PROPERTIES AND PHYSIOLOGICAL ROLE OF THE PEP-SYNTHASE OF *A. XYLINUM*

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1. Introduction

Previous studies have shown that the anhydroglucose carbon chain of cellulose arises from pyruvate in *Acetobacter xylinum* via phosphoenolpyruvate (PEP) [1]. The net synthesis of PEP from pyruvate was shown to be achieved in this organism by an enzyme which catalyzes the direct phosphorylation of pyruvate by ATP [2]. The stoichiometry we reported for this reaction was the same as that observed by Cooper and Kornberg [3] for the PEP- synthase from *E. coli*, namely:

$$\text{Pyruvate} + \text{ATP} \longrightarrow \text{PEP} + \text{AMP} + \text{Pi}\,.$$

In the present communication the partial purification and some properties of the *A. xylinum* enzyme are described. Evidence is presented that in addition to ATP, orthophosphate is also a reactant in the conversion of pyruvate to PEP. AMP and pyrophosphate (P–P) are the other products. Although the enzyme also catalyzes the reverse reaction, studies on factors affecting the formation of this enzyme in *A. xylinum* clearly indicate that its physiological function in this organism is related to the process of gluconeogenesis.

2. Methods and materials

Succinate-grown cells of *A. xylinum* were grown and harvested as previously described [4]. Glucose-grown cells were obtained according to Schramm et al. [5].

Purification of the enzyme. (Enzyme fractions during all steps were maintained at 15–25°C.) Succinate-grown cells, suepended in 5 mM Tris-H_2SO_4 buffer (pH 7.4) containing 1 mM $MgCl_2$, 5 mM EDTA and 0.1 ml dithiothreitol (TMED buffer) were ruptured by sonic oscillation. The homogenate was centrifuged for 15 min at 18000 × *g*, then for 60 min at 150000 × *g*, and the sediment was discarded. The supernatant containing 400 mg protein was placed on a column prepared from 7 g DEAE-cellulose (Whatman DE 52) previously equilibrated with TMED-buffer. The column was washed with 80 ml of 0.15 M KCl/TMED. The enzyme was then eluted by increasing the KCl concentration to 0.25 M, and 10 ml fractions were collected. Fractions rich in the enzyme were pooled and fractionated by addition of solid $(NH_4)_2SO_4$. The fraction which precipitated between 35 and 55% saturation was dissolved in TMED buffer to 1/10 the original volume of the crude extract. This fraction has a specific activity of 0.6 in the forward reaction and represented a 45 fold purification over the crude extract with 65% recovery of the total activity. It was free from pyruvate kinase, adenylate kinase, PEP-carboxylase and contained only traces of pyrophosphatase and ATPase activities. It was stable for 2–3 weeks, when kept at room temperature.

The standard assay system for determining PEP-synthase activity in the forward reaction (formation of PEP) was as described in table 1, but with 10 mM Pi, 5 mM ATP and 2 units of crystalline inorganic pyrophosphatase. Samples were withdrawn at various time intervals and assayed for pyruvate and PEP. A blank reaction mixture from which ATP was omitted was used as a control. The activity in the reverse reaction (formation of pyruvate) was assayed spectrophotometrically or fluorometrically. The reaction mixture was as in table 1, but with 0.15 mM NADH and 2 units

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Table 1
Stoichiometry of the forward and reverse reaction catalyzed by PEP-synthase.

Component measured	Changes (μmoles) in							
	Forward reaction:				Reverse reaction:			
	Complete system	−ATP	−Pyruvate	−Pi	Complete system	−AMP	−PEP	−PP
Pyruvate	−0.56	−0.05	0	−0.05	+0.90	0	0	0
ATP	−0.62	0	−0.10	−0.10	+0.92	0	0	0
Pi	−0.44	0	+0.10	+0.10	+0.93	+0.12	+0.15	0
PEP	+0.51	0	0	0	−0.88	0	0	0
AMP	+0.52	0	0	0	−0.88	0	0	0
PP	+0.49	0	0	0	−0.90	−0.05	−0.07	0
ADP	+0.08	0	+0.10	0.10	0	0	0	0

Complete systems (1 ml) contained the following in μmoles: for the forward reaction, Tris-H_2SO_4, pH 8, 2, 100; pyruvate, 2; ATP 4; Pi, 2; $MgCl_2$, 10; enzyme, 0.1 mg protein; for the reverse reaction, imidazole-HCl, pH 7, 0, 50; PEP, 2; AMP, 2; PP, 2; $MgCl_2$, 10; enzyme, 0.15 mg protein. Incubated for 15 min at 30°C. Reactions were terminated by 1 ml of 0.6 M $HClO_4$, followed by centrifugation. Protein-free supernatants were neutralized with 2 M K_2CO_3. Components were determined as described in Methods and Materials.

of crystalline lactic dehydrogenase. Gluconeogenetic activity was determined by incubating cells with uniformly labelled ^{14}C-pyruvate and measuring the radioactivity of the cellulose synthesized [1]. Activity is expressed as μmoles pyruvate converted into cellulose per hr. Products and reactants of the PEP-synthase reaction were determined as described previously [2]. P–P was determined with inorganic pyrophosphatase [6].

3. Results

When incubated in Tris-H_2SO_4 buffer at pH 8.2, with pyruvate, ATP and Pi, the purified enzyme catalyzed the formation of PEP, AMP and P–P. The reverse reaction was demonstrable in imidazole buffer at pH 7.0. The stoichiometry of the overall reaction as measured in both directions is shown in table 1. PEP synthesis was dependent on the presence of pyruvate, ATP, magnesium ions and orthophosphate. Aresnate could be substituted for phosphate, though at equimolar concentrations, the rate of PEP synthesis in the presence of arsenate was 65% the rate in the presence of phosphate. The rate of the forward reaction declined rapidly with time. However, when the assay system was supplemented with an excess of either pyrophosphatase, adenylate deaminase or adenylate kinase the reaction rate was linear with time and proportional to the amount of enzyme added over a range of 15 to 100 μg of protein. The reverse reaction was dependent on the presence of PEP, AMP, P–P and magnesium. ADP could not substitute for AMP. When coupled to the lactic dehydrogenase system the reaction was linear with time and with enzyme concentration up to 125 μg of protein.

When assayed in Tris-maleate buffer the optimum pH was 8.2 for PEP formation and 6.5 for the reverse reaction. To test the effect of the concentration of each of the substrates on enzyme activity, the appropriate assay methods were modified so that the non-variable components of the reaction were present at saturating concentrations and the substrate under examination was present at a rate-limiting concentration. The apparent Km values calculated from Lineweaver-Burk plots were: (forward reaction) pyruvate 2×10^{-4} M; ATP 3.5×10^{-4} M; Pi 8×10^{-4} M; Mg^{++} 2.2×10^{-3} M: (reverse reaction) PEP 10^{-4} M; P–P 7×10^{-5} M; AMP 1.6×10^{-6} M; Mg^{++} 8.7×10^{-4} M. AMP was found to inhibit PEP formation competitively with regard to ATP and a K_I value of 0.2 mM was calculated from a Dixon plot. The apparent

Table 2
Effect of carbon source in growth media on PEP-synthase formation by *A. xylinum*.

Growth conditions		Activity (μmoles/g dry wt of cells/min)			
		0	5 hr	15 hr	25 hr
A) Glucose-grown cells transferred to succinate medium	PEP-synthase	1.2	1.3	4.6	9.9
	Gluconeogenesis	0.3	0.4	2.9	8.9
B) Glucose added to cells growing in succinate medium	PEP-synthase	13.0	8.0	3.7	2.5
	Gluconeogenesis	10.5	4.2	2.1	0.8

A) Cells grown on glucose were harvested at their exponentially growing phase, washed and then inoculated at an initial density of 0.15 mg dry wt/ml into a succinate medium (zero time). At the times indicated, cells were harvested. One portion was assayed for gluconeogenic activity (expressed above as the amount of pyruvate converted into cellulose) and from another portion extracts were prepared and assayed for PEP-synthase (expressed above as the amount of PEP synthesized under the standard conditions of assay). Total mass of cells increased 4-fold by the end of the experiment.
B) Succinate cells were inoculated into succinate medium. When the cells had grown to 0.06 mg dry wt/ml, 50 mM glucose was added (zero time). At the times indicated, cells were harvested and assayed for gluconeogenic and PEP-synthase activities. By the end of the experiment total mass of cells increased 9-fold and glucose concentration decreased to 10 mM. PEP-synthase activity of a control culture, which received no glucose, remained unchanged after 25 hr of growth.

Km for ATP increased 10-fold in the presence of 1.5 mM AMP.

The effect of the carbon source in the growth-medium on enzyme formation, is shown in table 2. Enzyme formation was induced on transfer of glucose-grown cells to succinate or pyruvate-containing media. Enzyme formation did not occur if such media were supplemented with glucose, fructose or glycerol or in the presence of chloramphenicol (50 μg/ml). When glucose was added to cultures of cells growing on succinate, the growth of the organism was unimpaired but was accompanied by the virtual cessation of PEP-synthase synthesis. Induced enzyme formation was accompanied by a parallel increase in the ability of cells to convert pyruvate into cellulose.

4. Discussion

The previously reported stoichiometry for the PEP-synthase reaction in *A. xylinum* which indicated orthophosphate as a product of pyruvate phosphorylation [2], was most probably due to the presence of pyrophosphatase activity in the enzyme preparations used in those studies. It was only upon further purification of the enzyme that its activity was found to be dependent on the presence of orthophosphate and that pyrophosphate was also recognised as one of the products of pyruvate phosphorylation. The results reported here are compatible with a reaction proceeding as follows:

$$\text{pyruvate} + \text{ATP} + \text{Pi} \rightleftharpoons \text{PEP} + \text{AMP} + \text{P–P}\,.$$

It is suggested that in spite of the reversibility of the reaction, the physiological role of PEP-synthase in *A. xylinum* is to catalyze the formation of PEP from pyruvate. This will enable the organism to grow on citrate-cycle intermediates and to synthesize from such compounds the cellulose essential for its growth in a static liquid medium [7]. Evidence for the gluconeogenetic role of the enzyme comes from the induced formation of the enzyme during growth on citrate-cycle intermediates, from the effect of glucose in repressing enzyme formation and from the correlation between PEP-synthase activity and the ability of cells to convert pyruvate into cellulose. The maintenance of the required rate of PEP formation during gluconeogenesis is most probably accomplished in the cell, by the removal of pyrophosphate and AMP by pyrophosphatase and adenylate kinase, both of which are very active in these cells.

With regard to its physiological role our enzyme differs from a similar enzyme reported in *Entamoeba*

histolytica and *Bacteroides symbiosus*, where it is assumed to have a glycolytic function in catalyzing the conversion of PEP to pyruvate [3]. In *A. xylinum*, however, the dephosphorylation of PEP during carbohydrate oxidation is accomplished by pyruvate kinase which was found both in glucose- and succinate-grown cells [9]. In this connection it may be added that a possible mechanism for controlling the disadvantageous coupling of the pyruvate kinase reaction with the PEP-synthase reaction is the reciprocal effect of AMP and ATP on the activity of these two enzymes as reported here and elsewhere [9].

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