recalling words, basic units of natural language sentences. The number of words which an average native speaker is able to access readily is estimated[21] to be about 50,000. This number is rather small compared to the number of neurons and their interconnections in the segment of human brain which deals with speech. Therefore, the method of encoding words can be safely assumed to be sparse. Simulation studies of the two-component model outlined in the previous section may do well to adhere to this aspect of encoding.

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ACKNOWLEDGEMENTS. I thank Dr G. Venkataraman for his valuable suggestions towards improving the structure of this paper.

Received 15 September 1994; revised accepted 13 January 1995

RESEARCH ARTICLES

Overexpression of particulate phosphofructokinase in yeast by *PFK2*

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A glucose-negative strain *pfk1 pfk2* of *Saccharomyces cerevisiae* mutated in both the genes coding for the soluble phosphofructokinase brings about nearly 30-fold overexpression of the particulate phosphofructokinase (PFK II) when transformed with multiple copies of *PFK2*. The overexpression is independent of the dosage of *PFK1*-encoded β subunit. The elevated enzyme activity correlates directly with the increased particulate association of the polypeptide encoded by *PFK2*. Multiple copies of the *PFK2* gene appear to be the only requirement for overproduction of the particulate phosphofructokinase.

THE soluble phosphofructokinase of yeast (PFK I) is a hetero-octameric enzyme, composed of 4α and 4β subunits[1]. This contrasts with the phosphofructokinase found in the human muscle or in *Escherichia coli*, where the activity resides in a single polypeptide. Mutants in both the subunits have been isolated in various laboratories. This led to the identification and isolation of two genes *PFK1* and *PFK2* coding for the β and α subunits, respectively[2–10] (note: the genes *PFK1* and *PFK2* and the encoded polypeptides have been named differently in different laboratories. The nomenclature used here is according to Gayatri and Maitra[11]). Analysis of a large number of *pfk1* and *pfk2* mutants isolated in this laboratory has shown that mutations in the *pfk1* (β) gene abolish the enzyme activity while mutations in the *pfk2* (α) gene invariably give rise to an alteration of the regulatory properties of the enzyme. Thus, the two subunits have distinct roles to play in the hetero-octamer[12, 13]. Two mutants that do not conform to this rule are DFY70 (α) and DFY250 (β), which exhibit reverse phenotypes[4, 11]. Recently, it has been shown that single-amino-acid changes in either of the two subunits

the level of soluble-enzyme activity[14]. We believe that this reflects the multimeric form of the enzyme.

The *pfk1* mutants lacking the soluble-enzyme activity are able to grow on glucose. This is due to the presence of another isozyme called particulate phosphofructokinase (PFK II)[15]. It is expressed only during exponential growth on glucose and its synthesis ceases as the cells reach the stationary phase[16]. Unlike the soluble enzyme, which is present constitutively, the particulate enzyme is undetectable during growth on alcohol[15]. The *PFK2* gene, which codes for the regulatory subunit of PFK I, is also the structural determinant of PFK II[13], which further requires the function of *PFK3*, *PFK5*[17, 18] and *PFK7*[19]. None of these loci except *PFK2* has any dose-dependent effect on the particulate-enzyme activity[17], suggesting that *PFK2* is perhaps the sole structural determinant of PFK II while *PFK3*, *PFK5* and *PFK7* regulate its synthesis in a manner yet undetermined. Recently, we have demonstrated that the *PFK3* gene is identical to *TPS2*, a determinant of the trehalose biosynthesis complex[20].

Overproduction of the soluble PFK I requires multiple copies of both *PFK1* and *PFK2* genes, reflecting its α_4 β_4 constitution. We report here that the particulate enzyme in contrast requires for its overproduction only the *PFK2* gene in multiple copies. We also show that the *PFK2* gene product (α) is partitioned between the soluble and the particulate fractions in response to glucose. The particulate association of the α subunit is directly correlated with the activity profile of the PFK II enzyme.

Methods

Mutant allele *pfk1–1* is an ochre-suppressible nonsense mutation and *pfk2–2* is a missense mutant. Both of these have been described earlier[12]. The *pfk1* and *pfk2* deletion disruptions in the HD56-5A recipient strain were gifts from J. Heinisch. *pfk3–4* is an allele induced by EMS[17]. Transformation was done by either lithium acetate or spheroplast methods[21]. *PFK2* clone was isolated by transforming *pfk1–1 pfk2–2 ura3* strain using a YEp24 genomic library of *S. cerevisiae* (obtained through U. Vijayraghavan). The restriction map of the insert was similar to that of the corresponding clone described by Heinisch[7, 10]. The isolation and properties of the *PFK3* clone have been described[20]. This clone was obtained from a YCp50 library (courtesy Jane Robinson) using a glucose-negative *pfk1–1 pfk3–4 leu2* strain, complementing its growth defect on glucose. This was confirmed by integration and disruption[20]. It was subsequently subcloned into a YEp24 vector for overexpression studies. *E. coli* strain HB101 was used for making plasmids by standard procedures. Yeast strains for transformation were grown in YEP medium or in minimal medium supplemented with necessary amino acids[21]. Either ethanol (1%) or glucose (1%) was used as the carbon source. The cells were harvested during the mid-log phase of growth.

The particulate phosphofructokinase activity was estimated in toluene lysates[15] made with a cell suspension in 50 mM K-phosphate buffer (pH 7.5), 2 mM β-mercaptoethanol, 2 mM EDTA and 50 mM ammonium sulphate (KEE buffer). For preparing crude extracts and membrane fractions, mid-log phase cells were broken by French press. The cell debris were removed by spinning at 3000 rpm for 10 min. The supernatant was spun at 13,000 rpm for 15 min. The 13 K supernatant was used as the soluble fraction, the pellet was resuspended in KEE buffer and layered on top of a sucrose step gradient in SW 51 tubes (prepared by layering 2.5 ml of 45% sucrose with 2 ml of 20% sucrose) and spun at 20,000 rpm for 45 min in a Sorvall ultracentrifuge. The turbid layer at the interphase was collected and diluted into 5 ml of KEE buffer; this was then spun at 13,000 rpm for 15 min. The resulting pellet was used as the particulate fraction. Any contamination by the soluble fraction was checked by assaying both glucose 6-phosphate dehydrogenase activity and protein.

For differential centrifugation of the particulate fraction, spheroplasts were prepared by zymolyase 100 T treatment (0.25 mg/ml) and broken in 0.3 M buffered mannitol using a hand homogenizer. Cell debris and unbroken spheroplasts were removed by spinning the extracts at 3000 rpm for 10 min. The supernatant was spun at 13,000 rpm for 15 min. The resulting pellet was used as the 13 K pellet. The 13 K supernatant was spun at 36,000 rpm for 1 h and its pellet was used as the 36 K pellet.

Various enzymes were assayed in the following manner. The phosphofructokinase reaction mixture contained 5 mM fructose 6-P, 1 mM ATP and the coupling enzymes needed to assay fructose 1,6-bis-P[22]. Glucose 6-P dehydrogenase (G6PD) was assayed using 0.2 mM NADP and 1 mM glucose 6-P fluorometrically[22]. Toluene lysates were used for measuring the particulate phosphofructokinase. Coupling enzymes were from Boehringer Mannheim and the substrates used for the assays were from Sigma. The NADH oxidase and NADPH oxidase activities were assayed fluorometrically using 0.25 mM reduced pyridine nucleotide in 100 mM Tris buffer (pH 7.4). The ATPase activity was also measured fluorometrically in 10 mM Pipes buffer (pH 6.7) using 5 mM ATP, 5 mM $MgCl_2$ and the coupling enzymes required to estimate ADP[22].

For studying the *PFK2* polypeptide the proteins were electrophoresed on a 7.5% polyacrylamide gel[23]. The gels were stained with Coomassie Blue to visualize all the protein bands[24]. Western blot experiments used a rabbit antibody against purified phosphofructokinase from

yeast employing a published procedure[4].

The quantitation of proteins was done according to Zamenhoff[25] and Peterson[26] using bovine serum albumin (BSA) as a standard.

The reagents and detergents used in solubilization of the membrane fractions were obtained from Sigma or Boehringer.

Results

Overexpression of PFK2

The particulate phosphofructokinase is determined by a number of genes. Mutation in any one of them leads to a loss of this enzyme. These mutations, when present in a *pfk1* genetic background, confer glucose negativity. The effect of overexpression of two of these genes has been compared in Table 1. The transformant carrying multiple copies of *PFK2* has 40-fold more enzyme activity compared to the *pfk1* strain with a single chromosomal copy (Table 1, rows 3 and 1). In contrast, the enzyme activity in the *PFK3* transformant is not significantly different from that in the parent *pfk1 PFK2* strain (Table 1, row 7). This shows that the activity of this enzyme is related directly to the dosage of *PFK2* but not of *PFK3*. This is in contrast with the soluble PFK activity, whose elevated levels require the overexpression of both its genetic determinants *PFK1* and *PFK2*[7,10]. Both the *PFK1* and *PFK2* genes are needed to synthesize the soluble enzyme while the particulate enzyme is active without any contribution from the *PFK1* gene. Deletion of the locus *PFK1* has no effect whatsoever on the synthesis of the particulate phosphofructokinase or its overexpression by *PFK2* clone (Table 1, rows 4 and 5).

Table 1. Elevated expression of particulate phosphofructokinase in *PFK2* multicopy clone

Strain genotype	Growth on glucose	Enzyme activity (mU/E_{650})	
		PFK	G6PD
pfk1$_{ns}$ *PFK2*	+	4	25
pfk1$_{ns}$ *pfk2*	–	0	19
pfk1$_{ns}$ *pfk2 :: PFK2*[a]	+	200–225	22
pfk1▲ PFK2	+	8	20
pfk1▲ PFK2 :: PFK2[a]	+	200–250	24
pfk1$_{ns}$ *pfk3*	–	0	18
pfk1$_{ns}$ *pfk3 :: PFK3*[a]	+	8	26

Cells were grown to early exponential phase on glucose or alcohol as appropriate. Enzyme activity was measured in toluenized cells and expressed per unit E_{650} (extinction at 650 nm). The subscript *ns* refers to a nonsense mutant allele; *pfk1*▲ denotes the deletion strain from J. Heinisch. The genotype of the deletion strain is according to our nomenclature.

[a]The *PFK2* and *PFK3* genes were cloned in a high-copy episomal vector YEP24.

PFK, particulate phosphofructokinase; G6PD, glucose 6 P dehydrogenase.

In order to characterize the overexpressed PFK II enzyme, its activity profile was studied. The particle-bound enzyme responsible for the growth of *pfk1* mutants on glucose is glucose-induced; however, it is expressed only transiently[16]. The *PFK2* transformant behaves likewise (Figure 1). The specific activity of the enzyme in glucose culture peaks around $E_{650} = 2.0$ and decreases thereafter. Continued growth in the absence of glucose (unfilled circles, Figure 1) leads to disappearance of the specific activities of the enzyme (Figure 1) but no detectable loss of total enzyme activity per unit volume of the cell suspension. This indicates the absence of any active process of decay of particulate phosphofructokinase. The decrease of the activity is merely due to cessation of its synthesis in alcohol cultures as well as during the late growth phase as the cells approach the stationary phase.

Association of α polypeptide with the particulate fraction

Since *PFK2* is a determinant of both the soluble and the particulate forms of phosphofructokinase, we checked the distribution of its gene product α in cell fractions. We find that during growth on glucose the α subunit is partitioned between the soluble and the particulate fractions (Figure 2). In order to study the correlation between the particulate association of α and the PFK II activity, the levels of this polypeptide in the soluble and particulate fractions of the *pfk1 PFK2* and the *pfk1 pfk2 :: PFK2* strains grown on glucose were compared.

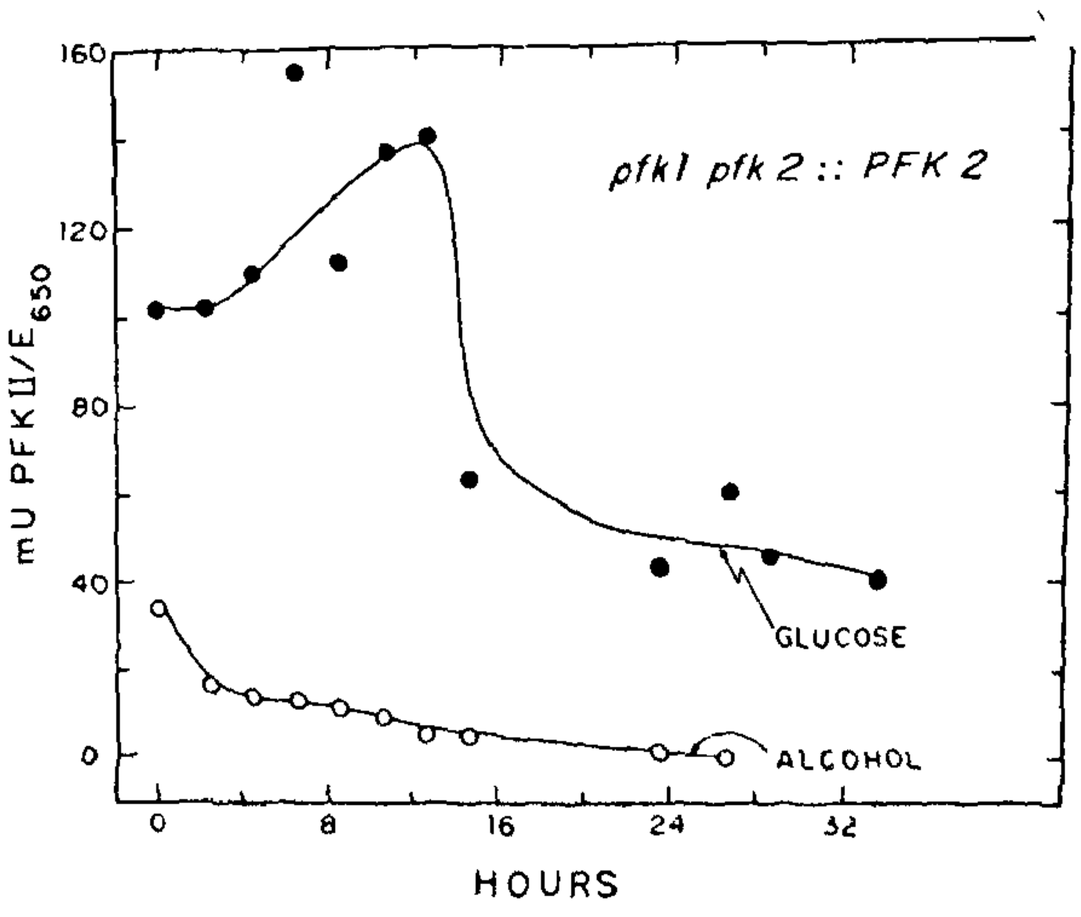

Figure 1. Rise and fall of particulate phosphofructokinase activity of a *multicopy PFK2 transformant* during growth on glucose. An overnight culture of a strain *pfk1 pfk2 , PFK2* grown on YEP glucose was washed and diluted in a fresh medium containing glucose or alcohol. After resumption of exponential growth, aliquots were assayed for the particulate enzyme (PFK II).

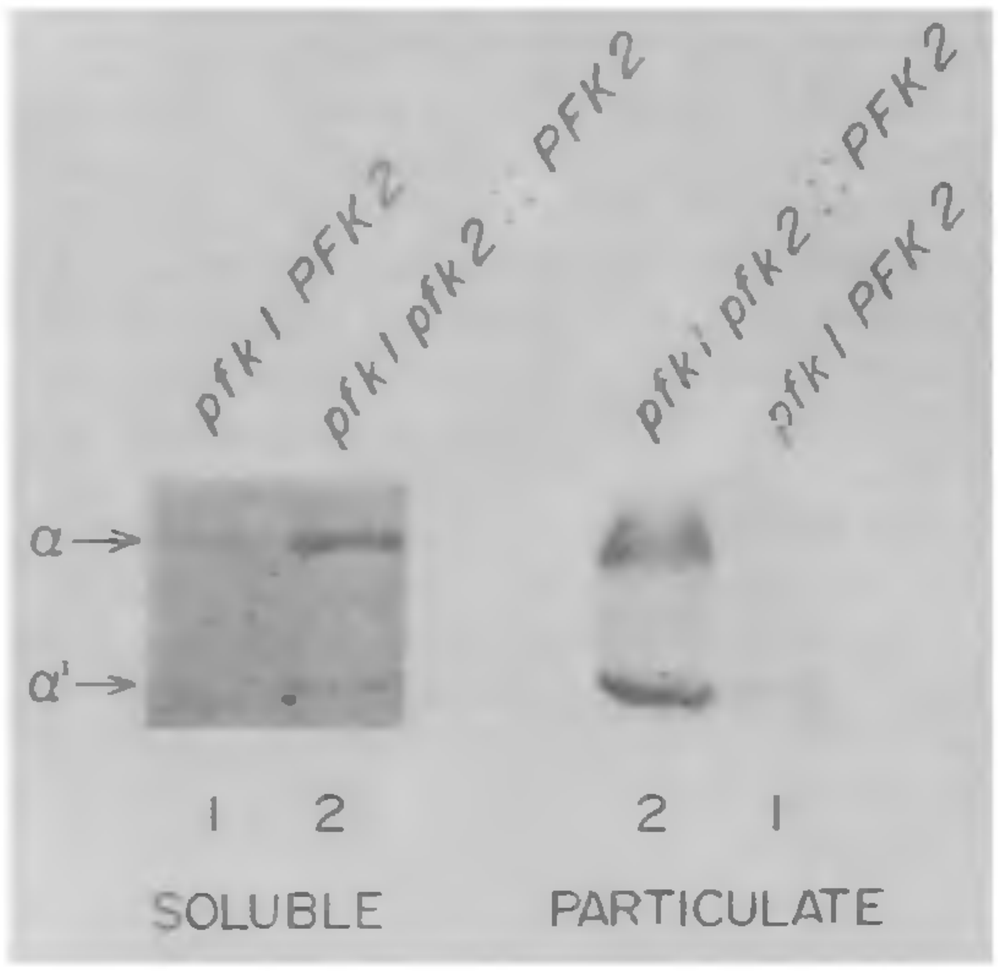

Figure 2. Western blot showing levels of the α subunit in the soluble and particulate fractions of *pfk1 PFK2* and *pfk1 pfk2 :: PFK2* strains. Glucose-grown cultures were analysed. 100 µg of protein was loaded in each lane. α' is a proteolysed form of the α subunit[4].

The *pfk1 pfk2 :: PFK2* transformant has 30- to 40-fold higher PFK II activity than the *pfk1 PFK2* strain due to the presence of the *PFK2* gene on a multicopy plasmid. The G6PDH activity remains constant in all the strains (Table 1, last column).

The results in Figure 2 show that the relative levels of the α polypeptide of two strains with a single chromosomal copy (*pfk1 PFK2*) versus a multicopy *PFK2* cloned in a *pfk1 pfk2* strain shows a marked difference in the particulate fraction. The particulate fraction of the *PFK2* transformant (particulate, lane 2) shows much higher levels of the α polypeptide than the strain of the genotype *pfk1 PFK2* (particulate, lane 1). Thus, in a *PFK2* transformant, the increase in PFK II activity appears to be causally related to increased association of the α subunit with the particulate fraction. Although *PFK2* is also a determinant of the soluble PFK I, the α polypeptide level in the soluble fraction is only 2- to 3-fold increased in the *PFK2* transformant (Figure 2, soluble, lane 2) compared to *pfk1 PFK2* (Figure 2, soluble, lane 1).

Glucose – induction of the particulate association of α polypeptide

The PFK II enzyme shows strong induction by glucose. It is present in log-phase cells grown on glucose, but totally absent in cells grown on alcohol. Is the particulate association of the subunit α of PFK II a prerequisite for its induction during growth on glucose?

The association of the α subunit with the particulate fraction is seen only in glucose-grown cultures (Figure 3, particulate, lane 2), which also show particulate-enzyme activity in toluene lysates (Table 1, row 3). Cells grown on alcohol as the carbon source show neither the enzyme activity nor appreciable amounts of the particle-bound α (Figure 3, particulate, lane 1). Thus, glucose causes the translocation of the α subunit from the soluble to the particulate fraction. This is perhaps the likely mechanism of the absence of particulate-enzyme activity during growth on alcohol and its induction by glucose.

To rule out the possibility of any contamination by the soluble fraction, the particulate fractions described in the earlier sections were checked for glucose 6-phosphate dehydrogenase (G6PD) activity; none could be detected while the soluble fraction had around 100 mU/mg protein. Further, the particulate fractions did not show any antigen corresponding to glucose 6-phosphate dehydrogenase (G6PD), when probed with G6PD antibody obtained from Sigma. We also used the cDNA clones of 6-P-gluconate dehydrogenase (GND) (Lobo and Maitra, unpublished) which overexpress the enzyme 50–60-fold to see if overexpression of a protein might make it particulate. In the GND transformants no GND antigen could be detected in the particulate fraction. We conclude from these results that the particulate-phosphofructokinase activity is directly correlated with the association of the α subunit with the particulate fraction.

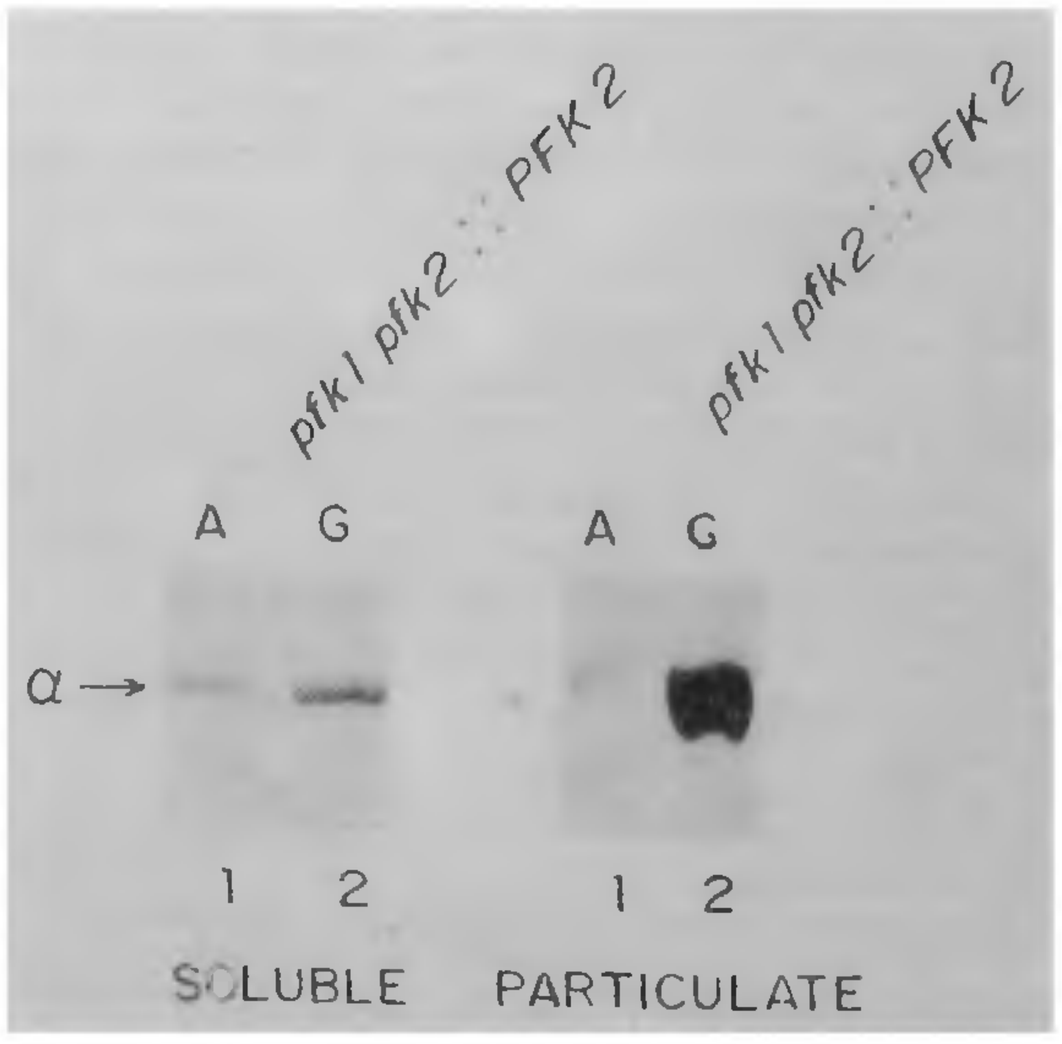

Figure 3. Western blot using PFK antibody showing relative levels of the α subunit in the soluble and particulate fractions. Alcohol-grown (A) and glucose-grown (G) cultures of *pfk1 pfk2 :: PFK2* strain carrying the cloned *PFK2* gene in high copies were used. 200 µg of soluble and 100 µg of the particulate-protein fractions were loaded

Although the *PFK2* gene product α gets associated with the particulate fraction under certain conditions as shown above, we do not detect any appreciable difference in size between the soluble and particulate forms on SDS-PAGE. In both the fractions the α subunit migrates as a species of molecular weight 116 kDa. However, the particulate fraction shows a broad band which might be a result of post-translational modification of α (Figures 2 and 3, particulate, lane 2).

The sequence of *PFK2* does not show any distinctive membrane-targetting signals or hydrophobic domains[9]. Probably, the cytoplasmic form of α undergoes post-translational modification in response to glucose, resulting in particle-bound activity. So, we checked the membrane association in our mutant isolates in other complementation groups that effect the PFK II activity. This was probed by using the mutants *pfk3* and *pfk7*. However, these mutants, when transformed with multi-copy of *PFK2*, do not restore growth on glucose in a *pfk1* background unlike the *pfk1 pfk2* strain. The absence of PFK II activity in *pfk3* and *pfk7*, therefore, necessitated the incorporation of *PFK1* gene on the recipient strain for it to grow on glucose. As a result, *PFK1 pfk3* and *PFK1 pfk7* were transformed with high-copy *PFK2* clone. The membrane association of α in *pfk3* and *pfk7* mutants is not altered (Figure 4, particulate, lanes 3 and 2) and stays the same as in wild-type transformants (particulate, lane 1), giving a broad band of α subunit exactly similar to that seen in Figure 2, particulate, lane 2. However, in the soluble fraction the amount of α is more or less the same as that of β (Figure 4, soluble, lanes 2 and 3), suggesting that the overexpressed α from the *PFK2* clone is targetted to the membrane fraction. So far, none of the mutants lacking PFK II was affected specifically in the particulate association of the α subunit.

We detected the presence of β subunits also in the particulate fraction in *PFK1* genetic background (Figure 4, lanes 2 and 3). This might be due to the inherent affinity of the subunits for each other rather than due to any role of β in the particulate-enzyme activity. The particulate activity and its elevated levels can be observed in strains lacking β polypeptide (Table 1).

To determine whether the observed particulate association of the α subunit is with a specific cellular membrane, the particulate fraction was subjected to differential centrifugation. Table 2 shows the relative distribution of the enzyme markers of various organelles after differential centrifugation – (a) mitochondrial: NADH oxidase[27], (b) plasma membrane: vanadate-sensitive Mg^{2+}–ATPase[28] and (c) microsomal: NADPH oxidase[27,29]. Most of the mitochondrial activity was observed in the 13 K pellet. The 13 K pellet also contained some amount of microsomal vesicles. The plasma membranes and the microsomes were detected in the 36 K pellet. The 13 K and 36 K pellets lacked the G6PD activity which was present in the cell-free extracts.

The PFK II activity is highly unstable *in vitro*, so its activity profile could not be tracked. We, therefore, checked the fractions for the α antigen using PFK antibody. The antigen was found in both 13 K and 36 K pellets. This indicates that the particulate association of PFK II is not specific to mitochondria or to plasma membrane. Another possibility is the association of PFK II with the cytoskeleton. Density gradient centrifugation of purified organelles might help to identify the specific structure inside the cell with which PFK II associates in response to glucose. The preliminary studies reported here only show that this structure is probably not the mitochondrial or plasma membrane.

We have tried to solubilize the particulate form of the *PFK2* gene product using spheroplasts from a high-copy clone of the *pfk1 pfk2 :: PFK2* strain. Spheroplasts

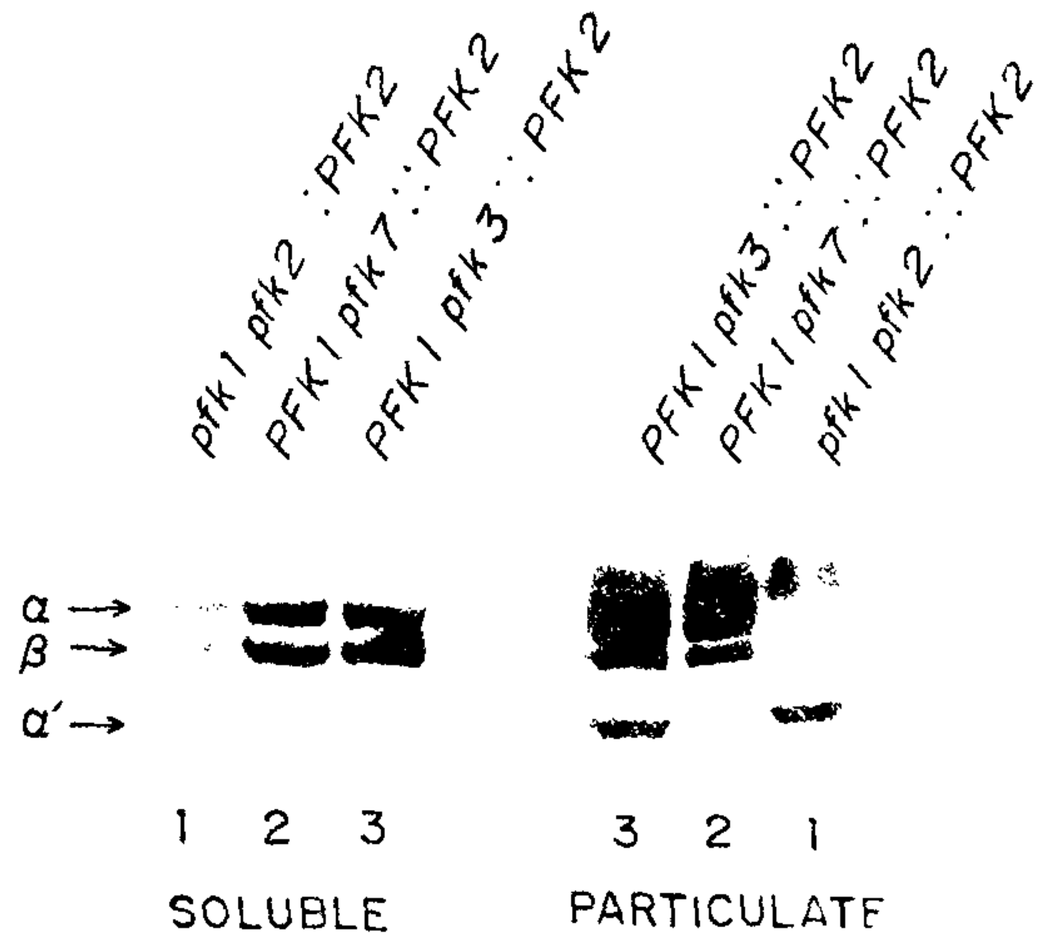

Figure 4. Western blot using PFK antibody showing the particulate association of the α subunit in *pfk3* and *pfk7* mutants. Glucose-grown cultures were used. 200 µg protein was loaded in each lane: 1, *pfk1 pfk7 :: PFK2*, 2, *PFK1 pfk7 :: PFK2*; 3, *PFK1 pfk3 :: PFK2*.

Table 2. Relative distribution of organelle marker enzymes and PFK II antigen α in 13 K and 36 K pellets after differential centrifugation of the particulate fraction

Marker enzyme	Activity in 13 K pellet (m Units)	Activity in 36 K pellet (m Units)
NADH oxidase	2560	46
Mg^{2+}-ATPase	0	248
NADPH oxidase	6	13
PFK II (α antigen)	+	+

A high-copy transformant of *PFK2* in a *pfk1 pfk2* recipient strain was used for this experiment

were prepared from cultures grown in glucose and lysed in 0.1 M Tris (pH 8.0), 10 mM $MgCl_2$, 2 mM β-mercaptoethanol containing 1 mM each of PMSF and pepstatin A. The pellet was washed, resuspended in the same buffer and incubated overnight with a variety of detergents to examine if solubilization of the PFK II had occurred. These include 1% Triton X-100, 2% Triton X-114, 0.5% Na deoxycholate, 20 mM *n*-dodecyl-β-D-maltoside, 15 mM octonyl-*N*-methyl glucamide, 20 mM decanoyl-*N*-methylglucamide, 2.5 M urea, 25 mM EDTA, 0.1 M Na carbonate (pH 11.5), 2 M KCl, 0.1 M NaOH, 1% Nicol, 0.2% CHAPS, 1% CHAPSO and 1% BIG CHAPSO. Despite the use of a variety of detergents and other reagents, it has not been possible to solubilize the active PFK II enzyme from the pellet fractions. Caustic soda (0.1 M) was the only reagent that could extract from the pellet fraction the α polypeptide that reacted positively in the dot blot assay for the α antigen.

Discussion

The *PFK2* gene plays a dual role in the *S. cerevisiae* cells. It is a structural determinant of the classical phosphofructokinase enzyme present in the soluble extracts, coding for the regulatory subunit. Apart from this, the *PFK2* gene product (α) has another function. This was deduced from the following observations: (a) it was found to be the genetic determinant of the particulate phosphofructokinase enzyme, and (b) the particulate-enzyme activity showed a dose dependence on PFK2.

The results described here show that the PFK II enzyme activity is dependent only on the dose of the *PFK2* gene. Thus, *PFK2* is the sole catalytic and the structural determinant of the particulate PFK II enzyme. Overexpression of the *PFK2* gene alone can support 30- to 40-fold increase in the PFK II activity. This enzyme activity is directly correlated with the particulate association of the *PFK2* gene product.

The transition of the soluble polypeptide to a particle-bound form seems to occur in response to glucose. During growth on glucose, the *PFK2* polypeptide is partitioned both in the soluble and in the particulate fractions, whereas growth on ethanol keeps it mainly in soluble form. This demonstrates biochemically its dual role as a structural determinant of the soluble PFK I as well as the particulate PFK II.

Overexpression studies described here show that the factors necessary for the transition from the soluble to the particulate forms are abundant inside the cell and can support a 30- to 40-fold increase in PFK II levels; it is, therefore, surprising that the soluble form of the α subunit still persists during growth on glucose. This suggests mechanisms that deny accessibility of a fraction of this polypeptide pool to a particulate fate. One explanation would be the presence of the *PFK1* polypeptide that would bind the *PFK2* product and thus prevent it from becoming particulate. An alternative explanation can be the synthesis of separate mRNA species from the *PFK2* locus that specify the two enzymes. So far, we have not been successful in identifying such forms of the *PFK2* mRNA.

Since the primary protein synthesized from the *PFK2* locus is soluble, it is not clear as to how the soluble-to-particulate transition occurs. Post-translational modification of the α subunit might play a role. However, neither *PFK3* nor *PFK7* appears to define such a process. Considering that for post-translational modification a general protein might be involved, e.g. a kinase, mutations in such genes might be lethal. A screen for temperature-sensitive PFK II determinants might identify such loci.

Regarding the nature of the particulate association, preliminary results reported here show that it is not specific to mitochondria or the plasma membrane. Our results, however, do not negate the possibility of microsomal membranes being the likely target, but this needs to be further analysed by density gradients. Another interesting possibility is that the particulate nature of the PFK II enzyme is due to an association with the cytoskeletal elements or another protein present in the membrane, rather than due to the anchoring into the membrane. The phosphofructokinase from human erythrocyte interacts with the band 3, the predominant integral protein and anion transporter of the erythrocyte membrane[30], and this interaction relieves the inhibition by ATP and 2,3-bis-phosphoglycerate, converting the sigmoidal fructose 6-P saturation curve to a hyperbolic form[31]. This phosphofructokinase also interacts with thin filaments of muscles[32] and with calmodulin[33]. Although in yeast there is no evidence for the association of the *PFK2* gene product with the particulate fraction mediating an allosteric control on the soluble enzyme, the possibility that the α subunit might associate with proteins either in the membrane or in the cytoskeletal matrix appears interesting.

What could be the requirement for the dual role of the *PFK2* polypeptide in the cell? The particulate phosphofructokinase contributes only 20% of the total phosphofructokinase activity in yeast and it appears only during the exponential phase of growth on glucose. We believe that the partitioning of this polypetide between the soluble and the particulate fractions could be involved in functions other than the phosphofructokinase activity. This assumption is borne out by our observation that one of the determinants of this enzyme, *PFK3*, is identical with *TPS2*[20], which codes for the 100 kDa subunit of the trehalose biosynthesis complex[34]. Another subunit of the same complex, *TPS1*[35,36], appears to be involved in the regulation of glycolytic flux at the level

of glucose transport. In view of this, the dual role of the *PFK2* gene product and the particulate nature of the PFK II enzyme assume significance outside the phosphofructokinase reaction. This is being investigated further.

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ACKNOWLEDGEMENTS. We thank K Wahane for providing the *pfk7* mutant, J Heinisch for providing the *pfk1* and *pfk2* disruptant strains, and D Lloyd for the helpful suggestions for differential centrifugation analysis. This work was supported by a grant from the Department of Biotechnology, Government of India.

Received 3 December 1994, revised accepted 8 March 1995