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Factors Affecting the Activity of Pyruvate Kinase of *Acetobacter xylinum*

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1. Extracts of *Acetobacter xylinum* were found to contain the glycolytic enzymes involved in the conversion of triose phosphate into pyruvate. Pyruvate kinase had the lowest relative activity. Phosphofructokinase activity was not detected in the extracts. 2. Only slight differences in the activity of pyruvate kinase were observed between cells grown on glucose and those grown on intermediates of the tricarboxylic acid cycle. 3. Pyruvate kinase, partially purified from ultrasonic extracts by ammonium sulphate fractionation, required Mg^{2+} ions for activity. It was not activated by K^+ or NH_4^+ ions. 4. The plots representing the relationship between initial velocity and phosphoenolpyruvate concentration were sigmoidal, suggesting a co-operative effect for phosphoenolpyruvate. The Hill coefficient (n) for phosphoenolpyruvate was 2. The rate of the reaction changed with increasing ADP concentrations according to normal Michaelis–Menten kinetics. 5. The enzyme was inhibited by ATP (K_i $0{\cdot}9 \times 10^{-3}$M). The inhibition was competitive with regard to ADP but not with regard to phosphoenolpyruvate. It was not relieved by excess of Mg^{2+} ions. 6. The possible relationship of the properties of pyruvate kinase to regulatory mechanisms for controlling gluconeogenesis and carbohydrate oxidation in *A. xylinum* is discussed.

The pyruvate kinase (EC 2.7.1.40) reaction has been postulated as one of the control points for the pathways of glycolysis and gluconeogenesis (Newsholme & Gevers, 1967). The conversion of carbohydrates into pyruvate, with the pyruvate kinase reaction as its final step, differs in *Acetobacter xylinum* from that in mammalian and yeast systems in that it involves only a segment of the glycolytic pathway, namely the oxidation of triose phosphate, formed from the sugars in the pentose phosphate cycle, via phosphoglyceric acids (Schramm, Gromet & Hestrin, 1957*a*). Phosphofructokinase (EC 2.7.1.11), generally believed to be a regulatory enzyme for glycolysis, could not be detected in *A. xylinum* (Gromet, Schramm & Hestrin, 1957). Gluconeogenesis in this organism, in the form of cellulose synthesis from tricarboxylic acid-cycle intermediates, differs from that in mammalian, avian, yeast and some bacterial systems (Wood & Utter, 1965) in that it involves the irreversible direct phosphorylation of pyruvate by ATP catalysed by PEP* synthase, a soluble enzyme (Benziman, 1966; cf. Cooper & Kornberg, 1965). Since the coupling of this reaction with the pyruvate kinase reaction would result in an energetically wasteful cycle, there should be some mechanism to control the opposing enzymic activities involved in the interconversion of PEP and pyruvate.

* Abbreviation: PEP, phosphoenolpyruvate.

The present paper describes some properties of the pyruvate kinase of *A. xylinum*, especially with regard to possible regulatory mechanisms for controlling gluconeogenesis and carbohydrate oxidation in this organism.

A preliminary report of this work has been published (Benziman, 1968).

METHODS AND MATERIALS

Cells. Succinate-grown cells of *A. xylinum* were grown and harvested as described by Benziman & Burger-Rachamimov (1962). Glucose-grown cells were obtained by the procedure of Schramm, Gromet & Hestrin (1957*b*).

Cell-free extracts. Cells were suspended to a final concentration of 30mg. dry wt./ml. in 50mM-tris–H_2SO_4 buffer, pH 7·5, containing 5mM-EDTA and 1mM-$MgCl_2$. Portions (15ml.) of this suspension were treated in a Raytheon model DF101 magnetorestrictive oscillator at 200w and 10kcyc./sec. for 5min. The cap was cooled with ice–water during the ultrasonic period. The ultrasonic extract was centrifuged at 9000 *g* for 15min. in the cold and the precipitate was discarded. The supernatant was centrifuged in a Spinco model L preparative ultracentrifuge

at 105000*g* for 90min. at 0°. Solid $(NH_4)_2SO_4$ to 40% saturation was added in the cold to the high-speed-supernatant fraction (containing 8–12mg. of protein/ml.). The salt was added slowly with stirring, and precipitation was allowed for 15min. before centrifugation at 15000*g* for 15min. The precipitate was discarded. To the supernatant solution a further amount of $(NH_4)_2SO_4$ was added to give 45% saturation, and the suspension was again left to stand for 15min. and then centrifuged. The precipitate was dissolved in one-third of the original volume of 0·05M-tris–H_2SO_4 buffer containing 5mM-EDTA and 1mM-$MgCl_2$. The specific activity of pyruvate kinase in this fraction was 12 times that of the crude extract. Unless stated otherwise, enzyme partially purified in this manner from succinate-grown cells was used. When stored frozen it retained most of its activity over a period of 1–2 weeks. For certain experiments this fraction was dialysed overnight against 3000vol. of the above buffer.

Enzyme assays. Enzyme assays that involved the measurement of extinction changes were carried out at room temperature (about 22°) in cuvettes of 1cm. light-path and 1·2ml. capacity with a Zeiss spectrophotometer or a Gilford automatic recording attachment for the Beckman DU spectrophotometer.

Pyruvate kinase activity was assayed by measuring the decrease in E_{340} concomitant with the oxidation of NADH in the coupled reaction with the lactate dehydrogenase (EC 1.1.1.27) system. The complete assay system consisted of: 100mM-tris–H_2SO_4 buffer, pH7·5, 20mM-$MgCl_2$, 0·15mM-NADH, 2 units of lactate dehydrogenase, amounts of PEP and ADP as indicated in the text, enzyme and water to a final volume of 1·0ml. NADH was omitted from the blank cuvette. The reaction was started by the addition of PEP.

For assay of the enzyme in crude extracts, the lactate dehydrogenase and NADH were omitted and the pyruvate formed was determined, after termination of the reaction, as described under 'Analytical methods' below.

Other enzymes tested were assayed by measuring the oxidation of NADH in the following coupled assay systems: (*a*) for glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12): 3-phosphoglycerate kinase (EC 2.7.2.3), ATP and 3-phosphoglycerate; (*b*) for 3-phosphoglycerate kinase: glyceraldehyde 3-phosphate dehydrogenase, ATP and 3-phosphoglycerate; (*c*) for phosphoglyceromutase (EC 2.7.5.3): enolase (EC 4.2.1.11), pyruvate kinase, lactate dehydrogenase, 3-phosphoglycerate and ADP; (*d*) for enolase: pyruvate kinase, lactate dehydrogenase, 2-phosphoglycerate and ADP. The conversion of 3-phosphoglycerate into pyruvate was determined by following the rate of pyruvate formation, as described by White & Wang (1964).

Analytical methods. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard. Pyruvate was determined either colorimetrically by the method of Friedemann & Haugen (1943) as modified by Kornberg & Morris (1965), or enzymically with lactate dehydrogenase. Oxaloacetate, PEP, ADP and ATP were determined as previously described (Benziman & Abeliovitz, 1964; Benziman, 1966).

Chemicals. ADP, ATP, AMP, NAD, PEP (tricyclohexylammonium salt), sodium pyruvate, oxaloacetic acid, glucose 6-phosphate, CoA, 2-phosphoglycerate, pyruvate kinase, 3-phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase, enolase and lactate dehydrogenase were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Glyceraldehyde 3-phosphate, fructose 1,6-diphosphate and *p*-hydroxymercuribenzoate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., and GDP and GTP from Pabst Laboratories, Milwaukee, Wis., U.S.A. Acetyl-CoA was prepared from acetic anhydride and CoA by the method of Stadtman (1957). Acetyl phosphate was prepared as the dilithium salt by the method of Stadtman & Lipmann (1950).

RESULTS

All the experiments reported here were carried out with both glucose-grown and succinate-grown cells. No significant differences in the quantity or the properties of the enzymes studied were observed between the two types of cells.

Activity of pyruvate kinase relative to the activities of other glycolytic enzymes. Extracts of *A. xylinum* were found to contain the glycolytic enzymes involved in the conversion of triose phosphate into pyruvate. The activities of the various enzymes are shown in Table 1. The activity of pyruvate kinase was much lower than that of any other enzyme in this pathway, and was of the same order as the overall rate of the conversion of phosphoglycerate into pyruvate.

Stoicheiometry. The stoicheiometry expected for the pyruvate kinase reaction was observed (0·66μmole of PEP and 0·72μmole of ADP were utilized, and 0·60μmole of pyruvate and 0·68μmole of ATP were formed by 1mg. of protein of the purified enzyme, in 10min. at 30°).

Optimum pH *and cation requirements.* The partially purified pyruvate kinase, assayed by the spectrophotometric procedure with saturating concentrations of PEP (6mM) and ADP (8mM), exhibited optimum pH 6·8–7·2 in tris–maleate buffer. Unlike the pyruvate kinase from yeast,

Table 1. *Activities of glycolytic enzymes in* A. xylinum

Assays were carried out with the crude extract from glucose-grown cells after high-speed centrifugation, as described in the Methods and Materials section.

Enzyme reaction	Activity (μmoles/hr./mg. of protein)
Glyceraldehyde 3-phosphate dehydrogenase	3·60
3-Phosphoglycerate kinase	9·00
Phosphoglycerate mutase	1·20
Enolase	3·00
Pyruvate kinase	0·33
3-Phosphoglycerate $\rightarrow$ pyruvate	0·36

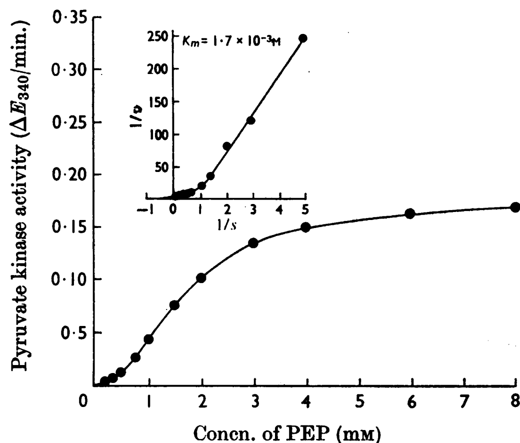

Fig. 1. Effect of PEP concentration on pyruvate kinase activity, assayed spectrophotometrically as described in the Methods and Materials section with 0·4mg. of protein, 4mM-ADP and PEP concentrations as indicated. The v values are expressed as ΔE_{340}/min.

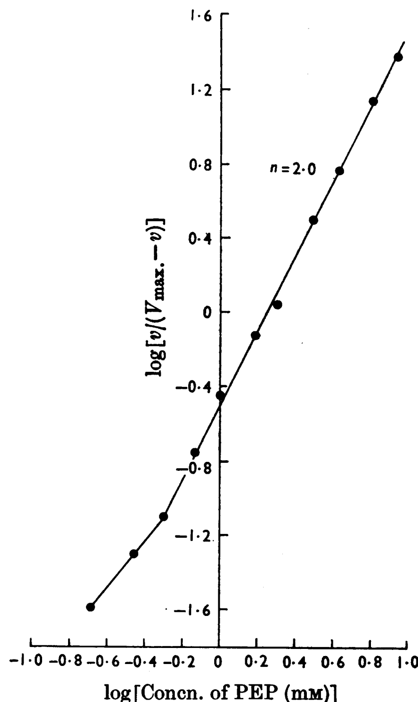

Fig. 2. PEP co-operativity in the pyruvate kinase reaction: Hill plot of the data shown in Fig. 1. $V_{max.}$ was determined from the reciprocal plot of velocity versus PEP concentration.

animals and plants (Boyer, 1962; Miller & Evans, 1957) the *A. xylinum* enzyme was not activated by K^+ or NH_4^+ ions. Addition of these ions did not increase the activity of an extensively dialysed enzyme preparation when a similarly dialysed lactate dehydrogenase preparation was used for the assay procedure.

Mg^{2+} ions were essential for pyruvate kinase activity, maximum enzyme activity being observed at 20mM-Mg^{2+}. Of the various bivalent cations tested only Mn^{2+} ions showed some activation of the enzyme (30% of that of Mg^{2+} ions at 10mM concentration).

Effect of PEP concentration. Fig. 1 shows the plot of enzyme activity as a function of the concentration of PEP. The activity was very low with concentrations of PEP below about 0·5mM, and increased abruptly above this concentration. The Lineweaver–Burk plot for PEP (the inset in Fig. 1) is a second-order curve rather than a straight line as expected from the sigmoidal rate–concentration curve. The apparent K_m for PEP was $1·7 \times 10^{-3}$M and did not vary with changes in the concentration of ADP in the range 2–16mM.

The saturation kinetics of the enzyme for PEP suggests that there is a co-operative effect in the binding of more than one PEP molecule to the enzyme. Fig. 2 shows the Hill plot for PEP calculated from the rate–concentration data by the procedure of Monod, Changeux & Jacob (1963). Two parts of the curve could be distinguished, which point to a change in the binding mechanism at higher PEP concentrations. From the slope of the upper part of the curve a Hill coefficient of 2 was calculated, suggesting a multipoint activation of the co-operative type.

Effect of ADP concentration. The rate–concentration data with ADP as the variable substrate yielded a Michaelis–Menten hyperbola (Fig. 3). The apparent K_m for ADP calculated from the Lineweaver–Burk plot was $1·54 \times 10^{-3}$M. The affinity of the enzyme for ADP did not change with the concentration of PEP in the range 2–6mM. Unlike the pyruvate kinase from rat liver (Tanaka, Harano, Sue & Morimura, 1967), the *A. xylinum* enzyme was not inhibited by excess of ADP up to a concentration of 16mM.

Effect of ATP on pyruvate kinase activity. ATP, a product of the reaction, was found to inhibit the pyruvate kinase activity. The inhibition appeared to be competitive with ADP, and a K_i value of $0·9 \times 10^{-3}$M was computed from Dixon and Lineweaver–Burk plots (Figs. 4 and 5). Thus, in the presence of 2mM-ATP, the apparent K_m for ADP increased 3·5-fold. On the other hand the addition of inhibitory amounts of ATP did not change the sigmoidal shape of the rate–concentration curve with regard to PEP.

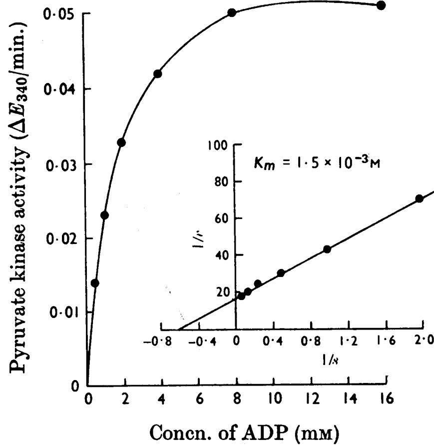

Fig. 3. Effect of ADP concentration on pyruvate kinase activity, assayed spectrophotometrically as described in the Methods and Materials section with 0·25mg. of protein, 2mM-PEP and ADP concentrations as indicated.

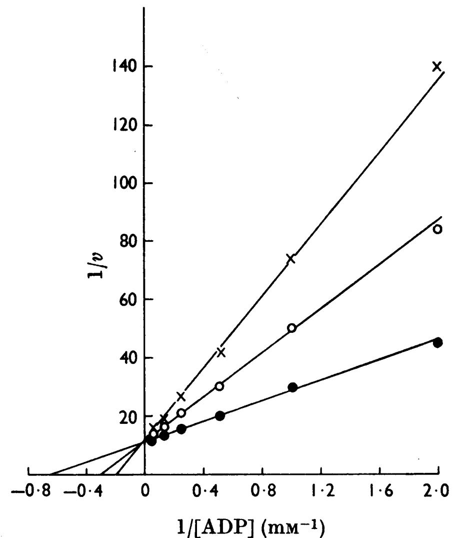

Fig. 5. Effect of ATP concentration on pyruvate kinase activity, assayed spectrophotometrically as described in the Methods and Materials section with 0·4mg. of protein, 3mM-PEP and ADP concentrations as indicated. ●, No added ATP; ○, 1mM-ATP; ×, 2mM-ATP.

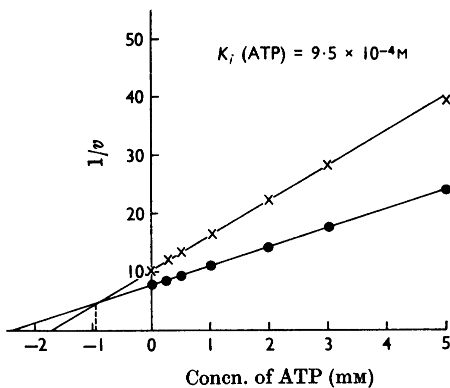

Fig. 4. Inhibition by ATP of pyruvate kinase activity, assayed spectrophotometrically as described in the Methods and Materials section with 0·17mg. of protein, 6mM-PEP and ATP concentrations as indicated. ×, 2mM-ADP; ●, 4mM-ADP.

The inhibition by ATP is unlikely to be due to formation of complexes with Mg^{2+}, since inhibition by it was not affected by raising the Mg^{2+} ion concentration to 40mM.

Effect of thiol inhibitors. The partially purified enzyme was sensitive to pretreatment with *p*-hydroxymercuribenzoate. At 4μM the mercurial compound inhibited the enzyme activity by 50%. The possibility that the binding site of this inhibitor on the enzyme is related to PEP co-operativity was examined by studying the relationship between PEP concentration and the activity of a partially inhibited enzyme preparation. The shape of the substrate-saturation curve of the treated enzyme did not, however, differ significantly from that of the untreated enzyme.

Effect of other metabolites. Unlike the pyruvate kinases from liver (Weber, Lea, Convery & Stamm, 1967), *Escherichia coli* (Maeba & Sanwal, 1968) and yeast (Hess, Haeckel & Brand, 1966), the *A. xylinum* enzyme was not affected by fructose 1,6-diphosphate (1–10mM) even at low concentrations of PEP. Glucose 6-phosphate and fructose 6-phosphate were similarly without effect. The hexose phosphates did not relieve the inhibition by ATP. Other metabolites that were found not to affect enzyme activity in the absence or presence of ATP were: AMP (0·1–10mM), acetyl-CoA (0·2–1mM), acetyl phosphate (0·2–2mM), glyceraldehyde 3-phosphate (0·2–2mM), 3-phosphoglycerate (0·2–2mM), acetate (2–20mM), inorganic phosphate (2–100mM) and citrate (2–20mM).

DISCUSSION

As proposed by Krebs & Kornberg (1957) the pacemaker enzymes of a metabolic pathway, characterized by catalysing non-equilibrium reactions and by their low activity relative to other enzymes of the same pathway, are the most susceptible points for metabolic control. The equilibrium of the reaction catalysed by pyruvate kinase lies

far to the side of pyruvate formation and is assumed to be practically irreversible under physiological conditions (Krebs & Kornberg, 1957; Utter, 1963). Because of its low activity relative to that of other glycolytic enzymes it is possible that the pyruvate kinase of *A. xylinum* is a pace-setter for that segment of the glycolytic pathway involved in the conversion of carbohydrates into pyruvate in these cells.

Growth of *A. xylinum* on glucose or intermediates of the tricarboxylic acid cycle was accompanied by only slight variations in the quantities of pyruvate kinase formed. It appears, then, that the regulatory mechanism for pyruvate kinase involving the control of enzyme synthesis during growth on different carbon sources, reported for the yeast enzyme (Ruiz-Amil, de Torrontegui, Palacián, Catalina & Losada, 1965; Gancedo, Gancedo & Sols, 1967), is absent from *A. xylinum*. On the other hand, the kinetic properties of the enzyme described above suggest the possible function in these cells of an alternative regulatory mechanism, involving the control of enzyme activity.

The sigmoid velocity–substrate-concentration relationship with regard to PEP, characteristic of regulatory enzymes, suggests a co-operative effect by this substrate. The kinetic data obtained could be fitted to the empirical Hill equation (Changeux, 1964) and a value of 2 was derived for n, which is a function for the number of interacting binding sites and of the strength of the interaction (Atkinson, Hathaway & Smith, 1965). Substrate co-operativity could have considerable regulatory significance by ensuring extreme sensitivity of enzyme activity to very narrow ranges of substrate concentration (Stadtman, 1966). In our case, PEP would then be both a substrate and a positive-feedback 'effector'. As the pace-maker enzyme for the conversion of carbohydrates into pyruvate, which in turn is oxidized in *A. xylinum* through the tricarboxylic acid cycle (Benziman & Burger-Rachamimov, 1962), the pyruvate kinase in these cells could decrease or increase the rate of oxidation of carbohydrates via this pathway, depending on the availability of PEP. Such a mechanism is compatible with the low affinity of the enzyme for PEP. The apparent K_m for this substrate obtained here ($1{\cdot}7\times10^{-3}$M) is much higher than the K_m for it of muscle pyruvate kinase (Boyer, 1962), but approximates to the values reported for the liver and yeast enzymes, which similarly show allosteric properties (Tanaka *et al.* 1967; Hess *et al.* 1966).

A second mechanism for controlling the activity of pyruvate kinase is suggested by the inhibitory effect of ATP. The inhibition was shown to be competitive with ADP (but not with PEP; cf. Reynard, Haas, Jacobson & Boyer, 1961) and the affinity of the enzyme for the inhibitor was similar to its affinity for the substrate (Fig. 3). Although a similar inhibition of other pyruvate kinases was attributed to the binding of Mg^{2+} ions by ATP (Wood, 1968), this does not appear to apply to our system, since the inhibition was observable even in the presence of excess of Mg^{2+} ions. ATP is one of the end products of the pyruvate kinase reaction. Since pyruvate kinase in *A. xylinum* is involved in the channelling of carbohydrate via pyruvate into the tricarboxylic acid cycle, the enzyme plays an important role in the generation of ATP both directly and indirectly. A high ADP/ATP concentration ratio will favour both the pyruvate kinase reaction and the oxidative pathways of the tricarboxylic acid cycle, with concurrent phosphate esterification (Benziman & Levy, 1966). On the other hand a rise in the ATP concentration would provide a regulatory action on pyruvate kinase, also acting as a negative feedback. An increase in the concentration of free ATP would then lead to a decrease in the production of this high-energy phosphate.

These postulated variations in pyruvate kinase activity in response to changes in the cellular concentration of the different adenine nucleotides might have additional physiological significance, considering the effects of similar changes on the activities of the key enzymes responsible for the channelling, in these cells, of pyruvate into the gluconeogenetic route (Benziman, 1966; Eisen, Palgi & Benziman, 1968) and of PEP into the anaplerotic pathway of oxaloacetate synthesis (Benziman, 1968).

The pyruvate kinase of *A. xylinum* as a possible regulatory enzyme differs from the enzyme from other sources in its insensitivity to fructose 1,6-diphosphate, even at low PEP concentrations. This observation, however, is compatible with the absence in *A. xylinum* of phosphofructokinase, the enzyme responsible for fructose diphosphate formation in the classical glycolytic pathway. It is noteworthy that the activation by fructose diphosphate of yeast pyruvate kinase was reported (Hess & Haeckel, 1967) to be related to the activation of this enzyme by K^+ and NH_4^+ ions. The *A. xylinum* enzyme, as noted above, was not only insensitive to fructose diphosphate, but was also unaffected by K^+ and NH_4^+ ions.

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