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Yeast Pyruvate Kinase: A Mutant Form Catalytically Insensitive to Fructose 1,6-Bisphosphate

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The paper describes some of the characteristic properties of an altered form of pyruvate kinase from a mutant of *Saccharomyces cerevisiae*. The partially purified enzyme does not require fructose 1,6-bisphosphate for activity but is stabilised in its presence both at low and at high temperatures. The enzyme displays in the absence of fructose 1,6-bisphosphate hyperbolic kinetics with phospho*enol*pyruvate (K_m, 0.11 mM), ADP (K_m, 0.12 mM) and K^+ (K_m, 11 mM). Sedimentation velocity experiments indicate that the mutated enzyme and the wild type enzyme have $s_{20,w}$ values of 8.9 and 8.6 S respectively.

The mutant with the pyruvate kinase insensitive to fructose 1,6-bisphosphate is capable of growing on synthetic media with alcohol or malate as the sole carbon source. The steady-state intracellular levels of phospho*enol*pyruvate in the mutant suggest mechanisms that prevent depletion of this metabolite despite an active pyruvate kinase. Spontaneous reversion of this mutant yields clones with normal enzyme activated by fructose 1,6-bisphosphate.

Pyruvate kinase of yeast is known to be controlled by fructose 1,6-bisphosphate. The enzyme is largely inactive in absence of this metabolite unless very large amounts of phospho*enol*pyruvate are present [1]. Fructose 1,6-bisphosphate is said to interact with this enzyme allosterically, converting it to a form that has more affinity towards phospho*enol*pyruvate. The intracellular kinetics of fructose 1,6-bisphosphate and phospho*enol*pyruvate is consistent with this idea [2, 3]. It has been suggested [4] that the positive control of pyruvate kinase by fructose 1,6-bisphosphate provides the switching mechanism by which rate-limiting amounts of cellular phospho*enol*pyruvate could be conserved for gluconeogenesis.

While searching for mutants of yeast lacking pyruvate kinase, we came across a mutant that had pyruvate kinase but the altered enzyme did not require fructose 1,6-bisphosphate for activity. Some of the properties of this enzyme have been compared with those from the wild type. Besides, we have examined the growth behaviour of the mutant bearing the altered enzyme under gluconeogenic conditions. This report describes some of these experiments.

MATERIALS AND METHODS

Strain of Yeast

A leaky pyruvate kinase mutant was first obtained by mutagenising a wild-type prototrophic strain of *Saccharomyces cerevisiae* with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to obtain a glucose-negative, alcohol-positive colony. The procedure was the same as described earlier [5]. This mutant, named PK3 had low levels of pyruvate kinase activity, approximately one-twentieth of that seen in the wild-type strain. It was unable to grow on a yeast extract peptone medium supplemented with glucose, fructose or mannose; however, it grew on alcohol nearly as well as the wild type. Prolonged incubation on solid media containing glucose gave rise to a smear of growth. This mutant PK3 was plated heavily on a yeast extract peptone medium containing glucose; following 10 days of incubation at 30° C revertant colonies appeared. PK3R121 was one such revertant that grew on glucose; it was purified by streaking on plates containing glucose medium. This strain, called R121 was used in this study. The level of pyruvate kinase activity in the mutant R121 was nearly a third to half of that of the wild type while the activity of phosphoglucose isomerase was comparable.

Assay of Enzymes and Substrates

Pyruvate kinase and other glycolytic enzyme activities were measured either in toluene lysates or in cell-free extracts by methods described earlier [6]. Since the pyruvate kinase from the mutant R121 did not require fructose 1,6-bisphosphate, the reaction was started with ADP or in a few cases with pyruvate-free phospho*enol*pyruvate; the order of addition of

Enzyme. Pyruvate kinase (EC 2.7.1.40)

reactants did not make any difference to the observed activity. Metabolites were measured in acidified cell-extracts following neutralisation [6]. For kinetic studies the stock enzyme solution was freed from fructose 1,6-bisphosphate by gel filtration on Sephadex G25 using a 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM 2-mercaptoethanol, 2 mM EDTA and 25% glycerol for stabilising the enzyme activity. The standard reaction mixture for assaying pyruvate kinase consisted of 50 mM triethanolamine buffer, pH 7.4, 10 mM $MgCl_2$, 50 mM KCl, 1 mM each of phospho*enol*pyruvate, ADP and fructose 1,6-bisphosphate, 0.02 mM NADH and 1 unit of muscle lactic dehydrogenase. In a number of experiments the pyruvate kinase activity was measured discontinuously by assaying the ATP produced.

RESULTS

Preparation of Pyruvate Kinase from Strain R121

The strain R121 bearing the altered enzyme was grown aerobically for 24 h in 2 l of a medium containing yeast extract, peptone and glucose. 25 g of washed cells were suspended in 50 ml of a buffer containing 50 mM potassium phosphate, pH 7.4, 2 mM EDTA, 2 mM 2-mercaptoethanol, 2 mM phenylmethanesulfonyl fluoride and 0.5 mM fructose 1,6-bisphosphate and the suspension passed through a French press. The broken cell suspension was centrifuged in the cold at $10000 \times g$ for 15 min yielding 50 ml of supernatant. This was fractionated with solid $(NH_4)_2SO_4$ in the cold at pH 7.4 and the fraction precipitating between 30% and 60% saturation was taken up in 7 ml of a buffer containing 5 mM potassium phosphate, pH 7.4, 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.5 mM fructose 1,6-bisphosphate in 25% glycerol; the enzyme solution was freed of $(NH_4)_2SO_4$ by passing it through a column of Sephadex G25 equilibrated with the same buffer. The filtered enzyme solution was adsorbed on a DEAE-cellulose column (100 ml packed volume) prepared in the same buffer at pH 7.4, washed with 200 ml of this buffer, and the pyruvate kinase eluted with a linear gradient of 2×250 ml of 0 to 0.3 M KCl in the buffer containing 25% glycerol and 0.5 mM fructose 1,6-bisphosphate. The peak of enzyme activity was eluted at 166 mM KCl. Pyruvate kinase was precipitated from the pooled eluate (60 ml) with $(NH_4)_2SO_4$, the fraction precipitating between 40% and 80% saturation having the bulk of the enzyme activity. The precipitated enzyme was dissolved in a buffer at pH 7.5 containing 50 mM Tris, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.5 mM fructose 1,6-bisphosphate and 25% glycerol. The enzyme solution (2.2 ml) was freed from $(NH_4)_2SO_4$ by gel filtration on Sephadex G25 equilibrated in Tris-glycerol buffer at pH 7.5 and the salt-free enzyme was adsorbed on a column of DEAE-Sephadex A50 (20 ml column volume) previously equilibrated with the same Tris-glycerol buffer containing 0.5 mM fructose 1,6-bisphosphate. A linear gradient of 0 to 0.3 M KCl in 2×100 ml of Tris/glycerol/fructose 1,6-bisphosphate buffer was used to elute the enzyme. Maximal activity of pyruvate kinase was eluted when the column became 160 mM with respect to KCl. The enzyme was precipitated from the pooled eluate (25 ml) as a fraction precipitating between 35% and 80% saturation of $(NH_4)_2SO_4$. This was dissolved in a minimum volume of the same buffer as the one used for the first DEAE-cellulose chromatography and stored in the refrigerator. Table 1 gives a summary of the purification procedure. The enzyme preparation was substantially free from enolase, aldolase, NADH oxidase, ATPase and adenylate kinase. Electrophoresis of the purified enzyme in polyacrylamide gel and subsequent visualisation of the enzyme band by staining for the reaction product ATP using phospho*enol*pyruvate, ATP-free ADP, Mg^{2+}, K^+, glucose, purified yeast hexokinase, glucose-6-phosphate dehydrogenase, NADP, phenazine methosulphate and nitroblue tetrazolium, as indicated earlier for the staining of hexokinase [6], showed a single band of pyruvate kinase activity near the cathodal end. Besides, a single, substantially symmetrical peak of enzyme activity was obtained during the chromatographic procedures.

Table 1. *Purification of pyruvate kinase from mutant yeast R121*

Fraction	Total activity	Total protein	Specific activity
	units	mg	units/mg protein
Crude extract	730	910	0.8
30–60% $(NH_4)_2SO_4$ precipitate	840	406	2.1
DEAE-cellulose fraction	374	29	12.9
DEAE-Sephadex fraction	240	3.9	61.5

Wild-Type Pyruvate Kinase

The mutant enzyme was compared to a preparation of pyruvate kinase from the wild type *S. cerevisiae* in respect of a number of properties. For this, the wild-type strain was grown up in yeast extract/peptone/glucose medium, the cells from the stationary phase were broken in a French pressure cell in a buffer containing 25% glycerol, 50 mM potassium phosphate, pH 7.4, 2 mM 2-mercaptoethanol, 2 mM EDTA and 2 mM phenylmethanesulfonyl fluoride. The bulk of pyruvate kinase activity was precipitated from the

supernatant by adding 30 g of solid $(NH_4)_2SO_4$ per 100 ml of the extract. The precipitated enzyme was dissolved in a buffer containing 25% glycerol, 10 mM potassium phosphate, pH 7.4, 2 mM 2-mercaptoethanol and 2 mM EDTA and the $(NH_4)_2SO_4$ removed by gel filtration on a column of Sephadex G25. The salt-free enzyme solution was stirred up for 1 h at 25 °C with 2 volumes of a DEAE-cellulose slurry in the same buffer; the suspension was centrifuged and pyruvate kinase which was not adsorbed by DEAE-cellulose under these conditions was precipitated with $(NH_4)_2SO_4$. The precipitated enzyme had a specific activity of 40 units per mg protein and this was used without any further purification.

PROPERTIES OF THE ALTERED PYRUVATE KINASE

Kinetic Properties

Unlike pyruvate kinase from wild-type yeast [1], the enzyme from the mutant was active in absence of any added fructose 1,6-bisphosphate irrespective of whether seen in toluene lysates, crude extracts or in purified preparations. Addition of this substance had no effect on the reaction velocity over the range 1 μM to 10 mM at all concentrations of phospho*enol*pyruvate or ADP examined. The enzyme displayed hyperbolic kinetics with respect to both these substrates. Results in Fig. 1 show that the reciprocal plots are linear and the affinity of the enzyme for either of the substrates is independent of the concentration of the other over a 20-fold range of concentration of the fixed substrate. The K_m values for phospho*enol*pyruvate and ADP are 0.11 and 0.12 mM respectively. The presence of ATP in the reaction mixture increases the K_m values for both the substrates. However, ATP inhibition is overcome by increase in the concentration of either ADP or phospho*enol*pyruvate, as shown in Fig. 2. ATP thus behaves as a competitive inhibitor of pyruvate kinase from the mutant.

The presence of monovalent cations is essential for the activity of pyruvate kinase from several sources [7], potassium being the most effective. Pyruvate kinase from wild type yeast is activated by K^+ or NH_4^+ ions, smaller concentrations being required in the presence of the allosteric activator fructose 1,6-bisphosphate [1, 8]. When such experiments were performed with the enzyme from the mutant R121, no dependence of K^+ requirement on the presence of fructose 1,6-bisphosphate was seen. The activation by K^+ or NH_4^+ ions of the mutant pyruvate kinase followed essentially Michaelis-Menten relationship (Fig. 3). The only effector found to have a positive homotropic effect on the catalytic activity of this enzyme was Mg^{2+} ion. Results in Fig. 4 illustrate this. The inset represents a Hill plot with $MgCl_2$ as a variable. The value of the Hill coefficient of interaction

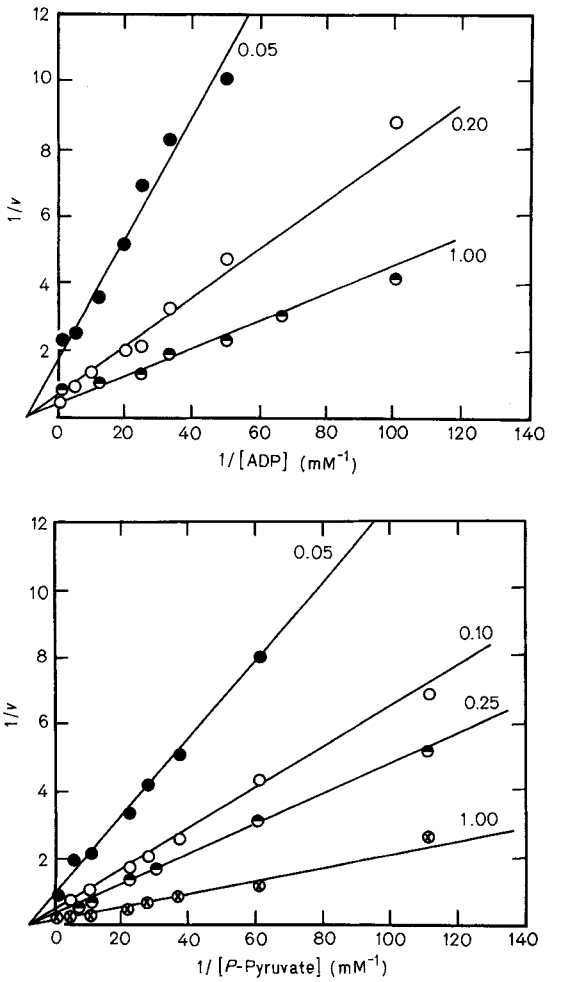

Fig. 1. *Double-reciprocal plots to show the effect of ADP and phospho*enol*pyruvate concentrations on the reaction velocity of pyruvate kinase from the mutant R121. v*, reaction velocity in arbitrary fluorescence units; *P*-pyruvate, phospho*enol*pyruvate. The upper curve shows the double-reciprocal plots with ADP as the variable substrate with the respective concentrations of phospho*enol*pyruvate in mM shown against each of the three plots. The lower curve shows the variation of the initial velocity as a function of the phospho*enol*pyruvate concentrations; the number against each line shows the fixed concentration of ADP in mM. The amounts of pyruvate kinase used in each assay were in the range of 0.025 to 0.05 unit. Assay conditions are as described in Materials and Methods

was 1.6 irrespective of whether fructose 1,6-bisphosphate was present. However, we have not determined whether the chelation of Mg^{2+} ion by ADP was responsible for the deviation of the Hill coefficient from unity.

Fig. 5 depicts the pH-profile of the pyruvate kinase activity from the mutant R121 at two concentrations of phospho*enol*pyruvate, one at approximately the concentration giving the half-maximal velocity, and the other at nearly 20-fold higher concentration. The assay was first order with respect to the amount of enzyme over the entire range of pH. The optimum

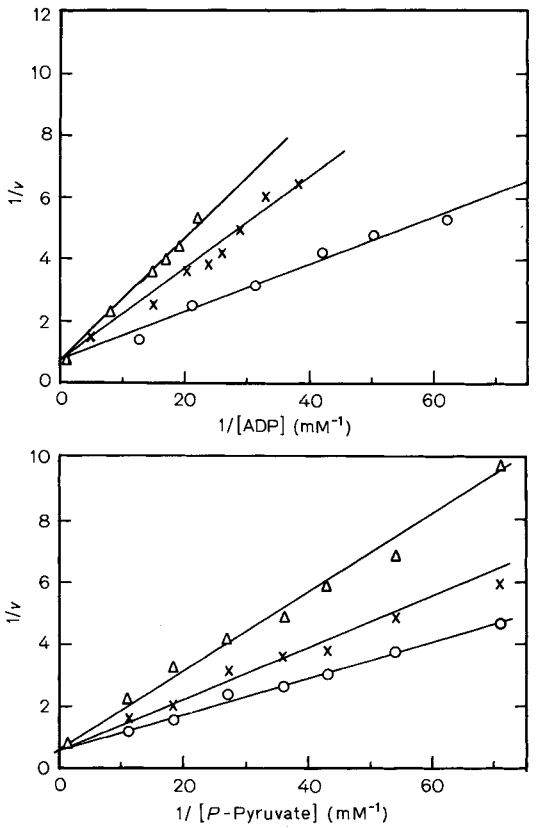

Fig. 2. *Competitive inhibition of pyruvate kinase by ATP.* The concentrations of the fixed substrates in the experiments described in the upper and lower panels respectively were phospho*enol*-pyruvate (*P*-pyruvate) 0.2 mM and ADP 0.27 mM. Other details are as in Fig. 1. (△) 5 mM ATP; (×) 2 mM ATP; (○) 0 mM ATP

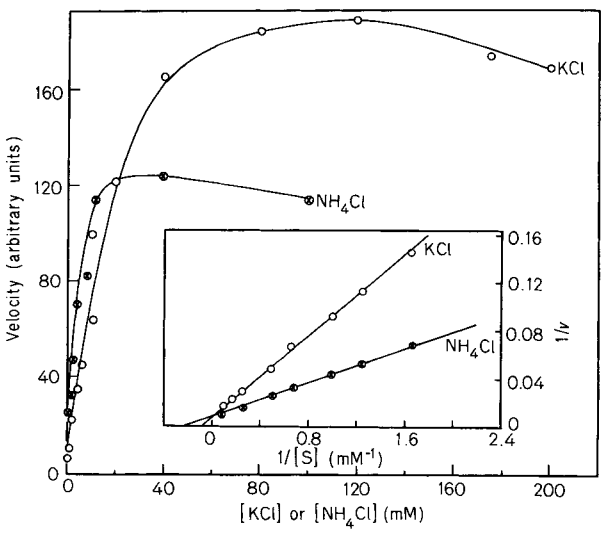

Fig. 3. *Dependence of pyruvate kinase activity from mutant R121 on monovalent cations.* Reaction mixtures contained 20 mM tetramethylammonium maleate buffer, pH 6.5, 5 mM $MgCl_2$, 0.03 mM NADH, 1 mM each of phospho*enol*pyruvate and ADP, obtained by neutralising the free acids with $(CH_3)_4NOH$ and 1 unit of lactic dehydrogenase dialysed against tetramethylammonium maleate buffer. The inset is a double-reciprocal plot; *v* and [S] are the initial reaction velocity and the salt concentration respectively

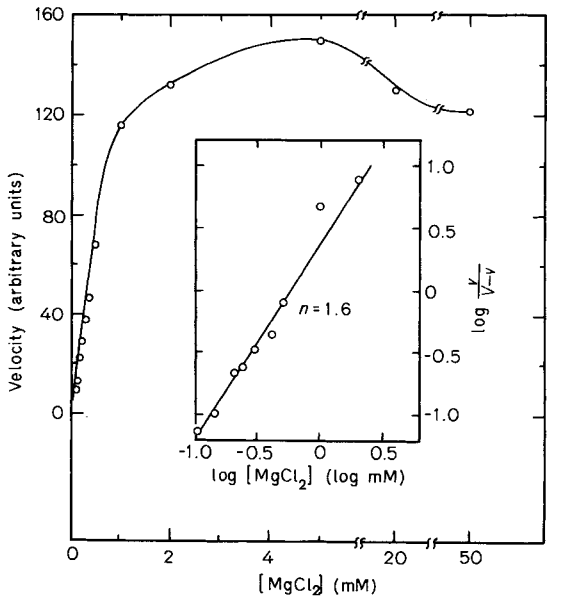

Fig. 4. *Relationship between the initial reaction velocity of mutant pyruvate kinase and $MgCl_2$ concentration.* Reaction mixtures contained 50 mM Tris buffer, pH 7.5, 100 mM KCl, 1 mM each of ADP and phospho*enol*pyruvate, 0.03 mM NADH, 1 unit of lactic dehydrogenase and varying concentrations of $MgCl_2$ indicated. The inset describes a Hill plot. *v*, observed velocity; *V*, maximum velocity (at 5 mM $MgCl_2$) and *n*, slope of the Hill plot

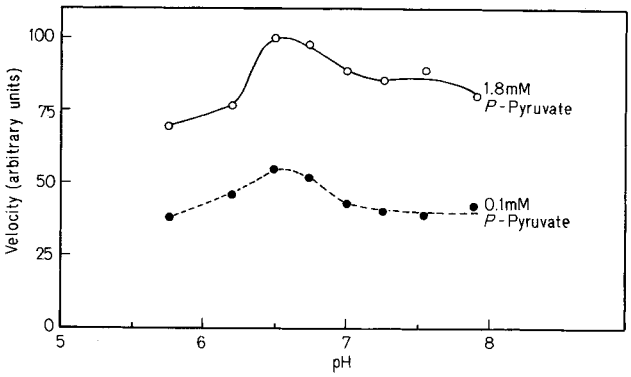

Fig. 5. *pH profile of mutant pyruvate kinase activity.* 50 mM potassium phosphate was used over the entire pH range using two different concentrations of phospho*enol*pyruvate (*P*-pyruvate), 0.1 and 1.8 mM. Other details are the same as described under Materials and Methods

activity is observed at pH 6.5; the ratio of the velocities at the two concentrations of the substrate remains nearly constant over the range of pH studied. Fructose 1,6-bisphosphate was without any effect on the activity of pyruvate kinase at any pH value using either of the two concentrations of phospho*enol*pyruvate.

Stability of the Enzyme

Kuczenski and Suelter [9,10] had reported on the destabilising effect on yeast pyruvate kinase of its allosteric activator fructose 1,6-bisphosphate. This

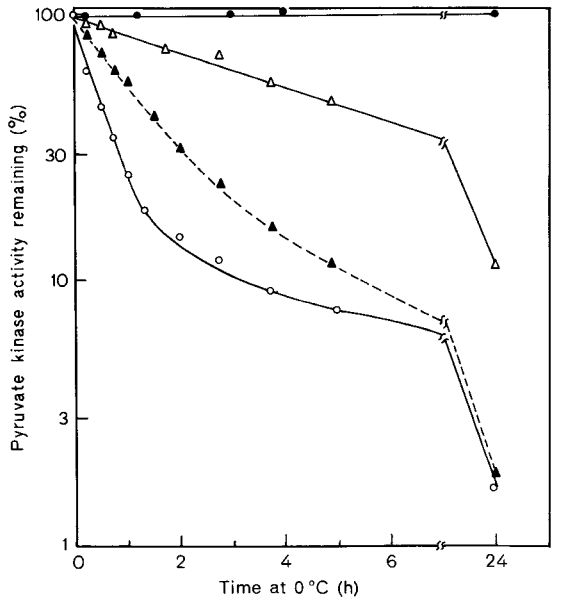

Fig. 6. *Effect of fructose 1,6-bisphosphate on cold lability of pyruvate kinase from yeast mutant.* The stock enzyme from the mutant was freed from glycerol and fructose 1,6-bisphosphate by passing through a column of Sephadex G25 equilibrated with a buffer containing 50 mM potassium phosphate, pH 7.4, 2-mercaptoethanol and 2 mM EDTA. The same buffer was used for eluting the enzyme. The enzyme from the wild type was treated similarly. Fructose 1,6-bisphosphate, when present, was at a concentration of 2 mM. Results are plotted as the fraction of initial activity in a logarithmic scale and time on linear scale. Assay conditions are described under Materials and Methods. Fructose 1,6-bisphosphate was omitted from the assay mixture for the mutant enzyme. (●) Mutant + fructose 1,6-bisphosphate; (△) wild type, no fructose 1,6-bisphosphate; (▲) wild-type + fructose 1,6-bisphosphate; (○) mutant, no fructose 1,6-bisphosphate

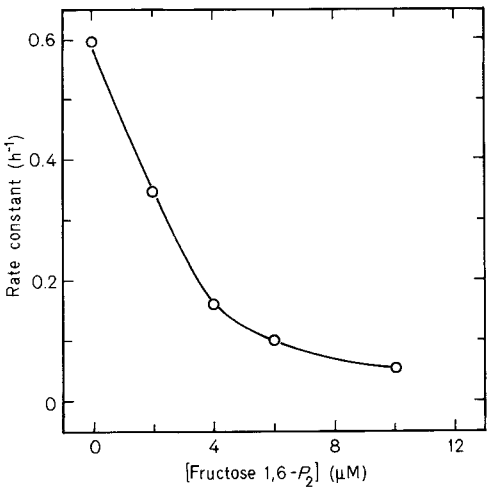

Fig. 7. *Effect of fructose 1,6-bisphosphate on the inactivation of mutant pyruvate kinase.* The first-order inactivation constant for the faster decaying component at each concentration of fructose 1,6-bisphosphate (fructose 1,6-P_2) was calculated in units of reciprocal h. Details are in text

substance was found to promote the inactivation of the wild-type enzyme at 0 °C as also at 23 °C. The mutant enzyme in contrast, did not require this metabolite for its activity, but in the absence of glycerol it was dependent on fructose 1,6-bisphosphate for maintaining itself in the catalytically active configuration. This protective effect of fructose 1,6-bisphosphate was distinct from its well-known activating effect on the enzyme reaction since the unprotected enzyme was fully active in its absence. High concentrations of glycerol or sucrose were just as effective in stabilising the enzyme. Protection against cold-lability was also afforded by a mixture of 5 mM Mg^{2+} and potassium phosphate (10 mM or higher). Results in Fig. 6 illustrate the effects of fructose 1,6-bisphosphate on the stability of the enzymes from the mutant and from the wild type in the cold. This metabolite confers instability on the enzyme from the wild type, but affords protection on the mutant enzyme; however, it cannot reactivate an enzyme preparation that has already undergone inactivation.

The protection offered by varying concentrations of fructose 1,6-bisphosphate on the mutant pyruvate kinase was examined at 25 °C. The enzyme was rendered free of this metabolite by gel filtration on Sephadex G25 using a buffer containing 25% glycerol; at zero time the enzyme was diluted 50-fold in 50 mM potassium phosphate buffer, 2 mM 2-mercaptoethanol, 2 mM EDTA and varying concentrations of fructose 1,6-bisphosphate to obtain an initial concentration of nearly 0.2 unit pyruvate kinase per ml. Aliquots were assayed for the enzyme activity as incubation was continued at 25 °C. The inactivation was biphasic; however, the rapid component of inactivation followed a single exponential curve. The first-order rate constant of rapid inactivation at each concentration of the protective agent, fructose 1,6-bisphosphate was plotted against its corresponding concentration (Fig. 7) by the method of Frieden [11]. Intrapolation from this curve for the concentration of fructose 1,6-bisphosphate at which the rate constant for rapid inactivation becomes half of that seen in absence of its protector gave the dissociation constant of the enzyme-fructose 1,6-bisphosphate complex. This was found to be 2.5 μM fructose 1,6-bisphosphate.

The stabilising effect of fructose 1,6-bisphosphate on the pyruvate kinase from the yeast mutant was observed also at higher temperatures. Results in Table 2 indicate that fructose 1,6-bisphosphate offers a remarkable protection to thermal inactivation of pyruvate kinase from the mutant, while having no effect on the enzyme from the wild type.

Table 2. *Effect of fructose 1,6-bisphosphate on the heat stability of yeast pyruvate kinase*
The enzyme preparations obtained as described in Materials and Methods were diluted in a buffer containing 50 mM potassium phosphate, pH 7.4, 2 mM 2-mercaptoethanol, 2 mM EDTA and 1 mg bovine serum albumin per ml. Diluted enzyme solutions were heated at 53 °C for periods varying from 10 s to 45 min, chilled in melting ice and assayed immediately thereafter. Inactivation was exponential in all the cases. Numbers refer to half-life ($t_{1/2}$) of the enzyme activity at 53 °C in min

Additions	Half-life of pyruvate kinase from	
	mutant R121	wild type
	min	
No fructose 1,6-bisphosphate	0.5	3.5
1 mM fructose 1,6-bisphosphate	18	3.8

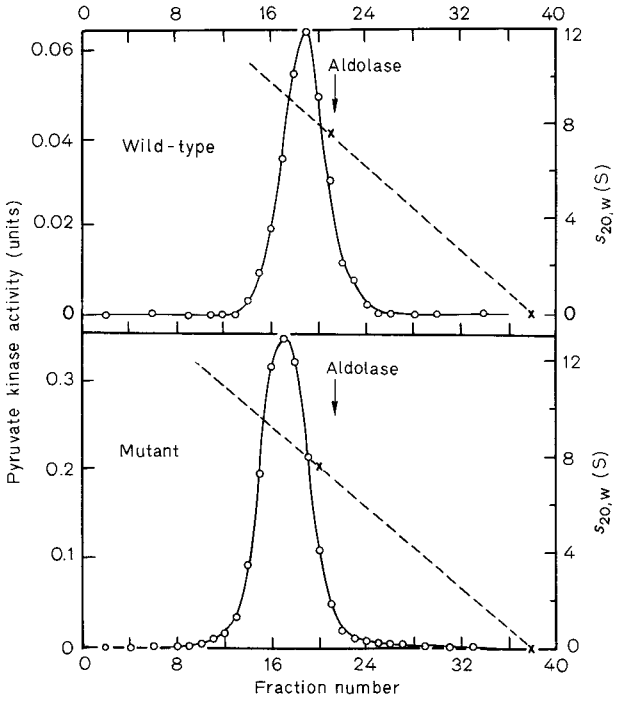

Fig. 8. *Sedimentation in a sucrose gradient of pyruvate kinase from the mutant and the wild type.* A linear gradient of 5% to 20% sucrose in 20 mM KCl in 10 mM potassium phosphate, pH 7.4 was layered on the top with purified preparations of pyruvate kinase from the wild type (1.2 units) and the mutant R121 (2.8 units) together with the marker enzyme. Centrifugation was at 33000 rev./min at 8 °C for 20 h in a SW39 rotor. Enzyme activities were determined in each of the 38 fractions collected after cenrtifugation. The direction of sedimentation was from right to left

Sedimentation Characteristics

Sedimentation velocity profile of the mutant pyruvate kinase was determined in a sucrose density gradient by the method of Martin and Ames [12]. Aldolase was used as the marker protein [13]. Results (Fig. 8) show that both the enzymes from the mutant and the wild type had nearly the same sedimentation coefficient of 8.9 S and 8.6 S respectively. The $s_{20,w}$ value for the mutant enzyme was not perceptibly altered when the sucrose gradient contained 1 mM fructose 1,6-bisphosphate.

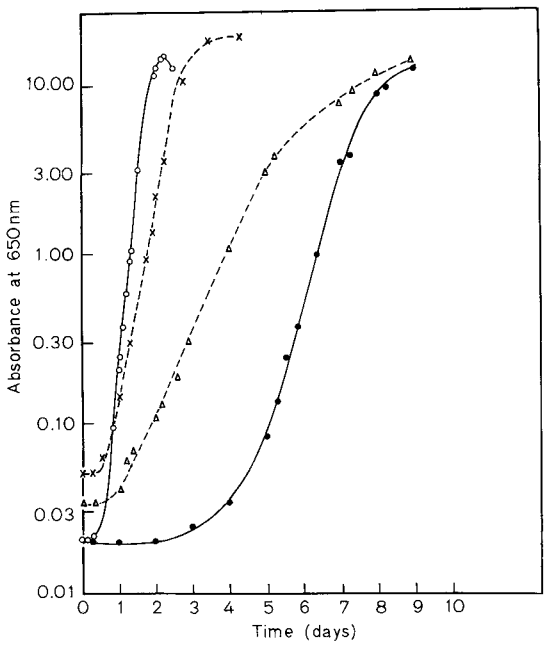

Fig. 9. *Kinetics of growth of the mutant R121 in minimal media containing alcohol or glucose.* The mutant and the wild type were inoculated in a salt-vitamin medium and incubated in shaken flasks at 30 °C. Absorbance at 650 nm was measured after suitable dilution to follow the cell density. (O——O) Mutant grown on glucose, doubling period 3 · 2 h; (×----×) wild type grown on glucose, doubling time 6 · 8 h; (Δ----Δ) wild-type grown on alcohol, doubling time 13 · 8 h; (●——●) mutant grown on alcohol, doubling time 8 · 8 h

Physiology of the Mutant

Growth. The rate of synthesis of phospho*enol*pyruvate is said to determine the pace of gluconeogenesis from amino acids and other carboxylic acid intermediates of the tricarboxylic acid cycle [14]. In *S. cerevisiae* the activating effect of fructose 1,6-bisphosphate on pyruvate kinase is considered as an essential feature of gluconeogenesis [4], since during growth on substances such as ethanol the low steady-state concentration of fructose 1,6-bisphosphate [15] keeps this enzyme practically inactive, thus sparing phospho*enol*pyruvate for gluconeogenic flux. The loss of this control of pyruvate kinase in the mutant R121 offered an occasion to examine the ability of this strain to grow on gluconeogenic carbon sources. A salt-vitamin medium described earlier [16] was chosen, omitting the yeast extract supplement. Results shown in Fig. 9 indicate that the mutant grows on glucose as also on alcohol somewhat faster than the wild type,

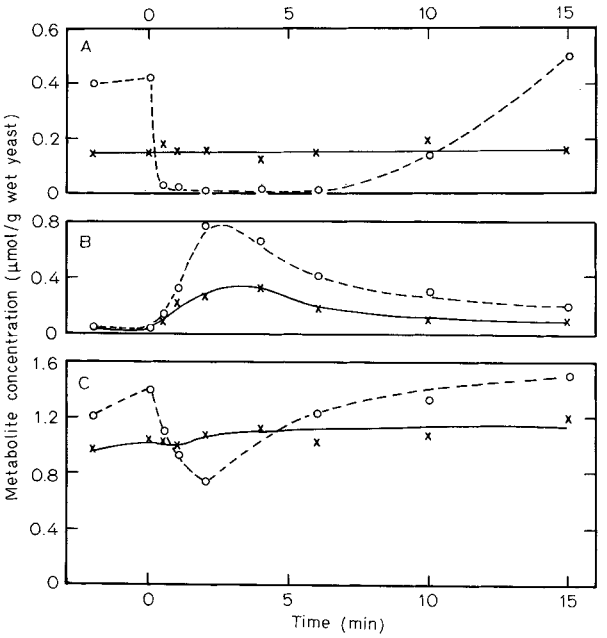

Fig. 10. *Time-course of several phosphorylated glycolytic intermediates during aerobic glycolysis of the yeast mutant.* Washed suspensions of the mutant R121 (102 mg wet cells/ml) and the wild type (78 mg wet cells/ml) in 50 mM potassium phosphate buffer were bubbled with a mixture of 95% O_2 and 5% CO_2 and at zero time were treated with 10 mM glucose. Reaction in aliquots was quenched in 5% perchloric acid and metabolites assayed fluorometrically as described in Materials and Methods. (A) Phospho*enol*pyruvate; (B) fructose 1,6-bisphosphate; (C) adenosine triphosphate. (O----O) Wild type; (×——×) mutant

although there is a considerable lag period before the exponential growth of the mutant ensues in media containing alcohol as the sole carbon source. This lag period could be reduced but could not be abolished altogether by prior growth in minimal media. The mutant also grew on the defined medium containing L-malate as the sole source of carbon. When pyruvate kinase activity was examined in cells collected either from the logarithmic or the stationary phase of growth of the mutant in alcohol medium, no requirement for fructose 1,6-bisphosphate in the catalytic reaction could be seen, suggesting that no selection occurred during the lag period. Such experiments also indicated that when the mutant was growing on alcohol, no inactivation of pyruvate kinase took place intracellularly by virtue of, for example, a low steady-state level of fructose 1,6-bisphosphate.

Intracellular Metabolite Concentrations. The ability of the mutant R121 to grow on ethyl alcohol as a sole carbon source suggested that the mutant was able to divert phospho*enol*pyruvate towards gluconeogenesis despite the presence of active pyruvate kinase. The intracellular levels of phospho*enol*pyruvate was measured in cell suspensions metabolising glucose aerobically. The results (Fig. 10) show that the level of phospho*enol*pyruvate in the mutant is nearly half of that in the wild type. However, glucose addition caused no change in its steady-state level, while in the wild type the level was inversely correlated with the concentration of fructose 1,6-bisphosphate that rose on the addition of glucose. Generally the metabolic transition brought about by glucose was rather feeble in the mutant; its rate of glucose utilisation (2.7 μmol glucose $\times$ min^{-1} $\times$ g $cells^{-1}$) also was nearly a third of that of the wild type.

DISCUSSION

The mutant pyruvate kinase described here differs from the wild type enzyme in respect of a number of distinctive features, the most notable one being the loss of allosteric properties and the attendant lack of activating effect of fructose 1,6-bisphosphate on the catalytic activity. We have failed to observe any stimulatory effect of this substance on the catalytic reaction at acidic or alkaline side of its pH optimum using either low or high concentrations of either of the reactants, phospho*enol*pyruvate or ADP, or of K^+ ion. For each of these substances the enzyme displayed hyperbolic kinetics. The complex pH-velocity relationship of the wild-type enzyme [1,8] was also transformed into that of an enzyme obeying typical Michaelis kinetics. The intersecting double-reciprocal plots for both the substrates and the competitive interaction of ATP with both ADP as also phospho*enol*pyruvate for the mutant enzyme are very similar to the kinetic characteristics of muscle pyruvate kinase studied earlier by Reynard *et al.* [17].

The other characteristic feature of the mutant enzyme is in regard to the protective action of fructose 1,6-bisphosphate against spontaneous inactivation at low and high temperatures. Pyruvate kinase from the wild-type yeast has been shown by Kuczenski and Suelter [9,10] to be labilised by this substance at substantially low concentrations. The dissociation constant of enzyme-fructose 1,6-bisphosphate complex was found to be nearly 60 μM. The enzyme from the mutant, in contrast, was found to be protected by this metabolite against inactivation at low as also at high temperatures. The concentration of fructose 1,6-bisphosphate providing half-maximal protection at 25 °C was 2.5 μM. It is not known whether the catalytic and the destabilising effects of fructose 1,6-bisphosphate are brought about by interaction with the same or different sites on the wild-type pyruvate kinase [9]. The present data with the mutant enzyme do not seem to shed any light on this question. Apart from the fact that the effects of fructose 1,6-bisphosphate on the stability properties of the two enzymes are qualitatively dissimilar, the higher association constant of this substance with the mutant pyruvate kinase than with the enzyme from the wild

type is unlikely to be brought about by a random mutational event.

The full activity of the enzyme in the absence of any externally added fructose 1,6-bisphosphate suggested the possibility that this compound was already present on the enzyme in a tightly bound state such that gel filtration or extensive dialysis failed to bring about dissociation. 1 mg of the purified enzyme of specific activity 60 units per mg protein was treated with 5% $HClO_4$ and the neutralised supernatant tested fluorometrically for the presence of fructose 1,6-bisphosphate. Such experiments showed that 1 mg of the enzyme had less than 0.1 nmol of fructose 1,6-bisphosphate. Assuming the enzyme to be of 25% purity, the fructose 1,6-bisphosphate content of the enzyme is less than 0.1 mol per mol enzyme. It is unlikely therefore that the mutant pyruvate kinase had any tightly bound fructose 1,6-bisphosphate.

It must be remembered that the mutant R121 was isolated from the wild-type strain by a two-step selection procedure. The primary mutant PK3 could be reverted spontaneously to give glucose-positive revertants with a wide spectrum of stability of their pyruvate kinase. This suggested that PK3 might have had a structurally defective pyruvate kinase. All such revertants examined required fructose 1,6-bisphosphate for catalytic activity except the strain R121. This strain, like the other glucose-positive revertants, is thus likely to carry a second corrective mutation leading to a catalytically active pyruvate kinase. The strain R121, defective in anaerobic growth on sugars, can be spontaneously reverted for this character on fructose, yielding further single-step revertants all of which have allosteric pyruvate kinase that cannot be distinguished from the wild-type enzyme in their requirement for fructose 1,6-bisphosphate and thermal stability. A single mutational alteration in R121 is thus able to change its pyruvate kinase to a form qualitatively indistinguishable from the wild-type enzyme.

The ability of the mutant R121 to grow on defined media containing gluconeogenic precursors such as ethanol or malate as the sole source of carbon was unexpected. The activity of phospho*enol*pyruvate carboxykinase, presumably the only enzyme responsible for the synthesis of phospho*enol*pyruvate from tricarboxylic acids in yeast is definitely much lower than that of pyruvate kinase [18]. Since the pyruvate kinase in this strain is not subject to the normal control by fructose 1,6-bisphosphate, the observed steady state level of phospho*enol*pyruvate in cells grown in either glucose or alcohol (0.2 to 0.3 μmol per g wet weight) indicates mechanisms that prevent its abortive conversion to pyruvate. A number of compounds was examined for their possible inhibitory effect on the mutant pyruvate kinase activity; acetyl CoA (0.02 mM), CoA (0.2 mM), glyoxylate (2 mM), L-malate (3 mM), L-alanine (4 mM) and L-aspartate (10 mM) were all found to be without effect. The only compound found to inhibit the enzyme activity was ATP. However, the steady-state intracellular concentration of this substance and those of phospho*enol*pyruvate and ADP do not indicate a strong inhibition of pyruvate kinase activity *in vivo*. This implies that other control parameters must operate in the cell that maintain the level of phospho*enol*pyruvate sufficient for gluconeogenesis.

REFERENCES

1. Haeckel, R., Hess, B., Lauterborn, W. & Wuster, K. H. (1968) *Hoppe-Seyler's Z. Physiol. Chem. 349*, 699–714.
2. Barwell, C. J., Woodward, B. & Brunt, R. V. (1971) *Eur. J. Biochem. 18*, 59–64.
3. Betz, A. & Chance, B. (1965) *Arch. Biochem. Biophys. 109*, 585–594.
4. Gancedo, J. M., Gancedo, C. & Sols, A. (1967) *Biochem. J. 102*, 23C–25C.
5. Maitra, P. K. (1971) *J. Bacteriol. 107*, 759–769.
6. Maitra, P. K. & Lobo, Z. (1971) *J. Biol. Chem. 246*, 475–488.
7. Rose, I. A. & Rose, Z. B. (1969) *Compr. Biochem. 17*, 93–161.
8. Hunsley, J. R. & Suelter, C. H. (1969) *J. Biol. Chem. 244*, 4819–4822.
9. Kuczenski, R. T. & Suelter, C. H. (1970) *Biochemistry, 9*, 939–945.
10. Kuczenski, R. T. & Suelter, C. H. (1971) *Biochemistry, 10*, 2867–2872.
11. Frieden, C. (1963) *J. Biol. Chem. 238*, 146–154.
12. Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372–1379.
13. Rutter, W. J. (1960) in *The Enzymes* (Boyer, P. D., Lardy, H. A. & Myrbäck, K., eds) 2nd edn, vol. 5, pp. 341–366, Academic Press, New York.
14. Krebs, H. A. (1963) *Adv. Enzyme Regul. 1*, 385–400.
15. Barwell, C. J. & Hess, B. (1971) *FEBS Lett. 19*, 1–4.
16. Maitra, P. K. (1970) *J. Biol. Chem. 245*, 2423–2431.
17. Reynard, A. M., Hass, L. F., Jacobsen, D. D. & Boyer, P. D. (1961) *J. Biol. Chem. 236*, 2277–2283.
18. Hansen, R. J., Hinze, H. & Holzer, H. (1976) *Anal. Biochem. 74*, 576–584.

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