The Phosphoenolpyruvate Carboxykinase of *Verticillium albo-atrum*

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SUMMARY

Partially purified phosphoenolpyruvate carboxykinase from *Verticillium albo-atrum* had a pH optimum of 6.2 and required manganese for maximum activity, having lesser activity with iron or cobalt. Sodium and potassium ions were slightly stimulatory. Adenosine-5'-diphosphate increased activity and inosine-5'-diphosphate supported low activity, but other nucleotides were ineffective. Inhibition of the enzyme by *p*-chloromercuribenzoate was partially reversed by dithiothreitol. Avidin had no effect on enzyme activity. Oxalacetate slightly stimulated the enzyme and NADP⁺ strongly inhibited, but aspartate and acetyl-CoA showed no effect. Low levels of phosphoenolpyruvate carboxykinase were present in cells grown on glucose, xylose, or glycerol. Aspartate, pyruvate, and acetate as carbon sources resulted in higher levels of activity and malate gave the highest. The data indicate that the enzyme functions physiologically in the gluconeogenic conversion of oxalacetate to phosphoenolpyruvate.

INTRODUCTION

Carbon dioxide was utilized in the synthesis of aspartate, glutamate and nitrogen bases when the phytopathogenic fungus *Verticillium albo-atrum* was grown on glucose (Hartman, Keen & Long, 1972). Growth did not occur in CO₂-free atmospheres on glucose or glycerol media, but normal growth occurred on succinate or acetate. The suggested role of anaplerotic CO₂-fixing enzymes was confirmed when Hartman & Keen (1973) subsequently demonstrated the presence of pyruvate carboxylase (EC. 6.4.1.1) and phosphoenolpyruvate carboxykinase (PEPCK, EC. 4.1.1.32) in *V. albo-atrum*. On the basis of its properties and sensitivity to inhibition by aspartate and acetyl coenzyme A, it was postulated that pyruvate carboxylase catalyses anaplerotic CO₂ fixation in the fungus (Hartman & Keen, 1974). To assess the role of PEPCK in the physiology of the fungus, we have studied its properties and determined the effect of various carbon sources on enzyme activity.

METHODS

Preparation of phosphoenolpyruvate carboxykinase. *Verticillium albo-atrum*, strain V3H (ATCC26289), was grown and disrupted as previously described (Hartman & Keen, 1973). Yeast-form cells were grown in a synthetic medium, washed, suspended in the tris breakage buffer (0.1 M-tris; 1.0 mM-dithiothreitol; 0.1 mM-Na₂EDTA; 0.1 M-KCl; and HCl to pH 7.6),

Table 1. *Effect of pH on the activity of phosphoenolpyruvate carboxykinase from Verticillium albo-atrum*

Reaction mixture: imidazole, 100 μ mol; MnSO_4 , 2 μ mol; KCl , 100 μ mol; phosphoenolpyruvate, 10 μ mol; ADP, 5 μ mol; NaHCO_3 , 10 μ mol; dithiothreitol, 5 μ mol; $\text{NaH}^{14}\text{CO}_3$, 5 μCi ; protein, 0.2 mg; NaOH to pH stated; total volume, 1.5 ml. Incubation time: 10 min.

pH	Radioactivity incorporated (c.p.m./0.5 ml)
5.2	1972
5.8	9233
6.2	13059
6.9	6617
7.3	3136

Table 2. *Effect of cations on enzyme activity*

Reaction mixture: imidazole, 100 μ mol; phosphoenolpyruvate, 10 μ mol; ADP, 5 μ mol; KCl , 100 μ mol; dithiothreitol, 5 μ mol; KHCO_3 , 10 μ mol; $\text{NaH}^{14}\text{CO}_3$, 5 μCi ; protein, 0.2 mg; NaOH to pH 6.2; total volume, 1.5 ml. Incubation time: 10 min.

Cation (μ mol)	Radioactivity incorporated (c.p.m./0.5 ml)
MnSO_4 (2)	21190
CoCl_2 (3)	5394
CaCl_2 (3)	26
FeSO_4 (3)	9663
ZnSO_4 (3)	72
MgSO_4 (3)	208
None	26

and disrupted for 1 min in a Bronwill MSK homogenizer. The preparation was centrifuged for 10 min at 4 °C and 34 000 g. All procedures were carried out at approximately 4 °C. To 10 ml of supernatant was added solid ammonium sulphate (3.13 g) to achieve 50 % saturation (23 °C). The precipitate was removed by centrifugation and 1.25 g of solid ammonium sulphate was added to achieve 70 % saturation (23 °C). The precipitate was dissolved in 4 to 5 ml of 0.01 M-tris, 0.1 mM- Na_2EDTA , 1.0 mM-dithiothreitol and HCl to pH 7.6, and dialysed against the same buffer. The preparation represented a fourfold purification of the enzyme compared with the dialysed crude cell extract and fixed approximately 0.12 μ mol of CO_2 (from HCO_3^-) per min per mg protein.

Assay methods. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as the standard. Enzyme activity was measured on the basis of the fixation of $\text{NaH}^{14}\text{CO}_3$ (Hartman & Keen, 1973). The specific activity of the $\text{NaH}^{14}\text{CO}_3$ was 50 mCi/mm. A 10 min incubation period was used to determine enzyme activity; fixation was shown to be linear with respect to time during the first 10 min.

RESULTS

The optimal pH for PEPCK from *Verticillium albo-atrum* was approximately 6.2 (Table 1). Substitution of uridine-5'-diphosphate (UDP) or guanosine-5'-diphosphate (GDP) for adenosine-5'-diphosphate (ADP) in the reaction mixtures gave no activity, while inosine-5'-diphosphate (IDP) supported only 12 % of the activity with ADP at the optimum pH.

Manganese was the most effective divalent cation in supporting PEPCK activity

Table 3. *Effect of pH on the relative effectiveness of magnesium and manganese on enzyme activity*

Reaction mixture: imidazole, 100 μ mol; phosphoenolpyruvate, 10 μ mol; ADP, 5 μ mol; KCl, 100 μ mol; dithiothreitol, 5 μ mol; KHCO_3 , 10 μ mol; $\text{NaH}^{14}\text{CO}_3$, 5 μ Ci; protein, 0.2 mg; NaOH to pH stated; final volume, 1.5 ml.

Divalent cation added (μ mol)	Reaction mixture pH	Radioactivity incorporated (c.p.m./0.5 ml)
None	6.1	140
MnSO_4 (2)	6.1	25453
MgSO_4 (3)	6.1	90
MnSO_4 (2)	6.8	15685
MgSO_4 (3)	6.8	902
MnSO_4 (2)	7.4	3870
MgSO_4 (3)	7.4	770

Table 4. *Effect of potassium, sodium and bicarbonate on enzyme activity*

Reaction mixture: imidazole, 100 μ mol; phosphoenolpyruvate, 10 μ mol; ADP, 5 μ mol; KCl, 100 μ mol; MnSO_4 , 2 μ mol; dithiothreitol, 5 μ mol; $\text{NaH}^{14}\text{CO}_3$, 5 μ Ci; protein, 0.2 mg; NaOH to pH 6.2; total volume, 1.5 ml.

Component(s) added (μ mol)	Radioactivity incorporated (c.p.m./0.5 ml)
None	12713
KCl (100)	14862
NaCl (100)	14891
NaHCO_3 (10)	15249
+ KCl	16020

Table 5. *Effect of biotin, avidin and acetyl coenzyme A on enzyme activity*

Reaction mixture: imidazole, 100 μ mol; phosphoenolpyruvate, 10 μ mol; ADP, 5 μ mol; MnSO_4 , 2 μ mol; KCl, 100 μ mol; KHCO_3 , 10 μ mol; $\text{NaH}^{14}\text{CO}_3$, 5 μ Ci; protein, 0.2 mg; NaOH to pH 6.2; total volume, 1.5 ml.

Component(s) added	Radioactivity incorporated (c.p.m./0.5 ml)
None	15063
Biotin (0.5 μ g)	15368
Biotin + avidin (0.5 units)	15050
Avidin	14924
CoASAc (0.5 μ mol)	15846

(Table 2). Less activity was observed with Fe^{2+} and Co^{2+} , and Mg^{2+} supported little. A relationship between pH and the relative effectiveness of Mn^{2+} and Mg^{2+} was observed (Table 3). With Mn^{2+} , enzyme activity decreased as the pH increased while with Mg^{2+} it increased.

The enzyme activity was slightly stimulated by the addition of either K^+ or Na^+ to the reaction mixture (Table 4). Acetyl coenzyme A, biotin or avidin did not affect activity (Table 5). Dithiothreitol had a slightly stimulatory effect on PEPCK, while *p*-chloro-mercuribenzoate completely inhibited activity (Table 6). The inhibition could be partially reversed by the addition of dithiothreitol. *N*-Methyl maleimide was only slightly inhibitory.

Of various glycolytic pathway intermediates tested, glyceraldehyde-3-phosphate and glycerate-2,3-diphosphate gave 35 % inhibition (Table 7), and the citric acid cycle inter-

Table 6. *Effect of sulphydryl reagents on enzyme activity*

Reaction mixture: imidazole, 100 μ mol; phosphoenolpyruvate, 10 μ mol; ADP, 5 μ mol; MnSO_4 , 2 μ mol; KCl, 100 μ mol; KHCO_3 , 10 μ mol; $\text{NaH}^{14}\text{CO}_3$, 5 μ Ci; protein, 0.2 mg; NaOH to pH 6.2; total volume, 1.5 ml. Where single reagents were added to the reaction mixture, they were added 3 min before the addition of $\text{NaH}^{14}\text{CO}_3$. In the reaction mixture containing the combination of reagents, the enzyme in the reaction mixture was exposed to *p*-chloromercuribenzoate for 3 min followed by the addition of dithiothreitol for 2 min before the addition of $\text{NaH}^{14}\text{CO}_3$.

Sulphydryl reagent(s) added (μ mol)	Radioactivity incorporated (c.p.m./0.5 ml)
None	13788
Dithiothreitol (20)	15715
N-Methyl maleimide (1.5)	11920
<i>p</i> -Chloromercuribenzoate (0.15)	15
+ dithiothreitol	6880

Table 7. *Effect of metabolic intermediates on phosphoenolpyruvate carboxykinase activity*

Reaction mixture: imidazole, 100 μ mol; phosphoenolpyruvate, 10 μ mol; ADP, 5 μ mol; MnSO_4 , 2 μ mol; KCl, 100 μ mol; dithiothreitol, 5 μ mol; KHCO_3 , 10 μ mol; $\text{NaH}^{14}\text{CO}_3$, 5 μ Ci; protein, 0.2 mg; NaOH to pH 6.2; total volume, 1.5 ml. Where DL compounds were used, 20 μ mol were added.

Intermediates added (10 μ mol)	Radioactivity incorporated (percentage of control)*	Intermediates added (10 μ mol)	Radioactivity incorporated (percentage of control)*
Aspartate	96	β -NAD ⁺	66
Oxalacetate	110	NADP ⁺	37
Malate	65	Glycerol	95
Succinate	83	DL-Glycerol-3-P	50
Fumarate	87	DL-Glyceraldehyde-3-P	66
Citrate	66	Glycerate-3-P	88
DL-Isocitrate	74	Glycerate-2-P	76
α -Ketoglutarate	82	Glycerate-2,3-P ₂	62
Glutamate	94	Fructose-6-P	85
Alanine	95	Fructose-1,6-P ₂	88
β -NADH	86	Glucose-1-P	95
NADPH	65	Glucose-6-P	92

* The control value was the radioactivity obtained in the reaction mixture in the absence of the added intermediates. The radioactivity averaged about 25500 c.p.m./0.5 ml in the controls for the several experiments from which these data were obtained.

mediates malate and citrate also caused 35 %. The greatest inhibition, 63 %, was exhibited by NADP⁺, while the related cofactors, NADPH and NAD⁺, caused 35 % inhibition. Although the stimulation by oxalacetate was only 10 %, it was consistent in several experiments (Table 7).

Malate was the most effective carbon source for activity of PEPCK in *Verticillium albo-atrum* (Table 8), followed by aspartate and then by pyruvate and acetate. The least effective carbon sources were glucose, xylose, and glycerol. When aspartate was tested, a small quantity of glucose was added to achieve an adequate quantity of cells.

DISCUSSION

The physiological role of PEPCK in *Verticillium albo-atrum* appears to be the gluconeogenic decarboxylation of oxalacetate to phosphoenolpyruvate. This hypothesis is supported by several observations. Although several glycolytic and Krebs-cycle inter-

Table 8. *Effect of carbon source on the activity of phosphoenolpyruvate carboxykinase in cells of Verticillium albo-atrum*

Cells were grown in the carbohydrate (5 g/l)–ammonium nitrate (2.43 g/l)–biotin medium, pH 6.3 (Hartman, Keen & Long, 1972) from a washed yeast-phase cell inoculum. Enzyme-assay reaction mixture: imidazole, 100 μ mol; phosphoenolpyruvate, 10 μ mol; ADP, 5 μ mol; MnSO_4 , 2 μ mol; KCl, 100 μ mol; dithiothreitol, 5 μ mol; KHCO_3 , 10 μ mol; $\text{NaH}^{14}\text{CO}_3$, 5 μ Ci; protein, 0.2 mg; NaOH to pH 6.2; total volume, 1.5 ml. Enzyme preparation was dialysed cell extract.

Carbon source(s) in growth medium (0.5 %)	Specific radioactivity (10^3 c.p.m./mg protein)
Expt A	
Glucose	43
Xylose	50
Pyruvate	129
Glycerol	34
Acetate	144
Expt B	
Glucose	50
Malate	285
Aspartate + glucose (0.02 %)	212

mediates inhibited the enzyme, the immediate gluconeogenic substrate oxalacetate was stimulatory. As in other micro-organisms (Shrago & Shug, 1969) and in liver tissue (Seubert, Henning, Schoner & L'age, 1968), elevated activity of PEPCK was observed under conditions that promote gluconeogenesis and reduced activity occurred when glucose was available. The high level of PEPCK activity when *V. albo-atrum* was grown on pyruvate could be correlated with the formation and decarboxylation of oxalacetate that is generally considered necessary for the conversion of pyruvate to phosphoenolpyruvate (Mahler & Cordes, 1971). Growth on malate and aspartate could similarly require a high level of cellular PEPCK because both compounds can readily be converted to oxalacetate. The increased synthesis of PEPCK during growth on acetate can be explained by the functioning of the glyoxylate pathway, which again results in the synthesis of oxalacetate through malate. Enzymic evidence indicates the presence of this pathway in *V. albo-atrum* (Tokunaga *et al.* 1969). The low level of PEPCK in cells grown on glycerol is also consistent with the gluconeogenic hypothesis since it has been reported that glycerol can be directly metabolized via the Embden–Meyerhof pathway with the formation of phosphoenolpyruvate (Thimann, 1963).

Verticillium albo-atrum has been shown to contain only two C_3 – C_4 carboxylation enzymes, viz. pyruvate carboxylase and PEPCK (Hartman & Keen, 1973, 1974). The pyruvate carboxylase appears to function in the anaplerotic conversion of C_3 compounds to C_4 Krebs-cycle intermediates (Hartman & Keen, 1974), while PEPCK seems to function in gluconeogenesis. The presence of these distinct enzymes would appear consistent with the metabolic flexibility required for growth of the organism on various carbon sources.

REFERENCES

- HARTMAN, R. E. & KEEN, N. T. (1973). Enzymes capable of anaplerotic carbon dioxide fixation in *Verticillium albo-atrum*. *Phytopathology* **63**, 947–953.
 HARTMAN, R. E. & KEEN, N. T. (1974). The pyruvate carboxylase of *Verticillium albo-atrum*. *Journal of General Microbiology* **81**, 15–19.
 HARTMAN, R. E., KEEN, N. T. & LONG, M. (1972). Carbon dioxide fixation by *Verticillium albo-atrum*. *Journal of General Microbiology* **73**, 29–34.

- LOWRY, O. H., ROSEBROUGH, N. T., FARR, A. R. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MAHLER, H. R. & CORDES, E. H. (1971). *Biological Chemistry*, 2nd. edn, pp. 520–528. New York: Harper & Row.
- SEUBERT, W., HENNING, H. V., SCHONER, W. & L'AGE, M. (1968). Effects of cortisol on the levels of metabolites and enzymes controlling glucose production from pyruvate. *Advances in Enzyme Regulation* **6**, 153–187.
- SHRAGO, E. & SHUG, A. L. (1969). Regulation of phosphoenolpyruvate carboxykinase in *Escherichia coli* by carbohydrate metabolism. *Archives of Biochemistry and Biophysics* **130**, 393–398.
- THIMANN, K. V. (1963). *The Life of Bacteria*, 2nd edn, p. 575. New York: Macmillan.
- TOKUNAGA, J., MALCA, I., SIMS, J. J., ERWIN, D. C. & KEEN, N. T. (1969). Respiratory enzymes in the spores of *Verticillium albo-atrum*. *Phytopathology* **59**, 1829–1832.