CO₂-fixing Enzymes in *Pseudomonas fluorescens*

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SUMMARY

Pseudomonas fluorescens grown on glucose or glutamate at 1 or 20 °C, or on acetate at 20 °C, as sole carbon sources, contained both pyruvate carboxylase and phosphoenolpyruvate carboxylase. Pyruvate carboxylase was insensitive to acetyl-coenzyme A and L-aspartate, and its level in cell-free extracts was markedly dependent on the carbon source for growth, the highest specific activity being attained in glucose-grown cells. Phosphoenolpyruvate carboxylase, on the other hand, although less dependent on the nature of the carbon source, showed its highest level in acetate-grown cells; the enzyme activity required acetyl-coenzyme A and was strongly inhibited by L-aspartate. The micro-organism had, in addition, a phosphoenolpyruvate carboxykinase, which showed its highest specific activity in cells grown on acetate, and a NADP-linked malate enzyme, apparently repressed by acetate and showing its highest specific activity in glutamate-grown cells.

INTRODUCTION

The anaplerotic synthesis of oxaloacetate from three-carbon precursors (Kornberg, 1966) is usually accomplished in bacteria through the action of one of the two CO₂-fixing enzymes, the biotin-dependent pyruvate carboxylase (EC. 6.4.1.1), which catalyses reaction 1:

or the biotin-independent phosphoenolpyruvate carboxylase (EC. 4.1.1.31), which catalyses reaction 2:

Phosphoenolpyruvate carboxykinase (EC. 4.1.1.32), which catalyses reaction 3, is also usually present in bacterial cells grown under gluconeogenic conditions:

Since both pyruvate and phosphoenolpyruvate carboxylases are supposed to fulfil the same function in cell metabolism (Kornberg, 1966), it was thought that they were not likely to be simultaneously present in the same cell. However, both carboxylases are present in *Pseudomonas citronellolis* and *Azotobacter vinelandii* (Scrutton & Taylor, 1974).

This paper describes the factors that regulate the levels and activity of these carboxylases in *Pseudomonas fluorescens*.

METHODS

Organism. *Pseudomonas fluorescens*, strain R12, was isolated from water of the Paraná River at Rosario, Argentina, and characterized according to Stanier, Palleroni & Doudoroff (1966) by O. Forchetti, Silvia Milrad de Forchetti, Angélica González and J. L. Parada, from the Departamento de Microbiología, Facultad de Ciencias Bioquímicas, Rosario.

Culture. *Pseudomonas fluorescens* was grown in 500 ml batches at 1 °C, in the cold room, with occasional shaking, or at 20 °C in a water bath; in the latter case the cultures were shaken and aerated by forcing air through the medium with the aid of a vacuum pump. The culture medium was that described by Sundaram, Cazzulo & Kornberg (1969), with the addition of 0.1 µg D-biotin/ml, and glucose, glutamate or acetate as sole carbon source (1 %, w/v). Growth was followed as the increase in extinction at 680 nm, determined in a Bausch & Lomb Spectronic 20 spectrophotometer. The cells grown on glucose or glutamate at 20 °C were harvested after 20 h, when the E_{680} reached about 1.4 (beginning of the stationary phase); those grown on the same carbon sources at 1 °C, or on acetate at 20 °C, were harvested at E_{680} values of about 0.3, after growth for 10 days or 3 days, respectively.

Preparation of cell-free extracts. The cultures were harvested by centrifuging at 6500 g for 20 min at 4 °C. The cells were washed with 0.05 M-tris-HCl buffer pH 7.6 containing 1 mM-disodium EDTA, 10 mM-MgCl₂ and 50 mM-KCl, and kept frozen overnight. The cells were suspended in the same buffer and disrupted by ultrasonic oscillation as previously described (Vidal & Cazzulo, 1972a). The homogenate was centrifuged at 37000 g for 20 min at 4 °C. The supernatant (cell-free extract) was dialysed for 2 h against 100 vol. of tris-HCl buffer pH 7.6 containing 1 mM-EDTA, at 1 °C.

Ammonium sulphate fractionation. A crude extract obtained from cells grown on glutamate at 20 °C (4.2 ml) was brought to 37 % saturation with saturated ammonium sulphate solution (adjusted to pH 7 with NH₄OH), with stirring, at 0 °C. The suspension was centrifuged at 37000 g for 20 min at 4 °C. The precipitate was dissolved in 1 ml of tris-HCl buffer (pH 7.6 containing 1 mM-EDTA, and dialysed for 2 h against 100 vol. of the same buffer solution at 1 °C. The supernatant was successively brought to 42.0, 46.6, 50.6, 54.3 and 75.0 % saturation with saturated ammonium sulphate solution, and the precipitates obtained were processed as described for the 37 % saturation fraction.

Assay methods. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

The standard reaction mixture for the assay of pyruvate carboxylase contained (in µmol) in a final volume of 0.4 ml: tris-HCl buffer pH 9.0, 20; sodium pyruvate, 2; ATP, 0.5; MgCl₂, 2; NaH¹⁴CO₃, (0.9 µCi), 1.8; NADH, 0.33; pig heart malate dehydrogenase (EC. 1.1.1.37), 0.5 i.u.; and 0.01 to 0.06 ml of enzyme preparation. The standard reaction mixture for phosphoenolpyruvate carboxylase was similar, except that potassium phosphoenolpyruvate (1 µmol) was substituted for pyruvate and ATP, and acetyl-coenzyme A (acetyl-CoA) (0.05 µmol) was added. The reactions were started by the addition of the enzyme and stopped after 15 min incubation at 30 °C by the addition of 1.2 ml of absolute ethanol. The samples were processed and counted, and the enzyme activities expressed, as previously described (Vidal & Cazzulo, 1972a).

Phosphoenolpyruvate carboxykinase was assayed at 30 °C by measuring the rate of exchange of radioactivity (from NaH¹⁴CO₃) into oxaloacetate, as previously described (Vidal & Cazzulo, 1972a).

NADP-linked malate enzyme (EC. 1.1.1.40) was assayed spectrophotometrically as

Table 1. *Requirements of the reaction catalysed by pyruvate carboxylase from Ps. fluorescens*

The basal reaction mixture was that described in the Methods, with the omissions or additions stated. A 0.02 ml portion of a cell-free extract obtained from cells grown on glucose at 20 °C was used per assay. When avidin was present, 0.09 ml of cell-free extract was preincubated for 25 min at 30 °C with 0.01 ml avidin (50 µg). When biotin and avidin were present, avidin was preincubated with 15 µg D-biotin at 30 °C (10 min) before 25 min preincubation at 30 °C with the enzyme.

Experimental conditions	Pyruvate carboxylase activity (nmol ¹⁴ CO ₂ fixed/ min/mg protein)
Basal	42.6
Plus 0.12 mM-acetyl-CoA	45.1
Plus 12.5 mM L-aspartate	39.5
Minus pyruvate	3.0
Minus ATP	0.5
Minus MgCl ₂ , plus 2.5 mM-EDTA	0.05
Plus avidin	2.4
Plus avidin, plus biotin	38.0

previously described (Vidal & Cazzulo, 1972*b*), except that the concentrations of L-malate-tris, NADP and NH₄Cl were 1, 0.15 and 105 mM, respectively.

Chemicals. Potassium phosphoenolpyruvate, NADH, NADP, ATP, ADP, L-malate, coenzyme A, avidin, D-biotin, 5,5'-dithiobis-(2-nitrobenzoic acid) [DTNB], malate dehydrogenase from pig heart (EC. 1.1.1.37) and pyruvate kinase from rabbit skeletal muscle (EC. 2.7.1.40) were obtained from Sigma. Oxaloacetic acid and pig heart citrate synthase (EC. 4.1.3.7) were purchased from Boehringer, Mannheim, Germany, and sodium pyruvate from Merck. NaH¹⁴CO₃ was obtained from the Comisión Nacional de Energía Atómica de la República Argentina. Acetyl-CoA was prepared from coenzyme A with acetic anhydride (Stadtman, 1957) and assayed with DTNB, oxaloacetate in excess and citrate synthase. All other chemicals used were analytical reagents of the highest purity available.

RESULTS

Preliminary experiments suggested the simultaneous presence of pyruvate carboxylase and phosphoenolpyruvate carboxylase in cell-free extracts of *Ps. fluorescens*. Table 1 shows the requirements of the pyruvate carboxylase, which was insensitive to acetyl-CoA and L-aspartate, and inhibited by avidin. The activation by monovalent cation (Milrad de Forchetti & Cazzulo, 1976) was not observed under these experimental conditions, since the reaction mixtures contained 7 mM-NH₄⁺ added with the malate dehydrogenase. The results shown in Table 1 were obtained with a crude extract from cells grown on glucose at 20 °C; similar results were obtained with cells grown on glucose at 1 °C, except that the enzyme activity assayed in the complete reaction mixture was somewhat lower (26.4 nmol ¹⁴CO₂ incorporated/min/mg protein).

The enzyme determined under the assay conditions for phosphoenolpyruvate carboxylase was identified as such because of its strict requirement of phosphoenolpyruvate and a divalent cation for activity, and the lack of effect of avidin. There was an increase in ¹⁴CO₂ fixation into oxaloacetate upon addition of ADP to the complete reaction mixture, reflecting the probable presence of phosphoenolpyruvate carboxykinase (see below). This does not imply, however, that the enzyme assayed as phosphoenolpyruvate carboxylase was phospho-

Fig. 1. Effects of acetyl-CoA and L-aspartate on the activity of phosphoenolpyruvate carboxylase from *Ps. fluorescens*. The enzyme assays were performed as described, except for the presence of variable concentrations of acetyl-CoA (○, ●) or L-aspartate (△, ▲). A 0.06 ml portion of a mixture of 0.95 ml of cell-free extract and 0.05 ml of avidin (250 μg), preincubated for 25 min at 30 °C, was used per assay. The activities of pyruvate carboxylase remaining after this treatment were 0.2 and 0.04 nmol ¹⁴CO₂ fixed/min/mg protein, for the acetate and glutamate cell-free extracts, respectively. Acetyl-CoA variable: the enzyme rate in the presence of 0.25 mM-acetyl-CoA (100 %), was 19.35 nmol ¹⁴CO₂ fixed/min/mg protein for the extract from the cells grown on acetate at 20 °C (○), or 2.94 nmol/min/mg protein for the extract from cells grown on glutamate at 1 °C (●). L-Aspartate variable, in the presence of 0.12 mM acetyl-CoA: the enzyme rate in the absence of the inhibitor (100 %), was 9.29 nmol ¹⁴CO₂ fixed/min/mg protein for the extract from cells grown on acetate at 20 °C (△), or 1.6 nmol ¹⁴CO₂ fixed/min/mg protein for the extract from cells grown on glutamate at 1 °C (▲).

enolpyruvate carboxykinase using traces of ADP which might still be present in the dialysed cell-free extract. In fact, the phosphoenolpyruvate carboxylase activity was only slightly reduced when a great excess of pyruvate kinase, sufficient to convert all the ADP possibly present into ATP, was added (in the presence of 25 mM-KCl and 5 mM-MgCl₂). Moreover, as shown in Fig. 1, the CO₂ fixation into oxaloacetate depended on the presence of acetyl-CoA and was strongly inhibited by L-aspartate; this was shown to occur in crude extracts obtained from cells grown on acetate at 20 °C or on glutamate at 1 °C. The curve for the activation by acetyl-CoA was sigmoidal, suggesting that the activation may be allosteric.

Pyruvate carboxylase and phosphoenolpyruvate carboxylase could be partially separated by ammonium sulphate fractionation (Table 2). The activity of phosphoenolpyruvate carboxylase was somewhat decreased in the samples preincubated with avidin (25 min at 20 °C); this was probably due to the lability of the latter enzyme, which lost 90 % of its activity when kept under conditions (4 days at 8 °C, in tris-HCl buffer pH 7.6, containing 1 mM-EDTA) in which pyruvate carboxylase was stable.

Table 3 shows that the levels of the two carboxylases, and those of phosphoenolpyruvate carboxykinase and malate enzyme, depended on the nature of the carbon source for growth.

Table 2. *Partial separation of pyruvate carboxylase and phosphoenolpyruvate carboxylase from Ps. fluorescens by ammonium sulphate fractionation*

Cell-free extract from cells grown on glutamate at 20 °C was fractionated with ammonium sulphate as described in Methods. The enzyme activities were assayed with 0.02 ml of enzyme preparation, in the standard reaction mixtures described. Preincubation with avidin was at 20 °C.

Fraction precipitated by ammonium sulphate (% saturation)	Specific activity (nmol ¹⁴ CO ₂ fixed/min/mg protein)			
	Pyruvate carboxylase		Phosphoenolpyruvate carboxylase	
	– Avidin	+ Avidin	– Avidin	+ Avidin
37.0	9.05	0.81	20.15	14.76
42.0	32.24	1.28	55.08	45.63
46.6	88.95	0.37	22.15	15.91
50.6	28.89	0.17	1.76	0.42
54.3	4.26	0.20	1.05	0.13
75.0	0.27	0.11	0.27	0.28

Table 3. *Levels of pyruvate carboxylase, phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase and malate enzyme in cell-free extracts of Ps. fluorescens grown on different carbon sources*

Cultures were grown at 20 °C, cell-free extracts obtained, and the enzyme activities determined as described in Methods. Phosphoenolpyruvate carboxylase was assayed in samples preincubated with avidin as described in the legend to Table 1.

	Specific activity (nmol ¹⁴ CO ₂ fixed/min/mg protein)		
	Glucose	Glutamate	Acetate
Pyruvate carboxylase	49.0	19.0	4.3
Phosphoenolpyruvate carboxylase	4.1	8.3	10.9
Phosphoenolpyruvate carboxykinase	5.6	8.5	23.0
NADP-linked malate enzyme	114.0	150.0	67.0

The highest level of pyruvate carboxylase was found in glucose-grown cells, and that of phosphoenolpyruvate carboxylase in acetate-grown cells. The levels of both carboxylases in cells grown on glutamate at 20 °C were similar when the cells were harvested either in the exponential phase or at the beginning of the stationary phase of growth; similar results were obtained with glucose as carbon source.

DISCUSSION

Pyruvate carboxylase and phosphoenolpyruvate carboxylase were present simultaneously in *Ps. fluorescens*, as has been previously reported for *Ps. citronellolis* and *A. vinelandii* (Scrutton & Taylor, 1974). The possibility that only one carboxylase was present in *Ps. fluorescens*, and the other belonged to a contaminant, was ruled out since similar levels of the two enzymes were found after repeated purification of the strain, with cultures arising from different single colonies. Moreover, similar results were obtained when the micro-organisms were grown on glucose or glutamate at 20 or 1 °C.

As with *A. vinelandii* (Scrutton & Taylor, 1974) the two carboxylases showed different regulatory properties. Pyruvate carboxylase was insensitive to acetyl-CoA and L-aspartate, but its levels were considerably affected by the nature of the carbon source used for growth.

Phosphoenolpyruvate carboxylase, on the other hand, showed little change in its levels with the variation in the carbon source; its activity, however, was affected by acetyl-CoA and L-aspartate, as has been reported for phosphoenolpyruvate carboxylases from other microbial sources (Cánovas & Kornberg, 1966; Maeba & Sanwal, 1965; Vidal & Cazzulo, 1972*a*). These regulatory differences suggest that the two carboxylases might have different functions in the living cell. The highest pyruvate carboxylase activity was found in *Ps. fluorescens* cells grown on glucose or pyruvate (not shown), namely under the growth conditions where the anaplerotic reaction is essential (Kornberg, 1966). Phosphoenolpyruvate carboxylase, on the other hand, showed its highest activity in acetate-grown cells, where the anaplerotic CO₂-fixation is supposed not to be required, and the glyoxylate cycle (Kornberg, 1966) is induced (unpublished results). These facts might suggest that the anaplerotic role was fulfilled by pyruvate carboxylase. However, phosphoenolpyruvate carboxylase was the enzyme showing the acetyl-CoA activation generally associated with the anaplerotic function.

Phosphoenolpyruvate carboxykinase showed a considerably higher level in acetate-grown cells than in glucose-grown cells, in good agreement with its gluconeogenic role (Sanwal, 1970). Malate enzyme, on the other hand, showed its lowest value in acetate-grown cells, suggesting that the enzyme might be repressed by acetate, as demonstrated for the malate enzyme from *Ps. putida* (Jacobson, Bartholomaeus & Gunsalus, 1966).

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