PYRUVATE KINASE FROM *HYDROGENOMONAS* H 16

J.W. DROZD

Department of Biochemistry, School of Biology, University of Leicester, University Rd., Leicester LE1 7RH, England

Received 1 August 1973

1. Introduction

The physiologically irreversible reaction catalysed by pyruvate kinase constitutes an important regulatory point in the glycolytic pathway in many bacteria [1, 2], yeasts [3], and animal tissues [4]. In many of these examples fructose 1,6-diphosphate, an early metabolite on the glycolytic pathway, is a positive effector of the enzyme. In this paper it is shown that glucose 6-phosphate, an early metabolite of the Entner–Douderoff pathway is a positive effector of the pyruvate kinase from *Hydrogenomonas* H 16. In this organism, for hexose degradation at least, the Entner–Douderoff pathway is operative [5]. The use of intact cells made permeable to substrates by treatment with toluene has been previously described [2, 6–8, 10].

2. Materials and methods

Hydrogenomonas H 16 was grown heterotrophically in batch culture at 30°C in minimal medium [9] supplemented with the carbon/energy source at 1%. Bacteria were harvested by centrifugation at 4°C when in the exponential phase, washed once in 10 mM phosphate buffer, pH 7.2, and ruptured by sonication (Branson sonifier). A typical purification of the enzyme from crude extract is outlined in table 1. Pyruvate kinase activity was assayed by a modification [10] of the colourimetric method of Cooper and Kornberg [11]. The reaction mixture contained per ml: Tris–HCl buffer, pH 7.4, 50 μmoles: ADP, 1 μmole; PEP (phosphoenolpyruvate), 1 μmole; $MgSO_4$, 5 μmoles. The pyruvate kinase activity in toluene-treated intact cells was measured by the above colourimetric method as previously described [10].

Table 1
Effect of carbon-energy source in batch culture on the level of pyruvate kinase from *Hydrogenomonas* H 16.

Carbon-energy source	μMoles pyruvate formed / mg protein/min
Fructose	0.06
Pyruvate	0.063
Acetate	0.05
Succinate	0.08

3. Results

3.1. *Purification*

The purification of the enzyme from fructose grown cultures is shown in table 1. All steps were carried out in 10 mM phosphate buffer pH 7.2. Enzyme activity was very rapidly and partially irreversibly lost in Tris–HCl or glycine buffers. No pyruvate kinase activity was detected in crude extracts of cells sonicated in 0.025 M Tris–HCl buffer, pH 7.4. The enzyme from fructose grown cultures was purified 100-fold to give one major and four minor bands on disc-gel electrophoresis.

3.2. *Approximate molecular weight*

From the elution pattern on Sephadex G-200, pyruvate kinase from fructose, succinate or pyruvate grown cultures had a mol. wt. of about 250 000. Elution of the 100-fold purified enzyme from fructose grown cultures on Sephadex G-200 calibrated with cytochrome *c*, catalase, lactate dehydrogenase and bovine serum albumin indicated a mol. wt. of 160 000.

3.3. *Stability*

The loss of activity of the enzyme in Tris–HCl was

Table 2
Typical purification of pyruvate kinase from fructose grown *Hydrogenomonas* H 16.

Preparation	Specific *(units/mg protein)	Total activity (units)	Overall recovery (%)
Crude extract	0.06	600	100
$(NH_4)_2SO_4$ ppt. 25–34% saturation	0.48	450	75
Sephadex G-200 column	2.40	290	48
$(NH_4)_2SO_4$ ppt. 30–35% saturation	4.10	260	43
Calcium phosphate gel adsorption	5.07	230	39

* 1 Unit; 1 μmole pyruvate formed/mg protein/min at 30°C.

partly reversible; if the 100-fold purified enzyme was diluted in 0.025 M Tris–HCl pH 7.4, activity was completely lost in 5 min. Re-incubation of the inactive preparation with 10 mM phosphate, 1 mM PEP or 1 mM ADP for 30 min at 30°C resulted in a complete restortation of activity provided the enzyme had not been in the inactive state for more than 3 hr. Dithiothreitol or EDTA were ineffective in restoring activity. After about 3 hr activity could not be completely restored, and after leaving overnight at room temperature or 4°C activity was completely and irreversibly lost. Addition of Tris to the enzyme in 10 mM phosphate buffer resulted in no loss of activity. The indications are that PEP, phosphate and ADP bind to and stabilize the enzyme.

3.4. *Constitutive nature*

Table 2 shows the specific activities of the enzyme in crude extracts of cultures grown with various carbon/energy sources. Previous work [10] showed that permeabilized intact fructose or pyruvate grown cells contained the enzyme, and the level was relatively independent of the growth rate.

3.5. *Regulation*

These studies were made, unless otherwise stated with the 100-fold purified enzyme from fructose grown cultures.

Mg^{2+} is an activator of the enzyme (fig. 1). Note

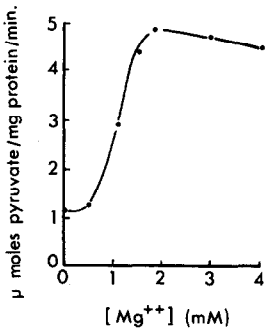

Fig. 1. Dependence of *Hydrogenomonas* H 16 pyruvate kinase activity on Mg^{2+} concentration. The activity is for the purified enzyme from fructose grown cells. ADP and PEP at 1 mM.

the activity in the absence of any added Mg^{2+}. The Hill plot has a slope (n-value) of 2. High Mg^{2+} concentrations (3 mM) caused a slight inhibition of activity. ATP appeared to act as an inhibitor by sequestering the Mg^{2+} in a 1:1 molar ratio. Na^+, K^+, Ca^{2+}, NH_4^+ were without effect on the enzyme activity or stability.

At saturating PEP (1 mM) and Mg^{2+} (5 mM) concentrations the enzyme exhibited normal Michaelis–Menten kinetics (n=1) with respect to ADP. The K_M was 80 μM ADP.

At saturating ADP (1 μM) and Mg (5 mM) the enzyme exhibited sigmoidal kinetics with respect to PEP (fig. 2), with a $S_{0.5}$ value of 300 μM and n-value in the Hill plot of 2.8. Glucose 6-phosphate (G 6-P)

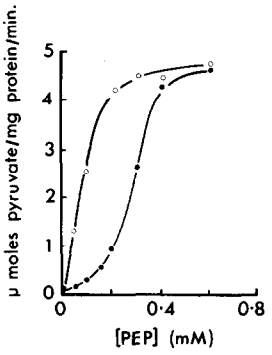

Fig. 2. Dependence of *Hydrogenomonas* H 16 pyruvate kinase activity on PEP concentration in the absence (●—●—●), and presence (○—○—○) of 1 mM glucose 6-phosphate. The activity is for the purified enzyme from fructose grown cells. ADP at 1 mM.

at 1 mM was a positive effector (fig. 2) and transformed the kinetics to a normal Michaelis–Menten type with n-value of 1 and K_M of 110 μM PEP. The sigmoidicity of the plot and activation by G 6-P were independant of pH over range pH 7.0–8.0. Glucose 1-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate and 3-phosphoglyceric acid showed no activity as activators. If the enzyme was stored (in 10 mM phosphate buffer, pH 7.2), frozen, at –5°C for more than four days, the sigmoidicity with respect to PEP was lowered ($S_{0.5}$ of 150 μM PEP) and the slope in the Hill plot halved to 1.4. G 6-P still activated the enzyme and kinetics with respect to ADP were unaltered.

Phosphate, as well as stabilizing the enzyme, inhibited the reaction. 3 mM phosphate buffer in the assay system caused a 30% inhibition. High Mg^{2+} concentrations (10 mM) lowered the inhibition to 10%. G 6-P was without effect on the phosphate inhibition.

AMP, up to 10 mM had no effect on the reaction kinetics. The effect of increasing concentrations of one of the two substrates (PEP or ADP) in the presence of a fixed concentration of the other substrate were estimated at pH 7.4 with 1 mM G 6-P. Double reciprocal plots were non-parallel which indicated that both substrates bound to the enzyme before the products were released, This is identical to the substrate binding for the *Neurospora crassa* pyruvate kinase [13].

4. Discussion

The activation of this Entner–Douderoff pyruvate kinase by glucose 6-phosphate is analogous to the activation of many of the glycolytic pyruvate kinases by fructose 1,6-diphosphate [1–3, 8]. The enzyme is probably regulated by changes in the pool sizes of activators and substartes. The pyruvate kinase in toluene treated 'permeabilized' intact cells of *Hydrogenomonas* H 16 was very similar in kinetic properties to the purified enzyme, and was similarly activated by G 6-P at low PEP concentrations [10].

The inhibition by phosphate, and stabilization by substrates is very similar to that of the *Azotobacter vinelandii* pyruvate kinase [12]. In *A. vinelandii* (which also utilizes the Entner–Douderoff pathway) a very wide range of substrates activated the enzyme at low PEP concentrations.

Acknowledgements

This work was supported by a Royal Society European Fellowship (1971–72) held at the Institut für Mikrobiologie, Gottingen, W. Germany. I would like to thank Professor H.G. Schlegel and Dr. J.H. Klemme for their advice and criticisms.

References

[1] Sanwal, B.D. (1970) Bacteriol. Rev. 34, 20.
[2] Kornberg, H.L. and Malcovati, M. (1973) FEBS Letters 32, 257.
[3] Barwell, C.J. and Hess, B. (1971) 19, 1.
[4] Bailey, E., Stirpe, F. and Taylor, C.B. (1968) Biochem. J. 108, 427.
[5] Gottschalk, G., Eberhardt, U. and Schlegel, H.G. (1964) Arch. Mikrobiol. 48, 95.
[6] Weitzman, P.D.J. (1973) FEBS Letters 32, 247.
[7] Reeves, R.E. and Sols, A. (1973) Biochem. Biophys. Res. Commun. 50, 459.
[8] Serrano, R., Gancedo, J. and Gancedo, C. (1973) J. Biochem. 34, 479.
[9] Schlegel, H.G., Kaltwasser, H. and Gottschalk, G. (1961) Arch. Mikrobiol. 38, 209.
[10] Drozd, J.W., Arch. Mikrobiol., in press.
[11] Cooper, R.A. and Kornberg, H.L. (1969) in: Methods in Enzymology (Colowick, P. and Kaplan, N.O., eds.), Vol. 13, p. 309, Academic Press, London and New York.
[12] Liao, C.L. and Atkinson, D.E. (1971) J. Bacteriol. 106, 37.
[13] Kapoor, M. and Tronsgaard, T.M. (1972) Can. J. Microbiol. 18, 805.