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Factors Affecting the Level and Activity of Pyruvate Kinase from *Coprinus lagopus sensu* Buller

By G. R. STEWART AND D. MOORE
Department of Botany, University of Manchester

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SUMMARY

The specific activity of *Coprinus lagopus* pyruvate kinase is high under conditions of glycolysis but low under conditions of gluconeogenesis. Only a single form of the enzyme was detectable, the properties of which resemble in a number of respects those of the allosteric forms of pyruvate kinase. These properties include co-operative interactions with phosphoenolpyruvate (PEP), ATP and fructose 1,6-diphospate. Fructose 1,6-diphosphate (FDP) almost completely reverses the inhibitory effects of ATP.

The decrease in substrate co-operativity in the presence of FDP, the allosteric activator and the increase in ATP co-operativity in the presence of activator suggests the enzyme can be classed as a 'K-system' allosteric protein. The regulatory properties of *Coprinus lagopus* pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40) are discussed in relation to differences in allosteric pyruvate kinases from other sources.

INTRODUCTION

It has been suggested that the pyruvate kinase reaction is an important control point for the pathways of both glycolysis and gluconeogenesis (Tanako, Harano, Sue & Morimura, 1967*a*). Evidence has been presented which indicates that the regulation of pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40) (PyK) may proceed through two mechanisms, one controlling its synthesis and the other its activity (e.g. Hess, Haeckel & Brand, 1966; Hommes, 1966; Gancedo, Gancedo & Sols, 1967; Tanako *et al.* 1967*a*; Malcovati & Kornberg, 1969).

Two main types of PyK have been distinguished in different organisms and tissues (Tanako, Harano, Sue & Morimura, 1967*b*) and also within particular tissues and cells (Pogson, 1968; Malcovati & Kornberg, 1969; Tanako *et al.* 1967*a*). One form usually exhibits kinetics of the simple Michaelis–Menten type (th M-form of Tanako *et al.* 1967*b*) while those of the other resemble in several respects at least those of allosteric or regulatory enzymes (the L-type of Tanako *et al.* 1967*b*). These regulatory properties include co-operative interactions with respect to one of the substrates phosphoenolpyruvate (PEP); specific allosteric activation by fructose 1,6-disphosphate (FDP) (Hess *et al.* 1966) and inhibition by ATP (Tanako *et al.* 1967*a*; Pogson, 1968). PyK from some sources is activated by other metabolites in addition to FDP. For example, the *Escherichia coli* enzyme is activated by AMP (Malcovati & Kornberg, 1969) and that from the loach by 3′,5′-(cyclic)-AMP (Milman & Yurowitzki, 1967). Studies of the FDP–ATP interaction indicate that FDP can reverse the ATP inhibition of the rat-liver enzyme (Tanako *et al.* 1967*b*) while FDP activation of loach PyK is almost completely abolished by ATP (Milman & Yurowitzki, 1967).

It has been postulated that the activation by FDP and the feedback inhibition by ATP may constitute an *in vivo* mechanism for the control of PyK activity. Less is known, however, regarding the nature of the factors controlling the synthesis of PyK. Gancedo *et al.* (1967) suggest that in some yeast species at least FDP might be an inducer of PyK.

The present paper reports a study of some of the factors affecting the level and activity of PyK in the basidiomycete *Coprinus lagopus*.

METHODS

Organism and growth conditions. The organism used in these studies was a strain of *Coprinus lagopus*, isolation number BC9/66; it is a haploid prototrophic laboratory wild-type originally prepared by D. H. Morgan at the John Innes Institute, Norwich. Oidial suspensions, prepared according to Moore (1968), were used to inoculate the culture medium. The basal medium used was the minimal medium described by Moore (1968), except that 0·5 mM-$MgSO_4$ was included. All carbon sources were autoclaved separately from other medium constituents, the medium being constituted after cooling. Mycelium for the partial purification of PyK was grown in 1 l. carrel flasks containing 250 ml. of basal medium made 75 mM with respect to glucose. These flasks were inoculated with freshly prepared oidial suspensions and were incubated at 37° as static cultures for 6 to 8 days. The effect of different carbon sources on total pyK activity was determined using mycelia grown in 50 ml. quantities of basal medium (+carbon source to the appropriate concentration) contained in 16 oz. medical flats. These were inoculated with identical amounts of the same oidial suspension and incubated statically at 37°.

Cell-free extracts. The mycelium was harvested by filtration through cheesecloth, washed with distilled H_2O, blotted and stored at −32° until required. These frozen mycelial pads were extracted by grinding in a mortar and pestle with acid-washed sand in an extraction buffer consisting of 50 mM-tris-HCl, pH 7·5, plus 0·5 mM-EDTA. In other experiments (the determination of total PyK activity) freshly harvested mycelial pads were ground to a powder with the aid of liquid N_2, the powder being taken up in a suitable volume of extraction buffer.

Extracts obtained by either of the above procedures were centrifuged at 30,000 *g* for 15 min., and in experiments where total PyK activity was determined samples of this supernatant were desalted on a small column of Sephadex G–25, equilibrated with extraction buffer. The temperature of the extracts, at all stages up to their assay, was maintained between 2° and 5°.

Assay of total PyK activity. PyK activity was determined at 35° in a Unicam SP 800 recording spectrophotometer equipped with a Honeywell external recorder. The assay medium contained 50 mM-triethanolamine-HCL; 8·0 mM-$MgSO_4$; 50 mM-KCl; 1 mM-PEP; 1 mM-ADP; 0·125 mM-NADH and 50 μg. lactate dehydrogenase; the final pH was 7·75 and the final volume 2·5 ml. The reaction was initiated by the addition of extract and the decrease in E_{340} was followed. The rates were linear over at least 2 to 3 min. and were directly proportional to the volume of extract added. Blank determinations without ADP were carried out. In these experiments PyK activity at low PEP (0·1 mM) and at low PEP in the presence of 0·5 mM-FDP was also determined. Protein was determined after precipitation with 10% (w/v) trichloroacetic acid by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard.

Kinetic experiments. Enzyme used in the kinetic studies was prepared from the crude supernatant. The supernatant solution was brought to 25% saturation with $(NH_4)_2SO_4$ and kept at 2° for 30 min., with occasional stirring. The mixture was centrifuged at 35,000 *g*

for 15 min. and the second supernatant was collected. This was brought to 40% saturation with $(NH_4)_2SO_4$, kept at 2° for 30 min., and then centrifuged at 35,000 *g* for 15 min.

Precipitated protein was dissolved in a small volume of extraction buffer and stored at −32° until required. The 25 to 40% $(NH_4)_2SO_4$ fraction contained 70 to 80% of the PyK activity of the crude supernatant. A further 7 to 12% was recovered in the 40 to 60% $(NH_4)_2SO_4$ fraction. Attempts to remove the $(NH_4)_2SO_4$ by dialysis or gel filtration resulted in an almost complete loss of PyK activity. The addition of various compounds including dithiothreitol, K^+, Mg^{2+}, FDP and ATP failed to prevent this loss of activity.

Activity of the partially purified enzyme was followed in a system similar to that described above but at various concentrations of ADP and PEP. At low PEP concentrations the rates fell off markedly after a linear phase of 30 to 60 sec.; experimental rates were therefore measured over the initial period.

Expression of results. Specific activities are expressed as μmoles/min./mg. protein under the standard assay conditions. In the kinetic studies data are expressed in arbitrary units and the lines drawn in the figures represent the best fit by eye.

Reagents. Biochemicals and enzymes were purchased from Sigma Chemical Co. (London) and other chemicals (analytical grade where possible) from British Drug Houses Ltd (Poole, Dorset). All solutions were made up in deionized water.

Table 1. *Effect of carbon source on PyK specific activity*

Carbon source (mM)		Specific activity (μmoles/min./mg. protein)
Glucose	1	0·066 (3)*
	3	0·120 (3)
	10	0·230 (3)
	30	0·569 (3)
	100	0·682 (3)
	300	0·924 (3)
	500	0·985 (3)
Mannose	100	0·512 (3)
Fructose	100	1·080 (3)
Acetate	100	0·200 (2)
	300	0·240 (3)
Glycerol	100	0·078 (3)

* Number of experiments are in parentheses. Specific activities for different experiments differed by not more than 10%.

RESULTS

Effect of carbon source on total PyK activity

The results in Table 1 show that as the initial concentration of glucose in the growth medium was raised so the specific activity of PyK increased. There was a 14- to 15-fold increase in specific activity when the glucose concentration was increased from 1 to 300 mM. When cultures were grown on other hexoses such as fructose and mannose the specific activity was again considerably higher than that of acetate- or glycerol-grown mycelia. Low specific activity of PyK under conditions of gluconeogenesis (Ruiz-Amil *et al.* 1965) and on low glucose (Hommes, 1966) has also been observed in some yeasts. The low activity observed in glycerol-grown mycelia contrasts with the results of Gancedo *et al.* (1967), who found that PyK activity in *Candida utilis* was high on glycerol and glucose. However, glycerol is, at best, an extremely poor source of carbon and energy for *Coprinus lagopus* (Moore, 1969) so that the low activity on this compound is not particularly surprising.

Molecular heterogeneity of PyK

In an attempt to examine the PyK of *Coprinus lagopus* for multiple molecular forms the ratio of activities at high (1·0 mM) and low (0·1 mM) PEP concentrations and the percentage stimulation by FDP were determined in extracts from mycelia grown under different conditions. The rationale behind the use of these parameters to characterize PyK is derived from a consideration of the properties of the L- and M-type PyKs from rat liver and muscle tissue (Tanako *et al.* 1967*a*). The M-type PyK showed a low ratio of activity at high to activity at low PEP concentration and was not activated by FDP, while the L-type exhibited a high ratio of activities at high and low PEP concentration and was markedly activated by FDP. Thus in crude extracts containing two such forms of the enzyme the ratio of activities at high and low PEP concentration and the percentage FDP stimulation depended on the relative proportions of the two forms.

Table 2. *Ratio of activities of PyK at high and low PEP* concentrations and percentage stimulation by FDP*†

Extracts and ammonium sulphate precipitates were prepared as described in Methods.

Treatment	Ratio of activities at high and low PEP concentrations	FDP stimulation (%)
Glucose (3 mM)	14·1	995
Glucose (100 mM)	14·2	1050
Mannose (100 mM)	14·3	1105
Fructose (100 mM)	14·6	1100
Acetate (100 mM)	14·6	1080
Glycerol (100 mM)	14·4	1065
Glucose (75 mM)		
25 to 40% $(NH_4)_2SO_4$ppt.	14·6	1050
40 to 60% $(NH_4)_2SO_4$ppt.	14·3	1100
Acetate (100 mM)		
25 to 40% $(NH_4)_2SO_4$ppt.	14·2	1050
40 to 60% $(NH_4)_2SO_4$ppt.	14·0	985

* Assayed at 1·0 mM-PEP (high), 0·1 mM-PEP (low).
† 0·5 mM-FDP at 0·1 mM-PEP.

It can be seen from the results in Table 2 that these two parameters remained very constant in mycelia grown on different carbon sources. A concentration of 0·1 mM-FDP was used in these experiments to ensure maximum activation of PyK since in acetate-grown mycelium an active fructose 1,6-diphosphatase was present. It is noteworthy that the ratio of activity at high to activity at low PEP concentration and the FDP stimulation of the enzyme in both the 25 to 40% and 40 to 60% $(NH_4)_2SO_4$ fractions were also very similar. Since the enzyme recovered in these two fractions accounted for up to 92% of the activity in the crude supernatant, judged by the criteria indicated above there is evidence for only a single form of PyK in *Coprinus lagopus*. This is similar to the situation reported for yeast (Hess *et al.* 1966) but contrasts with that in *Escherichia coli*. In the latter, Malcovati & Kornberg (1969) have reported two forms of PyK differing in their sensitivity to FDP; the relative amounts of the two being determined by the nature of the carbon source in the growth medium.

Kinetic experiments

In the experiments reported below the enzyme preparation used was partially purified from frozen mycelial pads (see under Methods and Materials).

Effect of PEP and FDP

Fig. 1 shows the relationship between initial reaction velocity and PEP concentration in the presence of different FDP levels. In the absence of FDP the rate-response curve for PEP was markedly sigmoid, with an apparent K_m for PEP of 0·6 mM. In the presence of increasing FDP concentrations the sigmoidicity of the PEP rate-response curve was reduced. In the presence of 0·5 mM-FDP the kinetics of the PEP rate response curve were of the 'normal' Michaelis–Menten type.

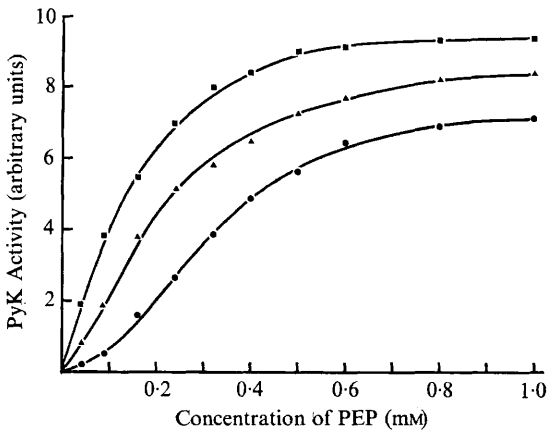

Fig. 1

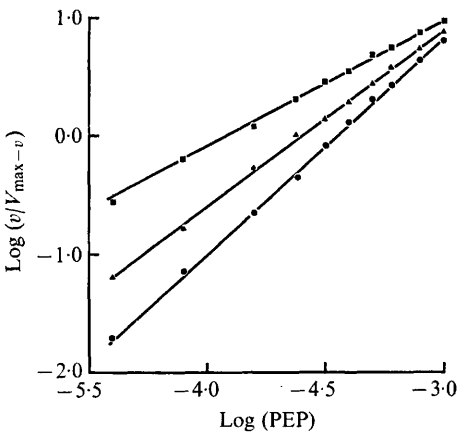

Fig. 2

Fig. 1. Effect of PEP concentration on PyK activity in presence of varying FDP levels. Assay system as described in text; 8·0 mM-$MgSO_4$; 50 mM-KCl; 1·0 mM-ADP. FDP concentration: ●, none; ▲, 0·1 μM; ■, 500 μM.

Fig. 2. Hill plots for PyK with various concentrations of PEP, with and without FDP. Assay system as in Fig. 1. FDP concentration: ●, none; ▲, 0·1 μM; ■, 500 μM.

The apparent K_m for PEP was reduced to 0·15 mM in the presence of 0·5 mM-FDP, a quarter that in the absence of FDP. In Fig. 2 it can be seen that increasing concentrations of FDP decreased substrate co-operativity as measured by the slope of the Hill plot of log v/V_{max-v} against log S. The Hill number (n) was reduced from 2·0 to 2·2 (in the absence of FDP) to unity in the presence of 0·5 mM-FDP. This change in substrate co-operativity has been observed with PyK preparations from several sources (e.g. Bailey, Stirpe & Taylor, 1968).

The effect of increasing concentrations of FDP on reaction velocity at low PEP (0·1 mM) is shown in Fig. 3. The rate-response curve is sigmoid with a tenfold increase in activity over the control rate. The concentration (1·2 μM) of FDP giving half-maximal stimulation was similar to that for L-type rat liver PyK (Bailey *et al.* 1968) but considerably lower, by almost two orders of magnitude, than that determined for the yeast enzyme (Hess *et al.* 1967). The sigmoid form of the response curve for FDP activation is similar to those reported for the yeast and L-type rat liver enzymes (Hess *et al.* 1967; Bailey *et al.* 1968) but contrasts with

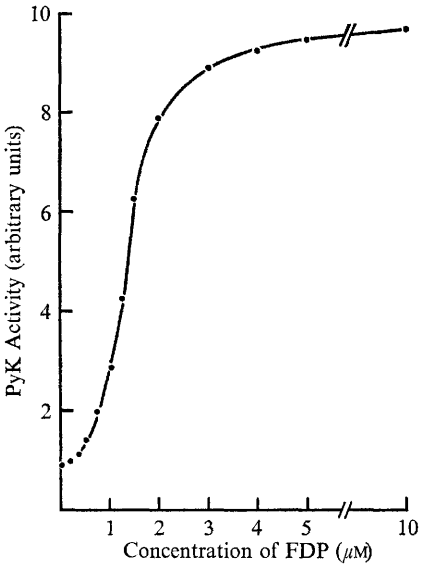

Fig. 3

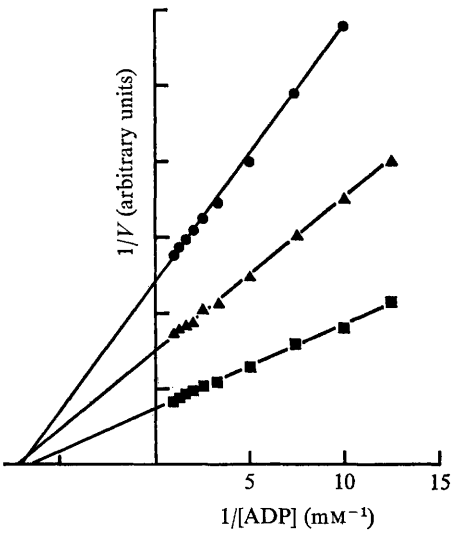

Fig. 4

Fig. 3. Effect of FDP on PyK activity at low PEP. Assay system as described in text: 8·0 mM-$MgSO_4$; 50 mM-KCl; 1·0 mM ADP; 0·1 mM PEP.

Fig. 4. Double-reciprocal plots for rates of PyK with various concentrations of ADP and fixed concentrations of PEP. Assay system as described in text; 8·0 mM $MgSO_4$, 50 mM KCl. The K_m for ADP was 0·16 mM. PEP concentration: ●, 0·15 mM; ▲, 0·25 mM; ■, 1·0 mM.

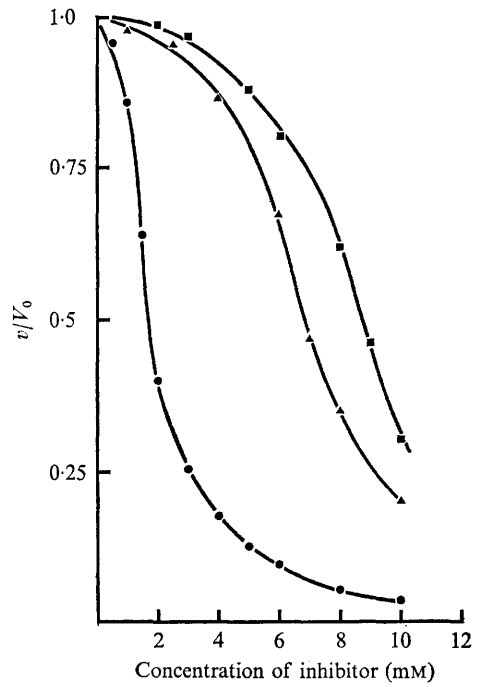

Fig. 5. Inhibitory effect of ATP, GTP and UTP on PyK. Assay system as described in text: 8·0 mM-$MgSO_4$; 50 mM-KCl; 1 mM-ADP; 0·25 mM-PEP. V_0, velocity in absence of ATP, etc.; v, velocity with ATP, etc. ●, ATP; ▲, GTP; ■, UTP.

that for PyK-A of adipose-tissue (Pogson 1968). The kinetics of the latter were of the Michaelis–Menten type.

Effects of ADP. Plots of rate against increasing ADP concentrations were of the Michaelis–Menten type. Lineweaver–Burk plots at different PEP concentrations are shown in Fig. 4, and it can be seen from these that the kinetic behaviour of the enzyme with respect to ADP and PEP was consistent with random binding of the two substrates. This is in agreement with the findings of Reynard, Hass, Jacobsen & Bayer (1961) and Pogson (1968).

Inhibition by ATP. It was found that ATP was a potent inhibitor of PyK and that CTP, GTP and UTP were also inhibitory. It can be seen (Fig. 5) that plots of reaction velocity

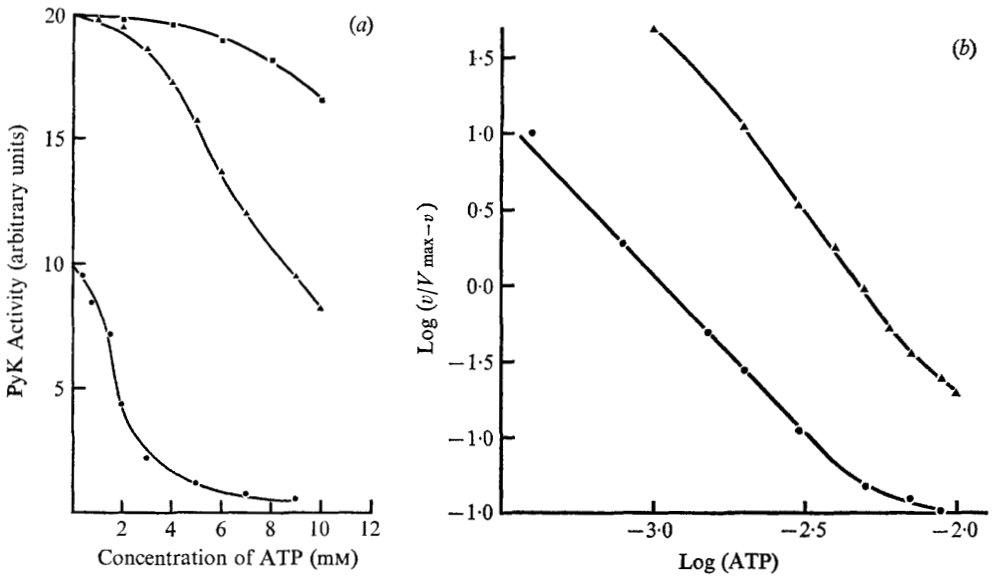

Fig. 6(*a*). Effect of FDP on the ATP inhibition of PyK. Assay system as described in text: 8·0 mM-$MgSO_4$, 50 mM-KCl; 1·0 mM-ADP; 0·25 mM-PEP. FDP concentrations; ●, none; ▲, 0·005 mM; ■, 0·5 mM. (*b*) Hill plots for PyK with various concentrations of ATP, with and without FDP. Assay system as described in text: 8·0 mM-$MgSO_4$; 50 mM-KCl; 1·0 mM-ADP; 0·25 mM-PEP. FDP concentration: ●, none; ▲, 0·005 mM; ■, 0·5 mM.

Table 3. *Compounds tested and found to have no effect* on PyK*

	Concentration range (mM)
Glucose-6-phosphate	0·1 to 5·0
Fructose-6-phosphate	0·1 to 5·0
6-Phosphogluconate	1·0 to 5·0
2-Deoxyglucose-6-phosphate	0·1 to 2·0
5-AMP	0·05 to 0·5
Citrate	0·1 to 1·0
Malate	0·1 to 5·0
Alanine	0·5 to 5·0
Aspartate	0·1 to 3·0
Glutamate	0·1 to 5·0
Phosphate	0·1 to 2·0
Co-A	0·05 to 0·3
Ethanol	0·1 to 5·0

* Tested at 1·0 mM-PEP; 0·1 mM-PEP; and 0·1 mM-PEP + 0·5 mM-FDP.

against ATP, GTP and UTP concentration are of the sigmoid form, with ATP being the most inhibitory. Inhibition by ATP is unlikely to have resulted from complexes formed with Mg^{2+} since the inhibition was not altered by increasing the Mg^{2+} ion concentration to 20 mM.

The effect of FDP on the ATP inhibition is shown in Fig. 6(*a*). It is evident that high concentrations of FDP virtually abolished the inhibitory effect of ATP. At 0·05 mM-FDP the Hill number for ATP inhibition was increased to 2·8 as compared with 2·1 in the absence of FDP (Fig. 6*b*).

Effect of other metabolites. A number of other metabolites were tested for their effect on PyK activity. A list of those used and the concentration range over which they were tested is shown in Table 3. None of these metabolites had any effect on reaction rates at low (0·1 mM) or high (1·0 mM) PEP or on the FDP activation at low PEP.

DISCUSSION

The changes in specific activity of PyK in response to carbon source together with the *in vitro* kinetic studies suggest that PyK activity in *Coprinus lagopus* may be regulated through changes in both concentration and activity. While the relative importance of control at the levels of synthesis and activity cannot be evaluated from experiments such as those described here, it is noteworthy that Gancedo *et al.* (1967) have argued that in yeasts they are alternative mechanisms of control. A species such as *Saccharomyces cerevisiae* does not show changes in PyK concentration with carbon source but is strongly stimulated by FDP. The activity of PyK from *Candida utilis* was not affected by FDP but total activity was dependent on the nature of the carbon source supplied for growth. In *C. lagopus* as in *Escherichia coli* and certain mammalian systems, PyK activity could clearly be regulated by both mechanisms. Some doubt can be thrown on the validity of alternative control mechanisms for yeast because Chapman & Bartley (1968) reported a 3- to 4-fold increase in PyK concentration in *S. cerevisiae* when the glucose concentration was increased from 0·9% to 10%. It has been suggested that PyK activity in *Can. utilis* (Gancedo *et al.* 1967) might be induced by a glycolytic intermediate, possibly FDP. The low specific activity observed here in acetate-grown mycelium seems to support this suggestion.

Using the criteria of the ratio of activities at high and low PEP concentrations and the degree of FDP stimulation the results in Table 2 indicate only a single molecular species of PyK. The use of these criteria in assessing PyK heterogeneity is justifiable on the assumption that possible multiple forms are discrete isoenzymes. Pogson (1968) has reported that PyK-A and PyK-B of adipose-tissue are interconvertible, the conversion of PyK-A into PyK-B being mediated by FDP and the reverse reaction by ATP, citrate or EDTA. Similarly Bailey *et al.* (1968) have shown an increase in co-operativity for PEP and FDP on pre-incubation at 25°, suggesting that there may be two forms of the L-type enzyme in rat liver. Clearly any attempt to demonstrate molecular heterogeneity of PyK using criteria derived from kinetic behaviour must take into account the possibility of interconvertible forms. Experiments have, however, been carried out to examine the effects of pre-incubation on *Coprinus lagopus* PyK. Pre-incubation was carried out in the presence of PEP, ADP, and ATP but in no instance was a change in rate observed. None of the pre-incubation treatments brought about any change in the ratio of activities at high and low PEP concentrations or in the degree of FDP activation. These observations confirm the suggestion that only a single form of PyK is detectable in *Coprinus lagopus*.

A detailed comparison of the kinetic behaviour of PyK from the numerous systems that have been investigated is difficult because such studies have been carried out on prepara-

tions of different degrees of purity and a variety of assay conditions have been employed. Some general observations can, however, be made. *Coprinus lagopus* PyK is similar in several respects to those forms of PyK which exhibit properties characteristic of allosteric or regulatory enzymes. The effect of the allosteric activator FDP on PEP substrate co-operativity, namely a loss of sigmoidicity in the rate-response curves, with an accompanying decrease in the slope of the Hill Plot to unity, and the opposite effect, an increase in ATP co-operativity in the presence of FDP, has been observed with other PyK preparations (e.g. Bailey *et al.* 1968; Bailey & Walker, 1969). While such kinetic data are insufficient in themselves to provide a molecular model to account for these homotropic and heterotropic interactions, they do indicate that the enzyme behaves in a manner consistent with that described for 'K-system' enzymes (Monod, Wyman & Changeux, 1965).

Most forms of PyK exhibiting sigmoid rate-response curves with PEP are strongly and specificially activated by FDP, one exception being the enzyme from *Acetobacter xylinum* (Benziman, 1969). Although co-operative interactions with PEP are shown it is unaffected by FDP. Another difference in the properties of the regulatory forms of PyK is in the effect of FDP on the ATP inhibition. *Coprinus lagopus* PyK responds in a similar way to the rat-liver enzyme (Tanako *et al.* 1967*b*) in that FDP reverses ATP inhibition, but contrasts with the ATP reversal of FDP activation shown by the loach enzyme (Milman & Yurowitzki, 1967). These differences in the regulatory properties of PyK from different sources may be related to differences in metabolism under conditions of glycolysis and gluconeogenesis. Certainly this could be the explanation in the case of PyK from *A. xylinum*. Here the lack of FDP activation might be related to the apparent absence of a key glycolytic enzyme, phosphofructokinase (EC 2.7.1.11) (see Benziman, 1969). Similarly differences in PyK with respect to affinity for PEP, FDP and ATP may be related to differences in the cellular concentration range of these intermediates in various organisms. In any case the differences in the regulatory characteristics of PyK from various sources illustrate the observation made on a number of other allosteric enzymes (see Stadtman, 1966) that regulatory properties seem to be subject to greater evolutionary selection that catalytic properties.

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