A single point mutation leads to an instability of the hetero-octameric structure of yeast phosphofructokinase

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Yeast phosphofructokinase is an oligomeric enzyme whose detectable activity *in vitro* depends on its hetero-octameric structure. Here we provide data demonstrating that an alanine residue at positions 874 (for the *PFK1*-encoded α -subunit) or 868 (for the *PFK2*-encoded β -subunit) is crucial to achieve this structure. Thus subunits carrying substitutions by either aspartate or lysine of this residue cause a lack of phosphofructokinase activity *in vitro* and signals of the subunits are poorly detectable in Western blots. Size-exclusion HPLC in conjunction with ELISA detection of the enzyme protein confirmed that no functional octamer is produced in such mutants. Our data suggest that the mutant subunits, not being assembled, tend to aggregate and subsequently become degraded. Substitution of the alanine by valine in either subunit leads to a reduction in specific activities, as expected from a conservative exchange. The

kinetic data of the latter mutant revealed a higher affinity to the substrate fructose 6-phosphate, a lower extent of ATP inhibition and a lower degree of activation by fructose 2,6-bisphosphate. In addition, the affinity of mutants carrying a valine instead of an alanine in either the α - or the β -subunit to fructose 2,6-bisphosphate was increased. As no X-ray data on eukaryotic phosphofructokinases are available yet, our data provide the first evidence that a non-charge amino acid at position 874 or 868 is essential for the formation of the functional oligomer. This conclusion is substantiated by comparison with the structure of the well-known prokaryotic enzyme.

Key words: allosteric regulation, glycolysis, mutagenesis *in vitro*, *PFK* genes, yeast metabolism.

INTRODUCTION

Phosphofructokinase (6-phosphofructo-1-kinase; EC 2.7.1.11) is regarded as one of the key enzymes regulating the flux through glycolysis in most organisms [1–3]. As the yeast *Saccharomyces cerevisiae* is extremely specialized in fermentation (reviewed in [4]), the biochemistry and genetics of its phosphofructokinase have been the subject of numerous investigations (reviewed in [5]). In contrast to the enzyme from most other organisms, which functions as homo- and heterotetramers, the yeast enzyme is a hetero-octamer composed of four α - and four β -subunits, which are arranged as a dimer of tetramers [6–8]. The subunits are each subject to limited proteolytic degradation *in vitro*, involving primarily the N-terminal region of about 200 amino acids [9]. Careful preparation of phosphofructokinase in the presence of protease inhibitors, however, yields exclusively unprocessed subunits [10].

The stability of the oligomeric structure has been studied by means of different methods [9,11,12]. In dilution, the native enzyme was found to be a stable octamer down to nanomolar protein concentrations in the presence of ammonium sulphate. However, by splitting off a C-terminal part from the α -subunit, the enzyme dissociates completely into two half-molecules of $\alpha_2\beta_2$ structure, which display full catalytic activity. This result gave rise to the assumptions that (i) the octameric structure is not essential for the catalytic function and (ii) the C-terminal region of the α -chain is the linking part between two tetrameric half-enzymes.

The genes *PFK1* and *PFK2*, encoding the α - and β -subunits of phosphofructokinase respectively [13], have been isolated by complementation of the glucose-negative phenotype of *pfk1/pfk2* double mutants, and their complete coding regions have been

sequenced [14,15]. Promoter studies revealed that *PFK1* and *PFK2* are constitutively transcribed at a relatively high level in *S. cerevisiae* [16]. From the deduced amino acid sequences of the open reading frames it was concluded that each of the genes evolved by duplication events from a prokaryotic-type ancestor. Therefore, one can assume that structural domains of the prokaryotic enzyme, which are known from X-ray analysis [17,18], have been preserved in both halves of the eukaryotic enzyme. This view has been substantiated by confirming the functional conservation of crucial amino acid residues in the yeast enzyme [19,20]. Unfortunately, no crystal structure of a eukaryotic phosphofructokinase is known, yet. It should be emphasized that, in phosphofructokinase from *S. cerevisiae*, both types of subunit carry N-terminal extensions of about 180 amino acids, which are not present in phosphofructokinase subunits from other eukaryotic organisms, with the exception of those of the closely related yeast *Kluyveromyces lactis* [21,22]. No significant similarities could be detected between the first 180 amino acids of either subunit. A similar result was found for the last 80 amino acids [15,21].

In contrast to *pfk1/pfk2* double mutants that lack the ability to grow on media containing glucose, *pfk* single mutants defective in either of the two genes obtained in different screenings were reported to grow on and ferment glucose [23–26]. Nevertheless, they lack detectable activity as assessed by assays *in vitro*. These phenotypes were confirmed by the construction of disruption and deletion mutants [15,27]. Different lines of evidence favour the hypothesis of the remaining subunit exerting a catalytic function *in vivo* that escapes detection after disruption of the cells [13,19]. However, enzyme activity could be regained *in vitro* under conditions where both subunits from single-deletion mutants could assemble to a stable octameric structure [28].

Abbreviation used: PBST buffer, 50 mM sodium phosphate buffer (pH 7.5) containing 155 mM NaCl and 0.05% Tween 20.

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In this study, the originally isolated *pfk* single mutants leading to a complete loss of phosphofructokinase activity in crude extracts [24,26] were investigated in further detail. They led to the discovery of a single amino acid exchange in the *PFK2*-encoded β -subunit, affecting the oligomeric structure and catalytic function. This mutation was further investigated by mutagenesis *in vitro* of both *PFK1* and *PFK2*, and expression of the mutant enzymes in yeast. Here we present an analysis of the catalytic and structural properties of these mutants.

EXPERIMENTAL

Yeast strains

MCH-2B (identical to HD56-5A: *MAT α ura3-52 leu2-3,112 his3-11,15 MAL SUC GAL*) and its *pfk* deletion derivatives, MCH-4B (*pfk1::HIS3*) and MCH-6B (*pfk2::HIS3*), were used for the biochemical analyses. The single *pfk* mutants carrying deletions of the coding regions, as described in [19], were used as recipient strains for transformation with CEN/ARS vectors (single-copy vectors with a centromeric chromosomal region as well as an autonomously replicating sequence, ensuring replication) carrying either the respective wild-type *PFK* alleles, or the mutant alleles obtained by mutagenesis *in vitro* (see below). The strain ABYSD CIII-51 (*MAT α pral prb1 prc1 prs1 prd1 ura3 leu2 his3 lys2 ade*) carrying multiple defects in the proteolytic apparatus, was kindly provided by D. Wolf (University of Stuttgart, Stuttgart, Germany). Deletions in each of the *PFK* genes were introduced by substitution for a *HIS3* marker and the resulting null mutants (ABYSD/*pfk1::HIS3* and ABYSD/*pfk2::HIS3*) were also used as recipients for the vectors described above. HD152-1C (*MAT α pfk1::HIS3 pfk2::HIS3 ura3-52 leu2-3,112 his3 Δ 1 trp1-289 MAL2-8⁺ SUC2 GAL*) is a derivative of VW1A [20]. This strain was used as a recipient to judge the ability of the *pfk* mutant alleles to restore growth on glucose when introduced on single copy vectors.

For work with *Escherichia coli*, strain DH5 α F' (Gibco-BRL) was used throughout.

Reagents and enzymes

Biochemicals and auxiliary enzymes for the phosphofructokinase assay were provided by Boehringer Mannheim (Mannheim, Germany). All other analytical reagent-grade chemicals used were purchased from Merck (Darmstadt, Germany) or Serva (Heidelberg, Germany). Bacto-peptone and yeast extract were obtained from Difco (Detroit, MI, U.S.A.) or Oxoid (Basingstoke, Hants, U.K.).

Media and culture conditions

Minimal medium containing 0.67% yeast nitrogen base and 2% glucose supplemented with amino acids and bases, as described in [29], but omitting uracil, was used. For growth on plates, 2% agar was added. Rich medium was based on 1% yeast extract/2% peptone, supplemented with either 2% glucose or 1% glycerol plus 2% ethanol.

Precultures for preparation of crude extracts for enzymic determinations were prepared in 20-ml tubes turned around on a rotary shaker overnight at 30 °C and then transferred into 500-ml Erlenmeyer flasks containing 200 ml of medium. All strains were grown at 30 °C with shaking at 250 revs./min to an attenuation at 580 nm (D_{580}) of between 4 and 7. Then the cells were centrifuged, washed twice with distilled water and resuspended in the respective buffer.

Genetic manipulations, plasmids and mutagenesis *in vitro*

Standard molecular-genetic procedures were followed in the preparation and handling of DNA [30,31]. *E. coli* was transformed by the method of Hanahan [32]. Transformation of yeast was carried out by a freeze method [33]. The *PFK* genes of strains carrying mutant alleles were sequenced by a combination of PCR and subcloning techniques. For this purpose, custom-made oligonucleotides were employed to amplify the complete *PFK* coding sequences and the products were subcloned into sequencing vectors pUC19 and pUK21 [34,35] using suitable restriction sites. If a mismatch to the wild-type sequence was detected, an independent PCR reaction was performed and the mutation was confirmed by sequencing the subcloned product.

For mutagenesis *in vitro* to introduce the aspartate and valine substitutions, the pSelect (= pAlter-1) system of Promega (Heidelberg, Germany) was employed according to the instructions of the manufacturer, with constructs containing the *PFK* genes as described in [20]. For mutagenesis of *PFK1* we used the oligonucleotide pP1-874 [5'-CTAAGACCGTGTgA-CC(G/C)(T/A)TTCCAGATTGCTG-3'], and for *PFK2* the oligonucleotide pP2-868 [5'-GATAGAACAAAGA(G/C)(T/A)TACTAGAATGGCtATTAAAGCTGTC-3'] was used. Underlined nucleotides designate the changes in the wild-type sequences leading to a substitution of the original alanine by either aspartate or valine; lower-case letters indicate a change in the wild-type sequence leading to no difference in the encoded amino acid residue, but to the creation of new *AspI* (*PFK1*) or *MscI* (*PFK2*) sites, respectively. For expression in yeast, the genes were subcloned as *SphI* and *PstI* fragments respectively, into the CEN/ARS vector YCplac33 [36] carrying *URA3* as a selectable marker.

For substitution of the alanine residues by lysine, the oligonucleotides pP1-674K and pP2-868K were used, with the sequences as described above, but exchanging an AAG codon (Lys) for the original GCT (Ala). In these cases, the PCR-based mutagenesis method described by Boles and Miosga [37] was employed, using the universal (−40) and reverse-sequencing primers as flanking oligonucleotides as required. For *PFK1*, a fragment 3' to the unique *BstEII* site within the coding region was subcloned from the PCR products, and for *PFK2* a fragment 3' to the *BglII* site was employed. The complete PCR-generated regions of the plasmids used in yeast transformations were confirmed to contain no PCR errors leading to any further amino acid exchanges, as compared with the wild-type *PFK* genes by sequencing (performed kindly in part by Lutz Kirchthath from Amersham-Pharmacia, Freiburg, Germany).

Sequence analysis

All mutants obtained by mutagenesis *in vitro* were sequenced at the site of mutation by the dideoxy chain-termination method [38] using the T7 sequencing kit from Pharmacia and custom-made oligonucleotides (MWG, Munich, Germany).

Preparation of cell-free extract

Yeast suspension (1 ml, cytocrit 25%) in 50 mM sodium phosphate buffer (pH 7.0)/5 mM 2-mercaptoethanol/0.5 mM PMSF with the protease inhibitor cocktail Complete[®] (Boehringer Mannheim) was shaken vigorously with 1 g of glass beads (0.5-mm diameter) using the Mini-Beadbeater (Biospec Products, Bartlesville, OK, U.S.A.) at 5000 revs./min for a total of six times at 10 s each. To prevent sample warming the tube was cooled on ice between each step for 1 min. The suspension was

Figure 1 Schematic representation of yeast *PFK* genes and localization of mutations investigated

Restriction sites for orientation and those used for subcloning are indicated. Sites depicted in bold italics are located within the vector sequences. Open boxes show the genomic regions of *PFK1* and *PFK2* and the black arrows inside them the open reading frames. Mutant *pfk* alleles are designated in italics, also showing the codons and respective nucleotide exchanges. Resulting exchanges in the deduced amino acid sequences are boxed. The large box contains a homology alignment of the deduced amino acid sequences in the region of interest from phosphofructokinase subunits of selected organisms (ScPfk1 and ScPfk2, α - and β -subunits of phosphofructokinase from *S. cerevisiae*; KIPfk1 and KIPfk2, α - and β -subunits from *K. lactis*; hmPfk, human muscle phosphofructokinase subunit; EcPfk, phosphofructokinase-subunit encoded by *E. coli PfkA* gene). The vertical arrow below highlights the alanine residue that was exchanged. The asterisk indicates the adjacent arginine residue shown to be involved in the formation of a salt bridge (see Figure 5).

centrifuged at 17000 *g* at 4 °C for 20 min and the supernatant, called cell-free extract, was used as described. For determination of the total amount of phosphofructokinase in the yeast extract (soluble and precipitated) 1 % SDS was added to the extraction buffer. The protein concentration was determined according to [39] using BSA as a standard. The presence of SDS in the samples was corrected for by diluting the standards with the same SDS concentration as in the samples.

Enzyme assays

The catalytic activity of phosphofructokinase was measured spectrophotometrically at 25 °C using a coupled enzyme assay containing 100 mM imidazole/HCl (pH 7.2), 3 mM fructose 6-phosphate, 0.6 mM ATP, 1 mM AMP, 5 mM MgSO₄, 5 mM (NH₄)₂SO₄, 0.2 mM NADH, aldolase (1 unit/ml), glycerol-

phosphate dehydrogenase (1.5 units/ml) and triosephosphate isomerase (5 units/ml). The reaction was started by addition of cell-free extract appropriately diluted with 50 mM sodium phosphate buffer (pH 7.0).

For the kinetic analysis of the mutated enzymes, probes were obtained by fractional precipitation of the cell-free extracts with polyethylene glycol 6000. The following assay was used: 100 mM imidazole/HCl buffer/100 mM KCl/10 mM MgCl₂/20 mM potassium phosphate/15 mM ammonium sulphate (pH 6.6)/0.2 mM NADH/aldolase (1 unit/ml)/glycerol phosphate dehydrogenase (1.5 units/ml)/triose-phosphate isomerase (5 units/ml). ATP, fructose 6-phosphate and fructose 2,6-bisphosphate were added as indicated in Table 2 (see below).

Glucose was assayed enzymically with glucose oxidase/peroxidase using the commercial kit of Boehringer Mannheim.

Western-blot analysis

Denaturation of protein and SDS/PAGE were carried out according to Laemmli, as described elsewhere [9], using 7.5 % acrylamide running gels. Proteins (0.3 μ g of purified enzyme or 10–15 μ g of cell extract per lane) were subjected to electrophoretic runs, followed by electrotransfer to a nitrocellulose membrane NC 45 (Serva). The membrane was washed, temporarily stained with Ponceau S (0.2 g of dye per 100 ml of 12 % trichloroacetic acid) and then treated with polyclonal rabbit anti-phosphofructokinase antibodies and anti-rabbit immunoglobulin/alkaline phosphatase conjugate (Boehringer Mannheim) as described in [13].

ELISA detection

The assay was performed in a sandwich mode. Nunc-Immuno-Plates I (96 wells with high binding capacity) were coated overnight at 4 °C with sheep polyclonal phosphofructokinase antibodies (1 μ g/ml in 0.1 M sodium carbonate buffer, pH 9.6), which were purified by immunosorption on phosphofructokinase–Sephacrose. After washing three times with 50 mM sodium phosphate buffer (pH 7.5) containing 155 mM NaCl and 0.05 % Tween 20 (PBST buffer), the wells were filled with 200- μ l samples in duplicate. The cell-free extract without SDS and the standard (purified phosphofructokinase) were diluted with PBST buffer containing 0.03 % SDS. Samples containing 1 % SDS were diluted with PBST buffer to adjust the SDS concentration to 0.03 %. Samples from the HPLC experiments were diluted twice with PBS buffer containing 0.1 % Tween 20.

After incubation for 1.5 h at 25 °C in a moisten chamber, the plates were washed three times as described above. Subsequently, 200 μ l of rabbit anti-phosphofructokinase-peroxidase conjugate (IgG fraction purified by immunosorption on phosphofructokinase–Sephacrose and labelled with horseradish peroxidase, diluted 1:6000 with PBST buffer) were poured into the wells. After a second incubation for 1.5 h at 25 °C in the moisten chamber and exhaustive washing, the plates were filled with 200 μ l of substrate solution [2 mM 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)/3.25 mM sodium perborate/39.8 mM citric acid/60 mM Na₂HPO₄ (pH 4.4)]. Then, the reaction was allowed to proceed at 25 °C. The incubation time with the substrate varied between 10 and 90 min to obtain measurable values of absorbance at 405 nm. Samples containing no antigen were used as a blank.

HPLC size-exclusion chromatography

Isocratic size-exclusion chromatography was performed at 20 °C on a Bio-Silex SEC 400-5 column with a guard column (Bio-

Rad, München, Germany) coupled to a HPLC chromatograph (Beckman, System Gold). The column was equilibrated with 50 mM sodium phosphate buffer (pH 7.0), containing 150 mM NaCl and 0.5 mM PMSF, and run at a flow rate of 1 ml/min. The absorbance of the effluent was monitored continuously at 280 nm and fractions of 0.25 ml were collected. For calibration, a mixture of standard proteins (Bio-Rad) and a sample of native octameric phosphofructokinase were used.

Determination of glucose consumption

Cells were grown to mid-logarithmic phase, harvested by centrifugation and washed twice with 20 mM sodium citrate buffer (pH 5.4). The suspension (approx. 25% cytocrit) was agitated slowly over night at room temperature, centrifuged again and resuspended in the same buffer, yielding a cytocrit of 4%. After addition of cycloheximide (final concentration 0.1 mg/ml), a stream of air was bubbled through the suspension for 30 min before the addition of glucose (initial concentration 20 mM). At time intervals of 15 min, with continuous bubbling, appropriate samples were removed, placed in 10% trichloroacetic acid and centrifuged. The clear supernatant (50 µl) was subjected to the glucose assay.

RESULTS

Sequencing of previously isolated mutant alleles

Mutants in any one of the yeast *PFK* genes retain the ability to grow on glucose. Thus a *pfk1* mutant, designated *pfk1-109*, was isolated originally in conjunction with another, yet unidentified, mutation, rendering the cells glucose-negative [24]. Later on, *pfk2* mutants were obtained by a selection procedure devised by Clifton et al. [23], which conferred the ability to grow in the presence of glucose on non-fermentable carbon sources to other glycolytic mutants [26]. The alleles isolated were designated *pfk2-3*, *pfk2-25* and *pfk2-41*.

Here, we set out to localize the mutations in these alleles. We started off by amplifying the mutant *pfk* alleles by PCR using chromosomal DNA of the respective mutant strains as templates. DNA fragments obtained were subcloned into suitable vectors and fragments obtained from two independent PCR reactions were analysed in each case. After sequencing, single point mutations could be identified (Figure 1). For *pfk1-109*, a G → A transition at position 1503 could be detected, resulting in a change of a tryptophan-encoding triplet (TGG) to a translational

stop codon (TGA). The deduced molecular mass of the truncated α -subunit (54.5 kDa) was in the range of the band observed in the Western blot (see below). For *pfk2-3*, an exchange at the translational start codon (ATG to ATA) impaired expression, as the next ATG sequence appearing further downstream was not in frame (note that in yeast the first ATG located on the transcript is commonly used to determine the translation start [40,41]).

To our surprise, *pfk2-25* and *pfk2-41* contained identical point mutations (Figure 1), indicating that they resulted from a single mutation event. As a regeneration step was included after mutagenic treatment and colonies were screened for their lack of phosphofructokinase activity, independent colonies may well arise after replication and cell division from one original mutant. Due to the mutation in *pfk2-25/41*, an alanine residue (Ala-868, encoded by GCC at positions 2602–2604) was replaced by an aspartate (encoded by GAC) in a highly conserved region of the deduced amino acid sequence (Figure 1).

Activity of phosphofructokinase after mutagenesis *in vitro* of both genes

To confirm that the exchange of Ala → Asp was indeed responsible for a complete loss of detectable enzyme activity *in vitro*, a mutagenesis *in vitro* approach was used to exchange the respective alanine in the wild-type sequence with either aspartate or valine in *PFK1* and *PFK2*, respectively. The mutant alleles obtained were placed on CEN/ARS plasmids to allow low-copy-number expression. These were then transformed into the respective single-deletion mutants (*pfk1::HIS3*, *pfk2::HIS3*), which lacked detectable enzyme activity in assays *in vitro*, selecting for uracil prototrophy to ensure plasmid maintenance. As expected, substitution by an aspartate did not restore significant phosphofructokinase activity as measured in crude extracts from the transformants (i.e. *pfk1-874Asp/ PFK2* and *PFK1/ pfk2-868Asp*; Table 1).

When constructs containing a substitution of the alanine by valine in each phosphofructokinase subunit were applied, activity was regained in both cases (i.e. *pfk1-874Val/ PFK2* and *PFK1/ pfk2-868Val*). Thus Ala-874 seems to serve an important function of maintaining the catalytic activity of the enzyme. As this residue is conserved in phosphofructokinase subunits from other organisms, a general importance for the stability may be deduced. To confirm this observation, we also substituted the alanine residue in each subunit for the positively charged amino

Table 1 Glucose consumption of yeast strains and specific activities of phosphofructokinase as determined in cell-free extracts from strains carrying the combination of *PFK* genes indicated

The values are means of at least three independent experiments.

Strain	Relevant genotype	Activity (m-units/mg)	Glucose consumption (µmol/ml of cells per h)
Plasmid-encoded α -subunit			
MCH-4B/YCp111u	<i>PFK1Ala/ PFK2</i>	720 ± 100	490 ± 40
MCH-4B/YCp1-874Asp	<i>pfk1-874Asp/ PFK2</i>	6 ± 4	294 ± 20
MCH-4B/YCp1-874Lys	<i>pfk1-874Lys/ PFK2</i>	< 1	294 ± 18
MCH-4B/YCp1-874Val	<i>pfk1-874Val/ PFK2</i>	550 ± 50	607 ± 50
Plasmid-encoded β -subunit			
MCH-6B/YCp223wt	<i>PFK1/ PFK2Ala</i>	850 ± 50	730 ± 40
MCH-6B/YCp2-868Asp	<i>PFK1/ pfk2-868Asp</i>	< 1	350 ± 30
MCH-6B/YCp2-868Lys	<i>PFK1/ pfk2-868Lys</i>	< 1	214 ± 20
MCH-6B/YCp2-868Val	<i>PFK1/ pfk2-868Val</i>	200 ± 30	640 ± 40

Figure 2 Growth of *pfk1/pfk2* double deletion transformed with plasmids carrying various mutant *pfk* alleles

Recipient strain HD152-1C (*pfk1::HIS3 pfk2::HIS3 ura3-52*) was transformed with derivatives of YCplac33 (CEN/ARS) carrying either the wild-type *PFK* sequences (wt) or the exchanges of Ala-874 (*PFK1*) or Ala-868 (*PFK2*) by the residues indicated. Transformants were selected on synthetic medium lacking uracil and containing 3% ethanol and 1% glycerol as carbon sources. Single colonies were picked on to fresh medium and again allowed to grow for 3 days. Cells were then streaked out for single colonies on synthetic media lacking uracil containing the carbon sources indicated and incubated for 5 days at 30 °C.

acid lysine. Strains carrying one of these subunits (i.e. *pfk1-874Lys/PFK2* and *PFK1/pfk2-868Lys*) lacked detectable enzyme activity in assays *in vitro* (Table 1).

In addition to these results, the extent of the glycolytic flux in the yeast mutants was determined by measuring the rate of glucose consumption under aerobic conditions (Table 1). Whereas the valine substitution in either of the subunits had no significant effect on the rate of glucose consumption, the re-

Figure 3 Western blot of yeast strains carrying the mutant alleles indicated, and the corresponding *PFK* genes encoded by a wild-type genomic copy

The respective cell-free extracts (50 μ l) were mixed with appropriate volumes of sample buffer [60 mM Tris/HCl (pH 6.8)/10% glycerol/2% SDS/0.5 M 2-mercaptoethanol/0.0001% Bromophenol Blue], to yield a protein concentration of about 1 mg/ml. The samples were heated to 95 °C for 4 min and 10–15 μ g of protein were loaded into each lane. The amount of purified phosphofructokinase was 0.3 μ g per lane. The gel was subjected to electrophoresis at room temperature for 60 min. For details on electrotransfer and immunodetection, see the Experimental procedures section. Positions of molecular-mass markers (Da) are given on the left. Calculated molecular masses of the wild-type α -subunits were 107984 Da and of the β -subunits were 104589 Da. Pfk, purified phosphofructokinase; control, MCH-4B/YCp111u and MCH-6B/YCp223wt.

placement of alanine by either lysine or aspartate reduced the rate to 30–50% of the control strain.

However, neither the substitutions of alanine by aspartate (*pfk1-874Asp* or *pfk2-868Asp*) or by lysine (*pfk1-874Lys* or *pfk2-868Lys*) were able to complement the glucose-negative phenotype of a *pfk1/pfk2* double-deletion mutant (Figure 2). Growth could be restored partially only by introduction of *pfk1-874Val*, but not by *pfk2-868Val*.

Kinetics of the phosphofructokinase mutants

In Table 2, the kinetic data of both point-mutated enzymes encoded by *pfk1-874Val/PFK2* and *PFK1/pfk2-868Val* are sum-

Table 2 Kinetic properties of yeast phosphofructokinase after single amino acid exchange (Ala $\rightarrow$ Val)

Assay conditions: 100 mM imidazole/HCl/100 mM KCl/10 mM $MgCl_2$ /20 mM potassium phosphate/15 mM $(NH_4)_2SO_4$ (pH 6.6), 25 °C. NADH₂ (0.2 mM), glycerol phosphate dehydrogenase (1.5 units/ml), triose-phosphate isomerase (5 units/ml) and aldolase (1 unit/ml) were used as auxiliary enzymes. Enzyme activity determined with 0.3 mM ATP in the absence of fructose 2,6-bisphosphate was taken as 100%. The values are means of at least two independent enzyme preparations and curve fittings. Fru 2,6-P₂, fructose 2,6-bisphosphate, Fru 6-P, fructose 6-phosphate.

Relevant genotype	Enzyme activity (%)			Fru 6-P		Fru 2,6-P ₂	
	0.3 mM ATP 20 μ M Fru 2,6-P ₂	5 mM ATP 0.3 mM Fru 6-P 0 μ M Fru 2,6-P ₂	5 mM ATP 20 μ M Fru 2,6-P ₂	$K_{0.5}$ (mM) 3 mM ATP 0–3 mM Fru 6-P 0 μ M Fru 2,6-P ₂	h	$K_{0.5}$ (μ M) 3 mM ATP 0.3 mM Fru 6-P 0–20 μ M Fru 2,6-P ₂	h
Plasmid-encoded α -subunit							
<i>PFK1Ala/ PFK2</i>	307 $\pm$ 20	15 $\pm$ 1	313 $\pm$ 18	1.11 $\pm$ 0.10	2.7 $\pm$ 0.2	1.22 $\pm$ 0.22	1.5 $\pm$ 0.2
<i>pfk1-874Val/ PFK2</i>	130 $\pm$ 5	43 $\pm$ 3	132 $\pm$ 5	0.38 $\pm$ 0.04	2.7 $\pm$ 0.2	0.25 $\pm$ 0.02	1.3 $\pm$ 0.1
Plasmid-encoded β -subunit							
<i>PFK1/ PFK2Ala</i>	245 $\pm$ 10	15 $\pm$ 1	252 $\pm$ 11	0.80 $\pm$ 0.07	2.7 $\pm$ 0.2	0.70 $\pm$ 0.07	1.6 $\pm$ 0.1
<i>PFK1/ pfk2-868Val</i>	134 $\pm$ 7	52 $\pm$ 4	135 $\pm$ 5	0.33 $\pm$ 0.02	2.2 $\pm$ 0.1	0.49 $\pm$ 0.09	1.2 $\pm$ 0.1

Figure 4 Size-exclusion HPLC of phosphofructokinase encoded by various combinations of mutant and wild-type *PFK* genes

(a) Panels A and B, profiles of MCH-4B/Ycp111u and MCH-6B/Ycp223wt respectively. The profiles of subclasses were obtained from strains carrying combinations, where the mutant allele was carried on a CEN/ARS vector, as above, with the respective allele being deleted at the genomic locus and the corresponding gene being genomic. ELISA substrate incubation times: 10 min (panels A, A1, B and B1); 25 min (panels A2 and A3); 45 min (panels B2 and B3). (b) Panel A1, *pfk1-874Val*/*PFK2*; panel B1, *PFK1/pfk2-868Val*; panel A2, *pfk1-874Lys*/*PFK2*; panel B2, *PFK1/pfk2-868Lys*; panel A3, *pfk1-874Asp*/*PFK2*; and panel B3, *PFK1/pfk2-868Asp*. ◇, Phosphofructokinase protein determined by ELISA; ●, phosphofructokinase activity (for details, see the Experimental procedures section).

marized in comparison with the control. Both enzymes exhibited co-operativity towards fructose 6-phosphate (with a Hill coefficient of around 2.5), but the affinity to this substrate was significantly higher in the mutants. The maximum inhibition by ATP of about 50 % was smaller than in the corresponding wild-type control, but could be abolished completely by the addition of fructose 2,6-bisphosphate. Also, the activation by the latter at non-inhibiting ATP concentrations was found to be similar for both mutants but lower than for the respective wild-type controls. The Ala → Val exchange in the α -subunit caused a significant

increase in affinity for fructose 2,6-bisphosphate without changing the co-operativity.

Structure of phosphofructokinase in strains expressing the mutant alleles

To prove that phosphofructokinase subunits are produced from the mutant alleles described above, we first performed a Western-blot analysis (Figure 3). As stated above, a strain carrying *pfk1-109* yielded a truncated α -subunit (≈ 50 kDa). The alleles carry-

Table 3 Total concentration and the partition of phosphofructokinase protein in the pellet and supernatant of yeast strains carrying the mutant alleles indicated

Cell-free extracts were prepared from a yeast suspension (cytocrit 25%) with and without 1% SDS as described in the Experimental procedures section. The values are means of at least two independent experiments. The total concentration of phosphofructokinase protein was determined by disruption of the cells in the presence of 1% SDS and by performing the ELISA in the presence of 0.03% SDS. The partition of phosphofructokinase protein between supernatant and pellet was calculated from measuring the enzyme protein in the presence (total) and absence of 1% SDS. Pfk, phosphofructokinase.

Combination of <i>PFK</i> alleles	Total concentration of Pfk protein ($\mu\text{g/ml}$)	Partition of Pfk protein (%)	
		Supernatant	Pellet
Plasmid-encoded α -subunit			
<i>PFK1Ala/PFK2</i>	71.8 $\pm$ 3.6	95 $\pm$ 5	3 $\pm$ 3
<i>pfk1-874Asp/PFK2</i>	58.6 $\pm$ 2.9	60 $\pm$ 3	40 $\pm$ 2
<i>pfk1-874Val/PFK2</i>	27.1 $\pm$ 1.4	95 $\pm$ 5	3 $\pm$ 1
<i>pfk1-874Lys/PFK2</i>	19.9 $\pm$ 1.0	34 $\pm$ 2	66 $\pm$ 3
Plasmid-encoded β -subunit			
<i>PFK1/PFK2Ala</i>	109.3 $\pm$ 5.5	63 $\pm$ 3	37 $\pm$ 2
<i>PFK1/pfk2-868Asp</i>	30.3 $\pm$ 1.5	59 $\pm$ 3	41 $\pm$ 2
<i>PFK1/pfk2-868Val</i>	16.4 $\pm$ 0.8	62 $\pm$ 3	38 $\pm$ 2
<i>PFK1/pfk2-868Lys</i>	19.2 $\pm$ 1.0	30 $\pm$ 2	70 $\pm$ 3

ing substitutions of alanine 874/868 by aspartate or lysine in either subunit produced only one significant signal, which corresponded to the subunit of the non-mutated gene. Traces of the mutated subunits can be observed only by overloading the polyacrylamide gels or by using recipient strains with multiple proteolytic defects. However, after substitution of alanine 874/868 by valine in both strains two bands became visible that corresponded to the subunits of the non-mutated MCH strains. In addition, traces of immunoreactive protein became visible in all mutants, which are most likely produced by minor proteolytic degradation of the subunits.

To investigate the effects of the mutations on the oligomeric composition of phosphofructokinase, we separated cell-free extracts of the different mutants by size-exclusion HPLC using a Bio-Select SEC400 column and detected the enzyme by activity and protein distribution using ELISA. As shown in Figure 4, phosphofructokinase of the control strain displayed a retention time of 10 min, which corresponded to a hetero-octameric structure of 800 kDa, where enzyme activity and phosphofructokinase protein coincide in one peak. A small amount of enzyme was found to be aggregated, particularly in the MCH-6B strain, and another minor protein fraction was of lower molecular mass. The Ala-874/868 $\rightarrow$ Val exchanges also resulted in the appearance of an octameric structure, as detected by activity and protein distribution (Figure 4b, panels A1 and B1). Both profiles were similar to the non-mutated strains. In contrast, both point mutations Ala $\rightarrow$ Lys and Ala $\rightarrow$ Asp revealed significantly different patterns. In the cases of *pfk1-874Lys/PFK2* and *PFK1/pfk2-868Lys*, a significant amount of the eluted enzyme protein was of lower molecular mass (of less than 100 kDa; Figure 4b, panels A2 and B2) apart from a small amount of aggregated forms. The mutants *pfk1-874Asp/PFK2* and *PFK1/pfk2-868Asp* displayed phosphofructokinase protein in the range of 100–800 kDa and some aggregates (Figure 4b, panels A3 and B3). The total amount of detectable phosphofructokinase protein after size-exclusion chromatography was significantly lower in strains containing a point mutation in the β -subunit, particularly indicated by the longer incubation time with the detection substrate in ELISA.

Distribution of soluble and precipitated phosphofructokinase protein in strains expressing different mutant alleles

Size-exclusion chromatography, as performed above, revealed that phosphofructokinase of several strains carrying point-mutated alleles either forms aggregates or undergoes proteolytic degradation in the cell extract. Therefore, it was of interest to measure the total phosphofructokinase protein in the mutants in comparison with their control strains and to determine the portion of the precipitated protein in the pellet. As seen in Table 3, the total phosphofructokinase protein decreased when either the α - or the β -polypeptide chains contained a mutation. However, both mutants showed differences in the ratio of soluble to non-soluble enzyme. Looking at the recipient strain MCH-4B, nearly all enzyme protein was found to be soluble. The same result was observed after an Ala $\rightarrow$ Val exchange in the α -polypeptide chain, whereas the Ala $\rightarrow$ Asp and Ala $\rightarrow$ Lys exchanges yielded significant amounts of non-soluble phosphofructokinase. In the MCH-6B strain, a third of the total enzyme was found precipitated, yet the amount of soluble enzyme protein remained equal to the MCH-4B strain, because of its higher quantity. The portion of precipitated phosphofructokinase was found to be highest (65–70% of the total amount) upon an Ala $\rightarrow$ Lys exchange.

DISCUSSION

In a previous study [26], mutant alleles in either of the genes encoding the hetero-octameric yeast phosphofructokinase were isolated as rendering cells without detectable activity, as measured by assays *in vitro*. As also found for single-deletion mutants, their growth on glucose as a sole carbon source was not affected. Therefore, missing phosphofructokinase activity could be indicative either of a lack of expression of the respective gene or be attributed to an impairment of the functional stability of the enzyme. In this work, we obtained evidence for both mechanisms.

The mutation observed in *pfk2-3* led to the loss of the original translational start codon. With the next ATG sequence not being in frame with the *PFK2* coding region, we presume that no

Figure 5 The main interaction sites between both subunits (A and B) of phosphofructokinase from *E. coli*

X-ray-structure data from phosphofructokinase prepared from strain K12 (KIECFA in PIR database) were used [18]. Between Arg-262 of helix 11 in subunit A and Asp-152 of helix 6 in subunit B a strong electrostatic interaction is generated. The distance between these amino acid residues is about 4 Å. Since the subunits are homologous and organized as a dimer of dimers, the salt bridge occurs twice.

functional β -subunit was produced in this case. For *pfk1-109*, the G $\rightarrow$ A transition yielded a truncated α -subunit of half of the molecular mass of the wild-type polypeptide chain. This subunit was obviously unable to form a functional enzyme that was stable *in vitro*, as judged by the lack of activity in crude extracts. Next, we found a point mutation leading to a single amino acid exchange, Ala $\rightarrow$ Asp, in the *pfk2-25/41* alleles. Although the mutation was localized within the second half of the β -subunit, presumed to serve primarily regulatory rather than catalytic functions [19], a lack of enzyme activity in the crude extract was observed.

As we could show in this study by mutagenesis *in vitro*, single exchanges of Ala-874/868 either in the α - or in the β -subunits by Asp or Lys led also to a loss of enzyme activity in cell-free extracts. This is reminiscent of mutations generated in proliferating-cell nuclear antigen, where a single amino acid exchange of a leucine to a serine residue renders the protein unable to assume its trimeric structure [42]. In the case of yeast phosphofructokinase, we could further show that activity was preserved when Val replaced Ala-874/868, although for the β -subunit mutation a reduction to about 25% of the respective control strain activity was measured. Thus as observed previously [19], the PFK2-encoded β -subunit seems to be less efficient in contributing to the activity *in vivo* and is more prone to mutational inactivation.

The Ala $\rightarrow$ Val exchange in both mutants caused significant alterations of the enzyme kinetics. The increase of the affinity towards fructose 6-phosphate was surprising, since the mutated site was not located near the substrate-binding region in the primary structure [19]. Therefore, conformational changes in the ternary structure may have been responsible for these alterations.

The lower inhibition by ATP may be explained by the higher affinity towards fructose 6-phosphate. The observed changes on the behaviour towards fructose 2,6-bisphosphate were probably due to a direct influence of the point mutation, because of the vicinity to the proposed binding site of this sugar phosphate [20]. The kinetic data found for the control-strain enzymes correspond to the results published by others [43].

It should be mentioned that substitutions of Ala-874/868 by Asp or Lys in only one subunit left yeast strains able to grow on glucose and to use the monosaccharide, although with a reduced rate (Table 1). However, strains carrying only one mutant allele and not the other corresponding wild-type subunit were not able to grow on glucose (Figure 2). These phenotypes indicate that not only the formation of the hetero-octameric structure in conjunction with the corresponding wild-type subunit was impaired, but also the correct folding of each subunit *per se*. Different lines of evidence indicate that each wild-type subunit can serve a catalytic function *in vivo* which cannot be detected by assays *in vitro* [19,28]. This function is also abolished in the mutant alleles, where Asp or Lys replaces the Ala residue on either subunit. However, function is partially retained in the α -subunits when valine is placed at this position.

Looking at the total phosphofructokinase protein and the partition of soluble and non-soluble fractions, one could see that all three exchanges (Asp, Lys and Val) reduced the steady-state enzyme levels. Western-blot analyses revealed that the Ala $\rightarrow$ Lys and Ala $\rightarrow$ Asp exchanges in either subunit led to the disappearance of the respective polypeptide chain, but both subunits could be detected after Ala $\rightarrow$ Val substitution. The poor Western-blot signal was at least partly due to high intracellular

degradation and/or aggregation (accompanied by precipitation) of misfolded and non-assembled subunits. The latter view is supported by the observation that in single-deletion mutants the amount of precipitated phosphofructokinase was found to be significantly higher than in wild-type strains [28]. It is noteworthy, however, that the pellet of mutants in the present study did not contain significant amounts of the mutated subunit that was lacking in the extract (results not shown). Therefore, cellular degradation of the mutated subunit becomes probable, substantiated by the observation that, in a protease-deficient strain, mutant subunits can be substantially stabilized.

As for the structural implications, one can assume that a non-charged environment at positions 874 and 868 is important for the stability of the enzyme. Indeed, size-exclusion chromatography revealed no stable hetero-octamer after the exchanges Ala → Lys and Ala → Asp, whereas Ala → Val did not interfere significantly with the activity and protein profile as compared with the controls.

Comparing the amino acid sequence of the yeast enzyme with phosphofructokinase from *E. coli*, Ala-874/868 corresponds to Ala-265 of the prokaryotic enzyme. This position is located on helix number 11 of each polypeptide chain A–D as a central part of a cleft formed by two subunits [18]. The adjacent Arg-262 on the same helix shows strong electrostatic interaction with a facing Asp-152 on helix number 6, located on the opposite subunit. Because of the geometry of the assembly (the tetrameric enzyme is arranged as a dimer of dimers with 222 symmetry) the inter-subunit salt bridge appears twice (Figure 5). As estimated from the X-ray-structure analysis, both interactions seem to be the most important binding forces between the two subunits. The mutations Ala → Asp and Ala → Lys may interfere with this interaction either directly by repulsion and attraction forces and/or by changing the orientation of the helices within the dimer. Arg-262 and Asp-152 are found to be conserved amino acids in most of the phosphofructokinases. In *S. cerevisiae* they correspond to Arg-871/865 and Asp-380/372 respectively. Therefore, it may be deduced that the electrostatic interaction between both helices is generally essential for the inter-subunit interaction of diverse phosphofructokinases and, in consequence, for the stability of their oligomeric structure. A detailed structure analysis of the recently crystallized yeast phosphofructokinase half-molecule [44] may soon provide an answer to these questions.

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REFERENCES

- Barnett, J. A. (1976) Adv. Carbohydr. Chem. Biochem. **32**, 126–234
- Hofmann, E. (1976) Rev. Physiol. Biochem. Pharmacol. **75**, 2–68
- Uyeda, K. (1979) Adv. Enzymol. **48**, 193–244
- Heinisch, J. J. and Hollenberg, C. P. (1993) in Biotechnology (Rehm, H. J. and Reed, G., eds.), pp. 469–514, VCH Verlagsgesellschaft, Weinheim
- Kopperschlager, G. and Heinisch, J. J. (1997) in Yeast Sugar Metabolism (Zimmermann, F. K. and Entian, K. D., eds.), pp. 97–118, Technomic Publishing, Lancaster, PA
- Kopperschlager, G., Bär, J., Nissler, K. and Hofmann, E. (1977) Eur. J. Biochem. **81**, 317–325
- Plietz, P., Damaschun, G., Kopperschlager, G. and Müller, J. J. (1978) FEBS Lett. **91**, 230–232
- Nissler, K., Hofmann, E., Stel'Maschchuk, V., Orlova, E. and Kiselev, N. (1985) Biomed. Biochim. Acta **44**, 251–259
- Kopperschlager, G., Bär, J. and Stellwagen, E. (1993) Eur. J. Biochem. **217**, 527–533
- Diezel, W., Böhme, H.-J., Nissler, K., Freyer, R., Heilmann, W., Kopperschlager, G. and Hofmann, E. (1973) Eur. J. Biochem. **38**, 479–488
- Kriegel, T., Ovadi, J., Behlke, J. and Kopperschlager, G. (1986) Stud. Biophys. **113**, 247–259
- Kopperschlager, G. and Naumann, M. (1997) Int. J. Biochromatogr. **2**, 269–282
- Clifton, D. and Fraenkel, D. G. (1982) Biochemistry **21**, 1935–1942
- Heinisch, J. (1986) Mol. Gen. Genet. **202**, 75–82
- Heinisch, J., Ritzel, R. G., Von Borstel, R. C., Aguilera, A., Rodicio, R. and Zimmermann, F. K. (1989) Gene **78**, 309–321
- Heinisch, J., Vogelsang, K. and Hollenberg, C. P. (1991) FEBS Lett. **289**, 77–82
- Evans, P. R. and Hudson, P. J. (1979) Nature (London) **279**, 500–504
- Rypniewski, W. R. and Evans, P. R. (1989) J. Mol. Biol. **207**, 805–821
- Arvanitidis, A. and Heinisch, J. J. (1994) J. Biol. Chem. **269**, 8911–8918
- Heinisch, J. J., Boles, E. and Timpel, C. (1996) J. Biol. Chem. **271**, 15928–15933
- Heinisch, J., Kirchrath, L., Liesen, T., Vogelsang, K. and Hollenberg, C. P. (1993) Mol. Microbiol. **8**, 559–570
- Bär, J., Schellenberger, W. and Kopperschlager, G. (1997) Yeast **13**, 1309–1317
- Clifton, D., Weinstock, S. B. and Fraenkel, D. G. (1978) Genetics **88**, 1–11
- Ciriacy, M. and Breitenbach, I. (1979) J. Bacteriol. **139**, 152–160
- Lobo, Z. and Maitra, P. K. (1982) FEBS Lett. **139**, 93–96
- Breitenbach-Schmitt, I., Heinisch, J., Schmitt, H. D. and Zimmermann, F. K. (1984) Mol. Gen. Genet. **195**, 530–535
- Heinisch, J. (1986) Curr. Genet. **11**, 227–234
- Klinder, A., Kirchberger, J., Edelmann, A. and Kopperschlager, G. (1998) Yeast **14**, 323–334
- Zimmermann, F. K. and Scheel, I. (1977) Mol. Gen. Genet. **154**, 75–82
- Sambrook, J., Fritsch, E. S. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor
- Sherman, F., Fink, G. R. and Hicks, J. B. (eds.) (1983) in Laboratory Manual (Cold Spring Harbor Laboratory for Quantitative Biology), Cold Spring Harbor Press, Cold Spring Harbor
- Hanahan, D. (1985) in DNA Cloning: a Practical Approach, vol. I (Glover, P. M., ed.), pp. 109–135, IRL Press, Oxford
- Dohmen, R. J., Strasser, A. W. M., Höner, C. B. and Hollenberg, C. P. (1991) Yeast **7**, 691–692
- Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene **33**, 103–119
- Vieira, J. and Messing, J. (1991) Gene **100**, 189–194
- Gietz, R. D. and Sugino, A. (1988) Gene **74**, 527–534
- Boles, E. and Miosga, T. (1995) Curr. Genet. **28**, 197–198
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
- Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
- Yoon, H. and Donahue, T. F. (1992) Mol. Microbiol. **6**, 1413–1419
- Huang, H. K., Yoon, H., Hannig, E. M. and Donahue, T. F. (1997) Genes Dev. **11**, 2396–2413
- Piardi, K., Baldacci, G. and Tratner, I. (1998) Nucleic Acids Res. **26**, 2598–2605
- Otto, A., Przybylski, F., Nissler, K., Schellenberger, W. and Hofmann, E. (1986) Biomed. Biochim. Acta **45**, 865–875
- Obmolova, G., Kopperschlager, G., Heinisch, J. and Rypniewski, W. R. (1998) Acta Crystallogr. **D54**, 96–98

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