PYRUVATE CARBOXYLASE OF Rhizopus nigricans AND ITS ROLE IN FUMARIC ACID PRODUCTION

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Summary: The chief carbon dioxide-fixing system in a fumaric acid-producing strain of Rhizopus nigricans is an acetyl coenzyme A - dependent pyruvate carboxylase. This enzyme is produced in high levels during the period of rapid glucose utilization, and precedes fumarate accumulation. It is proposed that this system is the principal anaplerotic CO_2 fixing mechanism in this organism, and its continued operation in high glucose media after biosynthesis has ceased results in production of fumaric acid.

A previous report from this laboratory (1) established that the bulk accumulation of fumaric acid by the mold Rhizopus nigricans in high glucose media under aerobic conditions took place via a $C_3 + C_1$ carbon dioxide fixation. Evidence was based on whole cell experiments, in which the direct incorporation of $NaH^{14}CO_3$ into fumarate was measured, and by the demonstration that the specific radioactivity of fumarate formed from uniformly labeled ^{14}C glucose in the presence of excess non-radioactive bicarbonate was decreased by 25 percent, in accordance with a $C_3 + C_1$ CO_2 fixation mechanism. The present investigation was undertaken to determine which of the numerous CO_2-fixing systems was operative in this organism under conditions of fumarate accumulation.

MATERIALS AND METHODS

Rhizopus nigricans 45, a fumaric acid-producing strain de-

scribed by Foster and Waksman (2), was grown in a medium containing, per liter: glucose, 50 g; $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $CaCl_2$, 0.01 g; $Fe_2(SO_4)_3$, 0.01 g; $ZnSO_4 \cdot 7\ H_2O$, 0.005 g. This medium was dispensed in 400 ml amounts to 2 liter baffled flasks, sterilized, inoculated with 8 ml of a spore suspension standardized to an O.D. of 0.3, and incubated at 32 C on a rotary shaker at 250 rpm. After 4 hrs to allow spore germination, 12 g sterile $CaCO_3$ was added.

For preparation of cell-free extracts, cells were harvested by filtration, washed, resuspended in 0.05 M Tris-HCl buffer (pH 7.0), and ruptured by agitation in an ice-jacketed Waring blendor with glass beads (0.2 mm) according to the method of Lamanna and Mallette (3). After centrifuging at 14,500 g for 30 min, the supernatant was used as the enzyme preparation.

Carbon dioxide fixation was determined radiochemically using the following reaction mixture (complete system): Tris-HCl buffer (pH 7.0), 50 µmoles; sodium pyruvate, 1.5 µmoles; ATP, 1.5 µmoles; acetyl CoA, 0.1 µmole; KCl, 10 µmoles; $KH^{14}CO_3$, 10 µmoles (0.25 µc/µmole); malic dehydrogenase, 10 units; enzyme preparation, 0.25 ml; water to a total volume of 3.0 ml. After incubation for 30 min at 30 C, the reaction was terminated by addition of 1 ml 2N HCl. The acid-stable ^{14}C radioactivity was determined by placing 0.5 ml of the mixture in a glass scintillation vial, drying at 80 C for 60 min, dissolving in 0.5 ml H_2O, adding 10 ml Bray's scintillation fluid (4), and counting in a Packard Liquid Scintillation Spectrometer. Specific activity is expressed as µmoles CO_2 fixed per hour per mg protein.

Protein in extracts was determined by the method of Lowry, et al. (5); residual glucose in the growth medium was determined by the method of Folin and Malmros (6), and fumaric acid by the method of Ölander (7).

Table I

Requirements of $^{14}CO_2$-fixation system

Omissions from complete system*	Additions to complete system	Percent of maximum activity
None	none	100.00
Pyruvate and ATP	PEP	14.30
Pyruvate	PEP	53.24
Pyruvate and ATP	PEP and ADP	27.69
Acetyl CoA	none	0
ATP	none	0

* Components listed in Materials and Methods.

RESULTS AND DISCUSSION

Maximum $^{14}CO_2$-fixing activity was found in a reaction mixture containing pyruvate, ATP, magnesium and potassium ions, and acetyl CoA, corresponding to pyruvate carboxylase (pyruvate: carbon-dioxide ligase (ADP); EC 6.4.1.1). Results are shown in Table I. If phosphoenolpyruvate (PEP) was substituted for pyruvate and ATP, there was much reduced activity; this residual activity was probably due to the formation of pyruvate from PEP through the action of pyruvate kinase, which was present in the extract. This residual activity in the presence of PEP was increased when ATP was included in the reaction mixture, again indicating the presence of pyruvate carboxylase, since ATP is required in this system but not in the PEP carboxylase system. The activity found when PEP and ADP were substituted for pyruvate and ATP can be explained by the action

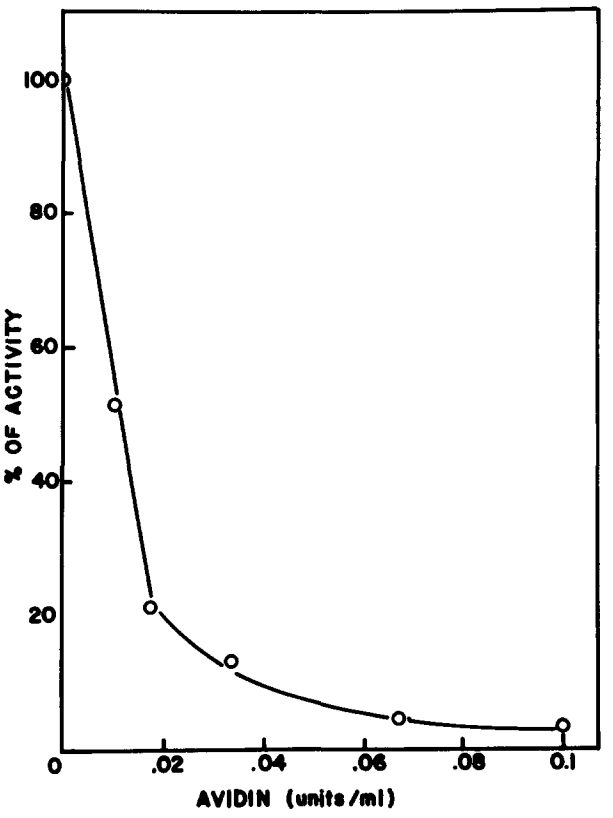

Figure 1. Avidin inhibition of pyruvate carboxylase activity.

of pyruvate kinase, which would generate pyruvate and ATP from PEP and ADP.

It is seen in Table I that acetyl CoA was essential for activity, and Table II shows that a concentration as low as 10 μM was sufficient for maximum activity. Thus, this system resembles that described in chicken liver mitochondria by Utter and Keech (8) with respect to its acetyl CoA activation, and is in contrast to the situation found in a citric acid-producing strain of Aspergillus niger (9) and in Pseudomonas citronella (10), where acetyl CoA is not required. In baker's yeast, acetyl CoA stimulates pyruvate carboxylase activity, but is not absolutely required (11).

Table II

Effect of acetyl CoA concentration on pyruvate carboxylase activity

Acetyl CoA (μM)	Counts per min per mg protein	μMoles CO_2 per hr
0	17	0.0004
10	4,800	0.139
30	4,400	0.127
60	4,237	0.122
80	4,753	0.137

Figure 1 shows that pyruvate carboxylase of R. nigricans is inhibited by avidin, confirming that it is a biotin-requiring enzyme.

The time course of pyruvate carboxylase formation in relation to glucose utilization and fumaric acid formation is shown in Figure 2. It is seen that the spore inoculum had a low level of pyruvate carboxylase; the enzyme was synthesized rapidly during the course of glucose utilization. Specific activity dropped off after glucose was exhausted. Fumaric acid accumulation began after significant levels of pyruvate carboxylase had been reached, and stopped after glucose had been exhausted and pyruvate carboxylase levels had dropped.

These data indicate that pyruvate carboxylase is the principal anaplerotic CO_2-fixing system in R. nigricans, serving to replenish C_4 intermediates of the citric acid cycle that are drawn off for biosynthesis during active growth. The high levels found during rapid glucose utilization and the

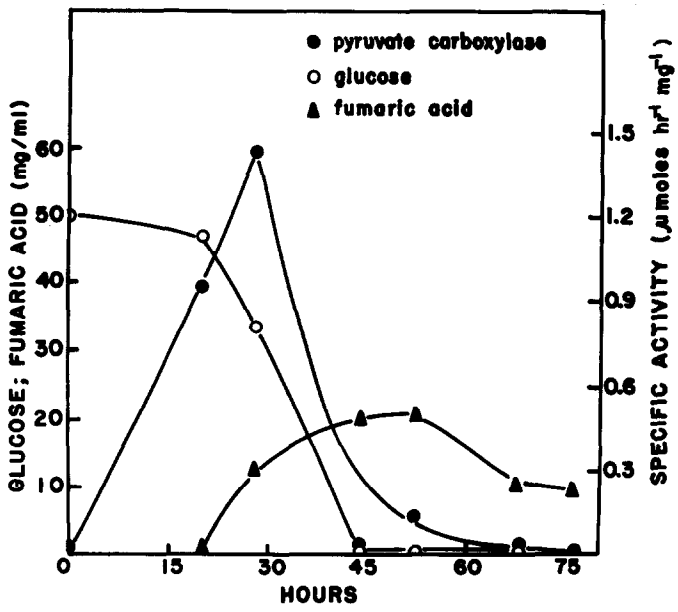

Figure 2. Time course of pyruvate carboxylase formation in relation to glucose utilization and fumaric acid production.

acetyl CoA activation are consistent with this anaplerotic function. It is hypothesized that under conditions of limiting nitrogen source where biosynthesis is restricted, excess sugar is metabolized to pyruvate, and continued operation of pyruvate carboxylase results in accumulation of C_4 compounds, principally fumarate. It is of interest that pyruvate carboxylase has also been assigned an essential role in citric acid production by Aspergillus niger (12).

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REFERENCES

1. Romano, A. H., Bright, M. M., and Scott, W. E., J. Bacteriol., 93, 600 (1967).

2. Foster, J. W., and Waksman, S. A., J. Bacteriol., 37, 599 (1939).

3. Lamanna, C., and Mallette, M. F., J. Bacteriol., 67, 503 (1954)

4. Bray, G. A., Anal. Biochem., 1, 279 (1960).

5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

6. Folin, O., and Malmros, H., J. Biol. Chem., 83, 115 (1929).

7. Ölander, A., Z. Physik. Chem. Abt. A, 144, 49 (1929).

8. Utter, M. F., and Keech, D. B., J. Biol. Chem., 238, 2603 (1963).

9. Bloom, S. J., and Johnson, M. J., J. Biol. Chem., 237, 2718 (1962).

10. Seubert, W., and Remberger, U., Biochem. Z., 334, 401 (1961).

11. Cazzulo, J. J., and Stoppani, A. O. M., Biochem. J., 112, 747 (1969).

12. Cleland, W. W., and Johnson, M. J., J. Biol. Chem., 208, 679 (1954).