Mutation of Monofunctional 6-Phosphofructo-2-kinase in Yeast to Bifunctional 6-Phosphofructo-2-kinase/Fructose 2,6-Bisphosphatase†

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ABSTRACT: We have shown previously that 6-phosphofructo-2-kinase in yeast has negligible fructose-2,6-bisphosphatase activity even though resembling in part of its C-terminal sequence the phosphatase domain of the bifunctional liver enzyme. Here we show that exchanging Ser-404 to His-404 in the yeast peptide creates a bifunctional enzyme with a fructose-2,6-bisphosphatase activity involving a phosphoprotein intermediate. Like mammalian bifunctional enzymes, the His-404 mutant protein is readily phosphorylated by fructose 2,6-P_2 with a half-saturation of 0.4 μM, the same K_m value as for its fructose-2,6-bisphosphatase activity. Protein phosphorylation by the C-subunit of cAMP-dependent protein kinase, presumably at a C-terminal consensus site, increases the K_m value to 1.5 μM. The newly created fructose-2,6-bisphosphatase is inhibited competitively by its product fructose 6-P with a K_i of 0.6 mM. No effect of the His-404 mutation was found on 6-phosphofructo-2-kinase activity, in line with the mutant yeast enzyme having independent kinase and phosphatase domains, like its mammalian wild-type counterparts. The results would fit with the evolution of the *PFK26* gene having involved fusion between kinase and phosphatase genes—as proposed for the mammalian enzyme—but with accompanying or later silencing of the fructose-2,6-bisphosphatase activity.

In yeast, synthesis of the 6-phosphofructo-1-kinase activator fructose 2,6-bisphosphate (fructose 2,6-P_2) is catalyzed by at least two 6-phosphofructo-2-kinases, with the recently described 96-kDa peptide specified by the *PFK26* gene accounting for >90% of cytosolic activity (Kretschmer & Fraenkel, 1991). Despite 42% identity between a continuous interior sequence comprising 455 residues of the 96-kDa peptide with almost the entire length (470 residues) of the bifunctional liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, the yeast enzyme does not show significant fructose-2,6-bisphosphatase activity and does not form a phosphoprotein intermediate when incubated with [2-^{32}P]-fructose 2,6-P_2 (Kretschmer & Fraenkel, 1991; Paravicini & Kretschmer, 1992). We speculated that this lack of fructose-2,6-bisphosphatase activity might be related to a missing key histidine residue [His-258 in rat liver; Ser-404 in yeast (Kretschmer & Fraenkel, 1991)], which in the bifunctional liver enzyme is the phosphoacceptor in the bisphosphatase reaction (Tauler et al., 1990). The corresponding residue is present (His-232) in the predicted 52.7-kDa product of the recently isolated yeast gene for fructose-2,6-bisphosphatase (*FBP26*). The latter polypeptide is also homologous to the entire rat liver sequence and is the only protein in yeast labeled by [2-^{32}P]fructose 2,6-P_2 and contributes no apparent 6-phosphofructo-2-kinase activity *in vivo* (Paravicini & Kretschmer, 1992).

Here we show that by exchanging Ser-404 → His-404, the major yeast 6-phosphofructo-2-kinase becomes a bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase which in its fructose-2,6-bisphosphatase reaction involves the formation of a phosphohistidine intermediate.

MATERIALS AND METHODS

Materials. Fructose 6-P, ATP, and most enzymes were from Boheringer (Mannheim, Germany). Acrylamide and *N,N'*-methylenebisacrylamide were from Bio-Rad Laboratories (Richmond, CA). Pyrophosphate:fructose-6-P phosphotransferase and the C-subunit of protein kinase A (bovine heart), fructose 2,6-P_2, PEG_{6000}, and all other chemicals were from Sigma (St. Louis, MO). Bactotryptone, yeast extract, yeast nitrogen base without amino acids, and casamino acids were obtained from Difco (Detroit, MI). Low molecular weight SDS–PAGE standards were from Pharmacia (Piscataway, NJ). [γ-^{32}P]ATP and deoxyadenosine 5'-(α[^{35}S]-thiotriphosphate) were purchased from New England Nuclear (Boston, MA), the GeneAmp polymerase chain reaction kit with AmpliTaq DNA polymerase was from Perkin Elmer Cetus (Norwalk, CT), the pMAL protein expression and purification system was from New England Biolabs (Beverly, MA), and the Sequenase version 2.0 DNA sequencing kit was obtained from U.S. Biochemicals (Cleveland, OH).

Plasmid Construction. For the introduction of the Ser-404 → His-404 mutation, the *PFK26* reading frame in pMK1 (Kretschmer et al., 1991) was amplified using a Perkin Elmer Cetus DNA thermal cycler. Primers A and B (see Figures 1 and 2) served to amplify the N-terminal half of the gene with primer B introducing the Ser-404 → His-404 mutation by exchanging AG → CA 1210 base pairs downstream from the *PFK26* start codon and also creating a new *Eag*I site by silent mutation T → C at position 1236. The same *Eag*I site-creating base exchange was introduced by primer C during amplification with primer D of the C-terminal half of *PFK26*. Primers A and D were designed to provide convenient *Bam*HI, *Sal*I and *Sac*I, *Hin*dIII sites, respectively. They were also used in a third polymerase chain reaction to amplify the wild-

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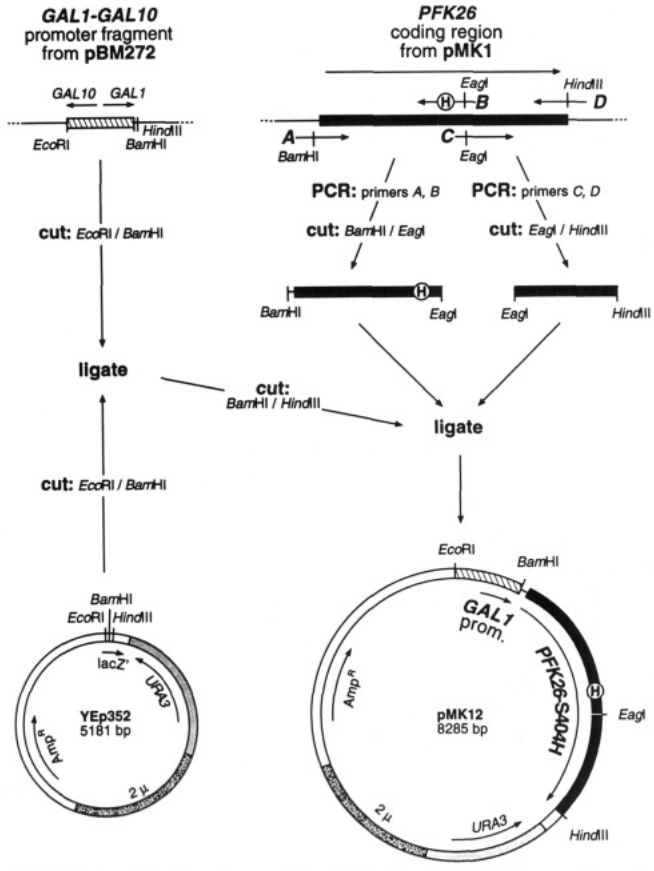

FIGURE 1: Construction of the YEp-derived multicopy plasmid pMK12 carrying *PFK26-S404H* under the control of the *GAL1* promoter. A circled "H" denotes the position of the basepair changes creating His-404. For the wild-type gene in pMK11, only primers A and D were used in one polymerase chain reaction.

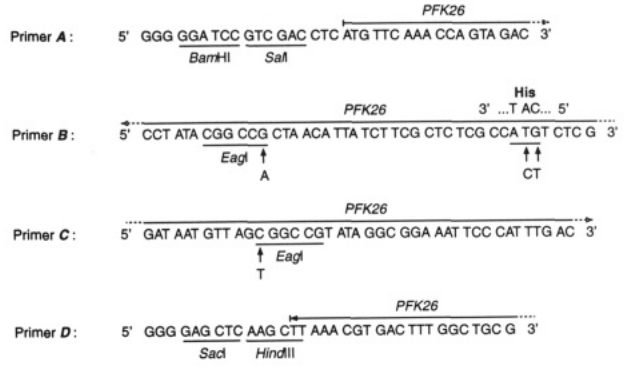

FIGURE 2: Primers used in polymerase chain reactions. The arrows denote basepair changes introduced into the *PFK26* gene. Coding sequence of *PFK26* is indicated by a solid line. Primers A and C are sense, and primers B and D are antisense strand.

type *PFK26* reading frame. The wild-type and the two fragments of the mutant gene, *PFK26-S404H*, were then ligated (see Figure 1) into a derivative of the yeast multicopy expression vector YEp352 (Hill et al., 1986) which also contained the yeast *GAL1-GAL10* promoter fragment subcloned from pBM272 [from Mark Johnston, contains the *GAL1-GAL10* promoter fragment between the *Eco*RI and *Bam*HI sites in the yeast centromere plasmid YCp50 (Rose et al., 1987)]. The resulting plasmid constructs, pMK11 and pMK12, contain the *PFK26* and *PFK26-S404H* genes fused to the *GALI* promoter. Analogous constructs (pMK9 and pMK10, carrying *PFK26* and *PFK26-S404H*, respectively) on a single-copy expression vector were obtained by subcloning into the *Bam*HI/*Hin*dIII sites of pBM272. *Escherichia coli* strain HB101 (Bolivar & Backman, 1979) was used as bacterial host throughout all cloning experiments. For expression in *E. coli*, the wild-type fragment was subcloned into the *Bam*HI/*Hin*dIII sites of the T7 polymerase based expression pT7/7 vector (Studier & Moffatt, 1986), with *E. coli* strain BL21D3 as host, or into the *Bam*HI/*Hin*dIII sites of pMALc-RI (New England Biolabs, Beverly, MA) with host strain TB1.

DNA Sequence Determination. After amplification by polymerase chain reaction and subcloning, the fidelity of *PFK26* and *PFK26-S404H* coding regions in pMK11 and pMK12 was confirmed by dideoxynucleotide sequencing (Sanger et al., 1977) of double-stranded DNA using primers A and D (see Figure 2), and primers originally applied for sequencing of the *PFK26* gene (Kretschmer & Fraenkel, 1991).

Expression in Saccharomyces cerevisiae and Purification. Strain DFY658 (*pfk26*::*LEU2*, *fbp26*::*HIS3*, *leu2*, *his3*, *ura3*), a segregant of a cross between GPY1126 [*a*, *fbp26*::*HIS3*, *leu2*, *his3*, *trp1*, *lys2*, *ura3*, *suc2* (Paravicini & Kretschmer, 1992)] and DFY650 [α, *pfk26*::*LEU2*, *leu2*, *his3* (Kretschmer & Fraenkel, 1991)], was transformed (Broach et al., 1979) with plasmids pMK9, pMK10, pMK11, and pMK12 and grown in enriched medium A (yeast nitrogen base containing casamino acids; uracil was omitted, except for strain DFY658 without plasmid), supplemented with 2% glucose or, for induction via the *GALI* promoter, with 2% galactose, or 1% glucose/1% galactose to an A_{580} of 6. Wild-type and mutant 6-phosphofructo-2-kinase were purified from 40 g wet weight of galactose grown cells in a three-step purification. Cells were harvested, washed, and disrupted using the French press with 100 mM potassium phosphate, pH 7.2, 1 mM EDTA, 4 mM mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride, and a 3–8% (mass/vol) poly(ethylene glycol) 6000 precipitate was collected as described (Kretschmer et al., 1991). The pellet was dissolved in 30 mL of 20 mM Tris-HCl, pH 7.5, 4 mM mercaptoethanol, and 300 mM KCl, and applied to a Sephacryl-S300 blue column (1.5 × 8.5 cm), equilibrated with the same buffer. After being washed with 150 mL, the column was developed with a linear gradient of 0.3–2 M KCl in 2 × 30 mL of column buffer. Active fractions (13 mL) were pooled, dialyzed twice for 1.5 h vs 300 mL of 50 mM sodium phosphate, pH 6.8, 5 mM magnesium chloride, 1 mM EDTA, and 4 mM mercaptoethanol, applied to a CM-Sephadex C-50 column (0.9 × 2.4 cm), equilibrated and washed (15 mL) with the same buffer, and eluted with 15 mM fructose 6-P and stored with 20% glycerol at −15 °C as described before (Kretschmer et al., 1991). For removal of fructose 6-P and glycerol prior to assays, fractions were desalted twice vs 50 mM Tris-HCl, pH 7.5, 5 mM magnesium chloride, 100 mM KCl, and 5 mM mercaptoethanol on a NAP10 column (Pharmacia, Piscataway, NJ).

Assay of Enzymes. 6-Phosphofructo-2-kinase was assayed in 50 mM Tris-HCl, pH 7.5, 8 mM ATP, 2 mM fructose 6-P, 6.5 mM glucose 6-P, 20 mM magnesium chloride, 2 mM potassium phosphate, and 5 mM mercaptoethanol. For kinetic measurements with purified enzyme, glucose 6-P was omitted, and the ATP and fructose 6-P content varied as indicated. Samples were withdrawn at 0, 2.5, and 5 min and assayed for fructose 2,6-P_2 as described (François et al., 1984). Fructose-2,6-bisphosphatase was assayed by the release of [^{32}P]P_i in a mixture containing 50 mM Tris-HCl, pH 7.5, 9 mM magnesium chloride, 5 mM mercaptoethanol, and [^{32}P]fructose 2,6-P_2 (2×10^{16} cpm/mol) as specified. Samples were taken at 0, 5, and 10 min, and [^{32}P]P_i was separated

from [2-^{32}P]fructose 2,6-P_2 on Dowex AG-1 as described (van Schaftingen et al., 1982) or the fructose 2,6-P_2 was assayed (François et al., 1984).

Equation 1 was used to describe the kinetic data for fructose-2,6-bisphosphatase. Parameters V_{max}, $K_{m\ F\text{-}2,6\text{-}P_2}$, and $K_{i\ F\text{-}6\text{-}P}$ were fitted by nonlinear regression analysis using Mathematica (Wolfram Research, Champaign, IL) on a NeXTstation TurboColor.

$$v = \frac{V_{max}[\text{F-2,6-P}_2]}{[\text{F-2,6-P}_2] + K_{m\ \text{F-2,6-P}_2}(1 + [\text{F-6-P}]/K_{i\ \text{F-6-P}})} \quad (1)$$

All enzyme activities given are the average of at least three determinations. One unit of enzyme is defined as 1 μmol of substrate converted/min at 25 °C.

Phosphohistidine formation was monitored as [^{32}P] in the 96-kDa peptide specified by the *PFK26-S404H* gene. The assay mixture (25 °C) was the same as described above for fructose-2,6-bisphosphatase. Samples were withdrawn at times indicated, treated for 1 min at 90 °C with 0.5 vol of a solution containing 4% SDS, 35% glycerol, 40 mM dithiothreitol, 0.04% bromphenol blue, and 125 mM Tris-HCl, pH 6.8, and immediately subjected to SDS–PAGE. The 10% acrylamide gel was then dried and ^{32}P-labeling quantified on a Molecular Dynamics Phosphorimager as described before (Paravicini & Kretschmer, 1992). For time dependencies of phosphoprotein formation (Figure 6), a numeric solution of the system

$$\frac{d[\text{E–P}]}{dt} = k_1[\text{F-2,6-P}_2][\text{E}] - k_2[\text{E–P}][\text{F-6-P}] - k_3[\text{E–P}] \quad (2)$$

$$\frac{d[\text{P}_i]}{dt} = k_3[\text{E–P}] \quad (3)$$

describing a simplified reaction scheme ($k_1 \approx k_2$)

$$\text{F-2,6-P}_2 + \text{E} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{E–P} + \text{F-6-P} \quad (4)$$

$$\text{E–P} \overset{k_3}{\rightharpoonup} \text{E} + \text{P}_i \quad (5)$$

(E stands for enzyme, E–P for phosphoenzyme) was fitted to the data by nonlinear regression using a program developed in Mathematica (Wolfram Research, Champaign, IL) on a NeXT TurboColorstation.

Galactokinase was determined as galactose-dependent ADP production by coupled assay with pyruvate kinase and lactate dehydrogenase in 50 mM Tris-HCl, pH 7.3, 5 mM magnesium chloride, 5 mM ATP, 1 mM phosphoenol pyruvate, and 20 mM NADH.

[2-^{32}P]fructose 2,6-P_2 was synthesized from fructose 6-P and [γ-^{32}P]ATP using yeast 6-phosphofructo-2-kinase as before (Paravicini & Kretschmer, 1992).

Protein concentration was determined with bovine serum albumin as described by Bradford (1976).

Enzyme Phosphorylation with Protein Kinase A. Purified wild-type or mutant 6-phosphofructo-2-kinase (1.5 μg) was incubated for 30 min at 30 °C in 50 mM Tris-HCl, pH 7.5, 5 mM magnesium chloride containing 0.33 mM [γ-^{32}P]ATP (1200 cpm/pmol) with and without 3 μg/mL C-subunit of protein kinase A from bovine heart, and then subjected to SDS–PAGE. Incorporation of ^{32}P was quantified on a Molecular Dynamics Phosphorimager as described (Paravicini & Kretschmer, 1992).

Table I: *GAL1* Promoter-Driven Expression of Wild-Type 6-Phosphofructo-2-kinase from Centromere Plasmid pMK9 and Multicopy Plasmid pMK11 in Yeast Strain DFY658[a]

	6-phosphofructo-2-kinase (milliunits/mg)			galactokinase (units/mg)	
	no plasmid	pMK9	pMK11	no plasmid	pMK11
2% Glu	<0.01	<0.01	<0.01	0.04	0.03
1% Glu, 1% Gal	<0.01	1.60	5.60	0.18	0.19
2% Gal	<0.01	14.00	60.00	0.80	1.00

[a] Cells were grown in medium A as specified (Materials and Methods).

For experiments involving phosphoprotein formation with [2-^{32}P]fructose 2,6-P_2 (Figures 4 and 6), unlabeled ATP was used for protein kinase A treatment.

RESULTS

Expression of Wild-Type and Mutant Enzymes. Attempts to produce yeast 6-phosphofructo-2-kinase from the pT7/7 expression vector in *E. coli* gave extracts with specific activities of only 0.2 milliunit/mg protein and were not further pursued. Higher extract activities (2 milliunits/mg protein) in *E. coli* were obtained with the pMAL-cRI protein fusion system (New England Biolabs, Beverly, MA), yielding a purified 136-kDa peptide of specific activity 500 milliunits/mg. Unfortunately, this material was unsuitable for studies of native size subunits since treatment with factor X protease to remove the N-terminal maltose-binding protein also cleaved within the 6-phosphofructo-2-kinase polypeptide chain (data not shown).

On the other hand, *S. cerevisiae* DFY658 (*pfk26::LEU2, fbp26::HIS3, ura3*), a strain with activities of <0.01 milliunit/mg 6-phosphofructo-2-kinase and no phosphoprotein-forming fructose-2,6-bisphosphatase, provided a convenient host for the expression of wild-type and mutant proteins, 6-phosphofructo-2-kinase activities up to 600 times wild-type level being obtainable with the enzyme expressed from *GAL1* promoter-driven plasmid constructs. The chromosomally coded galactose pathway genes themselves were normally expressed as indicated by assayed galactokinase activity (Table I).

With yeast strains overproducing 6-phosphofructo-2-kinase several hundred-fold, the purification was greatly simplified and gave a protein of ca. 7 units/mg specific activity, confirming the high value reported for partially purified yeast enzyme (François et al., 1988; Kretschmer et al., 1991). Both wild-type and Ser-404 → His-404 mutant 6-phosphofructo-2-kinase could be purified following the same protocol with Sephacryl-blue affinity chromatography and specific elution from CM-Sephadex as key steps. No differences in elution profiles or V_{max} values were observed. In addition, both enzymes showed the same affinity for fructose 6-P and ATP, with K_m values of 2.5 ± 0.5 mM and 65 ± 15 μM, respectively, when 6-phosphofructo-2-kinase was assayed at saturating concentrations of ATP or fructose 6-P. Treatment with the C-subunit of cAMP-dependent protein kinase increased the V_{max} values of both enzymes 2-fold and incorporated 1.0 ± 0.1 and 0.9 ± 0.1 mol of phosphate/mol of subunit of the wild-type and His-404 mutant protein, respectively.

In contrast to its lack of effect on 6-phosphofructo-2-kinase activity, the Ser-404 → His-404 mutation greatly changed the reaction with fructose 2,6-P_2. Confirming earlier results (Paravicini & Kretschmer, 1992), there was no peptide labeling detectable in wild-type 6-phosphofructo-2-kinase incubated with [2-^{32}P]fructose 2,6-P_2. By contrast, the Ser-404 → His-404 mutant was readily labeled (Figure 3). As with bifunctional mammalian 6-phosphofructo-2-kinases/fructose-2,6-bisphosphatases, the ability to form a labeled peptide with

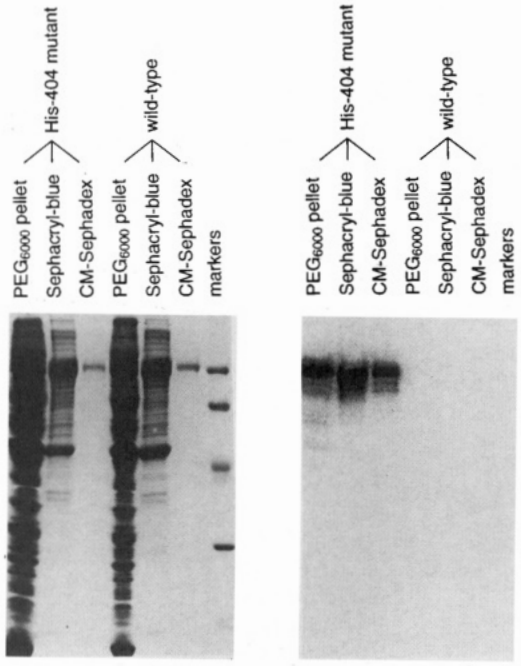

FIGURE 3: Purification and labeling with [2-^{32}P]fructose 2,6-P_2. (Left panel) Coomassie blue stain after SDS–PAGE of fractions from the purification of wild-type and His-404 mutant enzymes. Amounts of protein loaded for PEG_{6000} pellet, Sephacryl-blue pool, and CM-Sephadex pool were 250, 30, and 6 μg, respectively. Marker proteins were (top to bottom) phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa). (Right panel) Autoradiogram of the same gel after drying.

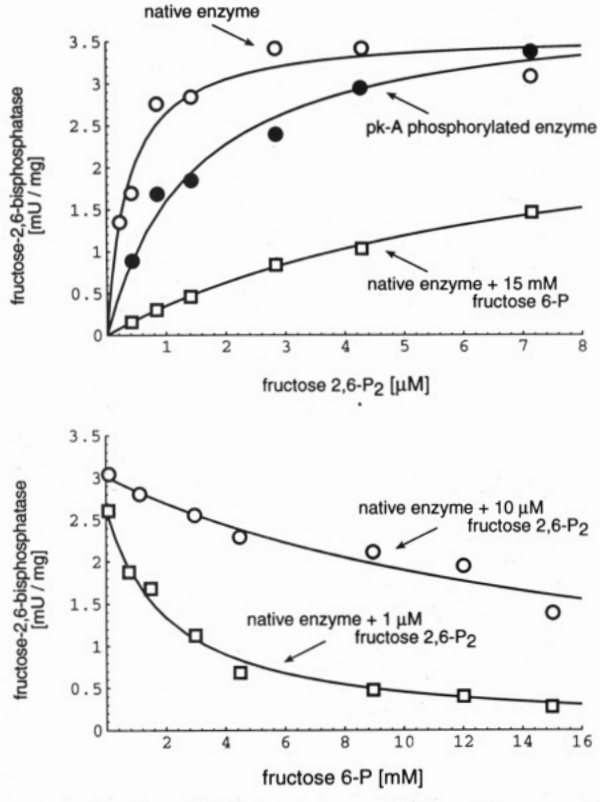

FIGURE 4: Fructose-2,6-bisphosphatase activity. (Upper panel) Substrate dependency of the mutant enzyme, untreated ("native") or treated with protein kinase A. (Lower panel) Inhibition by fructose 6-P.

[2-^{32}P]fructose 2,6-P_2 correlated with its newly gained fructose-2,6-bisphosphatase activity. The $K_{m\ F\text{-}2,6\text{-}P_2}$ (eq 1) for the latter activity of 0.4 ± 0.15 μM compared well with

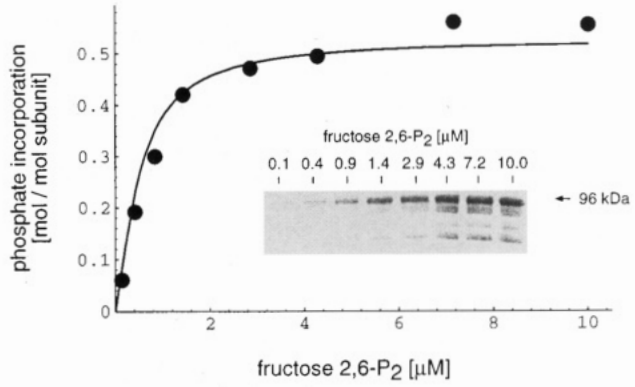

FIGURE 5: Phosphate labeling of the His-404 mutant enzyme as function of the fructose 2,6-P_2 concentration. Incubation was for 2 min with the indicated amounts of [2-^{32}P]fructose 2,6-P_2 (Materials and Methods). The insert shows the corresponding autoradiogram.

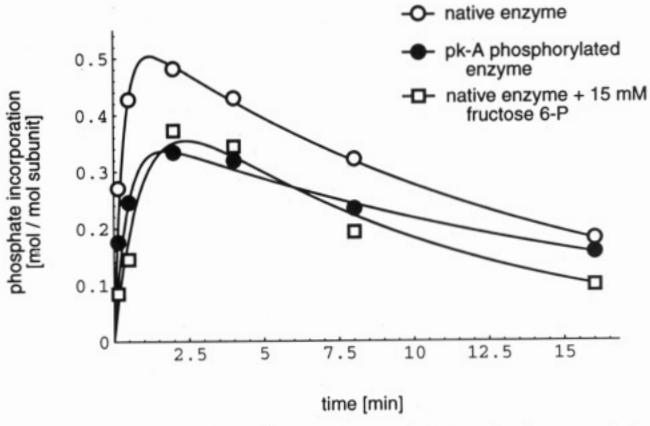

FIGURE 6: Time course of phospholabeling during the fructose-2,6-bisphosphatase reaction with the His-404 enzyme. Enzyme (0.3 μM) was incubated with 0.7 μM fructose 2,6-P_2 under the same conditions as in Figure 5. Curves were fitted as described (eqs 2–5, Materials and Methods) yielding:

$k_1 = k_2$ (μM^{-1} min^{-1})	k_3 (min^{-1})	
3.0 ± 0.5	0.2 ± 0.05	native enzyme
2.0 ± 0.6	0.2 ± 0.07	protein kinase A phosphorylated enzyme
0.6 ± 0.3	0.6 ± 0.3	nativeenzyme measured in the presence of 15 mM fructose 6-P

the concentration required for half-saturation in the labelling reaction with [2-^{32}P]fructose 2,6-P_2 (Figures 4 and 5). Fructose 6-P inhibited the activity competitively with its substrate [$K_{i\ F\text{-}6\text{-}P}$ = 0.6 ± 0.05 mM, eq 1] and reduced the amount of labeled protein formed (see Figure 6). Protein phosphorylation with the C-subunit of protein kinase A inhibited fructose-2,6-bisphosphatase by increasing its K_m value for fructose 2,6-P_2 to 1.5 ± 0.3 μM (Figure 4) and also reduced the formation of ^{32}P-labeled protein (Figure 6). When time curves of labeling with [2-^{32}P]fructose 2,6-P_2 were simulated by the solution of differential eqs 2–3 for a simplified Michaelis–Menten reaction scheme (eqs 4–5) (see Materials and Methods), reduced apparent rate constants for phospho-intermediate formation were obtained in the presence of fructose 6-P and with protein kinase A treated enzyme (Figure 6).

In contrast to the His-404 mutant protein, and confirming the separability in wild-type yeast of 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase activities (Kretschmer et al., 1987; François et al., 1988), the wild-type enzyme purified to homogeneity did apparently not hydrolyze fructose 2,6-P_2.

Table II: Purification of Wild-Type and His-404 6-Phosphofructo-2-kinase from Strain DFY658 Carrying the Plasmids pMK11 and pMK12, Respectively

	wild-type				His-404 mutant			
	protein (mg)	activity (units)	specific activity (units/mg)	purification (fold)	protein (mg)	activity (units)	specific activity (units/mg)	purification (fold)
PEG_{6000} pellet	533	56.0	0.1	1.0	396	25.1	0.1	1.0
Sephacryl-blue	32	53.6	1.7	15.9	24	20.2	0.8	13.3
CM-Sephadex	1.7	15.0	8.8	83.9	1.6	11.3	7.1	111.4

Table III: Amino Acids Known as Key Residues for Fructose 2,6-Bisphosphatase Activity of the Bifunctional Liver Enzyme[a]

		rat liver	yeast	
function in rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase		PFKFB1	PFK26	FBP26
interacts with the reactive C-2 phosphogroup of fructose 2,6-P_2	(i)	Arg-257	Arg-403	Arg-231
transiently phosphorylated in the fructose-2,6-bisphosphatase reaction	(ii)	His-258	Ser-404 (not phosphorylated)	His-232
as Arg-257, interacts with the reactive C-2 phospho group of fructose 2,6-P_2	(i)	Arg-307	Arg-474	Arg-282
maintains His-392 in protonated form	(iii)	Glu-327	Glu-497	Glu-302
part of the surface loop binding fructose 2,6-P_2	(iv)	Arg-352	Arg-522	Arg-327
	(v)	Lys-356	Lys-526	Lys-331
	(iv)	Arg-360	Arg-530	Arg-335
proton donor to leaving fructose 6-P	(ii)	His-392	His-565	His-367

[a] Data from (i) Lin, K., et al. (1992b), (ii) Tauler et al. (1990), (iii) Lin, K., et al. (1992a), (iv) Lin, L., et al. (1992b), (v) Lin, L., et al. (1992a). Corresponding residues for the yeast enzymes are according to sequence data for *PFK26* and *FBP26* (Kretschmer & Fraenkel, 1991; Paravicini & Kretschmer, 1992).

We earlier reported for partially purified enzyme a lower limit of 0.3 milliunit/mg which now, with the pure protein, is less than 0.05 milliunit/mg.

DISCUSSION

In order to study mutant 6-phosphofructo-2-kinase and labeling with fructose 2,6-P_2, an expression system in a background devoid of the kinase and of fructose-2,6-bisphosphatase was needed. Although this was not achieved with *E. coli* in the present work, the double null mutants, *pfk26*::*LEU2*, *fbp26*::*HIS3* in *S. cerevisiae* proved convenient for overexpression using *GAL1* promoter-driven plasmids. Expression from pMK11 of native 6-phosphofructo-2-kinase in amounts 600 times wild-type resulted in cellular concentrations of fructose 2,6-P_2 of 80 μM, which is ca. 40 times normal (Kretschmer & Fraenkel, 1991). Compared to strain DFY658 without plasmid, or with *PFK26* on single copy (pMK9 and pMK10), we observed a ca. 50% increased doubling time with multicopy plasmids pMK11 and pMK12 when induced on medium A containing 2% galactose. Since galactokinase was normally induced (Table I), this slow growth may be related to overexpression of 6-phosphofructo-2-kinase. The overexpressed wild-type and His-404 mutant 6-phosphofructo-2-kinase could be easily purified by a three-step procedure.

Table III lists amino acid residues known to play key roles in the fructose-2,6-bisphosphatase reaction of the bifunctional rat liver enzyme and the likely corresponding residues in the monofunctional enzymes 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase from yeast. Just two base pairs had to be changed in the *PFK26* gene for the monofunctional 6-phosphofructo-2-kinase to give the ability to also hydrolyze fructose 2,6-P_2 via a phosphoenzyme mechanism. The specific activity of the newly created function is in the range of 4 milliunits/mg protein, which compares to 20–40 milliunits/mg reported for purified mammalian enzymes (Kitamura et al., 1988; Kurland et al., 1992; Sakata et al., 1991; Ventura et al., 1992). Similar to those, the mutant enzyme has a low K_m (0.4 μM) for fructose 2,6-P_2 and is inhibited by fructose 6-P. Interestingly, this inhibition differs from the mammalian in being competitive with the substrate fructose 2,6-P_2 and, like fructose-2,6-bisphosphatase from spinach leaves (Macdonald et al., 1989), in requiring millimolar amounts of fructose 6-P for substantial inhibition of fructose-2,6-bisphosphatase activity.

c-AMP-dependent phosphorylation, presumably at C-terminal serine 645 in the only RRXS-type consensus sequence (Kretschmer & Fraenkel, 1991), causes 2–3-fold activation of the 6-phosphofructo-2-kinase activity (François et al., 1988; Kretschmer & Fraenkel, 1991). The phosphorylation increases the K_m value of the His-404 mutant enzyme for fructose 2,6-P_2 and, as does fructose 6-P, reduces the amount of phosphoprotein intermediate formed in the fructose-2,6-bisphosphatase reaction (Figure 6). These two effects resemble those on the wild-type heart enzyme which has a C-terminal consensus site for c-AMP-dependent phosphorylation (Kitamura & Uyeda, 1987; Kitamura et al., 1988; Sakata et al., 1991) and are opposite to liver where phosphorylation at a N-terminal site inhibits 6-phosphofructo-2-kinase (Van Schaftingen et al., 1981; El-Maghrabi et al., 1982; Furuya et al., 1982) and activates fructose-2,6-bisphosphatase (Van Schaftingen et al., 1982; Sakakibara et al., 1984; Stewart et al., 1986).

The finding that the Ser-404 → His-404 mutation exposes an active fructose-2,6-bisphosphatase site, without also influencing kinetics and activation by cAMP-dependent phosphorylation of 6-phosphofructo-2-kinase, suggests that yeast 6-phosphofructo-2-kinase, like the mammalian bifunctional enzymes (Bazan et al., 1989), may be the product of a gene fusion between kinase and phosphatase domains. However, a mutation in the active site has silenced its fructose-2,6-bisphosphatase activity. Our finding that several different wild-type yeast strains expressing the 96-kDa 6-phosphofructo-2-kinase did not contain a peptide of this size phosphorylated by fructose 2,6-P_2 (Paravicini & Kretschmer, 1992) excludes this serine mutation as being an artifact in the originally screened genomic library (Kretschmer et al., 1991). We do not know whether the silencing of the phosphatase domain of 6-phosphofructo-2-kinase represents an adaption to the specific

requirements of glucose metabolism in yeast. This question is particularly interesting in light of the seemingly opposite situation with yeast fructose-2,6-bisphosphatase, a peptide of 56 kDa, the only one phosphorylated by fructose 2,6-P_2 in wild-type yeast, which has no significant 6-phosphofructo-2-kinase activity and yet has the complete domain resembling the kinase part of the bifunctional mammalian enzyme. A mutational study of the lack of 6-phosphofructo-2-kinase in yeast fructose-2,6-bisphosphatase may also be informative.

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