The Pyruvate Carboxylase of *Verticillium albo-atrum*

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SUMMARY

The pyruvate carboxylase of *Verticillium albo-atrum* had a pH optimum of 7.8 and a specific requirement for ATP. At the optimum pH, magnesium ions were required for maximum activity, while at pH 6.8 manganese was more effective than magnesium. Potassium was stimulatory while sodium was ineffective. Avidin and *p*-chloromercuribenzoate strongly inhibited the enzyme while biotin and dithiothreitol, respectively, reversed the effect of the inhibitors. Aspartate and oxalacetate were inhibitory while acetyl-CoA and CoA reversed the inhibition by aspartate. These cofactors were ineffective in the absence of aspartate. None of the tested metabolic intermediates was stimulatory to pyruvate carboxylase activity while NADP⁺ and 2,3-diphosphoglycerate were the most effective inhibitors (75 %) at a concentration of 6.7 mM. Levels of pyruvate carboxylase in cells grown on glucose, acetate, malate, xylose, glycerol or aspartate differed only slightly. The data indicated that the physiological role of pyruvate carboxylase in *V. albo-atrum* is the anaplerotic biosynthesis of C₄ Krebs-cycle intermediates from pyruvate.

INTRODUCTION

Carbon dioxide was required for growth of *Verticillium albo-atrum* on glucose or glycerol but not on acetate or succinate (Hartman, Keen & Long, 1972). Since we also found that the CO₂ was used largely in the synthesis of aspartate, glutamate and nitrogen bases, it was hypothesized that anaplerotic biosynthesis of C₄ Krebs-cycle intermediates was an important role for CO₂ in the physiology of *V. albo-atrum*. The anaplerotic CO₂ fixation enzymes pyruvate carboxylase (EC. 6.4.1.1) and phosphoenolpyruvate carboxykinase (EC. 4.1.1.32) were shown to occur in *V. albo-atrum* (Hartman & Keen, 1973). This report describes studies to determine the properties of pyruvate carboxylase and the effect of various carbohydrates in the growth medium on the activity of this enzyme in *V. albo-atrum* cells.

METHODS

Preparation of pyruvate carboxylase. *Verticillium albo-atrum*, strain V3H (ATCC26289), was grown and cells disrupted as described previously (Hartman & Keen, 1973). Briefly, yeast-phase cells were suspended in the phosphate-breakage buffer, pH 6.6, disrupted in a Bronwill MSK homogenizer, and the supernant from centrifugation for 10 min at 34000g was the source of the enzyme. Dialysis, centrifugation, and fractionation were carried out at 3 to 5 °C. The supernatant (10 ml) was combined with 2 ml of the phosphate-breakage buffer containing

Table 1. *Effect of pH on the activity of pyruvate carboxylase from Verticillium albo-atrum*

Reaction mixture (μ mol): tris, 100; pyruvic acid, 10; ATP, 5; biotin, 4×10^{-5} ; KCl, 100; NaHCO_3 , 10; MgSO_4 , 3; $\text{NaH}^{14}\text{CO}_3$, 5 μCi ; protein, 0.2 mg; total volume, 1.5 ml.

pH	Radioactivity incorporated (c.p.m./0.5 ml)
7.1	3308
7.5	6078
7.8	6863
8.1	5679
8.4	2780

240 μ mol of oxalacetate and incubated for 30 min at 23 °C. Ammonium sulphate (2.81 g) was added slowly to 10 ml of the cooled solution (3 °C) to achieve approximately 45 % saturation (23 °C). The precipitate was removed by centrifugation and 1.25 g of ammonium sulphate was added to achieve 65 % saturation (23 °C). The precipitate was removed by centrifugation and suspended in 4 ml of a solution containing 0.1 M-potassium phosphate and 0.025 M-oxalacetate, pH 6.6, and then dialysed against 0.1 M-potassium phosphate buffer, pH 6.6, with four hourly changes of $15 \times$ volume. The enzyme preparation represented a fourfold purification over the oxalacetate-preincubated and dialysed cell extract. It generally contained 2 to 3 mg protein/ml and fixed 0.04 to 0.06 μ mol carbon/min/mg protein. The specific activity was determined at pH 7.8 under the conditions shown in Table 1. The total carbonate present was estimated to be 11 μ mol, based on the 10 μ mol added and the presence of about 1 μ mol of dissolved carbonate (Feir & Suzuki, 1969).

Assay methods. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as the standard.

The procedure for measuring the fixation of $\text{NaH}^{14}\text{CO}_3$ has been described in detail (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; enzyme activity had previously been shown to be linear with respect to time for the first 10 min.

RESULTS

The optimal pH for the activity of pyruvate carboxylase was approximately 7.8 (Table 1). Inosine-5'-triphosphate, guanosine-5'-triphosphate, or uridine-5'-triphosphate was not able to replace adenosine-5'-triphosphate in the reaction mixture at pH 7.8. When the divalent cations MgSO_4 , MnSO_4 , CoCl_2 , ZnSO_4 , CaCl_2 or FeSO_4 were used at 2 μ mol in the reaction mixture at pH 7.8, only MgSO_4 supported a high level of enzyme activity. Raising the MgSO_4 concentrations to 3 μ mol increased enzyme activity by 50 %.

When biotin was omitted from this reaction mixture there was no decrease in fixation. The importance of biotin in the reaction, however, was demonstrated by the complete inhibition of enzyme activity which resulted when biotin was replaced with 1 unit of avidin and by the reversal of this inhibition by the inclusion of 1 μ g biotin.

A relationship was found between pH and the relative effectiveness of Mg^{2+} and Mn^{2+} in supporting carboxylase activity. As the pH was decreased from 7.8 to 6.8 the relative effectiveness of Mg^{2+} decreased and that of Mn^{2+} increased, so that at pH 6.8 Mn^{2+} was more effective than Mg^{2+} (Table 2). Activity was maximal with Mg^{2+} at pH 7.8.

Table 2. *Effect of pH, magnesium and manganese on pyruvate carboxylase activity from Verticillium albo-atrum*

Reaction mixture (μmol): tris, 100; pyruvic acid, 10; ATP, 5; biotin, 4×10^{-5} ; KCl, 100; cation, 2; KHCO_3 , 10; $\text{NaH}^{14}\text{CO}_3$, 5 μCi ; protein, 0.2 mg; total volume, 1.5 ml.

Cation added	pH of reaction mixture	Radioactivity incorporated (c.p.m./0.5 ml)
None	7.9	248
MgSO_4	7.9	10888
MnSO_4	7.9	1078
MgSO_4	7.2	4442
MnSO_4	7.2	3888
MgSO_4	6.8	1702
MnSO_4	6.8	3823

Table 3. *Effect of sodium, potassium and bicarbonate on pyruvate carboxylase activity*

Reaction mixture (μmol): tris, 100; pyruvic acid, 10; ATP, 5; biotin, 4×10^{-5} ; MgSO_4 , 3; $\text{NaH}^{14}\text{CO}_3$, 5 μCi ; protein, 0.2 mg; total volume, 1.5 ml; pH 8.0.

Substance(s) added (μmol)	Radioactivity incorporated (c.p.m./0.5 ml)
None	16886
KCl (100)	29303
NaCl (100)	15998
KCl (100), NaHCO_3 (10)	11359

Table 4. *Effect of acetyl-CoA and CoA on pyruvate carboxylase*

Reaction mixture (μmol): tris, 100; pyruvate, 10; ATP, 5; biotin, 4×10^{-5} ; KCl, 100; MgSO_4 , 3; KHCO_3 , 10; $\text{NaH}^{14}\text{CO}_3$, 5 μCi ; protein, 0.3 mg; total volume, 1.5 ml; pH 7.9.

Substance(s) added (μmol)	Radioactivity incorporated (%)	Substance(s) added (μmol)	Radioactivity incorporated (%)
None	100*	Aspartate + CoA	98
CoASAc (0.5)	99	Oxalacetate (7.5)	30
CoA (0.5)	96	+ CoASAc	34
Aspartate (7.5)	27	+ CoA	34
+ CoASAc	93		

* The radioactivity incorporated here was 13675 c.p.m./0.5 ml.

At the concentration tested, potassium ions increased enzyme activity (Table 3) but sodium ions did not.

The addition of dithiothreitol (20 μmol) to the optimum reaction mixture did not enhance pyruvate carboxylase activity. The importance of free thiol groups for activity, however, was demonstrated by the complete inhibition that resulted when the enzyme was incubated in the reaction mixture containing *p*-chloromercuribenzoate (0.015 μmol) for 3 min before the addition of $\text{NaH}^{14}\text{CO}_3$. This inhibition was partially reversed (40 %) when dithiothreitol (20 μmol) was added to the reaction mixture containing the inactivated enzyme 2 min before the $\text{NaH}^{14}\text{CO}_3$.

Neither coenzyme A (CoA) nor acetyl coenzyme A (CoASAc) enhanced enzyme activity when added to the reaction mixture (Table 4). Both aspartate and oxalacetate inhibited the reaction approximately 28 % at 5.0 mM. The basis for inhibition by these compounds must

Table 5. *Effect of metabolic intermediates on pyruvate carboxylase*

Compound added* (10 μ mol)	Inhibition† (%)	Compound added* (10 μ mol)	Inhibition† (%)
Control†	0	NADP ⁺	74
Malate	18	NADP ⁺ + CoASAc (0.5 μ mol)	66
Succinate	15	Glycerol	0
Fumarate	14	DL-Glycerol-3-P	27
Citrate	14	DL-Glyceraldehyde-3-P	32
DL-Isocitrate	15	Glycerate-3-P	19
2-Oxoglutaric acid	18	Glycerate-2-P	29
Glutamate	4	Glycerate-2,3-P ₂	79
Alanine	7	Fructose-6-P	26
β -NADH	35	Fructose-1,6-P ₂	39
NADPH	44	Glucose-1-P	10
β -NAD ⁺	47	Glucose-6-P	17

* Reaction mixture (μ mol): tris, 100; pyruvate, 10; ATP, 5; biotin, 4×10^{-5} ; KCl, 100; MgSO₄, 3; KHCO₃, 10; KH¹⁴CO₃, 5 μ Ci; protein, 0.25 mg; total volume, 1.5 ml; pH 7.9. Where DL compounds were used, 20 μ mol was added.

† The control value (0% inhibition) was the radioactivity obtained in reaction mixtures in the absence of the added intermediates. The radioactivity fixed by controls averaged about 12500 c.p.m./0.5 ml in the several experiments from which these data were obtained.

Table 6. *Effect of carbon source on the synthesis of pyruvate carboxylase*

Cells were grown in the carbohydrate (5 g/l)-ammonium nitrate (2.43 g/l)-biotin medium, pH 6.3, (Hartman, Keen & Long, 1972). The enzyme preparation was an oxalacetate-preincubated, dialysed cell extract. The composition of the reaction mixture was as described in Table 1, pH 7.8.

Carbon source (0.5 %)	Specific radioactivity incorporated (10 ³ c.p.m./mg protein)
Expt A	
Glucose	24
Pyruvate	30
Expt B	
Glucose	20
Acetate	20
Malate	13
Xylose	18
Glycerol	18
Expt C	
Glucose	37
Aspartate + glucose (0.02 %)	25

differ because both CoA and CoASAc at a level of 5.0 mM reversed the inhibition by aspartate but had little effect on the inhibition by oxalacetate (Table 4). Perhaps the latter was simply the result of product inhibition.

The effect of various metabolic intermediates on enzyme activity is illustrated in Table 5. Among the glycolytic pathway intermediates, 2,3-diphosphoglyceric acid was the most inhibitory (79%) while fructose-1,6-diphosphate inhibited the enzyme by 39%. The nicotinamide cofactors tested were all significantly inhibitory, NADP⁺ being the most effective (74%). Acetyl-CoA only slightly reversed the inhibitory effect of NADP⁺.

Growth of the cells in media with various single carbon sources did not result in levels of pyruvate carboxylase activity that were significantly different from those in cells grown on glucose (Table 6).

DISCUSSION

The physiological role of the *Verticillium albo-atrum* pyruvate carboxylase appears to be the anaplerotic conversion of pyruvate to oxalacetate, since aspartate inhibits the enzyme and this inhibition is reversed by CoASAc. Such observations have generally been interpreted as denoting the importance of the enzyme in modulating levels of Krebs-cycle intermediates (Utter & Scrutton, 1969). The properties of the *V. albo-atrum* pyruvate carboxylase are similar to those of the enzyme from other sources with respect to pH range, the requirement for a divalent ion and a monovalent ion, and the importance of biotin and free sulphydryl groups for enzyme activity (Scrutton & Young, 1970).

Activity of the *Verticillium albo-atrum* pyruvate carboxylase does not appear to be modulated by repression since no significant changes in activity were observed by growing the cells on various carbon sources. This is similar to observations with other microbial systems. The enzyme activity showed little change when *Bacillus licheniformis* was grown on glucose, malate, glycerol, peptone-yeast extract, or pyruvate (Renner & Bernlohr, 1972). *Rhodotorula glutinis* also had approximately the same enzyme activity whether grown on glucose, pyruvate, malate, aspartate, or acetate (Ruiz-Amil *et al.* 1965). Activity in *Rhodopseudomonas spheroides* was the same on acetate, glucose, pyruvate, malate, or lactate in the dark; in light, however, the activity of pyruvate carboxylase was doubled on pyruvate or lactate as compared to glucose (Payne & Morris, 1969).

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