A Yeast Phosphofructokinase Insensitive to the Allosteric Activator Fructose 2,6-Bisphosphate

GLYCOLYSIS/METABOLIC REGULATION/ALLOSTERIC CONTROL*

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In this work we used in vitro mutagenesis to modify the allosteric properties of the heterooctameric yeast phosphofructokinase. Specifically, we identified two amino acids involved in the binding of the most potent allosteric activator fructose 2,6-bisphosphate. Thus, Ser³⁷⁴ was replaced by an aspartate and His⁸⁵⁹ by a serine in each of the enzyme subunits. Whereas the substitutions had no drastic effects when introduced only in one of the two types of subunits, kinetic parameters were modified when both subunits carried the mutation. Thus, the enzyme with His³⁸⁹ → Ser showed an increase in Kᵣ for binding of the activator, whereas the one with Ser³⁷⁴ → Asp failed to react to the addition of fructose 2,6-bisphosphate, at all. The enzymes still responded to other allosteric activators, such as AMP. Stabilities of the mutant subunits were not significantly altered in vivo, as judged from Western blot analysis. Phenotypically, strains expressing the mutant PFK genes showed a pronounced effect on the level of intermediary metabolites after growth on glucose. Mutants not responding to the activator at all (Ser³⁷⁴ → Asp) also displayed higher generation times on glucose medium. This could be suppressed by increasing the gene dosage of the mutant alleles. These results indicate that fructose 2,6-bisphosphate through its activation of phosphofructokinase plays an important role in regulation of the glycolytic flux.

The conversion of fructose 6-phosphate and ATP to fructose 1,6-bisphosphate and ADP catalyzed by phosphofructokinase (PFK; EC 2.7.1.11) is commonly viewed as a major checkpoint in the control of glycolysis (1, 2). Accordingly, defects in the muscle isoenzyme of human PFK cause a glycogen storage defect known as glycogen storage disease VII (Tarui’s disease) and several mutant alleles have been identified (see Refs. 3 and references therein). PFK activity in most organisms is subject to allosteric control mechanisms (reviewed in Ref. 4), with inhibition by ATP and activation by AMP being the most common ones for eukaryotic PFK. In addition, fructose 2,6-bisphosphate, now thought to be the most potent physiological activator of the majority of eukaryotic PFK enzymes, was not even included in the review cited above, as it was discovered fairly recently (5). All three effectors have been shown to act on the yeast enzyme as well (6, 7). As fructose 2,6-bisphosphate is thought to be crucial not only for the regulation of glycolysis but also of gluconeogenesis, its production has been thoroughly studied in yeast. Two isoenzymes have been identified that produce the activator from fructose 6-phosphate, and their encoding genes have been cloned and sequenced (8, 9). Double deletion mutants in these genes show no measurable levels of the activator (9) (see “Discussion” for further details).

Yeast PFK is a heterooctameric enzyme composed of α- and β-subunits, encoded by the genes PFK1 and PFK2 (10, 11). The latter have been cloned, sequenced, and deleted from the haploid yeast genome (12, 13). Whereas in most organisms homotetrameric enzymes are usually active, mutations in one of the yeast genes lead to a loss of in vitro detectable PFK activity (14–16). Yet, cells retain their ability to grow on and ferment glucose (17). On the other hand, double mutants carrying lesions in both PFK genes fail to ferment glucose and do not grow in its presence. In addition, mutants lacking either one of the PFK subunits accumulate the activator fructose 2,6-bisphosphate when shifted to glucose-containing medium (18, 19).

Yeast PFK shows striking similarities to both prokaryotic and higher eukaryotic PFKs, including the three human isoenzymes. Comparison of the primary sequence of Bacillus stearothermophilus PFK with the one from rabbit muscle led to the notion that the latter evolved through a gene duplication event from a prokaryotic ancestor (20). This was also postulated for each of the yeast PFK subunits, where apparently a second gene duplication followed, locating the two genes on different chromosomes (13).

For PFK from Escherichia coli and B. stearothermophilus, x-ray studies have been performed. There, specific amino acid residues involved in catalytic and regulatory (i.e. allosteric) functions could be defined (21, 22). It has been speculated that the N-terminal half of rabbit muscle PFK retained its catalytic function, whereas in the C-terminal half a former fructose 6-phosphate binding site has evolved to a fructose 2,6-bisphosphate binding domain with allosteric function (20). As highly homologous regions have been conserved from bacterial through yeast and human PFK (23) it seems feasible to deduce functions of specific amino acids in the eukaryotic enzymes. Straightforward genetic approaches make yeast the ideal organism to study the effect of point mutations at such sites both in vitro and in vivo, i.e. the physiological consequences of expression of an altered PFK in an organism specialized in glycolysis (see Heinisch and Hollenberg (24) for a review).
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In fact, we recently showed that amino acid residues implicated in catalysis in bacterial Pfk are functionally conserved in the yeast Pfk subunits (25). In addition, the E. coli PfkA isoenzyme and the human muscle isoform of Pfk can be expressed in yeast and complement its pkf defects, indicating that there are no specific metabolic requirements for a heteroctameric enzyme in Saccharomyces cerevisiae (26).

Here, we report on the results of mutants constructed by in vitro mutagenesis at two specific sites, altering the sensitivity of yeast Pfk to activation by fructose 2,6-bisphosphate. This provides the first direct evidence confirming the hypothesis that the binding domain for this activator evolved from a substrate binding site after an internal gene duplication.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions—Strain HD56-5A (MATa ura3-52 leu2-3, 112 his3-11, 15 MAL SUC GAL) and its isogenic pkf double deletion derivative HD114-8D (MATa pkf1::HIS3 pkf2::HIS3 ura3-52 leu2-3, 112 his3-11, 15 MAL SUC GAL) were used as recipients for transformations with plasmids carrying in vitro mutated pkf alleles for enzyme purification and determination of kinetic parameters. The exact construction of deletions in the PFK genes has been described in previous works. Briefly, an internal BglII fragment of PFK1 (13) and the same site in PFK2 (12) (between flanking BglII sites in BamHI sites by insertion of linker) (25) were each replaced by a 1.8-kilobase pair BamHI fragment carrying the HIS3 gene. The same deletion constructs were employed to construct an isogenic series of the strain VW1A (MATa ura3-52 leu2-3, 112 his3-11, 112 MAL-2 MAL-2 SUC2 GAL) which was employed in all other experiments described. Double deletion derivatives with each of the PFK genes substituted by the HIS3 marker were obtained by switching the mating type of one haploid deletion mutant after introduction of the HO gene, curing of the plasmid carrying the latter and crossing it to the strain carrying the other pkf deletion. Haploid segregants were then tested for their ability to grow on glucose-containing medium.

For amplification of plasmid DNA, the E. coli strain DH5αF’ (Life Technologies, Inc.) was used throughout. Standard media and growth conditions were employed as described in detail elsewhere (25).

Genetic Manipulations, Plasmids, and in Vitro Mutagenesis—Standard molecular genetic procedures were followed in the preparation and handling of DNA (27, 28). Transformation of yeast was carried out by a freeze method according to Dohmen et al. (29). For in vitro mutagenesis, the spheroplast transformation system (28) was employed according to the instructions of the manufacturer. Due to the high sequence conservation in the regions of interest, the same oligonucleotides were used for mutagenesis in both yeast Pfk genes. The oligonucleotides were numbered according to the amino acid to be altered, referring to the PFK1 encoded-a subunit: p724 (equivalent to position 724 in the α-subunit encoded by PFK2; introducing a new BglII recognition site): 5’-TTGATTCGACAGCCTCTTATAAYAAYGTTCCAGG-3’ and p859 (equivalent to position 859 in the β-subunit encoded by PFK2; introducing a new BamH1 recognition site): 5’-CGGATCCGACCGATCCGTGCAGGTG-3’. In the following, mutant alleles were numbered, accordingly, i.e. pkf-1-724 and pkf-2-724 or pkf-1-859 and pkf-2-859.

PFK1 was introduced into the vector for mutagenesis as a Sphl fragment containing the complete coding sequence and 5’ and 3’ flanking sequences necessary for expression. For PFK2 we first used a construction carrying the entire coding sequence and 150 base pairs of the 5’-noncoding sequence on a BamHI fragment. As with this construct different K2 deletion could either be introduced or that of an altered transcriptional regulation, PFK2 with all its relevant promoter sequences was also amplified from pPFK2-2 (12) by polymerase chain reaction (40 s/92°C; 2 min/60°C; 7 min/74°C) using the oligonucleotides P2END (5’-AGATCTTGAGATCCTCAGAGG-3’), in bold indicate PKF2 sequence from 3217 to 3242 relative to translation start codon and P23PS (5’-ATGAGATCCTGAGATCCTCAGAGG-3’), in bold indicate PKF2 sequence from 896 to 878 relative to the ATG translation start codon) and subcloned as a PstI fragment (recognition sequences are underlined). To avoid possible errors introduced by the Taq polymerase, all but the sequences upstream of the SpeI site (at 36 relative to the ATG translation start codon) in the promoter were then replaced by PFK2 sequences not resulting from polymerase chain reactions. For expression in yeast, the respective SpeI and PstI fragments were subcloned either into the CEN/ARS vector YCplac33 (30) or into the 2-µm based vector YEp352 (31), both carrying UR3 as a selectable marker. Plasmids carrying both PFK1 and PFK2 were derived from those carrying the respective PFK2 alleles by insertion of the PFK1 Sphl fragment into the unique Sphl site. For integration into the yeast genome we constructed a new integrative vector, pJH273, by inserting the 3-kilobase pair BglII fragment of YEp13 (32) carrying the LEU2 marker, into a PFK2 plasmid. The latter is a derivative of pUC19 (33), where a BglII linker was inserted into the unique NdeI site. pJH273 was used as a basis to integrate constructs carrying both PFK genes, or their mutant derivatives, at the LEU2 locus into the yeast genome, taking advantage of a unique NheI site in the plasmids, by the one-step procedure described by Rothstein (34).

PFK was purified for determination of kinetic parameters using a modification of the method of Kopperschläger and Johansson (36), based on precipitation with polyethylene glycol and affinity purification on Affi-Gel blue columns, as described elsewhere (25). Activation constants for fructose 2,6-bisphosphate were determined in 50 mM MES buffer at pH 6.4, containing 5 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, 0.5 units of triosephosphate isomerase, and 5 units of glyceraldehyde-3-phosphate dehydrogenase without other effectors. The ancillary enzymes were purchased from Boehringer Mannheim in ammonium sulfate dispersion and pelleted and dissolved in assay buffer prior to addition to the test mx in order to minimize allosteric activation by ammonium. For enzymatic determinations in crude extracts, 50 mM MOPS buffer at pH 7.0, containing 20 mM magnesium chloride and 2 mM dithiothreitil, was used with the same ancillary enzymes as described above, but with substrate concentrations of 2.5 mM fructose 6-phosphate and 1 mM ATP, unless indicated otherwise. 10 µM fructose 2,6-bisphosphate was added where indicated.

Immunological Detection—A polyclonal antiserum prepared against purified yeast Pfk was used for immunological detection of Pfk subunits in Western blots as described elsewhere (25), using the chemoluminescent detection kit of Tropix (Promega, Heidelberg) according to the instructions of the manufacturer. Cells were harvested in the late exponential growth phase for these experiments.

Sequence Analysis—All mutants obtained by in vitro mutagenesis were sequenced in the region of interest by the dideoxy chain termination method (37) using the T7 sequencing kit from Pharmacia and custom made oligonucleotides (MWG, Munich, FRG).

RESULTS

Exchange of Amino Acid Residues Involved in Activator Binding and Their Effect on Specific Pfk Activities—Due to a hypothesis on the evolution of eukaryotic Pfk enzymes based on sequence comparisons and deductions from x-ray studies on prokaryotic Pfk, a serine at position 724 and a histidine at position 859 (for numbering and designations of mutant alleles refer to “Materials and Methods”) could be important in binding of the allosteric activator fructose 2,6-bisphosphate by yeast Pfk (20, 25). To provide evidence for such a binding domain in an eukaryotic Pfk we altered the respective codons by in vitro mutagenesis in both yeast PFK genes (Fig. 1). Thus, Ser724 was substituted by an aspartate residue thought to prevent access of fructose 2,6-bisphosphate. The latter belongs to a domain similar to the one that binds the substrate fructose 6-phosphate at the catalytic site in the N-terminal half of both subunits. His859, presumably involved in coordinating the binding of the 6-phosphate group (22), was substituted by a serine residue. The mutant alleles were introduced into yeast strains being isogenic except for specific pkf deletions (see “Materials and Methods”). When transformed on single copy CEN/ARS vectors into derivatives of strain HD56-5A carrying a single deletion in the respective gene (e.g. pkf1-724 into a strain carrying a deletion in pkf1) significant effects were not observed on specific Pfk activity nor on the activation capacity of
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Mutations introduced into both PFK genes to alter the enzymes response to the allosteric activator fructose 2,6-bisphosphate. Schematic representation of the homology of the yeast PFK subunits at the amino acid level among each other and to those from human and bacterial origin (for the latter two, similarity values would be about 10% lower than those given for the comparison of the two yeast Pfk subunits). Arrows above indicate the presumed gene duplication event in the eukaryotic enzymes and the postulated function of the enzyme halves. Numbering of the amino acid exchanges introduced is according to the sequence of the α-subunit of the yeast enzyme encoded by PFK1. For the PFK2-encoded β-subunits Ser274 and His859 correspond to amino acids 718 and 847, respectively. Flanking amino acids to those exchanged are depicted in the box below, with stars indicating complete conservation and double points conserved exchanges. Corresponding amino acid residues at the catalytic site are also indicated. ScPFK5-encoded amino acid sequences of the yeast α- and β-subunits, respectively. HmpPFK = deduced amino acid sequence of the human muscle Pfk isomorph. BspPFK = amino acid sequence of the Pfk from B. stearothermophilus, for which x-ray analysis has been provided.

Fructose 2,6-bisphosphate as compared to a wild-type control (Table I). In addition, all but one construct were able to confer a glucose-positive phenotype to a pkf1 pkf2 double deletion mutant both on synthetic and on rich media. Only the CEN/ARS-vector carrying the pkf2-724 allele did not confer glucose growth. However, when the allele was carried on the multicopy vector YEp352, growth on glucose could be restored.

Unlike the introduction of single mutant pfk alleles in conjunction with the respective wild-type counterparts described above, enzymatic parameters for Pfk expressed from CEN/ARS plasmids carrying similar mutations in both PFK1 and PFK2 were different from wild type. Thus, the mutation Ser724 → Asp carried on both subunits led to a 2-fold reduction in specific Pfk activity in crude extracts when introduced on single copy vectors (Table I). It should be noted that in these assays Pfk activity was measured in the presence of 2,5 mM fructose 6-phosphate, ensuring a relatively high basal activity even in the absence of the activator. In these tests, wild-type activity could be exceeded, when the same alleles were carried on a multicopy vector (Table I). Only a moderate activation in crude extracts was observed for transformants with single copy vectors carrying the His859 → Ser mutation in both pfk genes as compared to the wild-type constructs (Table I).

In the following assays, low basal Pfk activity in the absence of effectors was ensured by lowering substrate concentration to 0.5 mM fructose 6-phosphate. To avoid possible plasmid effects, single-copy integrants of the PFK genes were employed (Table II). Again, the enzyme carrying the Ser724 → Asp substitution in both subunits was not activated, but even slightly inhibited, when fructose 2,6-bisphosphate was added. The His859 → Ser mutation could be activated depending on the concentration. In addition, we tested the effect of other allosteric activators on the mutant enzymes. Thus, the cumulative effect of addition of AMP and subsequently a variety of other activators could be observed (Table II). AMP was capable of activation in all cases and in the presence of further allosteric activators about wild-type activity could be obtained for the mutant enzymes. We take that as an indication that the overall quaternary structure of the enzyme was not significantly altered.

The stability of the Pfk subunits was also assessed by immunological detection to provide evidence that the hetero-octameric structure of the enzyme is not altered by the introduced amino acid exchanges. Previously, our results indicated that a failure to form a heterooctamer results in an increased instability of the Pfk subunits (25). Again, the single copy integrants carrying the respective mutations were employed. Western blots confirmed that subunits of expected sizes and similar stabilities are produced in the mutants as compared to the transformants carrying wild-type constructs (Fig. 2).

Effect of Fructose 2,6-Bisphosphate on Enzyme Kinetics—In order to study allosteric activation by fructose 2,6-bisphosphate, partially purified Pfk preparations from transformants carrying the PFK genes on CEN/ARS vectors were employed. When only one of the subunits was altered, K₅₀ values of 0.5–1.7 μM were determined for the Ser724 → Asp mutations and of 0.4–1.0 μM for the His859 → Ser mutations, not differing significantly from 0.5 to 1.3 μM measured in the wild-type controls. Thus, the allosteric behavior of Pfk from strains retaining one wild-type PFK gene did not significantly change. However, differences were observed for transformants of double deletion mutants with plasmids carrying similar mutant alleles for both PFK genes. Thus, the enzyme with the His859 → Ser substitution in both subunits showed an about 5-fold increase in K₅₀ for the effector (about 4 μM as compared to 0.4–1 μM for the wild-type control; Fig. 3). For transformants carrying the Ser724 → Asp mutation, no activation was observed under the same conditions when tested up to 50 μM fructose 2,6-bisphosphate. In fact, a weak inhibitory effect was observed at the higher concentrations tested.

Physiological Effects of Altered Allosteric Regulation—Three lines of experiments were followed to determine the in vivo effects of the mutations introduced. First, cells were shifted between glucose and nonfermentable carbon sources, such as glycerol and ethanol, and their growth behavior was followed. At first, transformants carrying the Ser724 → Asp mutations on a CEN/ARS vector grew more slowly than the wild-type controls and the transformants carrying His859 → Ser mutations on glucose medium. This was not observed for the Ser724 → Asp mutations carried on a multicopy vector. After storage at 4 °C and a series of fresh incubations, even the transformants with the single copy vectors displayed a normal growth behavior. We attributed this change to a possible elevation of plasmid copy number per cell and a concomitant increase in Pfk activity. To avoid these problems, vectors carrying both mutant pfk genes were integrated at the LEU2 locus in strains carrying deletions in both PFK1 and PFK2 at their original chromosomal locations. Single integrations were confirmed by Southern analysis for all constructs tested (data not shown). Growth kinetics of such strains are depicted in Fig. 4A. Indeed, strains carrying the Ser724 → Asp mutations in both PFK subunits were thus shown to grow significantly more slowly on either rich or synthetic media containing glucose.

To further evaluate the effects on metabolism, we also determined the rates of glucose consumption and ethanol production in the strains growing on rich media containing 1% glucose (Fig. 4, B and C, respectively). From these data we calculated rates of glucose consumption of 1.4, 0.8, and 1.3 (g of glucose/g of dry weight h⁻¹) for the wild type, the Ser724 → Asp and the His859 → Ser mutant, respectively. Accordingly, ethanol production rates were calculated to be 0.6, 0.3, and 0.6 (g of ethanol/g of dry weight h⁻¹), respectively. Thus, expression of a Pfk not activated by fructose 2,6-bisphosphate leads to a significant decrease both in glucose consumption and ethanol production.
Materials and Methods). For the Ser724 80–84% of theoretical maximum yield were obtained, 71–75% ATP for determinations of the effects of fructose 2,6-bisphosphate (added at a 10 mM final concentration), unless stated otherwise (see below).

In addition to the ancillary enzymes, the test mix contained the following effectors: ATP (1 mM), AMP (1 mM), fructose 6-phosphate (2.5 mM), and ADP (1 mM). The number in parentheses represents the activity after addition of fructose 2,6-bisphosphate at a 50 mM concentration.

Strain HD56-5A and its isogenic pfk deletion derivatives (see "Materials and Methods") were used in these experiments. Only the mutant pfk alleles introduced on plasmids are indicated, with the altered subunit produced designated in parentheses. For plasmid-borne PFK2 alleles constructs carrying the promoter up to −150 base pairs relative to the ATG translation start codon were employed (see "Materials and Methods"). PFK genes at their chromosomal locus not indicated in the recipient strain are always wild type.

Specific Pfk activities were determined in MOPS buffer at pH 7.0 as described under "Materials and Methods" at 2.5 mM fructose 6-phosphate concentration, unless stated otherwise (see below).

Cells were grown on synthetic medium containing 1% glucose. Enzymatic activities were determined at 0.5 mM fructose 6-phosphate and 3 mM fructose 2,6-bisphosphate (F-2,6-P2) was added at a final concentration of 10 μM.

**TABLE I**

| Relevant genotype of recipient strain | Mutant Pfk allele | Plasmid type | Specific activity† | Activation factor |
|--------------------------------------|-------------------|-------------|-------------------|------------------|
|                                      | PFK1              | CEN/ARS     | 153 ± 20          | 330 ± 50         | 2.2 |
| pfk1::HIS3                           | pfk1-724 (ΔSer724→Asp) | CEN/ARS     | 119 ± 20          | 321 ± 60         | 2.7 |
| pfk1::HIS3                           | pfk1-859 (ΔHis859→Ser) | CEN/ARS     | 201 ± 40          | 400 ± 80         | 2.0 |
| pfk2::HIS3                           | pfk2-724 (ΔSer724→Asp) | CEN/ARS     | 154 ± 50          | 241 ± 50         | 1.6 |
| pfk1::HIS3                           | pfk2-859 (ΔHis859→Ser) | CEN/ARS     | 121 ± 17          | 251 ± 50         | 2.1 |
| pfk1::HIS3 pfk2::HIS3                | PFK1 PFK2         | CEN/ARS     | 107 ± 10          | 271 ± 30         | 2.5 |
| pfk1::HIS3 pfk2::HIS3                | pfk1-724 pfk2-724 | CEN/ARS     | 57 ± 10           | 55 ± 10          | 1.0 |
| pfk1::HIS3 pfk2::HIS3                | pfk1-859 pfk2-859 | CEN/ARS     | 133 ± 14          | 186 ± 15         | 1.4 |
| pfk1::HIS3 pfk2::HIS3                | PFK1 PFK2         | 2 μm        | 1391 ± 85         | 2526 ± 175       | 1.8 |
| pfk1::HIS3 pfk2::HIS3                | pfk1-724 pfk2-724 | 2 μm        | 1604 ± 54         | 1350 ± 145       | 0.8 |
| pfk1::HIS3 pfk2::HIS3                | pfk1-859 pfk2-859 | 2 μm        | 2200 ± 70         | 2400 ± 34        | 1.1 |

- Strain HD56-5A and its isogenic pfk deletion derivatives (see "Materials and Methods") were used in these experiments.
- Only the mutant pfk alleles introduced on plasmids are indicated, with the altered subunit produced designated in parentheses. For plasmid-borne PFK2 alleles constructs carrying the promoter up to −150 base pairs relative to the ATG translation start codon were employed (see "Materials and Methods"). PFK genes at their chromosomal locus not indicated in the recipient strain are always wild type.

**DISCUSSION**

Phosphofructokinase is thought to be a major controlling step in yeast glycolysis (38). Yet, Pfk activity did not vary between cells grown on different carbon sources (see Fraenkel (39) for a review), and the encoding genes are not subject to transcriptional regulation (40). These findings supported the idea that control is exclusively exerted at the allosteric level. In an attempt to test this hypothesis we are beginning to employ specific mutations in the two yeast PFK genes encoding the subunits of the hetrooelameric enzyme. The results obtained with two mutants described here, Ser724→Asp and His859→Ser, indicate that both amino acid residues are involved in fructose 2,6-bisphosphate binding. Our data are also com-

![Fig. 2. Immunological detection of Pfk subunits in transformants.](image)

Fig. 2. Immunological detection of Pfk subunits in transformants. Strains carrying the PFK alleles indicated in single copy integrated at the LEU2 locus were grown either to late logarithmic phase or to mid logarithmic phase in rich medium containing 2% glucose (YEPD) or to mid logarithmic phase in synthetic medium with 1% glucose (SM), prior to preparation of crude extract. 30 μg of total protein were loaded in each lane, and the Western blot was performed as described under "Materials and Methods." After incubation with CSPD, filters were exposed to Kodak XR films for 5 min.

production. Nevertheless, the ethanol yields calculated from these data differ only slightly from wild type (for wild type, 80–84% of theoretical maximum yield were obtained, 71–75% for the Ser724→Asp mutant and 75–82% for the His859→Ser mutant).

As another measure of the effect of the mutations in vivo, we determined the concentrations of metabolites related to the Pfk reaction 2 and 6 h after addition of glucose to cultures pregrown on nonfermentable carbon sources (Table I). Concentrations of the substrate fructose 6-phosphate (and glucose 6-phosphate which is in equilibrium with it through the phosphoglucoisomerase reaction) were elevated in both mutants as compared to the wild type. Generally, higher concentrations were observed for the Ser724→Asp mutations than for the His859→Ser mutations. Accordingly, the product fructose 1,6-bisphosphate accumulated to about half the wild-type level in the mutant not responding to the activator (Ser724→Asp), whereas the mutant with the elevated Kₐ for fructose 2,6-bisphosphate (His859→Ser) displayed only slight variations as compared to wild type. ATP levels did not significantly change in any of the strains tested. Interestingly, levels of the activator fructose 2,6-bisphosphate itself increased about 10-fold in the strain carrying the His859→Ser mutation and more than 20-fold in the Ser724→Asp mutant as compared to the wild type.
ble with the idea that His859 is coordinating the 6-phosphate group in binding, as substitution of this amino acid leads to a decreased binding capacity, reflected by the elevated $K_a$. On the other hand, substitution of Ser724 by the charged aspartate completely abolished activation by fructose 2,6-bisphosphate, indicating that the 2-phosphate group may be localized near this residue, confirming its hypothetical role deduced from sequence comparisons (20). Previously, we have shown that mutations abolishing catalytic functions in only one of the genes reduced specific Pfk activity measured in crude extracts by about half (25). This was interpreted as both types of subunits contributing equally to catalysis. Thus, the discrimination into one type of subunit having preferentially a catalytic function as proposed earlier (15, 41) is highly unlikely. The data presented here also argue against one of the subunits functioning preferentially in allosteric regulation. If this was the case, mutations in either $PFK1$ or $PFK2$ should by themselves lead to a drastically altered susceptibility to fructose 2,6-bisphosphate activation.

In addition to these questions concerning enzyme structure and kinetic parameters, we were interested in the physiological implications of altering the allosteric properties of yeast Pfk. Metabolite determinations indicated that glucose metabolism was significantly altered with substrate concentrations of the Pfk reaction increasing in the mutants. Thus, steady state metabolite concentrations are indeed dependent on allosteric regulatory mechanisms. Concomitantly, the enzyme composed of subunits with the Ser724 → Asp substitution shows an increase in generation time for growth on glucose and a decrease in glucose consumption and ethanol production rates. The ethanol yield is only slightly lower in these mutants, indicating that they still follow a primarily fermentative metabolism. As normal growth is restored when multiple copies of the mutations are introduced, the phenotypes observed can be attributed to a reduced in vivo Pfk activity caused by the alteration in the allosteric behavior of the enzyme. We interpret these results as the concentration of the Pfk enzyme itself and that of its various activators being held at a crucial steady state in yeast cells growing on glucose. This ensures a minimum level of in vivo Pfk activity that may well be exceeded but cannot be lowered without serious effects on metabolism. Any disturbances of this steady state result in metabolic responses aimed at counteracting such deleterious effects. One such measure...
could be cooperative binding of fructose 6-phosphate to yeast Pfk leading to an increased glycolytic flux, as judged from the in vitro kinetic parameters of the enzyme. This notion would also be consistent with the high levels of fructose 2,6-bisphosphate at detectable levels (9). However, the enzyme either directly or indirectly control the production of fructose 2,6-bisphosphate itself. Fructose 6-phosphate is both a substrate for the enzymes producing the activator, but it also seems to inhibit at least two of the phosphatases implicated in its degradation (43, 44), adding another level of control. Along this line, moderate effects on metabolism have already been reported for strains not being able to produce fructose 2,6-bisphosphate at detectable levels (9). However, once growing on glucose, such strains displayed generation times similar to those of wild type. That the Ser724 Asp mutant described here shows a more drastic phenotype may be due to the inhibitory effect of fructose 2,6-bisphosphate on this enzyme at higher concentrations. Thus, in vivo Pfk activity not only lacks activation in the mutant, but could be further decreased by the accumulation of fructose 2,6-bisphosphate.

The results obtained here are supported by experiments on the heterologous expression of the Pfk from Dictyostelium discoideum in yeast (45). The latter enzyme is known to be subject to any kind of allosteric regulation (46), and yeast transformants carrying the gene on multicopy plasmids grow like wild-type cells on media containing either glucose or gluconeogenic carbon sources (45). Finally, our data underline the importance of allosteric regulation of Pfk for yeast metabolism, a notion established previously in in vitro experiments trying to mimic the in vivo situation as closely as possible (47). The genetic approach used here goes one step further in changing only one enzymatic parameter in vivo. Given these results, studies on the role of allosteric regulation of yeast Pfk by other effectors will be of obvious interest.

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