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Phosphofructokinase Mutants of Yeast

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Mutants of *Saccharomyces cerevisiae* completely lacking the soluble glycolytic enzyme fructose-6-P kinase are described. The mutations are semidominant, do not complement one another, and define a gene *PFK1* located 28-cm distal to *rna1* on the extended right arm of chromosome XIII. Of 10 independent mutants, 3 can be suppressed by ochre suppressors. All mutants examined synthesize proteins that cross-react to the antibody against the purified yeast P-fructokinase. The enzyme in spontaneous revertants is distinguishable from the wild type enzyme with respect to thermolability and ATP inhibition. The locus *PFK1* thus defines the structural gene of the enzyme.

The *pfk1* mutants are not leaky *in vivo*. All the glucose consumed by a double mutant lacking both P-fructokinase and 6-P-gluconate dehydrogenase ends up as 6-P-gluconate, yet the *pfk1* mutants can glycolyze and grow on glucose in air. The cell mass produced per unit of glucose also remains unchanged. Anaerobically, however, growth does not take place, nor does glycolysis. P-fructokinase is thus a dispensable enzyme for aerobic growth, but indispensable for anaerobic growth. The properties of *pfk1* mutants suggest that yeast has an alternative mechanism for the aerobic metabolism of fructose-6-P, presumably through the recently reported particulate P-fructokinase (Lobo, Z., and Maitra, P. K. (1982) *FEBS Lett.* 137, 279-282).

Mutants lacking P-fructokinase are potentially interesting. This enzyme catalyzes an irreversible step in the Embden-Meyerhof pathway and is said to be a flux-determining enzyme in glycolysis (1). The kinetic properties of the enzyme from diverse sources are consistent with this interpretation. P-fructokinase from yeast has been implicated in particular in the Pasteur effect (2) and in the generation of glycolytic oscillations (3). A study of mutants of this enzyme is likely to shed light not only on such questions of cellular physiology but on mechanisms underlying allosteric regulation in general.

Mutants of *Saccharomyces cerevisiae* lacking this enzyme have been reported from several laboratories (4-7). An unexpected feature of all of these mutants is their ability to grow on glucose. The location of P-fructokinase in the glycolytic pathway suggests that the pentose phosphate pathway may act as a bypass in *pfk1* mutants. The growth of these mutants on glucose might also reflect their leakiness *in vivo*. However, the recently discovered second P-fructokinase in *S. cerevisiae* (8) probably allows the growth of *pfk1* mutants on hexose sugars.

We describe the results of some of our experiments which have a bearing on the problem of growth of *pfk1* mutants on glucose. Genetic characteristics of the mutation are also described.

MATERIALS AND METHODS

Strains—A large number of *S. cerevisiae* strains which were used for mapping and suppression experiments, will be mentioned where appropriate in the text. All of these strains were given generously by the Yeast Genetic Stock Center at Berkeley. Other strains have been described: wild type strain haploid *S. cerevisiae* (9); pyruvate kinase mutants *pyk1-4* and *pyk1-5* (10); 6-P-gluconate dehydrogenase mutant *gnd* (11); the P-fructokinase mutant allele *pfk1-1* was isolated earlier (4) as a glucose-negative mutant. Mutants lacking pyruvate decarboxylase *pdc1* were isolated by H. M. Chikarmane of this laboratory.

Media—The minimal medium and the YEP medium containing yeast extract, peptone, and carbon source have been described (9). The *pfk1* and *gnd* mutants were maintained on YEP medium containing ethyl alcohol.

Chemicals—Fructose-6-P was from Sigma. It was essentially free of glucose-6-P and fructose-1,6-bisP. Other substrates and enzymes including yeast P-fructokinase were from Boehringer.

Enzyme Assays—P-fructokinase activity was assayed fluorometrically in either cell-free extracts or Toluene lysates as before (12) in a reaction mixture containing 5 mM fructose-6-P, 1 mM ATP, 0.03 mM NADH, 1 unit each of aldolase and α-glycero-P dehydrogenase, and 10 units of triose P isomerase. To avoid interference by the particulate enzyme activity which was present only in cultures grown on glucose but not on alcohol (8), glucose-grown cultures were examined only in cell-free supernatants for the soluble enzyme. Alcohol-grown cultures were assayed either in Toluene lysates or in centrifuged cell extracts. The segregation of P-fructokinase in tetrads was followed in Toluene lysates (9) in a discontinuous assay; 50 μl of the lysate were incubated overnight at 23 °C with 0.25 ml of an incubation mixture of 2 mM fructose-6-P, 0.5 mM ADP, and 1 mM ATP in 50 mM triethanolamine buffer containing 10 mM $MgCl_2$, pH 7.4. Samples were heated at 65 °C for 10 min and fructose-1,6-bisP was assayed fluorometrically in 5 to 10 μl of the supernatants. The particulate P-fructokinase was practically inactive under conditions that allowed accumulation of fructose-1,6-bisP (8). Other methods have been described (9, 12).

RESULTS

Isolation of Mutants Lacking P-fructokinase—The allele *pfk1-1* which has been used in most of the experiments unless mentioned otherwise was isolated accidentally as a glucose-negative mutant among clones resistant to 2-deoxyglucose (4). In crosses to wild type strains, the marker *pfk1* segregated independently of 2-deoxyglucose resistance and glucose negativity.

The majority of *pfk1* mutants were obtained as spontaneous glucose-resistant revertants of *pyk1* mutants (5) growing on YEP plates containing 150 mM ethanol and 50 mM glucose. The more than 120 independent *pfk1* mutants which have been isolated by this method have been designated *pfk1-2* through *pfk1-200*. A third method of isolation of *pfk1* mutants

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was to revert *pdc1* mutants on glucose media[1]; 3 mutants (alleles *pfk1-201* to *pfk1-203*) were used for complementation studies. The *pyk1* and *pdc1* markers were eliminated by crossing these isolates to a wild type strain; the resultant single gene mutants lacking P-fructokinase were all able to grow on glucose.

P-fructokinase Activity in Haploids and Diploids—The activity of P-fructokinase in the *pfk1* mutants was generally less than 0.1% of the wild type level. A few mutants which had detectable activity (about 1%) were suppressed poorly for growth on alcohol + glucose medium. The enzyme levels in homozygous and heterozygous diploids carrying the allele *pfk1-1* were examined in cell-free extracts from cultures growing exponentially on glucose. The P-fructokinase activity was as follows: *PFK1*, 290 nm/mg of protein; *pfk1-1*, <0.001 nm/mg of protein; *PFK1/PFK1*, 330 nm/mg of protein; *PFK1/pfk1-1*, 90 nm/mg of protein. In stationary cultures of glucose- or alcohol-grown cultures, the enzyme activity was about a third of these values. However, the specific activity of P-fructokinase in heteroallelic diploids was always less than 50% of that of the wild type haploids or homoallelic diploids. The mutation *pfk1* thus behaved as semidominant. Heterozygous diploids *pfk1/PFK1*, however, grew on glucose azide plates as did the wild type strains.

For complementation studies, a number of mutants were crossed to one another in paired combinations. The diploids were isolated either by micromanipulation of the zygotes or by prototrophic selection and examined for P-fructokinase activity after growth on alcohol. In most of the hybrids no enzyme activity higher than that seen in the parents could be seen. A few combinations which indicated slightly more enzyme activity than in the parents were sporulated and the activity of P-fructokinase was examined in the haploid progenies. No spore was found to be P-fructokinase-positive. The enzyme activity in such diploids presumably came about by interallelic complementation, *e.g.* in the diploid *pfk1-9/pfk1-6* (see "Nonsense Mutants"). Such experiments suggested that all the mutants tested lie in the same complementation group *PFK1*. We have, however, not examined all the paired combinations of mutants.

Nonsense Mutants—Of the 10 independent mutants examined in our collection, 3 could be suppressed by ochre suppressors *SUP7* or *SUP8*. Mutants were crossed to the suppressor-bearing strains X2182-3D (*a ade2-1$_0$ lys1-1$_0$ his5-2$_0$ trp5-48$_0$ met1-1 leu1-12 SUP7*) and X2318-26C (*αhis5-2$_0$ lys1-1$_0$ ade2-1$_0$ can1-100$_0$ trp5-48$_0$ ura3-1 SUP8*) separately and the tetrad analysis of the sporulated diploids indicated coincident suppression of *pfk1* along with known ochre-suppressible auxotrophic markers. Secondary crosses of *pfk1*-suppressed strains to wild type led to reappearance of P-fructokinase-negative spores as the suppressor was lost. We concluded from this that the following 3 alleles are nonsense mutants: *pfk1-1*, *pfk1-16*, and *pfk1-17*. No amber-specific suppression could be seen in any of the *pfk1* mutants using the strain 4380-2C from Prof. G. R. Fink (Cornell University, Ithaca, NY) which carried the following markers: *α met8-1a his4-644a leu2-1$_0$ lys1-1$_0$ SUP4-3a*.

These experiments had generated recombinants carrying several nonsuppressible mutant alleles of *pfk1* in conjunction with the nonsense suppressor *SUP7* or *SUP8* and a few of the suppressible auxotrophic markers. These strains were crossed to the test isolates of *pfk1* mutant to examine their suppressibility in the diploid. For example, the hybrid *pfk1-6* (nonsuppressible) *SUP7/pfk1-17* (nonsense) + was P-fructokinase-positive by enzyme assay, and its haploid progeny of the genotype *pfk1-17 SUP7* was also enzyme-positive. One other diploid of the constitution *pfk1-6* (nonsuppressible) *SUP7/pfk1-1* (nonsense) +, was, however, found not to have any P-fructokinase activity. When this diploid was sporulated and the spores examined for the enzyme activity it was found that haploids carrying *pfk1-1 SUP7* were in fact P-fructokinase-positive. In contrast, the enzyme-positive diploid *pfk1-9* (nonsuppressible) +/*pfk1-6* (nonsuppressible) *SUP7* failed to produce a spore that had P-fructokinase activity. We interpret these observations as reflecting interaction of subunits in this oligomeric protein, providing an instance of negative complementation in the second case, and positive complementation in the last. We have not encountered a *pfk1* mutant that is fully dominant with respect to its wild type allele *PFK1*.

Growth Properties—One of the features of *pfk1* mutants that distinguish them from most other glycolytic lesions is their ability to grow on glucose, fructose, and mannose much as the wild type yeast. Results in Table I illustrate this with respect to the nonsense allele *pfk1-1* growing, respectively, on alcohol and glucose as carbon sources. Loss of P-fructokinase reduced the rate of growth on alcohol only marginally, while on glucose it was halved. Restoration of enzyme activity by suppression of the nonsense mutation either by the unlinked tRNA suppressor *SUP7* or by an intragenic suppressor *R5* improved the rate of growth on glucose. However, the absence of any detectable enzyme activity in the soluble supernatants did not prevent growth.

How do *pfk1* mutants lacking any measurable enzyme activity of P-fructokinase in soluble supernatants continue to grow on glucose? A reasonable explanation is that the pentose phosphate pathway acts as a bypass of this enzyme. Because of the stoichiometry

$$\text{3-Glucose-6P} + \text{6-NADP} \rightarrow \text{2-fructose-6-P} + \text{glyceraldehyde-3-P} + \text{6NADPH} + 3CO_2 \quad (1)$$

operative during the use of this pathway, half the glucose ends up as CO_2 and is presumably lost from productive metabolism. The mutant allele *pfk1-1* was grown aerobically on limiting glucose to stationary phase and the yield of cell mass, measured as E_{650} was determined. Fig. 1 shows a plot of the saturating growth for several concentrations of glucose using both the YEP medium and a minimal medium. The linearity between the cell yield and glucose added showed that they were proportional at low concentrations. However, the loss of P-fructokinase made hardly any difference to the efficiency of cell synthesis at limiting concentrations of glucose. Except for a reduction in the rate of growth, *pfk1* mutants behaved very much like the wild type strain during aerobic growth on sugars.

Leakiness of the Nonsense Mutants—In order to examine

TABLE I

Comparison of P-fructokinase activity with growth rate

Haploid yeast cultures were grown on YEP glucose medium to the end of stationary phase and cell-free extracts were examined for P-fructokinase activity. The doubling periods were calculated during exponential growth on YEP medium containing glucose or ethanol. Spores T2A, T2B, and T2C were from a diploid heterozygous for *pfk1-1* and *SUP7*. The strain *pfk1-1* R5 was a spontaneous intragenic revertant of the *pfk1-1* mutant.

Strain	Genotype		P-fructokinase	Doubling period on	
				Alcohol	Glucose
			nm/mg protein	*h*	
T2A	*pfk1-1*	+	0	3.3	3.5
T2B	*pfk1-1*	*SUP7*	45	4.3	3.0
T2C	+	+	115	3.0	1.7
pfk1-1 R5	*pfk1-1* (R5)	+	140	3.2	1.8

[1] H. M. Chikarmane, personal communication.

if the *pfk1* mutants were leaky *in vivo*, the nonsense allele *pfk1-1* was used to construct a double mutant *pfk1 gnd* lacking the shunt pathway enzyme, 6-P-gluconate dehydrogenase, in addition to lacking P-fructokinase. The double mutant did not grow on glucose, but grew on alcohol, and possessed no more than 5% of the dehydrogenase activity of the wild type strains. P-fructokinase activity measured either in the cell-free extracts or in toluene lysates was undetectable since the particulate activity was not induced by growth on alcohol (8). It was grown in YEP alcohol medium to stationary phase and a washed suspension of cells was treated with glucose. Aliquots were withdrawn into acid for estimation of 6-P-gluconate, glucose-6-P, and glucose. Results are shown in Fig. 2. The

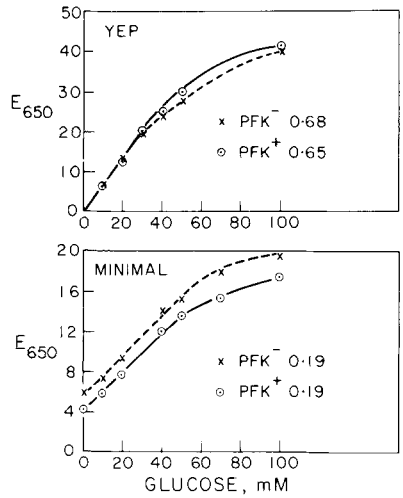

FIG. 1. **Yield of yeast cell mass as a function of glucose concentration during aerobic growth of a *pfk1-1* mutant and a wild type strain.** *Upper* and *lower*, YEP and minimal media, respectively. Cultures were grown on a rotary shaker containing YEP medium at 30 °C with either alcohol or glucose as the carbon source. Growth was followed by measuring the absorbance at 650 nm (E_{650}) in a 1-cm path. *PFK⁻* refers to the mutant allele *pfk1-1* and *PFK⁺* to the wild type strain. The observed slopes during the linear part of the plots are indicated for both strains.

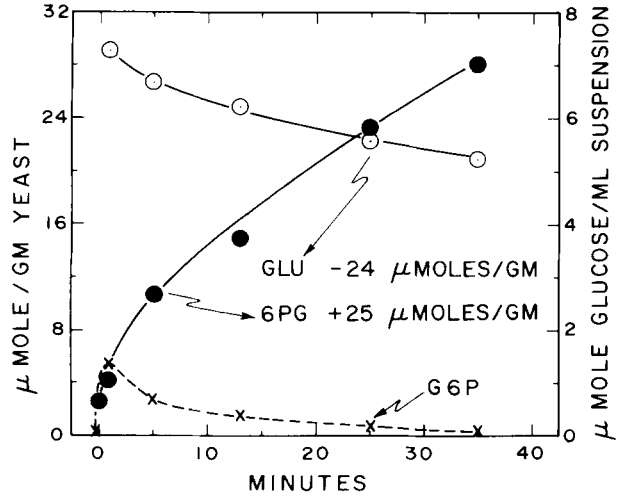

FIG. 2. **Accumulation of 6-P-gluconate during aerobic glucose utilization by a double mutant *pfk1 gnd* lacking both P-fructokinase and 6-P-gluconate dehydrogenase.** A washed suspension in 50 mM potassium phosphate buffer containing 100 mg, wet cells/ml was kept oxygenated with a mixture of 95% O_2 and 5% CO_2. Glucose was added at the instant indicated by 0 on the *abscissae*. At the end of the experiment, 25 μmol of 6-P-gluconate (*6PG*) were produced from the consumption of 24 μmol of glucose (*GLU*) for every gram of wet cells. *G6P*, glucose-6-P.

utilization of glucose started rapidly but slowed down considerably as 6-P-gluconate concentration kept on rising and that of glucose-6-P rose and fell. The total amount of glucose utilized at the end of the incubation was calculated from the extrapolated zero time value and found to be 24 μmol/g of yeast; that of 6-P-gluconate formed was 25 μmol/g. All the glucose utilized thus ended up as 6-P-gluconate suggesting that neither of the *pfk1* nor *gnd* blocks permitted any significant metabolic leak past these lesions.

Aerobic Glycolysis—Results in Fig. 3 depict a comparison of the profile of glycolytic intermediates in the *pfk1* mutant and the wild type yeast during aerobic metabolism. The cultures were grown in YEP glucose to stationary phase. The loss of P-fructokinase led to a nearly 3-fold drop in the rate of glucose consumption and a severe restriction in the outflow of this glucose into alcohol. As expected from a lesion in P-fructokinase, the level of fructose-1,6-bisP was much lower than in the wild type, while that of glucose-6-P was higher. Fructose-6-P levels (not shown) remained approximately at a quarter of those of glucose-6-P throughout. The kinetics of ATP indicated that the mutant strain was able to synthesize this substance, however, the turnover time was longer than in the wild type strain. The drop in the intracellular concentration of P-enolpyruvate was delayed unlike in the wild type strain mirroring a corresponding lag in the accumulation of fructose-1,6-bisP (13).

Anaerobic Glycolysis—Because of the involvement of P-fructokinase in Pasteur effect in many systems (1), the features of anaerobic glycolysis in *pfk1* mutant would be of interest. Our conclusion is that the mutation causes a very considerable, if not complete, reduction in the rate of anaerobic glycolysis. Results in Fig. 4 illustrate a typical experiment. Glucose was not utilized anaerobically; its addition led to a progressive loss of ATP and a monotonic accumulation of glucose-6-P. The levels of fructose-1,6-bisP (not shown) were low during the entire incubation period. The presence of oxygen on the other hand elicited a normal response. The effect of anaerobiosis was reversible. When N_2 gas was replaced with O_2, glucose utilization resumed and reached the aerobic rate. Mutants lacking P-fructokinase are thus characterized by having a negative Pasteur effect.

Anaerobic Growth—Due to the high affinity of cytochrome oxidase to oxygen (14) anaerobic experiments with complete exclusion of oxygen are technically difficult. We have used in these experiments three different but related methods to simulate anaerobic conditions of growth. In the first method *pfk1* strains were examined for growth on YEP glucose plates containing 0.3 mM sodium azide to inhibit respiration (9). Unlike the P-fructokinase-positive strains which grew on these plates within 1 day, *pfk1* mutants did not grow till the third day. This method was routinely used in conjunction with enzyme assay to follow the segregation of the marker *pfk1* in genetic crosses. In the second method the strain *pfk1-1* was crossed to a strain X3144-11A of the genotype *α leu2 trp1 rad1 arg9 ilv3 pet8 petx* and spores of the genotype *pfk1 pet8 petx* were isolated from 4-spore tetrads germinated on glucose plates. Although the viability of spores was normal, the unexpected observation was that these particular *pfk1* segregants could not be revived on further transfer to YEP glucose plates. Repeated transfers invariably led to the reappearance of P-fructokinase-positive clones. The third method of curing *pfk1* mutants of their mitochondrial genome by ethidium bromide (15) were also unsuccessful. The survivors were revertants for P-fructokinase activity. We conclude therefore that loss of P-fructokinase is incompatible with anaerobic growth.

Revertants—Since we were interested in revertants that were respiratory sufficient, the majority of the revertants were

isolated as clones able to form colonies on YEP glucose plates containing azide. The spontaneous reversion frequency varied from 1×10^{-7} to 5×10^{-7} depending on the *pfk1* allele. The nonsense mutant *pfk1-1* yielded both extragenic and intragenic revertants. On crossing to the wild type strain the revertant R1 gave enzyme-positive as also enzyme-negative spores as $4^+:0^-$, 2; $3^+:1^-$, 16; and $2^+:2^-$, 2 in tetrads. The generation of equal frequency of parental ditype and nonparental ditype asci showed that the suppressor locus in R1 was unlinked to the locus *pfk1*. The intragenic revertant R5, on the other hand, yielded no enzyme-negative progeny in 17 tetrads; its suppressor could not be segregated from the *pfk1* locus. No extragenic suppressor was found for the nonsuppressible mutant alleles tested.

The revertants from a number of *pfk1* mutants covered a wide spectrum of enzyme specific activity, thermolability, and kinetic property. Results in Fig. 5 illustrate this with respect to an intragenic revertant from a nonsense allele *pfk1-1* and a *SUP7*-suppressed derivative *pfk1-1 SUP7*, and intragenic revertants *pfk1-7* R13 and *pfk1-7* R22 obtained from the nonsuppressible allele *pfk1-7*. The enzyme from the revertant R22, for example, was not perceptibly inhibited by increasing concentrations of ATP unlike P-fructokinase from the revertant R13 or the wild type strain as seen in toluene lysates. When cell-free extracts were used, however, the reverted enzyme in R22 was found to be inhibited by high concentrations (4 mM) of ATP, while the wild type enzyme was inhibited at a much lower concentration of ATP (0.25 mM) under these conditions. No reverted enzyme was found to be completely insensitive to inhibition by ATP. The results in Fig. 5 also indicate that the reverted or the nonsense-suppressed enzyme could be either more stable or more labile to heat compared to the wild type enzyme. The inactivation was monophasic in every case studied.

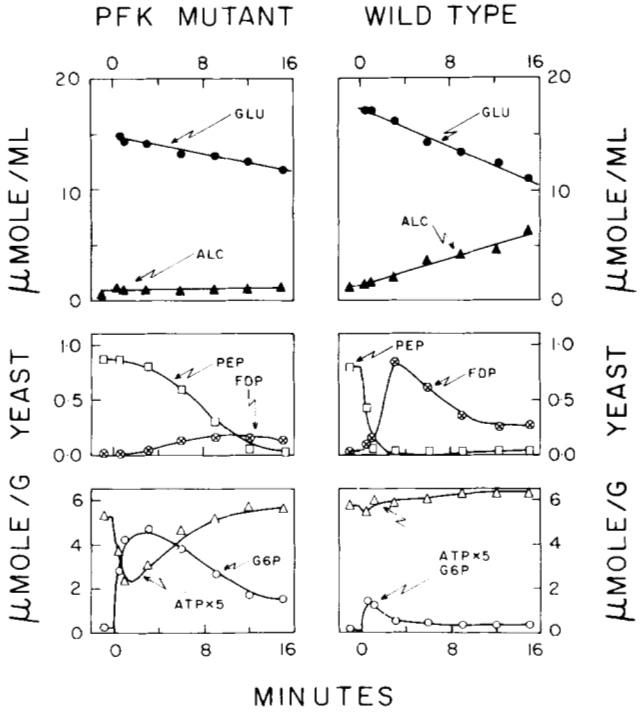

FIG. 3. **Comparison of aerobic glycolysis in P-fructokinase mutant and a wild type strain.** The suspensions contained per ml, 75 mg and 62 mg of the *pfk1-1* mutant and wild type strain, respectively. Other details are as in Fig. 2. *ALC*, alcohol; *PEP*, P-enolpyruvate; *FDP*, fructose-1,6-bisP; *G6P*, glucose-6-P. The values of ATP have been multiplied by 5.

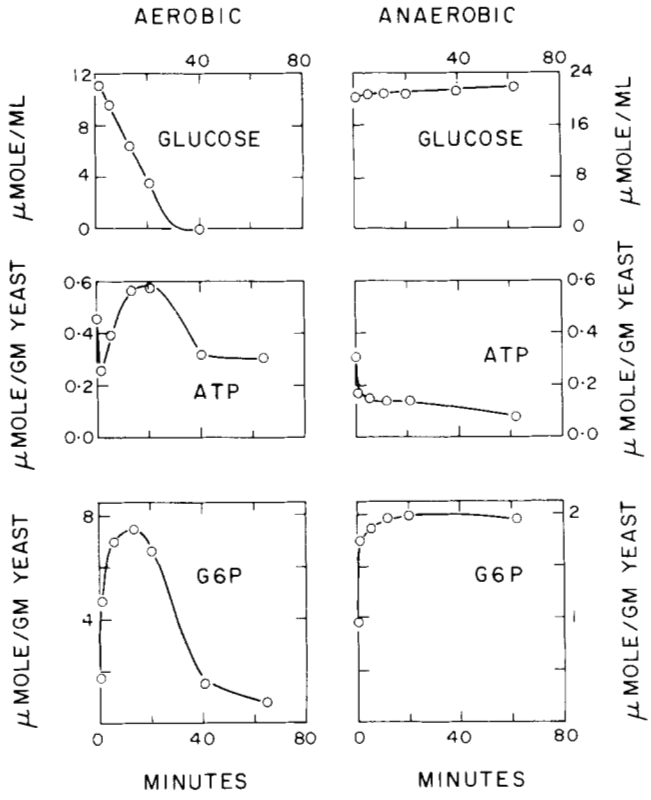

FIG. 4. **Aerobic and anaerobic glycolysis in a *pfk1* mutant.** Cells were grown on YEP glucose till the stationary phase and bubbled with a gas mixture containing 5% CO_2 and 95% O_2 (*left*) as the suspension was stirred over a magnetic stirrer in a 50-ml beaker. The anaerobic incubation was done in 50-ml test tubes with a slow stream of gas (95% N_2, 5% CO_2) gently agitating the suspension to minimize contamination from traces of O_2 present in the gas mixture. The cell concentrations were as: aerobic, 92 mg, wet yeast/ml; anaerobic, 200 mg/ml. Details are as in Fig. 3. *G6P*, glucose-6-P.

Cross-reacting Material—Unfractionated cell-free extracts from several mutants were tested for the presence of proteins immunologically similar to P-fructokinase. Ouchterlony double-diffusion plates (16) containing rabbit antiserum raised against purified P-fructokinase from yeast were used in the central well; the peripheral wells contained either the crude extracts from the mutants, their revertants, or the wild type strain or the purified enzyme. Strong, single precipitin lines were seen in every case suggesting the presence of cross-reacting materials in the mutants, including two independent nonsense alleles of *pfk1*. The lines merged with one another to produce a complete hexagonal figure when a hexagonal template was used. This suggested that mutants without enzyme activity synthesized P-fructokinase-like proteins. Since the soluble P-fructokinase of yeast is known to be composed of two nonidentical polypeptide chains (17, 18), it is possible that the precipitin lines were due not to the product of the gene *PFK1*, but reflected the presence of the second of the two constituent subunits.

Chromosomal Location—The mapping of *pfk1* proved laborious because its segregation had to be followed by assay of enzyme activity, and secondly, it was not linked to the centromere; the second division segregation frequency with respect to *trp1* was 0.69 (89/129). Table II gives the linkage analysis from meiotic data using three sets of diploids heterozygous for the marker pairs *pfk1-rna1*, *pfk1-SUP8*, and *rna1-SUP8* on chromosome XIII; *SUP8* was monitored by the homozygous ochre marker *lys1-1*. The nonsuppressible allele *pfk1-3*

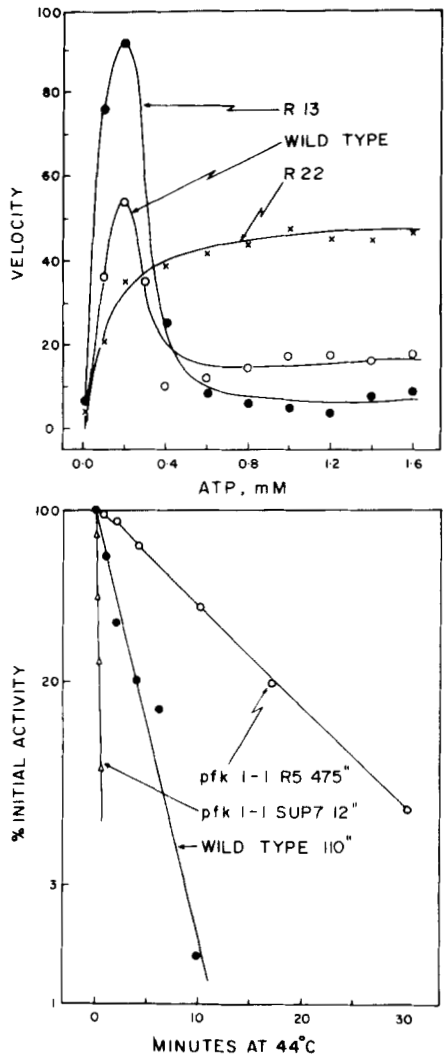

FIG. 5. **Effect on P-fructokinase of heat and ATP.** *Top,* effect of ATP on the activity of P-fructokinase from revertants and a wild type strain. Alcohol-grown cultures of revertants R13 and R22 from *pfk1-7* and a wild type strain haploid *S. cerevisiae* were examined at 1 mM fructose-6-P for P-fructokinase activity in toluene lysates. The enzyme activity is expressed in arbitrary units of NADH fluorescence. *Bottom,* heat-inactivation profile of P-fructokinase. Cell-free extracts of glucose-grown cultures of the intragenic revertant *pfk1-1* R5, the wild type strain haploid *S. cerevisiae,* and a suppressed strain *pfk1-1 SUP7* were heated at 44 °C for the periods indicated, chilled immediately in melting ice, and assayed for the remaining activity. The *numbers* indicate the time in seconds for the initial enzyme activity to be reduced by half.

TABLE II

Ascus type ratios for SUP8, rna1, and pfk1

Gene pair	Ascus type			Map distance
	Parental ditype	Nonparental ditype	Tetratype	
				centimorgan
rna1-pfk1	65	0	72	27.9
rna1-SUP8	8	0	22	37.0
pfk1-SUP8	24	7	28	59.3

was used when *SUP8* was segregating; the nonsense allele *pfk1-1* was used for the others. These results placed *pfk1* on the extended right arm of chromosome XIII 28 centimorgans from *rna1* in the order: centromere-*SUP8-rna1-pfk1*.

DISCUSSION

Aerobic and Anaerobic Growth of pfk1 Mutants—The property of *pfk1* mutants to continue to grow on sugars contrasts with that of most of the glycolytic mutants studied in yeast (19) and in *Escherichia coli* (20). The possibility that this was due to their leakiness *in vivo* can be ruled out in view of the experiment using the double mutant *pfk1 gnd* described in Fig. 2. The manner of their isolation suggested that loss of P-fructokinase allowed in some unknown manner the alleviation of growth inhibition caused by glucose in *pyk1* or *pdc1* mutants. It is likely that growth under these conditions was permitted not so much by attenuation of metabolic flux through glycolysis (5) as by modulation of negative control such as catabolite repression. Since the *pyk1* alleles constituted quite a tight metabolic block, the double mutant *pyk1 pfk1* somehow allowed the utilization of alcohol despite the presence of glucose. However, no relief of catabolic repression or catabolite inactivation could be seen in *pfk1* mutants with respect to isocitrate lyase and the glucose-repressible alcohol dehydrogenase (21) or of fructose-1,6-bis-phosphatase or P-enolpyruvate carboxykinase. It should, however, be noted that about two-thirds of the glucose-resistant mutants from *pyk1* strains continued to be positive for P-fructokinase activity.

In the absence of oxygen, however, *pfk1* mutants are unable to grow. Their rudimentary growth in standing cultures or in the presence of azide, the spontaneous selection of enzyme-positive revertants during their growth in the petite state (both nuclear and cytoplasmic), and the inability of stationary cultures of *pfk1* mutants to metabolize glucose in absence of oxygen justify the conclusion that the loss of this enzyme leads to impairment of anaerobic growth. If *pfk1* mutants grew anaerobically by way of the pentose phosphate pathway and obeyed the stoichiometry shown in Reaction 1, it is immediately apparent that anaerobic growth would not have been sustained; the shunt pathway produces too many reductant molecules for too few oxidant substrates. How then did single spores of the genotype *pfk1 pet8 petx* germinate on glucose to produce visible colonies? Was it due to some spore-specific P-fructokinase? Examination of spore suspensions of diploids homozygous for *pfk1* gave no indication of such an enzyme. Was it due to the diffusion of acetaldehyde as an electron acceptor from neighboring *PFK1* spores? This was ruled out by the observation that germination of spores was not affected by planting them far apart from one another. It is thus difficult to escape the conclusion that there must exist a *PFK1*-independent mechanism that allowed petite spores lacking P-fructokinase to undergo at least 20 cell divisions to produce a visible spore colony from a single micromanipulated cell. However, this was clearly inadequate to sustain the anaerobic growth of *pfk1* mutants. Perhaps the recently reported particulate P-fructokinase serves this purpose (8) and is also responsible for the normal growth yield of *pfk1* mutants from glucose.

Glycolytic Behavior—Two shades of behavior distinguished the P-fructokinase mutant from the wild type strain in aerobic incubation, one was the delayed rise of fructose-1,6-bisP and the attendant fall in the level of P-enolpyruvate as pyruvate kinase was activated (13). The other was the somewhat elevated level of fructose-6-P, the substrate of the blocked enzyme. Both of these features depended on the conditions of growth. When grown on alcohol the *pfk1* mutant showed a much higher level of fructose-6-P and a correspondingly reduced level of fructose-1,6-bisP due very likely to derepression of fructose-1,6-bisphosphatase (22). When cultures of the *pfk1* mutant growing exponentially on glucose were examined for the profile of glycolytic intermediates (data not shown), the distinction between the mutant and the wild type strain

practically disappeared. Under such conditions the mutant was almost as proficient in glycolysis as the wild type reflecting again the presence of the glucose-induced particulate enzyme (8).

Anaerobically, however, the situation was very different. Glucose metabolism was negligible and the kinetics of metabolites reflected blocked glycolysis (Fig. 4). The levels of ATP, unlike the aerobic experiment, were never restored following glucose addition. Reintroduction of oxygen reversed the changes seen in nitrogen.

Nature of pfk1 Mutation—The observation that *pfk1* mutants obtained by three different procedures lie in the same complementation group suggests that the catalytic activity of P-fructokinase is coded possibly by a single locus. There is ample evidence for the conclusion that this gene defines the structural determinant for this enzyme. The suppressibility of three of these mutants by tRNA suppressors showed that the gene is translated. Further, the P-fructokinase activity restored in the majority of the revertants was perceptibly different from that in the wild type strains: some were more thermolabile while some others more stable than the unmutated enzyme; some of them were altered with respect to ATP inhibition and sigmoidicity to fructose-6-P. Many of the reversion events leading to restoration of the enzyme activity mapped very close to or perhaps within the *PFK1* gene. These results accord with the conclusion that the locus *PFK1* specifies the structure of P-fructokinase. We have recently shown (18) that structural alterations in P-fructokinase come about by mutation in either of the genes *PFK1* or *PFK2* (8); lesions in the former cause loss of catalytic activity and those in the latter lead to loss of allosteric regulation (18). The observation that all the mutants described here were affected in the gene *PFK1* rather than in *PFK2* lends support to the idea that the former is the sole determinant that specifies the catalytic activity of P-fructokinase in *S. cerevisiae*.

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