MUTANTS OF *ASPERGILLUS NIDULANS* LACKING PYRUVATE KINASE

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1. Introduction

Pyruvate kinase (EC 2.7.1.40) is a key regulatory enzyme in glycolysis in a number of organisms including *Escherichia coli* [1], *Saccharomyces cerevisiae* [2], *Neurospora crassa* [3], *Coprinus lagopus* [4] and higher organisms [5,6]. In *E. coli* and higher organisms there is more than one enzyme species present. However in both *N. crassa* and *S. cerevisiae* there is only one which is regulated primarily by allosteric effectors. The same situation exists in *C. lagopus* where the enzyme is thought essentially glycolytic and to have only a minor role in gluconeogenesis, however no mutants lacking pyruvate kinase were available to examine whether or not the enzyme is required in gluconeogenesis [4].

A previous publication from this laboratory [7] described mutants of *Aspergillus nidulans* lacking NADP-linked malic enzyme (EC 1.1.1.40) and their failure to grow on acetate or glutamate. It was suggested that malic enzyme is required to supply pyruvate from malate during growth on carbon sources which are precursors of tricarboxylic acid cycle intermediates, and that pyruvate kinase is unable to supply pyruvate from PEP under these conditions. The low levels of pyruvate kinase found in mycelium grown upon such gluconeogenic carbon sources supported such an interpretation [7].

We now report the isolation of mutants of *A. nidulans* lacking pyruvate kinase. The mutants do not grow on glycolytic carbon sources but grow normally on gluconeogenic carbon sources. The mutations are recessive in diploids and have been assigned to a single gene *pKi*A located in linkage group V. These results indicate the presence in *A. nidulans* of a single species of pyruvate kinase which is required solely for growth on glycolytic carbon sources.

2. Methods

The strain of *A. nidulans* used was R21 (*paba*A1, *y*A2). Media were those previously described [8] and routine genetic techniques were according to Pontecorvo et al. [9].

To grow mycelium for enzyme assays, cultures were inoculated with suspensions of conidia to give 10^6 conidia/ml of medium and incubated with vigorous shaking at 37°C. Mycelium was harvested by filtration, washed with distilled water and resuspended in 50 mM potassium phosphate buffer pH 7.0. Cell free extracts were prepared by sonication (150W MSE Ultrasonic Disintegrator, peak to peak amplitude 12 μm) at 0°C for 1 min and centrifuged at 4°C and 12 000 g for 15 min to remove debris. Pyruvate kinase was assayed at 37°C in an SP1800 recording spectrophotometer by measuring NADH disappearance at 340 nm according to the method of Kapoor and Tronsgaard [3]. The reaction cell contained the following in 1 ml: PEP (5 mM), ADP (5 mM), $MgCl_2$ (3 mM), LDH (16 units; from pig heart supplied by Sigma), NADH (1.25 mM) and potassium phosphate buffer, pH 7.0 (50 mM). Cell-free extract was added to give absorbance changes of about 0.1 A/min. The reaction was started by addition of PEP to the test cell. Protein concentrations were determined using the Folin reagent [10].

3. Results

3.1. *Pyruvate kinase activity in R21 grown on different carbon sources*

Pyruvate kinase levels during growth on different carbon sources are shown in table 1. Levels are high on glycolytic carbon sources, but low on gluconeogenic carbon sources. On mixtures of glycolytic and gluconeogenic carbon sources the enzyme levels are intermediate. The low levels on a mixture of acetate and glucose are consistent with the observation that *A. nidulans* uses acetate preferentially from a mixture of acetate and glucose, though to a lesser extent from a mixture of acetate and sucrose [11].

3.2. *Mutants lacking pyruvate kinase*

Mutants unable to utilize hexose for growth were isolated from mutagenised suspensions of conidia following filtration enrichment [8]. Mutagenesis was performed using either *N*-methyl-*N*-nitro-*N*nitroso-guanidine [12] or ultraviolet irradiation. Most of the hexose non-utilizing mutants grew on glycerol, but amongst those unable to grow on glycerol ten were found to lack pyruvate kinase activity. The assays were performed on extracts of mycelium grown for 20 h in liquid minimal medium containing acetate as sole carbon source and then transferred to liquid minimal medium plus sucrose for a further 6 h incubation. Under these conditions the wild-type produced 50% of the activity found following 18 h growth on sucrose. No pyruvate kinase activity was detected in the ten mutants which are designated *pki*.

The ten *pki* mutants fail to complement when combined in heterokaryons thus identifying a single gene *pki*A which has been located in linkage group V.

The pyruvate kinase mutants were compared with other glycolytic mutants having similar phenotypes in a series of growth tests (table 2) and were clearly distinguished from mutants lacking either pyruvate dehydrogenase, *pdh*A [11] or pyruvate carboxylase, *pyc*A [13]. The growth tests on alanine, provided as a source of pyruvate, distinguish between mutants blocked before pyruvate (*pki*A) and mutants blocked after pyruvate (*pyc*A and *pdh*A). Growth tests on butyrate distinguish between the *pyc*A and *pdh*A mutants. The inability of *pyc*A mutants to grow on butyrate is a surprising result which is not fully understood.

The lack of growth of the *pki*A mutant on sucrose plus acetate may well be due to the accumulation of toxic phosphorylated intermediates as has been shown in other systems [14,15].

4. Discussion

The results presented here indicate that *Aspergillus nidulans* contains only one species of pyruvate kinase which is required solely for growth on glycolytic carbon sources. Mutants lacking the enzyme activity are unable to grow on any hexose or on glycerol, but grow normally on alanine (as a source of pyruvate) and

Table 1
Pyruvate kinase activity during growth of wild-type *A. nidulans* on different carbon sources

Carbon source	Conc. (M)	Time of incubation (h)	Specific activity (μmoles NADH oxidised h^{-1} mg^{-1})
Sucrose	0.02	18	70
Glycerol	0.04	24	26
Acetate	0.10	24	1.0
Glutamate	0.02	24	9.0
Sucrose and Acetate	0.02 0.10	18	30
Glycerol and Glutamate	0.04 0.02	24	13
Glucose and Acetate	0.02 0.10	18	13

Table 2
The growth of certain glycolytic mutants on a variety of carbon sources

Strain	Enzyme lesion	Chromosome location	Carbon sources for growth				
			Sucrose 0.02 M	Acetate 0.1 M	Alanine[a] 0.05 M	Butyrate[a] 0.1 M	Sucrose 0.02 M + Acetate 0.1 M
*pdh*A1	pyruvic dehydrogenase	I	–	++	+	++	++
*pyc*A3	pyruvic carboxylase	III	–	++	–	–	++
*pki*A1	pyruvic kinase	V	–	++	++	++	–
R21 wild type	–		+++	++	++	++	++

No growth is indicated –, and increasingly better growth as +, ++, and +++.

[a]Growth tests in liquid medium were performed as previously described [8,16]

also upon gluconeogenic carbon sources such as acetate or glutamate. In the latter situation pyruvate is supplied from malate by the action of malic enzyme [7]. Ten such mutants each lacking pyruvate kinase activity identify a single genetic function on the basis of their non-complementarity when combined in heterokaryons. The mutants are also recessive and most probably identify the structural gene for pyruvate kinase (designated *pki*A) which has been located in linkage group V. The *pki* mutants can be distinguished from certain other glycolytic mutants on the basis of growth tests (table 2).

Though the *pki* mutants grow normally on gluconeogenic carbon sources such as acetate, addition of a glycolytic carbon source such as sucrose inhibits growth. We intend to use the *pki*A mutants to screen for second site mutations affecting sugar uptake by selection for revertants escaping inhibition of growth by sucrose in the presence of acetate.

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