GENETIC EVIDENCE FOR DISTINCT CATALYTIC AND REGULATORY SUBUNITS IN YEAST PHOSPHOFRUCTOKINASE

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1. Introduction

Phosphofructokinase has been implicated as the rate-determining enzyme in glycolysis in diverse systems [1]. One of its substrates (ATP) inhibits the enzyme allosterically by decreasing the affinity for the other substrate (fructose 6-phosphate). Is this mediated through a distinct regulatory subunit in the manner of aspartate transcarbamylase [2]? In [3] the yeast enzyme was suggested to be composed of separate catalytic and regulatory subunits on the basis of its profile of binding to nucleotide effectors. In [4] this conclusion was reinforced from studies on the protection afforded by fructose 6-phosphate to the sulphydryls of only one of the constituent polypeptides of the enzyme.

We present here genetical evidence of the existence of a regulatory subunit* of phosphofructokinase from *Saccharomyces cerevisiae* and demonstrate its interaction with the catalytic subunit. Of particular interest is the observation that the gene coding for the regulatory subunit is also involved in specifying the reported particulate phosphofructokinase from this organism [5].

2. Materials and methods

The mutant allele *pfk1-1* is an ochre-suppressible nonsense mutation [6]. A second class of mutations *pfk2* renders *pfk1* mutants glucose-negative [5]. Two alleles of this mutation have been used in this study, *pfk2-1* and *pfk2-2*, the latter being temperature-sensitive for growth on sugars in a background of *pfk1* negativity. The double mutant using the other allele, viz. *pfk1-1 pfk2-1*, is unable to grow on glucose both at 23°C and at 36°C. Strains were grown on yeast extract (0.3%)–peptone (1%) medium containing 150 mM ethanol alcohol on a rotary shaker at 30°C unless stated otherwise. Fructose 2,6-bisphosphate was a kind gift from Dr E. van Schaftingen. Fructose 6-phosphate was from Sigma. All other enzymes and substrates were from Boehringer.

*The genetic symbols used are: *PFK1* and *pfk1*, the wild-type and mutant forms, respectively, of the catalytic subunit of the soluble, allosteric phosphofructokinase; *PFK2* and *pfk2*, the wild-type and mutant alleles of the particulate phosphofructokinase that is also the regulatory determinant for the soluble enzyme

3. Results

A large number of mutants of *S. cerevisiae* lacking the soluble phosphofructokinase have been isolated in this laboratory. The majority of these arose as suppressor mutations allowing the growth of pyruvate kinase mutants on alcohol + glucose [7]. They had <1% of the wild-type enzyme activity in the soluble supernatants. All of them define a single complementation group *PFK1*. These *pfk1* mutants are glucose-positive since yeast has another phosphofructokinase that is particulate [5]. Two unlinked genes *pfk2* and *pfk3* are involved in the synthesis of this particulate phosphofructokinase rendering the double mutants *pfk1 pfk2* or *pfk1 pfk3* glucose-negative.

When the double mutant *pfk1 pfk2* was crossed to a wild-type strain the generation of sugar-positive recombinant classes carrying each of the single mutations was signalled by the appearance of $3^+:1^-$ and $4^+:0^-$ tetrads on glucose plates. Results in table 1 illustrate this in respect of spores from a tetratype

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Table 1
Meiotic segregation of catalytic and regulatory subunits of the soluble phosphofructokinase from yeast

Spore	Genotype		Growth on glucose	Phospho-fructokinase activity (milliunits/mg protein)		
	PFK1	*PFK2*		0.2 mM ATP	4 mM ATP	2,6-FDP
A	+	+	+	10	0	45
B	–	–	–	0	0	0
C	+	–	+	80	140	150
D	–	+	+	0	0	0

A glucose-negative double mutant *pfk1-1 pfk2-1* having neither the soluble nor the particulate phosphofructokinase was crossed to a wild-type strain positive for both the enzymes, the diploid sporulated and a tetratype tetrad analysed for the soluble phosphofructokinase. The spores were grown in alcohol until the end of log phase, washed cells suspended in 50 mM potassium phosphate (pH 7.4), 5 mM 2-mercaptoethanol and 2 mM EDTA and extracted with a French press. The centrifuged supernatants were used directly as the source of the enzyme in 0.8 ml assay mixture containing 0.5 mM fructose 6-phosphate, ATP at the indicated concentrations, 0.03 M NADH, aldolase, α-glycero-P dehydrogenase, triose-P-isomerase and 2–10 milliunits of phosphofructokinase in 50 mM triethanolamine buffer (pH 7.4), 20 mM KCl and 10 mM $MgCl_2$; 5 μM fructose 2,6-diphosphate (2,6-FDP) was added after the velocity with 4 mM ATP had reached a constant value

ascus from such a cross. Except spore B which was negative for both the soluble and the particulate phosphofructokinases all the other 3 spores were glucose-positive. Spore A was typical of a wild-type character, having the allosteric phosphofructokinase that was completely inhibited by high concentrations of ATP. Fructose 2,6-diphosphate reversed this inhibition [8]. Having inherited the mutant allele *pfk1* of the soluble phosphofructokinase, spore D was negative for this enzyme, but grew on glucose by virtue of the particulate phosphofructokinase. The other glucose-positive spore C did not have the wild-type allele of the gene *pfk2* as was indicated by:

(i) The appearance of $3^+:1^-$ and $2^+:2^-$ tetrads on glucose plates after it was crossed to a soluble phosphofructokinase-negative strain *pfk1*;
(ii) The expected $2^+:2^-$ segregation for glucose positivity when it was crossed to the double mutant *pfk1 pfk2*.

Spore C synthesized the soluble phosphofructokinase but unlike the enzyme from spore A, it was not inhibited by 4 mM ATP or 2 mM citrate, was stable to heating at 44°C and had on an average 2–3-times the specific activity as the allosteric enzyme. Under these assay conditions it was however, partly stimulated by fructose 2,6-diphosphate. Based on the above method of assigning spore genotypes, the allosteric and the non-allosteric phosphofructokinases showed the following pattern of segregation in 12 complete tetrads: 1 PD, 2 NPD and 9 T asci. The same result was obtained independent of whether the parental configuration in the diploid was

$$\frac{pfk1\ pfk2}{+\quad +} \text{ or } \frac{pfk1\quad +}{+\quad pfk2}$$

Fig.1 shows the effect of ATP on the velocity of the soluble phosphofructokinase from spores A and C. The enzyme from the wild-type spore A was inhibited sharply by increasing concentrations of ATP while that from spore C ($PFK1^+\ pfk2^-$) was not inhibited. When the concentration of fructose 6-phosphate was varied keeping that of ATP fixed at 2 mM, the enzyme from spore A displayed the typical sigmoidal response while that from C presented a hyperbolic profile. Spore C thus appeared to synthesize a non-allosteric phosphofructokinase.

We then examined if the loss of allosteric behaviour in the enzyme from the spore $PFK1^+\ pfk2^-$ could be restored by mixing with it the extracts of $pfk1^-\ PFK2^+$. Based on preliminary experiments on mixing, an $(NH_4)_2SO_4$ fraction from spore D extracts was

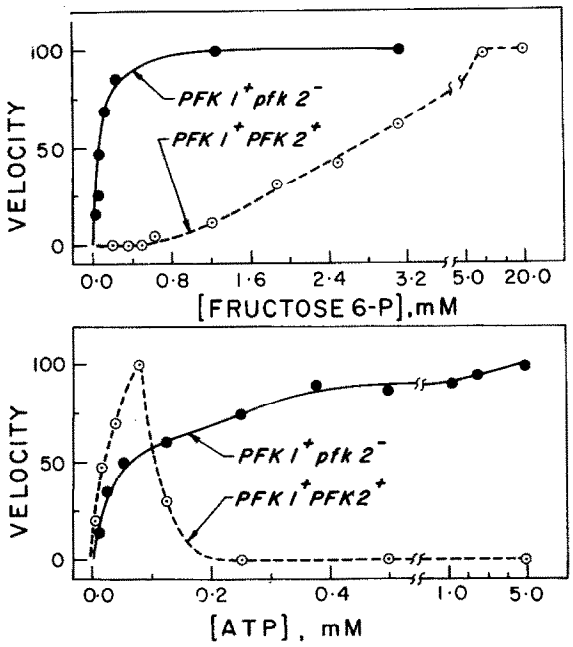

Fig.1. Effect of fructose 6-phosphate and ATP on the velocity of the soluble phosphofructokinase. The enzyme in the centrifuged supernatants from spores A and C (table 1) was precipitated in presence of 2 mM phenylmethane sulfonyl fluoride with ammonium sulphate (35–55% saturation), dissolved in the buffer mentioned in table 1 and used directly. Experimental details were the same as in table 1. The curve labelled *PFK1*$^+$ *PFK2*$^+$ refers to the enzyme from spore A and that labelled *PFK1*$^+$ *pfk2*$^-$ to that from spore C. The fixed concentration of ATP in the experiments in the upper figure was 2 mM and that of fructose 6-phosphate in the lower figure was 1 mM.

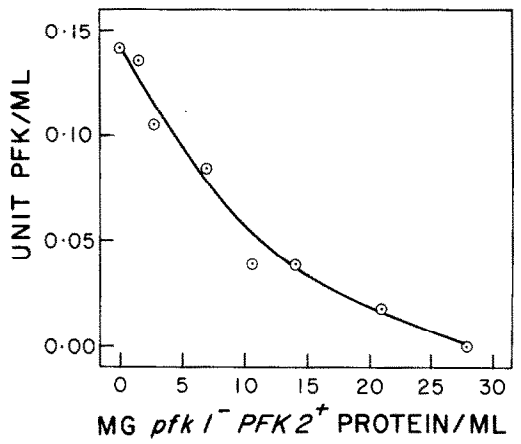

Fig.2. Restoration of ATP inhibition in phosphofructokinase from the *PFK1*$^+$ *pfk2*$^-$ strain by addition of extracts from a *pfk1*$^-$ *PFK2*$^+$ strain. An $(NH_4)_2SO_4$ fraction from crude extracts of the spore C precipitating between 35–55% saturation was the source of the phosphofructokinase and a similar fraction from spore D precipitating between 50–70% saturation the source of the regulatory subunit. Varying amounts of the latter (0–28 mg protein) were incubated with a fixed amount of the former (0.5 mg protein) in an ice bath for 30 min in 0.2 ml total vol. and aliquots were assayed as in table 1 using 1 mM fructose 6-phosphate and 2 mM ATP.

prepared as the source of the regulatory subunit. Results in fig.2 show that the extract from the strain *pfk1*$^-$ *PFK2*$^+$ (spore D) indeed confers on the unregulated phosphofructokinase from spore C the property of being inhibited by ATP. Addition of 5 μM fructose 2,6-diphosphate relieves this inhibition to a large extent [8]. The thermolability of this substance from spore D as also its non-dialysable nature make it unlikely to be anything but a protein. Further, experiments using the temperature-sensitive allele *pfk2-2* indicated that it was the product of *PFK2* gene itself that conferred on the catalytic subunit the property of ATP inhibition. Growth on alcohol of a strain *PFK1 pfk2-2* at 30°C (the non-permissive temperature for a strain *pfk1-1 pfk2-2* to grow on glucose was 36°C) yielded an enzyme that was non-allosteric, while growth at 23°C produced a variant enzyme that was somewhat sensitive to ATP inhibition. On warming the extract containing the altered enzyme to 36°C for various periods the enzyme became progressively unresponsive to the inhibitory action of high concentrations of ATP.

4. Discussion

These results presented here provide strong evidence for the conclusion that the allosteric property of yeast phosphofructokinase derives from the interaction of the catalytic subunit specified by the gene *PFK1* with a regulatory subunit specified by the gene *PFK2*. This is supported by experiments in vivo where a heterozygous pair of these genes reshuffle to produce a combination (spore C in table 1) in which the catalytic subunit is exchanged free from the regulatory subunit giving an enzyme that saturated with fructose 6-phosphate in a hyperbolic manner and was not at all inhibited by ATP. Mixing experiments in vitro supported this conclusion. Although the wild-type enzyme is known to consist of four each of two non-identical polypeptide chains [4], the amount of *PFK2* gene product required to

convert a fixed amount of the *PFK1* gene product to an ATP-inhibited form was nearly 50–100-times as much in terms of protein equivalents. The reason for this aberrant stoichiometry lies in the missense nature of the *pfk2-1* mutant allele whose product possibly remained bound to the wild-type product of the *PFK1* locus in the spore C. On the other hand the subunit specified by the *PFK2* gene present in the extracts of spore D was in all likelihood present in the free state as the allele *pfk1-1* was a nonsense mutation. The exchange process can be represented by the following reaction:

$$\alpha^+\beta^- + \beta^+ \rightleftharpoons \alpha^+\beta^+ + \beta^-$$

where α^+ is the product of the wild-type allele of the *PFK1* gene, β^+ the corresponding product of the *PFK2* gene, and β^- is the missense product of the *PFK2-1* allele. The mutant α^- subunit produced by the nonsense allele *pfk1-1* was very likely degraded [9]. The results of sucrose density gradient experiments were consistent with this interpretation. The enzyme in the crude extracts of spore A as also of spore C migrated as a heavy species of 15.5 $s_{20,w}$, while in the extracts of spore D alone, a peak was present for an activity presumably of *PFK2* gene product which conferred on the non-allosteric phosphofructokinase (the 15.5 $s_{20,w}$ peak from spore C) the property of ATP inhibition; this peak was light, migrating as 8.1 $s_{20,w}$ species corresponding to a dimeric form of a partially proteolysed regulatory subunit [3]. The prediction then would be that a mixture of extracts from single, nonsense mutants each of *pfk1* and *pfk2* strains would contain a heavier species of phosphofructokinase than the extract from the strain *pfk2* alone.

The gene *PFK2* is thus involved in the specification of both the phosphofructokinases of *S. cerevisiae*, as a regulatory subunit for the soluble, allosteric enzyme and as an essential determinant for the synthesis of the particulate enzyme. It is not known how a soluble subunit could be involved in the determination of an insoluble enzyme. The observation that a third gene *pfk3* is also necessary for the activity of the particulate phosphofructokinase raises interesting questions on the interaction of the products of genes *pfk2* and *pfk3*.

These experiments also demonstrate the ability of yeast cells with non-allosteric phosphofructokinase to grow on a gluconeogenic substrate such as ethyl alcohol as well as on glucose.

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