarithmic phase in rich medium with 1% glycerol and 3% ethanol as carbon sources. They were harvested by centrifugation, washed twice with distilled and sterile water, and resuspended in 50 ml of synthetic complete medium with 1% glucose at an *A*~405~ of about 0.5. Cultures were incubated with shaking at 30 °C, and 1-m1 aliquots were removed every hour, cells separated by centrifugation for 3 min in a microcentrifuge, and supernatants frozen at −20 °C for later determinations. Growth was followed by determination of the *A*~600~ in an Ultraspec 4000 photometer (Amersham Pharmacia Biotech).

For determination of intermediate metabolite concentrations, a method described in Ref. 33 was modified. Cells were grown overnight in 50 ml of synthetic complete medium containing 1% glycerol and 3% ethanol as carbon source. Cells were harvested by centrifugation and resuspended in 100 ml of synthetic complete medium with 4% glucose, and 50-ml aliquots were harvested by centrifugation after incubation at 30 °C for the times indicated. They were immediately resuspended in ice-cold 1 M HEPES buffer, 0.5% (p/v) PMSF, 1 mM diithiothreitol, and injected into 11.5 ml of boiling ethanol. After incubation for 3 min at 80 °C, suspensions were cooled on ice and dried to completeness under vacuum. After resuspension in 1.5 ml of distilled water, cell debris was separated by a 15-min centrifugation in a microcentrifuge and the supernatant was used for NADP- or NADH-coupled enzymatic determinations. Fructose 2,6-bisphosphate levels were determined via activation of pyrophosphate-dependent phosphofructokinase from potatoes as described previously (21). Calculations were based on the assumption that an *A*~520~ of 1 equals 4.0 μg/ml cell dry weight (34).

**Immunological Detection—**A polyclonal antiserum prepared against purified yeast Pfk was used for immunological detection of Pfk subunits in Western blots as described (11), visualized by treatment with alkaline phosphatase-coupled anti-rabbit-antibodies using 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium staining.

**Sequence Analysis—**Sequences were obtained from SeqLab (Göttingen, Germany) with standard oligonucleotide primers or primers custom-synthesized by MWG Biotech (Munich, Germany).
RESULTS

Yeast Phosphofructokinase Mutants

Sequence of the Point Mutation Leading to ATP-insensitive Yeast Pfk Activity—Previously, a pfk1 mutant could be isolated by its failure to grow on glucose as a sole carbon source in a strain already carrying a pfk2 defective allele (22). This mutant showed a phosphofructokinase activity insensitive to allosteric inhibition by ATP when crossed back into a pfk2 wild-type background. To localize the mutation, we used PCR to amplify the complete coding region of PFK1 with its flanking sequences from the genome of the mutant strain gTI-4C (22). Two fragments were obtained from the PCR product by digestion with appropriate restriction endonucleases and used for subcloning in conjunction with wild type flanking PFK1 sequences in a yeast multicopy vector (Fig. 1; see “Experimental Procedures” for details). Transformation into a yeast multicopy vector (Fig. 1; see “Experimental Procedures”) designated sequences derived from a PCR reaction using genomic DNA from the originally isolated PFK1atp mutant (gTI-4C) as a template. Restriction sites used for subcloning as explained under “Experimental Procedures” are indicated.

![Diagram](image)

**FIG. 1.** Identification of the PFK1 mutation conferring insensitivity to ATP inhibition and amino acid alignments of phosphofructokinases from different organisms in the region of interest. Boxes on top represent the PFK1 locus, with black arrows indicating the coding sequence. Gray shaded boxes indicate sequences derived from a PCR reaction using genomic DNA from the originally isolated PFK1atp mutant (gTI-4C) as a template. Restriction sites used for subcloning as explained under “Experimental Procedures” are indicated. + and − indicate the presence and absence, respectively, of ATP inhibition in Pfk assays performed in crude extracts, when the respective constructs were carried on multicopy plasmids in a pfk1 deletion background. Positions of amino acids substituted by in vitro mutagenesis of the encoding genes to alter the response to allosteric regulation by ATP (position 728 in ScPfk1p) and by fructose 2,6-bisphosphate (position 724 in ScPfk1p) are indicated at bottom. Sc, S. cerevisiae; Bs, Bacillus stearothermophilus; hm, human muscle.

| Strain | Relevant genotype | Without effectors* | With effectors\(^b\) | With effectors\(^b\) |
|--------|------------------|--------------------|---------------------|---------------------|
| HD264–10A | PFK1 PFK2 | <5 | 716 |
| HD264–7C | PFK1atp PFK2atp pfk1::TRP1 pfk2::URA3 | 241 | 480 |
| HD264–10B | PFK1atp PFK2atp pfk1::TRP1 pfk2::URA3 | 277 | 551 |
| HD264–9B | PFK1atp PFK2 pfk1::TRP1 | 313 | 677 |
| HD264–1C | PFK2atp PFK1atp pfk2::URA3 | 367 | 857 |
| gTI-4C | PFK1atp PFK2 | 103 | 412 |

*Activities were determined in 50 mM K-phosphate buffer, pH 7.0, with 20 mM MgCl\(_2\) at 1 mM ATP, 0.2 mM fructose 6-phosphate.

b Activities were determined in 50 mM K-phosphate buffer, pH 7.0, with 20 mM MgCl\(_2\) at 1 mM ATP, 2.0 mM fructose 6-phosphate, 0.5 mM AMP, 0.5 mM ADP, 10 \(\mu\)M fructose 2,6-bisphosphate (note that specific activities in various determinations did not vary by more than 20%, whereas relative activities of the mutants as compared to the wild type varied by less than 10%).

In order to confirm that indeed a single exchange of proline 728 for leucine is responsible for the unusual allosteric behavior of the octameric Pfk enzyme, we proceeded by introducing the same exchange in the coding region of both PFK1 and PFK2 by in vitro mutagenesis. We then constructed single-copy integrants of the mutant PFK alleles with only part of the sequence obtained from the PCR products, confirmed by sequencing to contain only the mutation introduced. For this purpose, single deletion mutants pfk1::TRP1 and pfk2::URA3 of opposite mating type were used to integrate PFK1atp at the HIS3 locus and the PFK2atp mutant allele at the LEU2 locus, respectively. The resulting strains were crossed and subjected to tetrad analysis to obtain all possible combinations of PFK alleles (segregants from HD264, Table I (see supplemental material)).

The strains were first tested for their specific phosphofructokinase activities under inhibitory and non-inhibitory conditions. Under optimized test conditions (i.e. at 1 mM ATP, 2.0 mM fructose 6-phosphate and in the presence of positive effectors), all strains displayed comparably high specific Pfk activities (Table III). It should be noted that the original non-isogenic mutant strain gTI-4C reaches only about 60% of the respective segregant (HD264-9B) in our isogenic series. Whereas Pfk activity was below the level of detection in crude extracts from wild-type cells under inhibitory test conditions (3 mM ATP, 0.2 mM fructose 6-phosphate, without effectors), all mutants retained considerable activities under such conditions.
FIG. 2. Growth of strains carrying PFKatp mutant alleles. A, growth on non-fermentable carbon sources and on glucose. Strains were pregrown on liquid-rich medium containing glycerol and ethanol as carbon sources and then streaked out onto either YEPGE or YEPD plates, as indicated. Growth was assessed after incubation at 30 °C for the times indicated. Strains tested are: HD264-10A (PFK1 PFK2), HD264-9B (PFK1atp PFK2), HD264-7C (PFK1atp PFK2atp), HD264-3B (pfk1::TRP1 PFK2atp), and HD266–1C–3B (PFK1atp pfk2::URA3). Note that HD264-3B forms colonies on YEPD when incubated for another 3 days, whereas HD266–1C and HD266–3B do not (data not shown). B, colony morphology displayed after prolonged incubation. Strains as in A were streaked out on YEPD for single colonies and allowed to grow for 7 days at 30 °C. C, serial dilution patch test of strains as in A and of the single deletion mutants AST3–2B (pfk1::TRP1 PFK2) and AST8–6C (PFK1 pfk2::URA3). Strains were grown to early stationary phase in YEPD, diluted in fresh medium to an \( A_{600} \) of 0.1, and allowed to grow for another 4 h at 30 °C. They were then diluted in series, and aliquots of 5 \( \mu l \) were spotted onto the media indicated. Plates were incubated for 3–5 days at 30 °C if not indicated otherwise.

**TABLE IV**

| Metabolites | SC | YEPD | ATP | Glc-6-P | Fru-6-P | Fru-1,6-P₂ | Fru-2,6-P₂ |
|-------------|----|------|-----|---------|---------|------------|------------|
| PFK allele | \( h^{-1} \) | pmol/mg dry weight | pmol/mg dry weight |
| PFK1 PFK2   | 0.40 | 0.38 | 3.17 | 0.87 | 0.82 | 5.10 | 3.5 |
| PFK1atp PFK2atp | 0.40 | 0.46 | 3.32 | 0.92 | 0.87 | 6.53 | 1.9 |
| PFK1atp PFK2 | 0.40 | 0.40 | 3.30 | 0.80 | 0.84 | 4.72 | 2.9 |
| PFK1 PFK2atp | 0.38 | 0.44 | 3.25 | 0.56 | 1.07 | 6.40 | 5.4 |

*Strains used in this experiment were: HD264–10A (PFK1 PFK2), HD264–7C (PFK1atp PFK2atp), HD264–9B (PFK1atp PFK2), and HD264–1C (PFK1 PFK2atp).*
As expected from previous works, strains carrying only one mutant \textit{PFK} allele and being deleted for the gene encoding the other subunit did not show detectable Pfk activity in crude extracts. However, there were distinct growth phenotypes associated with the strains of our isogenic series (Fig. 2A). Thus, all strains carrying two active \textit{PFK} alleles (i.e. \textit{PFK1} \textit{PFK2}, \textit{PFK1\textit{atp} PFK2}, \textit{PFK1} \textit{PFK2\textit{atp}}, or \textit{PFK1\textit{atp} PFK2\textit{atp}}) grew normally on media containing glucose as a carbon source (compare also Table IV). When \textit{PFK2\textit{atp}} was the only functional \textit{PFK} allele being present (\textit{pfk1:TRP1 PFK2\textit{atp}}), strains grew much more slowly on YEPD, whereas strains with only \textit{PFK1\textit{atp} (PFK1\textit{atp pfk2::URA3}) failed to grow on this medium. Mutant strains carrying either one or both \textit{PFK\textit{atp}} alleles showed a reduced capacity to grow on medium containing non-fermentable carbon sources (Fig. 2, A and C). This was not observed when the other \textit{PFK} allele was deleted (\textit{pfk1:TRP1 PFK2\textit{atp} PFK1\textit{atp pfk2::URA3}}).

Prolonged incubation on YEPD of the mutant strains resulted in distinct colony morphologies (Fig. 2B). Whereas the wild-type strain formed smooth round colonies, the mutants displayed various degrees of sectoring, being most pronounced in the double mutant (\textit{PFK1\textit{atp PFK2\textit{atp}}}), to a lesser degree in the \textit{PFK1\textit{atp PFK2}} mutant, and almost undetectable in the \textit{PFK1 PFK2\textit{atp}} mutant. Microscopic examination did not reveal any tendency to pseudohyphal growth at the colony borders.

An altered colony morphology could be caused by a defect in ammonium signaling (35). We therefore tested the sensitivity of our mutants to increasing concentrations of ammonium in the medium. In order to establish a possible connection of the regulation of the central glycolytic pathway with cell wall biosynthesis, we also tested drugs like caffeine and Congo Red. As evident from Fig. 2C, all strains carrying \textit{PFK} mutant alleles conferring insensitivity to ATP inhibition were more resistant to increasing concentrations of ammonium chloride. This also held true when we used ammonium sulfate (data not shown). Sodium chloride at similar concentrations did not show any inhibitory effect. With respect to caffeine, the \textit{PFK1\textit{atp PFK2}} mutant and the single \textit{pfk1} deletion showed an increased resistance as compared with the wild type and the other mutants. In contrast, the double mutant (\textit{PFK1\textit{atp PFK2\textit{atp}}}) was much more sensitive to Congo Red in the medium than the wild type. In the course of these experiments, we also noted that the \textit{pfk2} deletion causes a strong temperature-sensitive phenotype not growing at 37 °C (Fig. 2C).

This observation prompted us to determine the viability of \textit{pfk} null mutants upon long term storage at different temperatures with the isogenic series \textit{MCH-2B} (wild type), \textit{MCH-4B} (\textit{pfk1::HIS3 PFK2}), and \textit{MCH-6B} (\textit{PFK1 pfk2::HIS3}). Whereas the wild-type control showed only a marginal loss of viability in YEPD medium when stored at 4 °C for 50 days, viability decreased more than 5 orders of magnitude for \textit{pfk1::HIS3} and more than 2 orders of magnitude for \textit{pfk2::HIS3} with a half-life of about 3 days and 6 days, respectively. Similar increased losses of viability were observed upon incubation at 30 °C and 37 °C for the null mutants as compared with the wild-type control. A decreased viability for the deletion mutants was also observed in a different isogenic strain series (data not shown).

Finally, we determined the dependence of \textit{pfk} null mutants on respiratory capacity. It has been observed previously that \textit{pfk2} null mutants are inhibited for growth by the addition of inhibitors like sodium azide or antimycin A to the medium (15, 36). Here we used a genetic approach to investigate this relation. For this purpose a strain carrying a \textit{mtfl:LEU2} deletion (HALY2-2-B, causing a loss of respiratory capacity; Ref. 37) was crossed to a \textit{pfk1::HIS3 pfk2::URA3} null mutant (AST13-2C), and 17 tetrads were separated and allowed to germinate on YEPD at 30 °C. Although 11 of the segregants were prototrophic for histidine and leucine (\textit{mtfl pfk1 double null mutants}), no segregant being prototrophic for uracil and leucine (which would result from a \textit{mtfl pfk2} double null mutant) could be obtained. This clearly indicates that respiration is essential for viability of \textit{pfk2} null mutants.

After another 5 days of incubation at room temperature, two spores germinated carrying the \textit{mtfl pfk2} double mutation. These strains grew extremely slowly on glucose media at room temperature, indicating that a respiratory defect exacerbates the temperature-sensitive phenotype of \textit{pfk2} null mutants.

\textbf{Kinetic Properties of Mutant Pfk Enzymes—}As expected from the data on specific enzyme activities and the growth behavior, Pfk subunits were detectable in about equal amounts in Western blots using crude extracts from strains carrying either one or both \textit{PFK} alleles mutated (Fig. 3), indicating an intact oligomeric structure (see "Discussion" for details).

To determine the effects of the above mutations on the allosteric properties of yeast Pfk, we purified the enzyme from crude extracts of the respective strains and tested their response to various effectors (Fig. 4). In contrast to other mutants with allosteric or catalytic defects in Pfk, where we detected pronounced effects only if the mutation was introduced simultaneously in both genes encoding the Pfk subunits, mutation of proline 728 (using the numbering in Pfk1p) resulted in a loss of ATP inhibition over a broad concentration range when introduced in either one of the encoding genes. Thus, the wild-type enzyme was inhibited in the absence of positive effectors by 0.5 mM ATP, whereas the mutant enzymes retained more than 70% activity even at 3 mM ATP (Fig. 4). On the other hand, the wild-type enzyme was effectively activated by millimolar concentrations of AMP and micromolar concentrations of fructose 2,6-bisphosphate, but the mutant enzymes did not react to these effectors. Similarly, increasing amounts of ammonium chloride correlated with an activation of the wild type but not of the mutant enzymes (Fig. 4).

Enzyme stability was then determined in crude extracts prepared from the same segregants and adjusted to a concentration of 1 mg/ml total protein by dilution in phosphate buffer. Incubation at 50 °C resulted in a rapid loss of activity for the wild-type enzyme with different levels of resistance to heat inactivation displayed by the mutant combinations (Fig. 5). The double mutant proved to be most resistant to heat inactivation, retaining more than 90% activity after 20 min of heat treatment, when the wild-type activity already decreased to...
less than 40% of the initial value. The single mutants displayed an intermediate behavior.

Physiological Effects of Altered Allosteric Regulation—In order to gain some insight into the physiological consequences of altering the allosteric inhibition of yeast Pfk, we first determined growth, glucose consumption, and ethanol production in the mutant strains pregrown on non-fermentable carbon sources and then shifted to glucose containing media (Fig. 6). As stated above, all strains carrying mutant PFKatp alleles grow well on glucose media. Accordingly, glucose consumption and ethanol production were found to be very similar in all strains. These results coincide with the data obtained on the concentrations of intermediary metabolites in such strains, again after transfer from non-fermentable carbon sources to glucose containing media (Fig. 6). As stated above, all strains carrying mutant PFKatp alleles grow well on glucose media. Accordingly, glucose consumption and ethanol production were found to be very similar in all strains.

These results coincide with the data obtained on the concentrations of intermediary metabolites in such strains, again after transfer from non-fermentable carbon sources to glucose containing media (Table IV). Glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-bisphosphate levels are similar in all strains. These findings indicate a similar in vivo activity of the mutant Pfks, coinciding with the unaltered ATP levels measured (Table IV). In contrast to our experience with other Pfk mutants being either deleted for one of the encoding genes or altered in their allosteric properties, the levels of the activator fructose 2,6-bisphosphate remained low near the level of detection in all strains tested here (Table IV).

FIG. 4. Sensitivity of yeast Pfk variants to allosteric regulation. Crude extracts were prepared from the strains indicated after growth on YEPD and Pfk was purified by affinity chromatography as described under “Experimental Procedures.” Appropriate amounts of Pfk were used for enzymatic determinations at 30°C and maximal activities for each preparation under the conditions indicated were set at 100%. Note that for ammonium chloride maximal activities were not reached for the wild type even at a non-physiological concentration of 100 mM.

FIG. 5. Heat inactivation of Pfk from different mutant strains. Crude extracts were prepared from strains grown in YEPD and adjusted to a total protein concentration of 1 mg/ml with phosphate buffer. They were placed in a water bath at 50°C, and aliquots were taken at the time points indicated. Activities measured in 50 mM potassium phosphate buffer, pH 7.0, with 10 mM MgCl₂ at 1 mM ATP, 2.0 mM fructose 6-phosphate, and 10 μM fructose 2,6-bisphosphate were set at 100% at time 0.

DISCUSSION

Although phosphofructokinase is an enzyme of central metabolic importance that has been studied for decades, several questions as to the evolution of its subunits and the location of functional domains remain to be answered especially for the eukaryotic enzymes. In this context, the physiological significance of the complex allosteric regulation is subject to ongoing controversies. Among a variety of effector molecules influencing eukaryotic Pfk activity (see Ref. 4), allosteric inhibition by ATP seems to be one of the basic regulatory mechanisms. Earlier biochemical approaches determined the number of ATP binding sites per yeast Pfk subunit as either 1 or 2 (38, 39). Our previously published work in conjunction with the data presented here strongly argues in favor of two independent binding sites localized on each yeast Pfk subunit. One serves a catalytic and the other an allosteric function. Thus, we previously showed that, by altering specific amino acid residues thought to be involved in binding of ATP at the putative active site of the enzyme, we could abolish catalytic activity (11). These residues, together with the putative binding site for the substrate fructose 6-phosphate, are localized in the N-terminal half of each Pfk subunit. Further analysis showed critical residues for activator binding to be localized in the C-terminal halves of
the subunits (21). Here, we localized a mutation obtained in the PFK1-encoded a-subunit by classical genetic screening (22) as abolishing ATP inhibition also in the C-terminal half of the subunit (P728L). The effect of this mutation was confirmed by the results of site-directed in vitro mutagenesis of either PFK1 or PFK2 or both. Taken together, this strongly suggests that ATP can be bound at the catalytic site in the N-terminal half as well as at an allosteric site in the C-terminal half of each yeast Pfk subunit. Interestingly, Pro-728 is located only four amino acids downstream of Ser-724 in the deduced protein sequences of each subunit, which was shown to be involved in fructose 2,6-bisphosphate binding (21). These findings confirm a hypothesis by Poorman et al. (20), who proposed that eukaryotic Pfk subunits evolved by a gene duplication event and that domains formerly serving catalytic functions evolved in the C-terminal duplicate to allosteric binding sites. Thus, the substrates ATP and fructose 6-phosphate must be bound by the catalytic center for the Pfk reaction to proceed. For allosteric purposes, this site would have evolved to now bind the activator fructose 2,6-bisphosphate and the inhibitor ATP. However, it should be noted that a direct involvement of Pro-728 in ATP binding seems unlikely. Rather, the fixation of the three-dimensional structure of the neighboring domains by a bent induced by proline could lead to a conformation favoring effector binding. In this context, relaxation of secondary structure by substitution for a leucine could also result in localized conformational changes within the entire hetero-octamer, explaining that substitution in either subunit is sufficient to abolish ATP inhibition. Clearly, such speculations would profit from the revelation of the x-ray structure of a eukaryotic Pfk, which still is in its preliminary stages (40).
The increased resistance of the PfK produced by the mutants to heat inactivation could be explained by their altered allosteric properties. It has been observed that the presence of ATP protects PfK in vitro from proteolytic inactivation (41). This has been proposed to be caused by conformational changes upon ATP binding. If the latter takes place at the active site, one can assume that, due to the lack of ATP inhibition in our mutants, this binding is favored. Therefore, the mutant enzymes would be in a conformational state rendering them more resistant, as has already been observed for the original PFK1atp PK2 mutant (22).

The kinetic behavior of the yeast mutants described fits well with ATP-inhibition as a basic allosteric mechanism. Thus, the P728L substitution not only abolishes to a large extent the inhibitory effect of ATP (about 70% of maximal activity even at unphysiologically high ATP concentrations of 3 mM) but also renders the enzyme insensitive to activation by AMP or fructose 2,6-bisphosphate. This observation is in agreement with the work reported in Ref. 7, showing that these effectors act by reversing ATP inhibition. Physiological ATP concentrations (which are around 1 mM; Ref. 42) might well be inhibitory in the absence of positive effectors, as confirmed by our determinations of wild-type PfK kinetics (see Fig. 4). In order to get an indication of in vivo PfK activity in our mutants, we determined the concentrations of intermediary metabolites 2 h after a shift from non-fermentable carbon sources to glucose-containing media. These revealed no major differences between different combinations of mutant and wild-type subunits. It is also likely that the PfK produced in the mutants is still a heterotetramer, since a loss of the quaternary structure in the yeast enzyme usually results in aggregation and degradation of the subunits, leading to a drastic decrease in Western blot signals (see Ref. 43, and references therein), which is not observed in the PFK1atp mutants. Given these data, it is not surprising that the mutant strains did not show any obvious defect for growth on glucose media or in respect to glycolytic flux.

Nonetheless, abolishing allosteric inhibition led to a sustained slow growth of the mutants on gluconeogenic carbon sources instead of just difficulties in adaptation previously reported for other mutants (21). The sectored colony morphology could thus be caused by the ethanol produced during growth, which may be inhibitory for cells within the colony. Alternatively, ammonium signaling observed between neighboring colonies (35) could be defective within the colonies due to the insensitivity of the mutant PfK to ammonium activation. It would be interesting to see if our mutants display the typical morphological changes observed by scanning electron microscopy reported for starved colonies, i.e. the formation of connecting fibrils (44).

With respect to drugs impairing cellular integrity, the PFK1atp PFK3atp double mutant proved to be hypersensitive to Congo Red. Although no drastic differences in fermentative growth and in glycolytic flux were observed for this mutant, this sensitivity indicates a significant metabolic alteration. One possible explanation would be that a slightly reduced flux through the PfK reaction may result in a change in cell wall biosynthesis and/or cell wall composition, as the substrate fructose 6-phosphate is also providing the substrate for mannose synthesis as a constituent of cell wall mannoproteins.

In the course of this work, we also found some new phenotypes associated with single pfk deletions. The pfk1 null mutant (and this mutant transformed with the PFK1atp allele) proved to be more resistant to caffeine than the wild type and the pfk2 null mutant. In contrast, the latter showed a temperature-sensitive phenotype not observed for all other mutant combinations. This further substantiates the view that strains harboring only one of the PfK subunits ferment glucose through the usual glycolytic pathway despite of the lack of in vitro detectable PfK activity (9, 21). The homomeric enzyme formed from only the PfK1p subunits would be more sensitive to heat inactivation in vivo and the homomeric enzyme formed from only PfK2p subunits would be less susceptible to allosteric regulation in vivo, i.e. to activation by fructose 2,6-bisphosphate whose level will rise if caffeine inhibits phosphodiesterase (45), leading to an increased level of intracellular cAMP resulting in an increased activation of phosphofructo-2-kinase (46).

In summary, our results indicate that the heterooctameric structure and the allosteric properties of yeast PfK are important for the response of the cells to changing substrate environments rather than for growth and glycolytic flux under optimal fermentation conditions.

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