Particulate phosphofructokinase of yeast: physiological studies

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1. INTRODUCTION

A particulate phosphofructokinase has been reported in *Saccharomyces cerevisiae* [1]. It catalyses the phosphorylation of fructose 6-phosphate using ATP as the phosphate donor in accordance with the following reaction:

Fructose 6-phosphate + ATP →
Fructose 1,6-diphosphate + ADP

This enzyme serves a glycolytic function and allows yeast mutants *pfk1* lacking the soluble allosteric enzyme phosphofructokinase I to grow on glucose. Two unlinked nuclear genes *PFK2* and *PFK3* specify the particulate enzyme, called phosphofructokinase II. Gene *PFK2* specifies a soluble protein which is also responsible for conferring the property of ATP inhibition on the allosteric enzyme, phosphofructokinase I [2].

Here, we describe certain physiological characteristics of the particulate enzyme, phosphofructokinase II. Of the two gene products constituting this enzyme, one, the soluble component β [2] is made constitutively while the particulate component γ is made only in response to growth of yeast on sugars. We further demonstrate that the activity of phosphofructokinase II varies during growth.

2. MATERIALS AND METHODS

2.1. *Strains*

The nonsense mutant allele *pfk1–1* completely lacking the soluble allosteric phosphofructokinase I has been described [1]. Another allele *pfk1–7* was also used for centrifugation experiments. This was a missense mutant with no detectable activity of phosphofructokinase in soluble supernatants. The strains were grown aerobically with shaking at 30°C in YEP medium containing 0.3% yeast extract and 1% peptone using either ethyl alcohol or glucose as a carbon source. Cultures were tested for phosphofructokinase activity to see if any reversion had occurred during growth.

2.2. *Enzyme assays*

Aliquots of growing cultures were centrifuged, cells washed in 150 mM KCl and disrupted with toluene. The particulate phosphofructokinase was assayed in toluene lysates by monitoring the rate of NADH oxidation on a recorder. The soluble enzyme was assayed in cell-free extracts by the same method. Details have been described [1].

For quantitative measurement of the activity of β, the *PFK2* gene product, a fixed amount of the catalytic subunit α from a *PFK1 pfk2* strain was incubated at 0°C for 15 min with an aliquot of the fraction containing the β-subunit [2]. The incubated mixture was assayed for phosphofructokinase activity using 1 mM fructose 6-phosphate and 4 mM ATP.

2.3. *Other methods*

The procedure in [3] was followed for the sedimentation velocity experiments. Enzyme solutions were centrifuged in a 5–30% sucrose gradient (15 ml) using a SW41 rotor in a Beckman centrifuge model L5-50 at 30 000 rev./min for 16.5 h

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at 5°C. β-Galactosidase from *Escherichia coli* and pyruvate kinase from rabbit muscle were used as gradient markers corresponding to $s_{20,w}$-values of 16.0 [4] and 10.0 [5], respectively.

For the assay of glycolytic activity, aliquots of washed cell suspensions were examined periodically in neutralised perchloric acid extracts for glucose consumed and alcohol produced [6]. The suspensions were bubbled with either N_2 or O_2 gas containing 5% CO_2 during the incubation.

3. RESULTS

3.1. *Glycolytic behaviour of* pfk1 *mutants in exponential and stationary cultures*

Toluenized cells from alcohol-grown cultures of *S. cerevisiae* were found to have no phosphofructokinase II activity [1]. To see if this reflected absence of the enzyme in vivo, we examined the glycolytic capacity in intact cells. Buffered suspensions of stationary cultures of *pfk1* mutants neither ferment glucose nor produce ethanol during anaerobic incubation. Aerobically, glucose is utilised but no alcohol is made. Results in fig.1 illustrate this for the nonsense mutant *pfk1–1*. The rate of aerobic glucose consumption by the stationary-phase cells was ~2.5 μmol glucose · min^{-1} · g wet $yeast^{-1}$, while no alcohol formation could be detected. When the same experiment was repeated with cells in the exponential phase of growth (lower panel, fig.1), the *pfk1* mutant displayed a much higher glycolytic activity both in regard to the utilisation of glucose and formation of ethanol. The anaerobic rates were 8.8 μmol glucose consumed and 9.4 μmol alcohol produced · min^{-1} · g^{-1}. During aerobic conditions glucose utilisation was stimulated while the rate of alcohol formation slowed down. The same result was obtained with several other isolates of *pfk1* mutation.

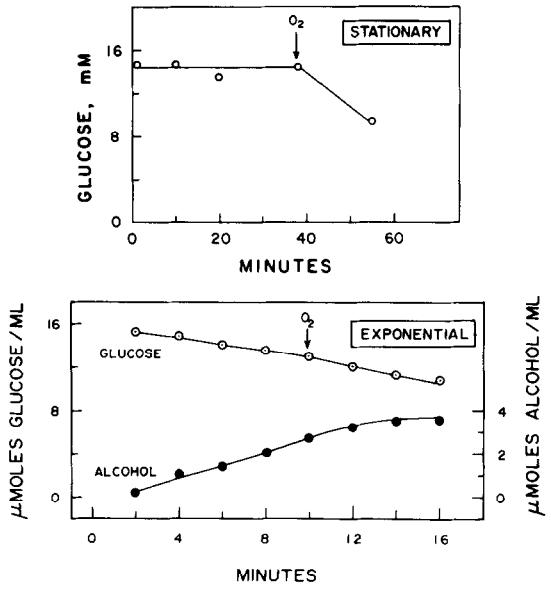

Fig.1. Anaerobic and aerobic glycolysis in *pfk1* mutant. The strain *pfk1–1* was grown in YEP medium containing 50 mM glucose for 64 h (stationary) or for 19 h (exponential). Washed cells were suspended in 50 mM potassium phosphate buffer (pH 7.4) and N_2 gas was passed for 5 min before 15 mM glucose was added. At the instants indicated by the vertical arrowheads N_2 gas was replaced with O_2. The suspensions contained: upper panel, stationary phase cells, 130 mg wet yeast/ml; lower panel, exponential phase cells, 34 mg/ml.

The failure of stationary cultures of *pfk1* mutant to produce alcohol was found to be strongly correlated with the absence of the particulate phosphofructokinase in such cultures. Cells growing exponentially on glucose had in contrast, a high activity of this enzyme. Growth on gluconeogenic substrates such as ethanol or acetate failed to induce the enzyme in stationary as also in exponential phases; this was paralleled by their inability to produce ethanol from sugars. A similar attenuation of glycolytic flux was observed in stationary cultures of *pfk1* mutant [7].

3.2. *Particulate phosphofructokinase activity requires both* PFK2 *and* PFK3 *gene products*

A mutation in either of the genes *PFK2* or *PFK3* prevents *pfk1* mutants from growing on glucose [1]. That this is due to the loss of the particulate activity can be seen by measuring the enzyme in presence of high concentrations of ATP. The phosphofructokinase activity of a wild-type strain grown on glucose and assayed in toluene lysates using 1 mM fructose 6-phosphate and 4 mM ATP was due entirely to the particulate enzyme; alcohol-grown cells or centrifuged supernatants of glucose-grown crude extracts showed no PFK I activity under the same conditions, due to inhibition by ATP. When the single-gene mutant *PFK1 PFK2 pfk3* was so tested after growth on glucose, the ATP-insensitive activity was extinguished. Phosphofructokinase II activity segregated as $2^+:2^-$ in 13 four-

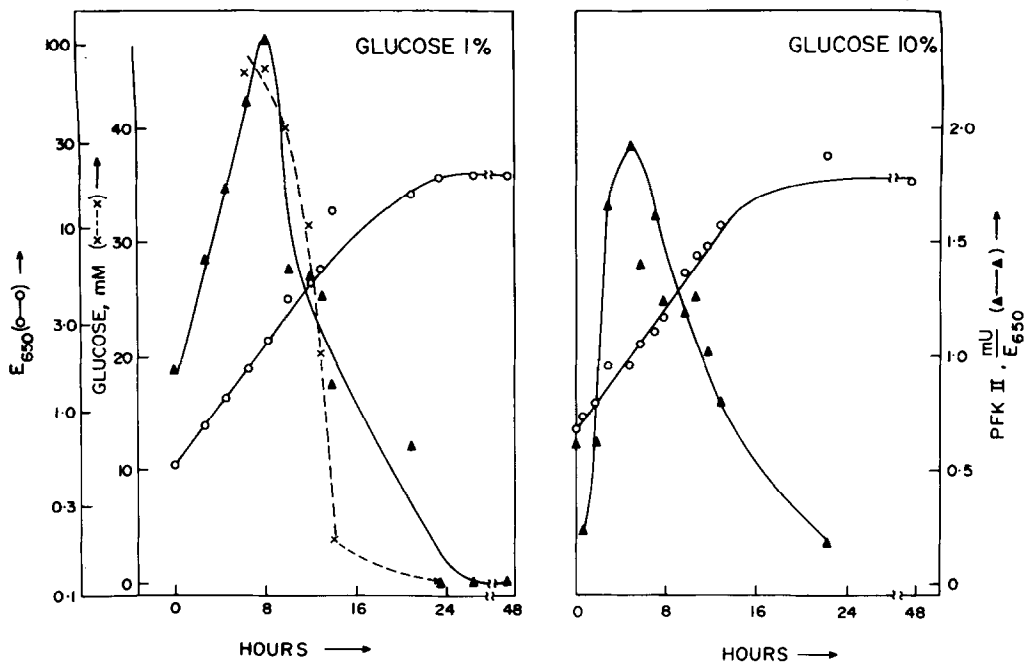

Fig.2. Variation of phosphofructokinase II activity during growth on glucose. The nonsense mutant *pfk1–1* was inoculated in YEP medium supplemented with 1% (left) or 10% (right) glucose and the growth kinetics followed by measuring E_{650}, absorbance at 650 nm in a 1 cm path. Aliquots of the growing cultures were examined for the particulate phosphofructokinase (PFK II) in toluene lysates and the results expressed as specific activity (mU/E_{650} referring to milliunits PFK II per E_{650}). The discontinuous line in the left panel shows the glucose remaining in the centrifuged supernatants.

spore tetrads obtained from the sporulation of a diploid heterozygous for *pfk3*. This showed that the product of the gene *PFK3* is required for expression of the particulate phosphofructokinase.

To show that the gene *pfk2* contributed to the particulate enzyme activity a glucose-positive diploid $\frac{pfk1\ PFK2\ pfk3}{pfk1\ pfk2\ PFK3}$ was constructed by mating two haploids both of which were unable to grow on glucose and failed to show the enzyme activity. The diploid in contrast had detectable activity of the particulate phosphofructokinase. About 25% of the meiotic progeny (6 out of 28 spores) was positive both for the enzyme activity as also growth on glucose. This showed that *PFK2* was the other determinant of the particulate enzyme activity.

3.3. *Synthesis and decay of particulate phosphofructokinase during growth*

An overnight culture of *pfk1* mutant growing on glucose in YEP medium was examined periodically for the particulate phosphofructokinase activity (fig.2). The specific activity of the enzyme kept on increasing until the culture density reached $E_{650} \simeq 2$; henceforth the specific activity of the enzyme started decreasing until it became almost undetectable towards the end of exponential growth. Of particular interest is the observation that the onset of the decreasing specific activity was independent of the initial concentration of glucose in the medium. That is, the presence of glucose was not a sufficient condition to maintain enzyme activity. Separate experiments (not shown) indicated that exhaustion of glucose followed, rather than preceded the complete decay of the enzyme. The particulate phosphofructokinase activity is thus only transiently expressed during growth of *S. cerevisiae* on sugars.

3.4. *Constitutive synthesis of β, the* PFK2 *gene product*

Washed cells of an alcohol-grown stationary cul-

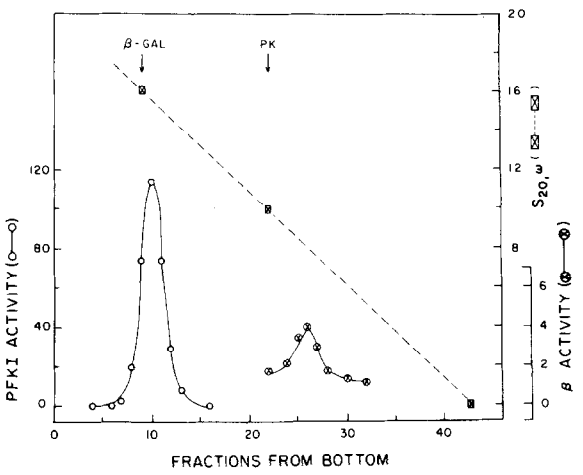

Fig.3. Separation of excess regulatory subunit β from the soluble, allosteric phosphofructokinase I in alcohol-grown cells of a wild-type strain of *S. cerevisiae*. Crude cell-free extract from a *PFK1 PFK2* strain was centrifuged in a sucrose density gradient as in section 2. The direction of sedimentation was from right to left. Fractions were analysed for soluble phosphofructokinase (PFK I) activity using 1 mM fructose 6-phosphate and 0.5 mM ATP (o—o). The peak corresponding to the β-subunit was scanned by mixing 25 μl of these fractions with 2 mU of a non-allosteric phosphofructokinase I from a *PFK1 pfk2* strain as in [2], and determining the extent of inhibition by 4 mM ATP. The activity of β is plotted as the ratio of phosphofructokinase activities in absence of β fractions to that in its presence.

ture of a wild-type strain of *S. cerevisiae* were broken with a French press in a medium containing 50 mM potassium phosphate (pH 7.4) and 2 mM each of 2-mercaptoethanol, EDTA and phenylmethane sulfonyl fluoride. The cell-free supernatant was centrifuged in a sucrose density gradient and the fractions analysed for the activities of phosphofructokinase I as also of β subunit as in fig.3. The results show the presence of two separate peaks; the heavier one with $s_{20,w} = 15.5$ corresponded to the partially proteolysed form of the phosphofructokinase I oligomer $\alpha_4\beta_4$ [8]; the lighter β peak had an $s_{20,w} = 8.1$ as had been seen earlier in a *pfk1 PFK2* strain [2]. When instead of the wild-type strain, a missense mutant of α (*pfk1–7 PFK2*) was used, two distinct β peaks were resolved, one at $s_{20,w} = 16$ and another at about $s_{20,w} = 8$ (not shown). The subunit β produced by the gene *PFK2* was thus present in excess over the amount required to form a 1:1 complex $\alpha_4\beta_4$ with the catalytic subunit α specified by the gene *PFK1*. Under the particular condition of growth using ethanol as a carbon supplement no particulate phosphofructokinase II activity was detectable. This suggested that the product γ of the gene *PFK3* was lacking in alcohol cultures. The appearance of the particulate phosphofructokinase activity during growth on glucose is therefore likely to reflect the presence of the *PFK3* gene product.

4. DISCUSSION

Although phosphofructokinase II functions as a glycolytic enzyme [1], it is distinct from all others in regard to its appearance during exponential growth on glucose and its absence in stationary cultures on glucose and in alcohol cultures, exponential or stationary. During the growth of yeast on gluconeogenic compounds all the glycolytic enzymes are present while glucose causes their enhanced synthesis [6]. Particulate phosphofructokinase activity in contrast is completely absent during growth on alcohol and is present only when yeast is grown on sugars. Assuming that the enzyme is made of two subunits β and γ specified by the genes *PFK2* and *PFK3*, respectively, it is only the particulate component γ that requires glucose for its appearance since the β subunit is present constitutively, regardless of carbon source or stage of culture. A more accurate method of assay of the β subunit would be required in order to test whether presence of γ helps to reduce substantially the β subunit level in the cytoplasm. Because phosphofructokinase I is an allosteric enzyme, the intracellular concentration of the β subunit is expected to be one of the principal determinants of the glycolytic rate in vivo.

The spontaneous decay in the activity of phosphofructokinase II during growth is another unique characteristic of this enzyme. The specific activity of the enzyme reaches a maximum when the culture attains ~ 10% of the saturating growth and falls sharply thereafter. The decay begins very much before any appreciable decrease has taken place in the level of glucose in the medium. The drop of specific activity is not simply due to a ces-

sation of synthesis in the growing culture as an examination of the total activity of phosphofructokinase II indicates loss of enzyme activity with time at a progressively accelerating rate. This suggests the decay to be an active process. The observation that an inhibitor of protein synthesis such as cycloheximide prevents this fall of the particulate phosphofructokinase activity reinforces this conclusion. The continued presence of the β subunit in soluble supernatants from stationary cultures of glucose-grown or alcohol-grown cells of *S. cerevisiae* lends strong support to the idea that the activity of phosphofructokinase II reflects the steady state level of the γ subunit.

The appearance of the particulate phosphofructokinase during growth of yeast on glucose and the decay of the enzyme activity while glucose continues to be present suggests that the catalytic role may not be its only function in glycolysis.

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