Mutant studies of phosphofructo-2-kinases do not reveal an essential role of fructose-2,6-bisphosphate in the regulation of carbon fluxes in yeast cells

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The effect of the allosteric regulator fructose-2,6-bisphosphate (F2,6bP) on the regulation of carbohydrate metabolism was investigated *in vivo* with *Saccharomyces cerevisiae* mutants containing no, very high or unregulated 6-phosphofructo-2-kinase activity. Simultaneous overproduction of F2,6bP and 6-phosphofructo-1-kinase activity did not increase the glycolytic flux to ethanol. Overexpression of fructose-1,6-bisphosphatase during growth on glucose in a mutant strain devoid of F2,6bP did not cause pronounced effects on the cells. Moreover, high levels of F2,6bP during growth on ethanol in a strain with a highly active 6-phosphofructo-2-kinase enzyme did not affect either carbon flux to glycogen or growth rate. Site-directed mutagenesis of 6-phosphofructo-2-kinase (Pfk26) revealed that serine 644 is involved in the activation of Pfk26 by protein kinase A phosphorylation, but that, additionally, the enzyme can be further activated by phosphorylation of another amino acid residue. The results demonstrate that F2,6bP is not needed to sustain an adequate glycolytic flux under fermentative conditions, but rather is concerned with the homeostasis of metabolite concentrations. Moreover, they fail to indicate a physiological significance for inhibition of fructose-1,6-bisphosphatase by F2,6bP.

Keywords: *Saccharomyces cerevisiae*, fructose-2,6-bisphosphate, 6-phosphofructo-1-kinase, glycolytic flux, futile cycling

INTRODUCTION

Since its discovery fructose-2,6-bisphosphate (F2,6bP) has been proposed to be the predominant effector of carbohydrate metabolism in *Saccharomyces cerevisiae*. Yeast 6-phosphofructo-1-kinase (6PF-1-K) (EC 2.7.1.11) is activated and fructose-1,6-bisphosphatase (F-1,6-bPase) (EC 3.1.3.11) is inhibited *in vitro* by nanomolar concentrations of F2,6bP (Lederer *et al.*, 1981; Bartrons *et al.*, 1982; Noda *et al.*, 1984; François *et al.*, 1987; Van Schaftingen, 1987). In yeast cells

growing on respiratory carbon sources (gluconeogenic conditions), F2,6bP concentrations are low but increase rapidly after the addition of fermentable sugars. Therefore, F2,6bP is thought to be crucial for the activation of glycolysis and to be a main determinant in preventing the energy-wasting cycling between fructose-6-phosphate (F6P) and fructose-1,6-bisphosphate (F1,6bP) (Hers & Van Schaftingen, 1982; François *et al.*, 1987, 1988; Navas *et al.*, 1993).

In yeast cells, two isoenzymes of 6-phosphofructo-2-kinase (6PF-2-K) (EC 2.7.1.105) catalyse the synthesis of F2,6bP from F6P and ATP. One of these isoenzymes, encoded by the gene *PFK26* (Kretschmer & Fraenkel, 1991), is activated by protein kinase A phosphorylation, while synthesis of the second isoenzyme, encoded by the gene *PFK27* (Boles *et al.*, 1996), is induced by fermentable carbon sources. F2,6bP is degraded by a specific fructose-2,6-bisphosphatase, encoded by the

Abbreviations: F2,6bP, fructose-2,6-bisphosphate; 6PF-1-K, 6-phosphofructo-1-kinase; F-1,6-bPase, fructose-1,6-bisphosphatase; F6P, fructose-6-phosphate; F1,6bP, fructose-1,6-bisphosphate; 6PF-2-K, 6-phosphofructo-2-kinase.

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gene *FBP26* (Paravicini & Kretschmer, 1992), and by unspecific phosphatases.

Recently, it has been demonstrated that a yeast *pfk26 pfk27* double-deletion mutant strain that is unable to synthesize F2,6bP (Boles *et al.*, 1996) and a yeast mutant strain containing a 6PF-1-K insensitive to F2,6bP (Heinisch *et al.*, 1996) are able to grow normally on fermentable sugars and have flux rates for glucose utilization and ethanol production similar to a wild-type strain. These results question the role of F2,6bP as an important determinant of glycolytic flux regulation in *S. cerevisiae*. To further investigate the physiological relevance of F2,6bP in yeast carbon metabolism, we have constructed mutants affected in the amount and the control of 6PF-2-K, 6PF-1-K and F-1,6-bPase activity. Investigations with these mutant strains confirm that the level of F2,6bP does not regulate glycolytic flux rates during glucose fermentation and fail to indicate a major role for F2,6bP in the prevention of futile cycling of the 6PF-1-K/F-1,6-bPase enzyme pair.

METHODS

Strains, media and growth conditions. All yeast strains used in this study were derived from strains VW-1A (*MAT α leu2-3,112 ura3-52 trp1-289 his3- Δ 1 MAL2-8 $^{\circ}$ SUC2*), VW-1B (*MAT α leu2-3,112 ura3-52 trp1-289 his3- Δ 1 MAL2-8 $^{\circ}$ SUC2*) or the corresponding diploid strain (VW-1D) unless otherwise stated. The strains CEN.PK113-9D (*MAT α LEU2 ura3-52 trp1-289 HIS3 MAL2-8 $^{\circ}$ SUC2*) and CEN.PK113-3C (*MAT α LEU2 URA3 trp1-289 HIS3 MAL2-8 $^{\circ}$ SUC2*) (kindly provided by P. Kötter, Institut für Mikrobiologie, Frankfurt, Germany) are isogenic to strain VW-1A. Strains VW.EB-3A (*pfk26 Δ*), VW.EB-9A and 9B (*pfk1 Δ pfk26 Δ*), and VW.EB-13A and 13B (*pfk26 Δ pfk27 Δ*) were described previously (Boles *et al.*, 1996). Yeast cells were grown at 28 °C in batch cultures as described by Boles *et al.* (1996). *Escherichia coli* strains JM101 and SURE (Stratagene) were used for the propagation of plasmids.

Molecular biology techniques. DNA was prepared and manipulated as described by Sambrook *et al.* (1989). Yeast-specific techniques were as described by Guthrie & Fink (1991). DNA was transformed into yeast according to Schiestl & Gietz (1989) and *in vitro* mutagenesis was as described by Boles & Miosga (1995).

Construction of plasmids and mutant strains. Plasmid pAN11 containing the *FBP1* gene behind the *ADH1* promoter, plasmid pPFK-D1.2 containing the genes *PFK1* and *PFK2*, and plasmid pMK2 containing the *PFK26* gene were described by Navas *et al.* (1993), Schaaff *et al.* (1989), and Kretschmer *et al.* (1991), respectively. The wild-type and different mutant *PFK26* alleles obtained after *in vitro* mutagenesis and sequencing (Boles & Miosga, 1995) were recloned into YIplac211 (Gietz & Sugino, 1988), linearized with *EcoRV* and, after transformation, integrated once or severalfold, [$\times$], into the *ura3* locus of strain VW.EB-13A (*pfk26 Δ pfk27 Δ*), resulting in strains VW.EB-14B (*Pfk26^{Ser644}*), VW.EB-14C (*Pfk26^{Ala644}*), VW.EB-14D (*Pfk26^{Asp644}*), and VW.EB-14E (*[Pfk26^{Asp644}] $\times$*). An *EcoRI*/*SalI* digest of plasmid pIJdel2 (Boles *et al.*, 1993) was used to replace the majority of the *PFK2* gene in the strain VW-1B with the yeast *URA3* gene, resulting in strain VW.EB-2B (*pfk2 Δ*). This strain was crossed with strain VW.EB-3A (*pfk26 Δ*), the diploid strain was sporulated and the tetrads

dissected, resulting in the *pfk2 pfk26* double-deletion mutant strain VW.EB-5B (*MAT α pfk2 Δ ::URA3 pfk26 Δ ::LEU2*). VW.EB-5B and VW.EB-9B were crossed with VW.EB-13A and after sporulation they were used to construct the *pfk1 pfk26 pfk27* and *pfk2 pfk26 pfk27* triple-deletion mutants.

Determination of enzyme activities and metabolite levels.

Crude extracts of the cells were prepared according to Ciriacy & Breitenbach (1979). 6PF-2-K activity was assayed according to Kretschmer *et al.* (1991). F2,6bP concentrations were determined after 0, 3 and 5 min with pyrophosphate-dependent fructose-6-phosphate kinase from potato tuber (Sigma) as described by François *et al.* (1984). F-1,6-bP activity was determined according to Gancedo & Gancedo (1971) in 50 mM imidazole buffer pH 7.0, containing 10 mM MgCl₂, 100 mM KCl and 0.1 mM EDTA. 6PF-1-K activity was determined as described by Heinisch *et al.* (1989). The protein concentrations were determined by the microbiuret method (Zamenhoff, 1957) using bovine serum albumin as a standard. Metabolite extracts were prepared as described by Boles & Zimmermann (1993). F2,6bP concentrations were determined according to Van Schaftingen *et al.* (1982) and the concentrations of glycolytic metabolites were assayed enzymically (Bergmeyer, 1974). Yeast cultures to be used for glycogen determination were chilled quickly by addition of an equal volume of a methanol and H₂O mixture (1:1) at -50 °C. Cells were collected by centrifugation at 4 °C and washed three times with distilled water at 4 °C. Glycogen was determined after nitrogen starvation as described by Lillie & Pringle (1980). The experiments were repeated at least once, with similar results.

Determination of growth rates, glucose consumption and ethanol production.

A Beckman DU-40 spectrophotometer and 10 mm cuvettes were used for all spectrophotometric analyses. Growth rate, glucose consumption and ethanol production were determined as described previously (Boles *et al.*, 1996). The experiments were repeated at least once, with similar results.

RESULTS AND DISCUSSION

Increasing both F2,6bP concentrations and 6PF-1-K activity does not accelerate glycolytic flux

Although it has been thought for a long time that 6PF-1-K is the rate-limiting step of glycolysis, a more than threefold overexpression of 6PF-1-K did not cause an effect on the anaerobic glycolytic flux to ethanol (Heinisch, 1986; Schaaff *et al.*, 1989). However, Davies & Brindle (1992) observed that yeast cells overexpressing 6PF-1-K showed a compensatory decrease in the concentration of F2,6bP, which is a potent activator of 6PF-1-K. They proposed that this decrease could significantly lower the activity of 6PF-1-K *in vivo* and compensate for the increased amount of 6PF-1-K. Nevertheless, it has been demonstrated recently (Boles *et al.*, 1996) that a yeast mutant strain unable to synthesize F2,6bP had flux rates for glucose utilization and ethanol production similar to those of the wild-type strain, indicating that F2,6bP is dispensable *in vivo* for the activation of 6PF-1-K. To further investigate the role of F2,6bP in the regulation of glycolytic flux, we constructed a yeast strain that simultaneously overproduced F2,6bP and overexpressed 6PF-1-K. Plasmid YIplac-PFK26^{Asp644}

Table 1. Glucose-consumption rates and ethanol-production rates of the wild-type (VW-1A) and a mutant strain overproducing F2,6bP and 6PF-1-K (VW.SM-2A)

The cultures were pre-grown to the mid-exponential phase in synthetic complete medium with 2% glucose lacking leucine, and then washed once with and transferred to the same pre-warmed medium. The data presented are the means of two or three different experiments $\pm$ deviation from the mean or $\pm$ SD, respectively (also applies to Tables 2 and 3).

Strain	Genotype	F2,6bP*	6PF-1-K†	$V_{\text{Glc}}‡$	$P_{\text{EtOH}}§$
VW-1A	Wild-type	7.5 $\pm$ 1.0	240 $\pm$ 30	5.1 $\pm$ 0.5	8.0 $\pm$ 0.7
VW.SM-2A	YIplac-PFK26 ^{Asp644} pPFK-D1.2	17.4 $\pm$ 1.9	650 $\pm$ 50	4.8 $\pm$ 0.5	7.6 $\pm$ 0.6

* F2,6bP concentration in pmol (mg dry weight)⁻¹.

† 6PF-1-K activity in nmol min⁻¹ (mg protein)⁻¹.

‡ Glucose-consumption rate in mM h⁻¹ per OD₆₀₀ unit.

§ Ethanol-production rate in mM h⁻¹ per OD₆₀₀ unit.

encoding a constitutively activated Pfk26^{Asp644} enzyme (see below) was integrated severalfold into the genome of the wild-type strain VW-1A. A transformant producing high concentrations of F2,6bP was selected and transformed with multi-copy plasmid pPFK-D1.2 containing the two genes encoding the subunits of 6PF-1-K. Both the concentration of F2,6bP and the 6PF-1-K activity in this strain (VW.SM-2A) when grown in a synthetic medium with 2% glucose were elevated two- to threefold as compared with the corresponding wild-type strain VW-1A (Table 1). Nevertheless, glucose-consumption and ethanol-production rates of this strain were identical to the wild-type strain (Table 1), indicating that neither the concentration of F2,6bP nor the activity of 6PF-1-K was the rate-limiting factor of glycolytic flux.

These results together with our previous observations (Boles *et al.*, 1996) undermine the traditional role of the allosteric effector F2,6bP as a regulator of metabolic flux. Instead, they support the model that allosteric effects are more likely to be concerned with homeostasis of metabolite concentrations than with the control of metabolic fluxes (Fell & Thomas, 1995; Thomas & Fell, 1996).

The Pfk2 but not the Pfk1 subunit of 6PF-1-K is sensitive to the absence of F2,6bP

In contrast to *pfk1 pfk2* double mutants, which do not grow on glucose medium, *pfk1* or *pfk2* single mutants grew well on all kinds and concentrations of carbon sources, although no 6PF-1-K activity could be detected in crude extracts of these cells (Breitenbach-Schmitt *et al.*, 1984). It has been proposed that each of the subunits can serve both catalytic and regulatory functions (Arvanitidis & Heinisch, 1994; Heinisch *et al.*, 1996). To test whether both subunits are also equivalent with respect to their susceptibility to F2,6bP, *pfk1 pfk26 pfk27* and *pfk2 pfk26 pfk27* triple-deletion strains were constructed by crossing strain VW.EB-13A (*pfk26 pfk27*) with strains VW.EB-9B (*pfk1 pfk26*) and VW.EB-

5B (*pfk2 pfk26*), respectively, followed by sporulation and tetrad dissection. This analysis revealed that mutant cells containing only the PFK2-encoded subunit of 6PF-1-K but unable to produce F2,6bP (*pfk1 pfk26 pfk27*) no longer grew on a glucose-containing medium but did grow on an ethanol-containing medium, indicating that the Pfk2 subunit alone is not active or not stable in the absence of F2,6bP. In contrast, the PFK1-encoded subunit is not sensitive to the absence of F2,6bP as the growth rates of *pfk2 pfk26 pfk27* triple mutants on 0.1–2% glucose-containing media were indistinguishable from *pfk2* single mutants. These results indicate that although both the two 6PF-1-K subunits can serve catalytic and regulatory functions, the subunits are not completely equivalent.

The absence of F2,6bP in yeast cells overexpressing F-1,6-bPase does not have deleterious effects on the cells

F2,6bP is a strong inhibitor of the gluconeogenic enzyme F-1,6-bPase *in vitro*, (Gancedo *et al.*, 1982; Noda *et al.*, 1984). It has been argued that this very strong inhibition of F-1,6-bPase by F2,6bP is responsible for the prevention of futile cycling between F6P and F1,6bP in a strain expressing high levels of gluconeogenic F-1,6-bPase during growth on glucose (Navas *et al.*, 1993). Interestingly, an increase in glucose utilization and ethanol production of 10–20% could be observed in this strain.

We investigated whether high-level expression of F-1,6-bPase under glycolytic growth conditions in a *pfk26 pfk27* mutant strain without F2,6bP would impair growth and ethanol production due to the expected futile cycling or whether it was possible to increase glycolytic flux even more under such conditions. The multi-copy plasmid pAN11 (Navas *et al.*, 1993), carrying the yeast *FBP1* gene (encoding F-1,6-bPase) controlled by the strong *ADH1* promoter was transformed into the *pfk26 pfk27* double mutant. However, no significant differences in the generation times on glucose media between this strain and the corresponding wild-type

Table 2. Growth rate, ethanol-production rate, F-1,6-bPase activity and concentrations of F6P and F1,6bP in the wild-type strain CEN.PK113-3C, in CEN.PK113-9D transformed with pAN11, and in the corresponding *pfk26 pfk27* double-deletion strain

Cultures of the wild-type strain CEN.PK113-3C, this strain transformed with pAN11 (containing the *FBP1* gene behind the *ADH1* promoter), and the corresponding *pfk26 pfk27* double-deletion strain VW.EB-13A, lacking F2,6bP, were pre-grown to the mid-exponential phase in synthetic complete medium with 2% glucose lacking uracil, leucine and histidine, and then washed once with and transferred to the same pre-warmed medium. See Table 1 for details of reproducibility.

Strain	Plasmid	P_{EtOH}^*	F-1,6-bPase†	$\mu^\ddagger$	F6P§	F1,6 bP§
CEN.PK113-3C (wild-type)	–	12.6 ± 0.4	<2	0.38 ± 0.02	2.5 ± 0.1	4.6 ± 0.1
CEN.PK113-9D (wild-type)	<i>ADH1-FBP1</i> (pAN11)	14.3 ± 0.4	190 ± 10	0.41 ± 0.01	3.3 ± 0.2	5.7 ± 0.1
VW.EB-13A (<i>pfk26 pfk27</i>)	<i>ADH1-FBP1</i> (pAN11)	12.6 ± 0.5	230 ± 10	0.39 ± 0.01	5.4 ± 0.2	2.6 ± 0.3

* Ethanol-production rate in mM h⁻¹ per OD₆₀₀ unit.

† F-1,6-bPase activity in nmol min⁻¹ (mg protein)⁻¹.

‡ Maximum specific growth rate, μ_{max} (h⁻¹).

§ F6P and F1,6bP concentrations in nmol (mg dry weight)⁻¹.

strains were observed (Table 2), indicating either that F2,6bP inhibition of F-1,6-bPase is not essential for the growth and survival of the cells or that other inhibitors such as AMP effectively substitute for F2,6bP (Noda *et al.*, 1984). Moreover, it has been shown recently that the overexpression in yeast cells of a nearly uncontrolled bacterial mutant F-1,6-bPase caused cycling between F6P and F1,6bP of about 14% but did not cause deleterious effects to the yeast cells (Navas & Gancedo, 1996).

Nevertheless, the increase in the ethanol-production rate of the wild-type strain overexpressing F-1,6-bPase returned to normal levels in the mutant cells without F2,6bP and simultaneously overexpressing F-1,6-bPase (Table 2). This suggests that, at least under these specific conditions, activation of 6PF-1-K by F2,6bP is necessary to enhance glycolytic flux. Alternatively, it could be speculated that in the absence of F2,6bP inhibition the F-1,6-bPase contribution to a futile cycle is too high so flux is decreased back to the wild-type rate. Interestingly, the concentration of F6P was very high and that of F1,6bP was low (Table 2). This has already been observed in *pfk26 pfk27* double mutant cells not overexpressing F-1,6-bPase (Boles *et al.*, 1996). Therefore, the latter explanation is not very likely as it has been shown that a high rate of futile cycling in fact increases the fermentation rate (Navas & Gancedo, 1996).

Regulation of 6PF-2-K activity

It has been described that Pfk26 is activated by fermentable sugars via the RAS-adenylate cyclase signalling pathway (François *et al.*, 1984; Kretschmer & Fraenkel, 1991; Thevelein, 1991). The serine residue at

amino acid position 644 of Pfk26 has been thought to be phosphorylated by protein kinase A after glucose addition, leading to activation of the enzyme (Kretschmer & Fraenkel, 1991). To test this hypothesis, mutant alleles of *PFK26* at this position were constructed by site-directed mutagenesis as described by Boles & Miosga (1995). One of these alleles contained an alanine at amino acid position 644 (Pfk26^{Ala644}) instead of the wild-type serine residue. Another allele contained an aspartate at position 644 (Pfk26^{Asp644}). An alanine residue can no longer be phosphorylated by protein kinase A, and aspartate has been shown in some cases to mimic a phosphorylated serine residue (Marcus *et al.*, 1988). The wild-type and mutant alleles were stably integrated into the genome of the *pfk26 pfk27* double-deletion strain. In the strain carrying the wild-type allele, 6PF-2-K activity increased about fourfold 10 min after the addition of glucose to ethanol-growing cultures (Table 3). Similarly, 10 min after addition of 10 mM cAMP to a culture of an ethanol-growing *pde2* deletion strain, which is sensitive to externally added cAMP (Wilson *et al.*, 1993), 6PF-2-K activity increased about fourfold (data not shown). No increase in 6PF-2-K activity after glucose addition could be observed in the strain carrying the Pfk26^{Ala644} allele (Table 3). However, the basal activity during growth on ethanol was already approximately twofold higher as compared to the wild-type strain. 6PF-2-K activity in the cells containing the Pfk26^{Asp644} allele was already very high during growth on ethanol but, surprisingly, was still activatable after glucose addition (Table 3). Activity was even higher in cells containing several copies of the Pfk26^{Asp644} allele. Similar results were obtained after addition of cAMP to crude extracts prepared from the different strains grown on ethanol-containing medium (Table 3). These results

Table 3. Activation of wild-type and mutant forms of Pfk26 by glucose or cAMP

The different strains were grown overnight on a yeast extract/peptone medium with 1% ethanol to an OD₆₀₀ of 2–5. 6-PF-2-K activity was determined in crude extracts from these cultures (EtOH) and from the same cultures 10 min after the addition of 2% glucose. Additionally, 6PF-2-K activity was determined 5 and 10 min after the addition of 100 µM cAMP to crude extracts prepared after overnight growth on ethanol medium. ND, Not determined. See Table 1 for details of reproducibility.

Strain	Genotype	6-Phosphofructo-2-kinase activity [$\mu\text{U (mg protein)}^{-1}$]			
		EtOH	Glucose (10 min)	cAMP (5 min)	cAMP (10 min)
VW.EB-14B	Pfk26 ^{Ser644}	4.1 ± 0.5	16.2 ± 0.5	29.4 ± 1.0	29.5 ± 1.0
VW.EB-14C	Pfk26 ^{Ala644}	14.0 ± 2.9	9.1 ± 2.0	20.2 ± 1.9	26.2 ± 1.8
VW.EB-14D	Pfk26 ^{Asp644}	19.5 ± 0.5	30.2 ± 0.6	39.5 ± 1.0	42.0 ± 1.4
VW.EB-14E	[Pfk26 ^{Asp644}] _x	42.0 ± 1.6	44.2 ± 1.8	ND	ND

Fig. 1. F_{2,6}bP levels in strains containing the wild-type or mutant alleles of Pfk26 (VW.EB-14B: Pfk26^{Ser644}; VW.EB-14C: Pfk26^{Ala644}; VW.EB-14D: Pfk26^{Asp644}; and VW.EB-14E: [Pfk26^{Asp644}]_x) after overnight growth on a yeast extract/peptone medium with 1% ethanol and at different times after the addition of 2% glucose to the cultures.

indicate that serine 644 is involved in the activation of Pfk26 by protein kinase A phosphorylation. Additionally, the enzyme can be further activated by phosphorylation of another amino acid residue. However, the second activation step is dependent on the efficient phosphorylation of serine 644.

The different enzymic activities of the mutant Pfk26 alleles were reflected in the levels of F_{2,6}bP after growth on an ethanol medium and after the addition of glucose (Fig. 1). In the wild-type strain the F_{2,6}bP concentration was very low after growth on an ethanol medium but increased to about 5 pmol (mg dry weight)⁻¹ 15 min

after glucose addition, whereas it had already reached levels of about 6 pmol (mg dry weight)⁻¹ during growth on ethanol in the strain containing several copies of the highly active Pfk26^{Asp644} allele. On the other hand, intermediate levels of F_{2,6}bP were observed in the strains containing one copy of the Pfk26^{Asp644} or the Pfk26^{Ala644} allele (Fig. 1). Surprisingly, in the case of the strain with one Pfk26^{Asp644} allele, F_{2,6}bP levels decreased after glucose addition although this mutant enzyme is more active than the wild-type form *in vitro*. This observation suggests that *in vivo* additional regulatory mechanisms which are also affected by the amino acid exchange might operate on 6PF-2-K activity.

In contrast to our results, it has been reported recently (Keßler & Eschrich, 1996) that serine 644 is not involved in cAMP-dependent phosphorylation of yeast Pfk26 but is important for catalytic activity. Keßler & Eschrich (1996) also used a mutant allele of Pfk26 that had substituted serine 644 for alanine. We do not understand the reasons for the discrepancies between that and our work. However, in contrast to our work, which uses genomically integrated single copies of the different mutant alleles, the study of Keßler & Eschrich (1996) used high-copy expression vectors and a nearly thousandfold overexpression of 6PF-2-K enzyme activity, which especially in the case of a tightly regulated enzyme like Pfk26 may cause misleading results.

Increased levels of F_{2,6}bP do not affect F-1,6-bPase activity *in vivo*

To test the physiological relevance of inhibition of F-1,6-bPase by F_{2,6}bP, we investigated the mutant strain containing several copies of the highly active Pfk26^{Asp644} allele and exhibiting high concentrations of F_{2,6}bP [about 6 pmol (mg dry weight)⁻¹] even under gluconeogenic conditions during growth on ethanol (Fig. 1). From data obtained *in vitro* (Noda *et al.*, 1984; Marcus *et al.*, 1988) it can be calculated that such high concentrations of F_{2,6}bP should inhibit F-1,6-bPase

activity nearly completely. However, growth rates of the mutant strain on synthetic or yeast extract/peptone medium with 3 % ethanol as the sole carbon source were identical to those of the wild-type strain. Moreover, we could not observe an increase in the concentration of F1,6bP or a decrease in the levels of glucose-6-phosphate, F6P or glycogen in the mutant strain as compared to the wild-type strain during growth on a yeast extract/peptone medium with 3 % ethanol, as would have been expected if F-1,6-bPase was inhibited by the high concentrations of F2,6bP. Therefore, our results indicate that F2,6bP does not inhibit F-1,6-bPase significantly *in vivo*. This discrepancy from the *in vitro* measurements might be explained by assuming that other regulatory mechanisms counteract the effect of F2,6bP. Alternatively, it would be tempting to speculate that F2,6bP is not freely available in the cytosol but is restricted to specific compartments (Müller *et al.*, 1996) and therefore is not accessible to F-1,6-bPase *in vivo*.

On the other hand, it must be concluded from these data that F2,6bP per se is not able to prevent the energy-wasting futile cycling during the transition from gluconeogenic to fermentative conditions until F-1,6-bPase is inactivated and its synthesis is repressed (Entian & Barnett, 1992). However, it has been shown that inhibition of F-1,6-bPase by F2,6bP is synergistic with that of AMP and is enhanced after phosphorylation of the enzyme (Noda *et al.*, 1984; Marcus *et al.*, 1988). Therefore, it seems that under normal conditions all three inhibitory mechanisms must collaborate to inhibit F-1,6-bPase activity efficiently.

When compared to the wild-type cells, the VW.EB-14E mutant cells containing high concentrations of F2,6bP in an ethanol medium seemed to adapt better to a shift from an ethanol medium to a glucose medium; however, this observation was difficult to quantify. This observation is in accordance with the opposite finding that a strain without F2,6bP exhibited a delay in growth, glucose consumption and ethanol production after such a shift (Boles *et al.*, 1996). During the exponential growth phase in a yeast extract/peptone medium with 2 % glucose, the growth rate of strain VW.EB-14E was identical to that of the wild-type strain ($\mu = 0.56 \text{ h}^{-1}$). Similarly, glycogen accumulation after nitrogen starvation of this strain was comparable to that of the wild-type strain when incubated in synthetic medium containing 3 % glucose but with no source of nitrogen [13.6 and $13.2 \text{ } \mu\text{g (mg wet weight)}^{-1}$, respectively, after 24 h].

According to our results, presented here and elsewhere (Boles *et al.*, 1996; Heinisch *et al.*, 1996), it could be suggested that one 6PF-2-K enzyme with a high, constitutive activity should be enough to fulfil all the demands of the yeast cells. Why then did *S. cerevisiae* develop such an elaborate system for regulation of its F2,6bP concentrations? The answer is difficult but one could imagine that precisely adjustable levels of F2,6bP might be favourable to the cells in specific natural situations which do not occur normally under laboratory conditions or are difficult to quantify.

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