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Complementation of soluble phosphofructokinase activity in yeast mutants

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We describe here the genetic and biochemical analyses of two classes of mutations in the soluble phosphofructokinase (PFK I) of *Saccharomyces cerevisiae*: those leading to the loss of activity and those giving rise to a kinetically altered enzyme. Complementation and allele-testing between these two classes of mutants show that loss of enzyme activity *in vitro* can come about not only by mutations in the catalytic subunit but also in the regulatory subunit. Also, a mutation in the catalytic subunit can give rise to an enzyme altered in its kinetic properties in a manner phenomenologically similar to that caused by a mutation in the regulatory subunit.

The results of the complementation studies in diploids suggest that, in spite of their distinct functions, both the subunits are essential for activity to be detected *in vitro*. This is confirmed by the reconstitution of an active PFK I enzyme by mixing cell-free extracts of two complementing parents, each of which lacks the enzyme activity. PFK activity appears in the mixture, reaching a maximum value of 60–100% of that of the diploid in 15–30 min at 24°C. Unlike the catalytic subunit which exists in various multimeric states in cell-free extracts of the mutant bearing only this subunit, the regulatory subunit exists largely as a monomer in a mutant devoid of the catalytic subunit. The reconstituted enzyme, however, is indistinguishable from that of the wild type, as analysed by sedimentation studies and Western blot analysis, demonstrating that only the heteromeric complex of the two subunits is active, while neither of the individual subunits displays activity *in vitro*.

The yeast, *Saccharomyces cerevisiae*, possesses two distinct phosphofructokinases: a soluble form (PFK I) and a particulate form (PFK II) (for a recent review see [1]). The presence of either of these isozymes is sufficient for growth on glucose [2]. The soluble phosphofructokinase is an octamer with a native molecular mass of about 800 kDa and is composed of four α subunits and four β subunits [1]. Analyses of mutants (reviewed in [3]) and molecular cloning of the genes involved [4] have shown that *PFK1* and *PFK2* are the structural determinants of the β and α subunits, respectively (for nomenclature see Materials and Methods). The gene *PFK2* is known to be shared between the soluble and the particulate forms of phosphofructokinase [5].

Evidence provided by substrate binding studies and protection experiments with fructose 6-phosphate (Fru6*P*) has led to the suggestion that the two subunits might be functionally distinct, β being the catalytic and α the regulatory subunit (see [1]). Our laboratory has provided genetic evidence supporting the above proposal, based on the finding that nearly 200 independent mutants lacking soluble PFK activity had lesions in the *PFK1* gene [6]. Mutations in the gene *PFK2*, on the other hand, invariably gave rise to only an alteration of the regulatory properties of the enzyme [7].

It was therefore surprising that a mutant DFY70, devoid of the soluble phosphofructokinase activity [8], was found to be non-allelic to *PFK1* [9]. This mutant in fact lacks the α subunit [10] and therefore seems to be affected in *PFK2*. Another mutant DFY250, possessing a soluble phosphofructokinase activity similar to that in a *pfk2* mutant [7], does not seem to be allelic to *PFK2* [10].

This paper reports a genetic and biochemical study of these two mutants *vis a vis* those reported from this laboratory, to resolve the contradiction between the phenotypes of these two mutant classes. Our results confirm that DFY70 has a mutation in the *PFK2* gene coding for the regulatory subunit, while DFY250 is affected in the gene *PFK1* encoding the catalytic subunit. Complementation in diploids as well as reconstitution of the soluble phosphofructokinase enzyme *in vitro* demonstrate that, although the β subunit can function by itself *in vivo*, association of the two subunits is essential for the detection of activity. Characterisation of the kinetically altered soluble enzyme synthesized by the *pfk1* mutant DFY250, as compared to an apparently similar enzyme from a *pfk2* mutant revealed differences between the two. The relevance of these findings to the roles played by the individual subunits in enzyme activity is discussed.

MATERIALS AND METHODS

Strains used and nomenclature

The genes *PFK1* and *PFK2* and the encoded polypeptides have been named differently in various laboratories (see [3]).

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Abbreviations. Fru6*P*, fructose 6-phosphate; Fru(2,6)P_2, fructose 2,6-bisphosphate; Fru(1,6)P_2, fructose 1,6-bisphosphate; PFK I, soluble phosphofructokinase of *Saccharomyces cerevisiae*; PFK II, particulate phosphofructokinase of *S. cerevisiae*; P, N and T refer to parental, non-parental and tetra-type tetrads respectively.

Enzymes. Alkaline phosphatase (EC 3.1.3.1); aspartate carbamyltransferase (EC 2.1.3.2); β-galactosidase (EC 3.2.1.23); 6-phosphofructokinase (EC 2.7.1.11); pyruvate kinase (EC 2.7.1.40).

We adhere to the following nomenclature (which is different from that followed in the previous publications from our laboratory): *PFK1* mapping on chromosome XIII and *PFK2* mapping on chromosome VII [11] encode, respectively, the catalytic subunit β (112 kDa) and the regulatory subunit α (118 kDa) of the soluble phosphofructokinase of yeast. Other groups use an inverse genetic nomenclature [4, 9, 10, 20, 22, 23] and, while referring to their work, we have reversed the gene names for the sake of clarity. The mutants DFY70 and DFY250, obtained through the kind courtesy of Dr Dan Fraenkel, have been designated for convenience as *pfk2-70* and *pfk1-250*, respectively, according to the complementation and allele-testing described in this paper.

The ochre-suppressible, nonsense mutation *pfk1-1* has been described [6]. The *pfk1-3* allele is missense with no detectable soluble PFK activity. The *pfk2* alleles and their effect on the allostery of this enzyme have been discussed [7]. Briefly, *PFK1 pfk2-1*, *PFK1 pfk2-5* and *PFK1 pfk2-6* synthesize a soluble PFK not inhibited by ATP while the enzyme from *PFK1 pfk2-4* is active only under de-inhibited conditions. The strain MC3, used as the wild-type control, was obtained from Dr G. R. Fink.

Media and growth

Strains were grown on an enriched medium containing 0.3% yeast extract and 1% peptone (YEP) supplemented with either 150 mM ethanol or 50 mM glucose, as carbon source, on a rotary shaker at 30°C.

Enzyme assays

PFK assays were done with either cell-free extracts or toluene lysates as described by Lobo and Maitra [6]. The segregation of PFK activity in tetrads was followed by discontinuous assays of toluenised cells, which were then tested for ATP inhibition by continuous assays. The soluble PFK was specifically measured in cell-free extracts, while toluenised cells were used for soluble as well as particulate enzyme measurements. Enzymes and substrates for assays were from Boehringer or Sigma.

Other procedures

The soluble PFK enzyme was purified using a slightly modified version of the protocol by Welch and Scopes [12], in which the acetone and protamine sulfate precipitation steps were replaced by ammonium sulfate precipitation and desalting before loading onto the affinity column. The purified preparation was used as the immunogen for obtaining rabbit antiserum by standard procedures [13].

The method for Western blotting was that of Towbin et al. [14] except for two changes: the blocking solution used was 5% non-fat dry milk; diaminobenzidine was used for the peroxidase colour reaction.

Sedimentation experiments were done according to the procedure followed by Nadkarni et al. [15]. The markers used were β-galactosidase and alkaline phosphatase of *Escherichia coli* and pyruvate kinase from rabbit muscle, corresponding to $s_{20,w}$ values of 16.0 S [16], 6.1 S [17] and 10.0 S [18], respectively.

Protein concentration in extracts was measured by Bradford's method according to Peterson [19], using crystalline bovine serum albumin as standard.

Table 1. *Complementation of DFY70 with* pfk1 *mutants*
Cultures were grown on YEP-glucose and extracted by a French press in 50 mM potassium phosphate pH 7.4 containing 2 mM EDTA and 2 mM 2-mercaptoethanol (buffer A). Non-stimulated PFK I activity was assayed with 2.5 mM Fru6*P* and 1 mM ATP; for stimulated activity, 5 μM Fru(2,6)P_2 was also added. Activity is expressed per mass of total protein in the extract; 1 mU is the amount catalyzing the production of 1 nmol Fru(1,6)P_2 min^{-1}

Strain	PFK I activity	
	non-stimulated	stimulated
	mU mg^{-1} protein	
Wild-type	76	196
DFY70	3	3
pfk1-1 PFK2	1	0.7
DFY70 X *pfk1-1 PFK2*	36	98
pfk1-3 PFK2	1.6	2.9
DFY70 X *pfk1-3 PFK2*	32	94

RESULTS

Characterisation of the mutant DFY70

The mutant DFY70 lacks PFK activity in cell-free extracts and is also devoid of the membrane-bound PFK II. However, it can grow on glucose both aerobically and anaerobically (data not shown) [8].

DFY70 was crossed with *pfk1* mutants and the diploids assayed for phosphofructokinase activity in their cell-free extracts. As seen in Table 1, neither DFY70 nor the *pfk1* mutants show any PFK activity in their crude extracts, even under Fru(2,6)P_2-stimulated conditions. The diploids, however, complement to give rise to about half the activity seen in the wild type. Results are shown for two *pfk1* alleles: *pfk1-1*, a nonsense allele and *pfk1-3*, a missense allele. Both are equally proficient in complementation with DFY70 *in vivo*.

PFK2 mutations affect the kinetic properties of the soluble PFK enzyme in at least two ways: they render the enzyme insensitive to the inhibitory effects of ATP (e.g. *pfk2-1*, *pfk2-5*, *pfk2-6*) or give rise to an enzyme that is active only under totally de-inhibited conditions (e.g. *pfk2-4*) [7]. In a *pfk1* mutant background the *pfk2* mutations confer glucose negativity [5]. Since DFY70 was seen to complement *pfk1* mutants for the soluble PFK activity, it was of interest to see whether it complements glucose-negative *pfk1 pfk2* double mutants and, if so, examine the nature of the complemented enzyme in the light of the specific properties of the *pfk2* alleles mentioned above. Diploids of DFY70 with *pfk1-1 PFK2*, *pfk1-1 pfk2-1* or *pfk1-1 pfk2-4* mutants were therefore assayed for PFK I activity under inhibited and de-inhibited conditions (Table 2). The diploid bearing the wild-type *PFK2* gene has the classical wild-type enzyme showing inhibition at high concentrations of ATP which can be relieved by Fru(2,6)P_2. The other two diploids display activities as described for the two types of *pfk2* alleles. Thus, the regulatory properties of the complemented enzyme seem to be dependent on the nature of the *PFK2* allele contributing to the α subunit in the diploid.

Complete tetrads of the diploid obtained by crossing DFY70 with the *pfk1-1* mutant were analysed for growth on glucose and PFK I activity. That the two mutations were free to recombine was marked by the appearance of glucose-negative spores as well as spores showing soluble PFK enzyme. The tetrads showed a P:N:T ratio of 2:2:10. Each tetratype

Table 2. *Effect of* PFK2 *alleles on the soluble PFK activity in diploids* Strains were grown on YEP-glucose. PFK I activity was measured in cell-free extracts prepared in buffer A (see Table 1). Non-inhibited activity was assayed with 1 mM Fru6*P* and 0.25 mM ATP, inhibited activity with 1 mM Fru6*P* and 5 mM ATP, de-inhibited activity with 1 mM Fru6*P*, 5 mM ATP and 5 μM Fru(2,6)P_2

Genotype	PFK I activity		
	non-inhibited	inhibited	de-inhibited
	mU mg^{-1} protein		
PFK1 pfk2-70 / *pfk1-1 PFK2*	55	0	110
PFK1 pfk2-70 / *pfk1-1 pfk2-1*	47	62.5	84
PFK1 pfk2-70[a] / *pfk1-1 pfk2-4*	0	0	0

[a] The extract of this diploid when assayed with 0.25 mM ATP, 5 μM Fru(2,6)P_2, and concentrations of Fru6*P* of 2.5, 5 and 10 mM, showed a PFK activity of 22, 60 and 85 mU mg^{-1}, respectively.

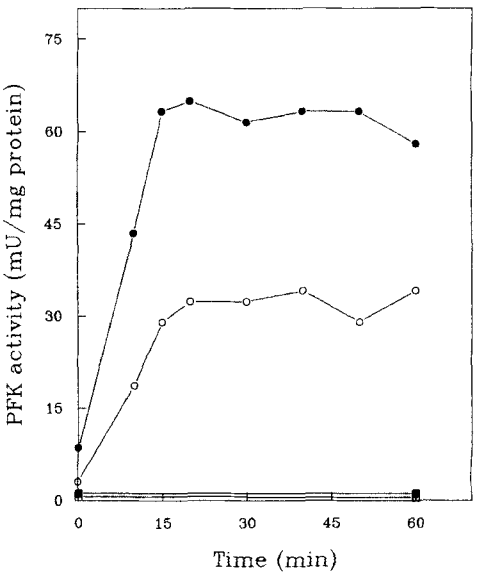

Fig. 1. *Kinetics of reconstitution of the soluble PFK.* Cell-free extracts of DFY70 (*PFK1 pfk2-70*) and the *pfk1-1 PFK2* mutant were prepared in 50 mM potassium phosphate pH 7.4 containing 5 mM EDTA, 5 mM 2-mercaptoethanol, 30 mM ammonium sulfate, 1 mM phenylmethylsulfonyl fluoride and 1 mM pepstatin A (reconstitution buffer). These were mixed in a 1:1 proportion and activity was measured at various time periods during incubation at 24°C. Assay conditions were: (○) 2.5 mM Fru6*P* and 1 mM ATP; (●) 2.5 mM Fru6*P*, 1 mM ATP and 5 μM Fru(2,6)P_2. Unmixed extracts were assayed under Fru(2,6)P_2-activated conditions. (□) DFY70 extract; (■) *pfk1-1 PFK2* extract. Results are expressed in mU mg^{-1} total protein in the mixture

tetrad of this cross contained one spore bearing soluble PFK activity, making an enzyme like the wild-type strain (data not shown). The other three spores were devoid of the soluble enzyme activity, one also being glucose-negative. The segregation pattern is thus that of two non-allelic mutations, each giving rise to the loss of soluble PFK activity and both together leading to glucose-negativity. Similarly, all of the eleven four-spore tetrads from the diploid obtained by crossing DFY70 and the *pfk2-5* mutant displayed parental phenotype for glucose-growth and PFK activity: that is, two of the spores from each tetrad had a soluble enzyme activity not inhibited by ATP (*PFK1 pfk2-5*) and two others had no other detectable activity *in vitro* (*PFK1 pfk2-70*) (data not shown). These results are in agreement with the interpretation that the mutation in DFY70 is allelic to *PFK2*.

The allele *pfk2-70* from DFY70 is seen to behave in the complementation studies described here as a null mutation, thus supporting the finding that it lacks the α polypeptide in its soluble extract [10]. All the *pfk2* mutants reported by Nadkarni et al. [7] however, seem to synthesize an altered α subunit. The observation that these latter kind can complement the mutant DFY70 for the soluble PFK activity implies that even the mutant α subunits suffice in this regard.

Reconstitution of the soluble PFK

Since DFY70 lacks the α subunit, it seemed likely that its complementation with *pfk1-1* mutant lacking the β subunit represents a physical association of the two subunits in the diploid, each coming from the two parents. Indeed when cell-free extracts of DFY70 and the null *pfk1-1* mutant were mixed, a dramatic rise in PFK activity was found. The controls of the unmixed extracts had negligible, if any, PFK activity. The amount of PFK activity obtained was on an average 60–100% of that in the diploid obtained by crossing the above two mutants. Fig. 1 shows the kinetics of the reconstitution process with the extracts of DFY70 and the *pfk1-1* mutant. Activity seen at zero time is likely to be due to the reconstitution during the assay. Activation of the enzyme by Fru(2,6)P_2 permitted the detection of activity as early as 5 min after mixing. The process is slow and shows a temperature dependence: the time taken to reach half-maximum activity at 24°C being 7–15 min. At 0°C or at 36°C, the reconstitution was either very slow or did not take place at all (data not shown).

In order to confirm the results with the diploids, reconstitution was carried out with the cell-free extract of DFY70 and that of the *pfk1-1 pfk2-4* double mutant. The kinetic properties of the enzyme so formed were examined along with those of the enzyme obtained in the mixture of homogenates of DFY70 and the *pfk1-1 PFK2* mutant. Results (Table 3) were similar to those seen with complementation studies *in vivo*, namely, that the wild-type enzyme was obtained in the mixture with the extract of the *pfk1-1 PFK2* mutant, while activity was seen only under de-inhibited conditions in the case of the extract of the *pfk1-1 pfk2-4* mutant. Thus, the regulatory properties of the reconstituted enzyme also reflected the nature of the *PFK2* allele. The specific activity of the reconstituted enzymes in these cases was significantly lower than that of the diploids. No reconstitution could be seen upon mixing of extracts from DFY70 and the double mutant *pfk1-1 pfk2-1*, although the diploid was enzyme-positive (Table 2). We have not ascertained if this was due to the instability of the α subunit specified by *pfk2-1*.

A comparison of the reconstituted enzyme with the wild-type enzyme was done. As seen in Fig. 2, sucrose density-gradient centrifugation of the two enzymes showed them to have similar sedimentation characteristics ($s_{20,w}$ values being 20.0 S for the wild-type PFK and 20.3 S for the reconstituted PFK), indicating a similar molecular mass. Fractions containing the reconstituted enzyme and corresponding to the peak

Table 3. *Effect of* PFK2 *alleles on the reconstituted soluble PFK*
Extracts were prepared in reconstitution buffer (see legend for Fig. 1) from logarithmic phase cells of the mutants mentioned below. Aliquots were mixed in equal proportions and activity measured after incubation at 24° C for 1 h as described in Table 2. Unmixed extracts were also assayed under the same conditions and were found to be negative for PFK activity (data not shown). n.d., not detectable

Extracts mixed	PFK activity in the mixture		
	non-inhibited	inhibited	de-inhibited
	mU mg^{-1} protein		
PFK1 pfk2-70 + *pfk1-1 PFK2*	19.9	2.0	29
PFK1 pfk2-70[a] + *pfk1-1 pfk2-4*	1.4	n.d.	n.d.

[a] This mixture when assayed with 0.25 mM ATP, 5 µM Fru(2,6)P_2 and concentrations of Fru6*P* of 2.5, 5 and 10 mM, showed a PFK activity of 14.6, 20.8 and 33.3 mU mg^{-1}, respectively.

of its activity were subjected to SDS/polyacrylamide gel electrophoresis, transblotted onto a nitrocellulose filter and probed with an antiserum raised against the purified soluble PFK enzyme. Fig. 3A shows the results of such an experiment. The crude extract from the wild-type strain and the purified PFK preparation both show the presence of the same two bands, the faster corresponding to β and the slower to the α subunit. The antiserum is seen to react more strongly to the β band than to the α band despite equal protein concentrations of each. Crude extract of the mutant DFY70 shows only the β subunit band as reported by Clifton and Fraenkel [10], whereas the nonsense *pfk1-1* mutant has only the band corresponding to the α subunit. The reconstituted enzyme reveals the presence of both the α and the β subunits, the amount of the two subunits in each fraction tallying with the amount of enzyme activity present in it. The results imply that association of the two subunits can take place spontaneously to give rise to an enzyme like that in the wild-type strain and that this is a prerequisite to the detection of PFK activity *in vitro*.

Sedimentation experiments were also performed with the unmixed extracts of the above two mutants in order to find out the oligomeric state of the α and the β subunits when present alone in the respective mutants. The relative amounts of the subunits in the fractions were estimated visually on the basis of the intensity of the peroxidase reaction on the Western blot. Fig. 3B shows the distribution profile of the α protein from the *pfk1-1* mutant on the gradient. A clear peak (fraction 30) in its quantity can be observed. Besides the band corresponding to the β protein, there is another band seen in the unfractionated crude extract of the *pfk1-1* mutant. This, as well as the additional faint band in the fractions, are probably degradation products of the α polypeptide, not found in a freshly prepared extract. A further analysis of fractions 27–32 enabled an accurate determination of the fraction containing the maximum amount of the α polypeptide. Based on this, the $s_{20,w}$ value of the free α subunit was found consistently to be between 6.4–6.6 S. Since these values correspond to an approximate molecular mass of about 130 kDa, free α exists largely as a monomer.

With the β protein of DFY70, on the other hand, there was no such sharp peak in its distribution (Fig. 3C). This polypeptide is seen to be present over a large number of

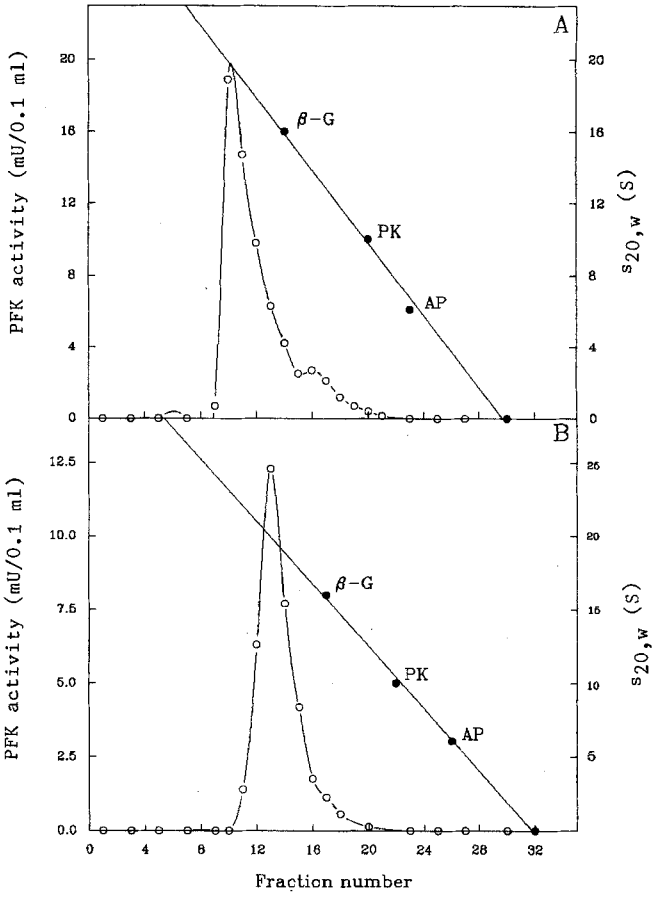

Fig. 2. *Comparison of reconstituted and wild-type PFK I enzymes by sucrose density-gradient centrifugation.* Cell-free extracts of *PFK1 pfk2-70* and *pfk1-1 PFK2* mutants in reconstitution buffer (see Fig. 1) were mixed in equal amounts and allowed to stand at 24° C for 1 h. 500 µl of the mixture was layered on 12 ml of a 5–20 % sucrose density-gradient in reconstitution buffer. Cell-free extract of the wild-type strain MC3 was also treated in the same manner and both the samples centrifuged using SW41 rotor in a Beckman centrifuge model L8-80 at 30000 rpm for 16.5 h at 4° C. Fractions were assayed for PFK activity using 2.5 mM Fru6*P* and 1 mM ATP. The markers used were: β-galactosidase (β-G) and alkaline phosphatase (AP) of *E. coli* and pyruvate kinase (PK) from rabbit muscle. (○) PFK activity; (●) $s_{20,w}$ value. The $s_{20,w}$ values for the wild-type PFK I (A) and the reconstituted PFK I (B) can be extrapolated as 20.0 S and 20.3 S respectively

fractions (5–25) which correspond to $s_{20,w}$ values of 24–10 S. This indicates that the free β subunit exists in an aggregated state extending from a dimer to an octamer (molecular mass ranging from 250 kDa to 922 kDa). In another experiment (data not shown), the β subunit was found to be distributed over fractions corresponding to $s_{20,w}$ values best suited for an octameric to a monomeric state of this subunit. Despite this variability, the catalytic subunit β, unlike the α subunit, exists not only in the monomeric form but also as higher-order aggregates.

It was also noticed during these experiments that the free β subunit, as it exists in DFY70, is more prone to degradation than the same in the $\alpha\beta$ heteromeric form. In spite of the antibody recognition being stronger against the β subunit, it became undetectable in the fractionated extract of DFY70 at 0° C, while at the same time it could be easily detected in fractions of the extract of the wild-type strain as also in freshly prepared extracts of DFY70. The free α subunit, on the other

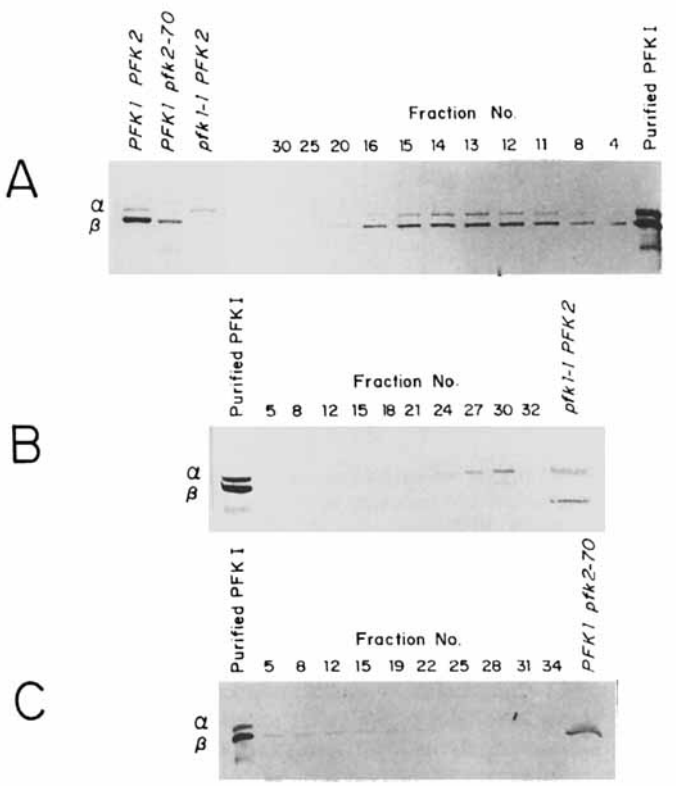

Fig. 3. *Western blot analysis of the sucrose density-gradient fractions of the reconstituted PFK I enzyme and of the unmixed extracts of the complementing mutants.* Crude extracts of YEP-glucose-grown cultures of the mutants *pfk1-1 PFK2* and *PFK1 pfk2-70* were subjected to sucrose density-gradient centrifugation (see legend to Fig. 2 for details). Equal volumes of the fractions obtained, along with the active fractions of the sedimentation experiment for the reconstituted PFK I (Fig. 2) were electrophoresed on a 7.5% SDS/polyacrylamide gel. Purified PFK I as well as the unfractionated extracts of a wild-type strain and of the two mutants were used as controls. The separated proteins were transferred onto a nitrocellulose filter and probed with anti-PFK I antiserum. (A) The left panel shows the subunits in the unfractionated crude extracts. The right panel shows the subunit bands of the purified PFK I and of the fractions of the reconstituted enzyme following sedimentation. (B) Fractionation of the α subunit of *pfk1-1 PFK2*. (C) Fractionation of the β subunit of *PFK1 pfk2-70*. In both B and C the first lane is the purified PFK I and the right most lane shows the unfractionated crude extracts of the respective mutants. The $s_{20,w}$ values for the individual subunits were determined with the help of markers (see legend to Fig. 2)

hand, was at least as stable as in the wild-type enzyme. Attempts to stabilise the β subunit *in vitro* were unsuccessful.

Characterisation of the mutant DFY250

The mutant DFY250 possesses a modified soluble PFK enzyme, which gives activity only when measured under high concentrations of the substrate Fru6*P* or in the presence of the activator Fru(2,6)P_2 [20]. It does not display any growth defect on glucose [10]. In this respect, it is similar to the *PFK1 pfk2-4* strain [7]. DFY250, however, complements the mutant DFY70 in synthesizing an enzyme similar to that in the wild-type strain [10], thus indicating the former to be non-allelic to *pfk2*.

In order to determine its genotype the strain DFY250 was crossed to both *pfk1* and *pfk2* mutants and the meiotic progeny analyzed for glucose-growth and soluble phosphofructokinase activity. Amongst five four-spore tetrads dissected from the cross of DFY250 and the *pfk1-1* mutant, all were of the parental type, two of the spores making an enzyme similar to that in DFY250 and the other two spores PFK-negative (data not shown), indicating that the mutation in DFY250 is allelic to *pfk1*.

The reciprocal cross of DFY250 with a *PFK1 pfk2-6* strain showed an interesting result. Firstly, all the segregants of the 10 tetrads examined were glucose-positive and enzyme-positive, indicating that the *pfk1-250 pfk2-6* double mutant did possess PFK activity. Different types of tetrads could be distinguished on the basis of the allosteric nature of the PFK I activity in the segregants and the segregation pattern for this cross was found to be 3P:1N:6T tetrads. The allosteric nature of the PFK activity in the spores of a typical tetratype tetrad of this cross was as follows. Two of the spores showed the presence of the parental enzymes, one bearing the enzyme insensitive to ATP as specified by the *pfk2-6* allele and the other having a PFK I similar to that of DFY250. Allosteric, wild-type enzyme was recovered in one of the spores indicating it to be the recombinant possessing wild-type copies of the two genes. The fourth spore, which was the *pfk1-250 pfk2-6* double mutant, possessed an enzyme similar in properties to that from DFY250 (data not shown). This double mutant was distinguished from the parental spore *pfk1-250* by back-crossing both to a wild-type strain. The segregants were examined for the presence of an ATP-insensitive PFK enzyme specified by the *pfk2-6* allele, expected to be seen in cell-free extracts of the segregants of the double mutant alone (data not shown).

It was thus of interest to examine the double mutant bearing the *pfk1* allele of DFY250 in combination with the *pfk2-4* allele. All the segregants of the cross between DFY250 and the *pfk2-4* mutant were proficient for glucose growth. The segregation pattern for this cross was 2P:1N:6T tetrads, distinguished on the basis of the kinetic properties of the PFK I enzyme of the spores. It was found that the soluble PFK enzyme in the double mutant *pfk1-250 pfk2-4* is similar to that in both DFY250 and the *pfk2-4* mutant in showing activity only under de-inhibited conditions (data not shown). This mutant could therefore be identified unambiguously only in the non-parental tetrad in which the two spores not bearing the wild-type enzyme were the double mutants.

The mutant DFY250, though grossly similar in phenotype to the mutant *pfk2-4*, is thus different from it in being a *pfk1* allele. It is epistatic to the *pfk2-6* allele, as the soluble PFK enzyme from *pfk1-250 pfk2-6* mutant is similar to that from DFY250 (*pfk1-250*).

The soluble PFK enzymes of both the mutants, DFY250 as well as *pfk2-4*, show a 'high K_m' for Fru6*P* [7, 20]. The allele *pfk2-4*, in addition, is known also to render the enzyme more susceptible to the inhibitory effects of ATP [7]. In an attempt to ascertain the differences between the two enzymes, if any, their ATP-inhibition patterns under normal and Fru(2,6)P_2-activated conditions were compared (Fig. 4). The wild-type enzyme becomes inhibited by ATP at concentrations above 0.5 mM (Fig. 4A). In the presence of Fru(2,6)P_2, the activity of this enzyme is increased by about fivefold, with very little inhibition by ATP even at a concentration of 5 mM of this ligand. The enzymes from both the mutants show hardly any activity when assayed without Fru(2,6)P_2 (Fig. 4B, C). With increasing concentration of ATP in the presence of 10 μM Fru(2,6)P_2, the enzyme of DFY250 (Fig. 4B) shows, like the wild-type enzyme, a relief of the inhibitory effects of ATP, at least up to a concentration of 5 mM ATP. There is a considerable difference in the absolute specific activities of these two

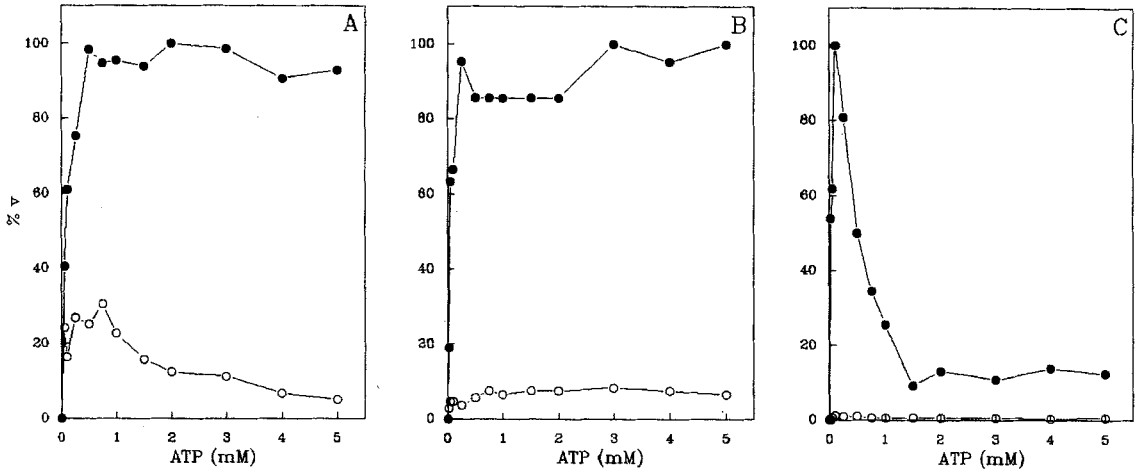

Fig. 4. *Effect of ATP on the soluble PFK activities from the* pfk1-250 PFK2 *and* PFK1 pfk2-4 *mutants.* Crude cell-free extracts in buffer A (see Table 1) were used as the source of the enzymes. The wild-type extract was also assayed. v indicates velocity of the reaction; values are percentages of the maximum. (A) Wild-type PFK; 100% = 207 mU mg^{-1} protein; (B) PFK from *pfk1-250 PFK2*; 100% = 32 mU mg^{-1} protein; (C) PFK from *PFK1 pfk2-4*; 100% = 75 mU mg^{-1} protein. (○): Assayed with 1 mM Fru6*P*; (●): assayed with 1 mM Fru6*P* and 10 μM Fru(2,6)P_2

enzymes: the velocity maxima of the wild-type being 207 mU mg^{-1} while that of the enzyme from DFY250 being 32 mU mg^{-1} under identical assay conditions. As compared to these two enzymes, a striking difference is observed in the ATP-saturation curve of the enzyme from the *pfk2-4* mutant (Fig. 4C). This enzyme fails to become deinhibited by Fru(2,6)P_2 at concentrations of ATP greater than 0.25 mM and remains practically inactive beyond an ATP concentration of 1.5 mM; the enzymes from the wild-type and DFY250 stay fully active under these conditions. The mutation *pfk2-4* therefore could be causing either an increase in affinity for ATP at the inhibitory site and/or a decrease in the affinity for the activator, Fru(2,6)P_2; either situation is expected to give rise to an enzyme 'hypersensitive' to inhibition by ATP. DFY250, on the other hand, shows a decrease in its catalytic efficiency. The increase in the $S_{0.5}$ value for Fru6*P* reported for the enzyme from the *pfk2-4* mutant [7] could then be attributed to the interdependence of the two substrates.

DISCUSSION

We have presented here the characterisation of two mutants of *Saccharomyces cerevisiae*, DFY70 and DFY250, affected in the soluble phosphofructokinase, in comparison with mutants of similar phenotypes obtained in this laboratory. Mutants lacking this enzyme activity were shown to constitute a single complementation group, *pfk1*, while *pfk2* mutants were known to synthesize a kinetically altered form of the enzyme [5]. Complementation and allele-testing reported here show that DFY70 and DFY250 do not conform to this observation and in fact show a reversal of these phenotypes. The results nonetheless conclusively establish that all the mutants affected in the soluble phosphofructokinase define only two complementation groups, *PFK1* and *PFK2*.

Unlike the well-studied enzyme aspartate transcarbamylase of *E. coli*, where the regulatory subunit is totally dispensable for enzyme function [21], association of the catalytic and regulatory subunits is essential for the functioning of the PFK I enzyme, at least *in vitro*, as established by the complementation and reconstitution studies reported here.

Besides DFY70, only one other mutant has been reported as of now (although poorly characterized) [9], which lacks the soluble PFK activity, by virtue of a mutation in the *pfk2* gene. As against this, a similar isolation procedure has provided more than 200 alleles of *pfk1* that result in soluble phosphofructokinase-negativity [6]. This disparity probably reflects the different functions of the two subunits. Point mutations in the *pfk2* gene might not lead to a loss of PFK I activity unless resulting in an inability to synthesize the polypeptide or giving rise to an α subunit incapable of association with β, since this seems to be essential for the enzyme activity. Indeed DFY70 is unique in lacking the α subunit as against all the other *pfk2* alleles studied which invariably bear missense mutations and appear to possess altered forms of this subunit [7]. The inability to suppress the mutation in DFY70 by nonsense suppressors (this laboratory, unpublished results) and the report that the *PFK2* transcript is reduced in this mutant [4] seem to indicate that it could be affected in the transcription of the *PFK2* gene.

That a single gene mutation affects both the PFK isozymes in DFY70 is not surprising, since the gene *PFK2* is known to be a common determinant governing them [5]. In spite of a lack of both the enzymes, this mutant ferments glucose under aerobic and anaerobic conditions. The observation that even a mutant bearing a disrupted copy of *pfk2* is proficient for glucose utilisation [22] shows this gene to be dispensable for glucose-growth. Metabolite accumulation studies for both DFY70 and the *pfk2*-disruptant mutant show only a partial block at the PFK step [10, 22]. Therefore the PFK reaction, though undetectable in assays, seems to function in both these mutants *in vivo*. This reaction has been attributed to the presence of the intact catalytic subunit in these mutants [10]. If indeed the β subunit functions *in vivo*, the inability to assay it in crude extracts may be because this subunit is less stable by itself than when associated with the α subunit. It is, however, not clear why there is a failure to detect activity in toluene-treated whole cells and in fresh extracts of DFY70 which do show the presence of the β subunit.

The existence of inactive mutants lacking either of the subunits or possessing an altered form of a single subunit has allowed easy shuffling of the subunits to give rise to phosphofructokinase enzymes of various subunit combinations in diploids. Such an exercise has also been performed *in vitro* enabling the reconstitution of this enzyme from the

free subunits. The reconstitution is found to be a slow process, requiring 20–30 min to reach completion at 24° C. Whether this is due to the fact that the reaction was carried out in crude extracts has not yet been ascertained. However another factor which could be responsible for the slowing down of this process is the existence of the subunits in a form not suitable for immediate association in the $\alpha_4\beta_4$ structure. While the β subunit displays a whole range of aggregated forms, the α subunit exists mainly as a monomer.

The mutant DFY250 possesses a modified β subunit which is unable to support growth on glucose in the absence of the α subunit (*pfk1-250 pfk2-70* is glucose-negative [10]). Even a mutant α subunit (as in *pfk1-250 pfk2-6* or *pfk1-250 pfk2-4*) restores detectable PFK I activity and renders the allele *pfk1-250* capable of supporting growth on glucose. This again implies the necessity of the $\alpha\beta$ association for PFK I activity especially for the defective β subunit, as occurs in DFY250.

The regulatory function of α is seen in the dependence on the *PFK2* allele for the allosteric properties of the soluble PFK enzyme in diploids as well as in the mixed extracts of DFY70 and the *pfk1 pfk2* mutants. The difference between the kinetically altered enzyme from the 'β mutant', DFY250, versus that from the 'α mutant', *pfk2-4*, also demonstrates the same. While the former is affected only in its catalytic efficiency, the latter has an increased sensitivity to the inhibitory effects of ATP.

It should be noted that though these and other studies indicate distinct roles for the two subunits, the amino acid sequences of both the α and β subunits, as deduced from the nucleotide sequences of the respective genes, show the presence of binding sites for Fru6*P* as well as for ATP [23]. Also, since all substrate-binding studies were performed with the whole enzyme, the possibility of a binding site being formed upon $\alpha\beta$ association cannot be ruled out. In fact, the impaired PFK activity in DFY70 as well as in the *pfk2* disruptant-mutant as compared to that in the wild-type strain indicates that the associated form is more efficient. Conclusive evidence resolving the issue has to come from site-directed mutagenesis at the predicted substrate-binding sites for each of the subunits to assess their participation in enzyme function.

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