Formation and Dissimilation of Oxalacetate and Pyruvate in Pseudomonas citronellolis Grown on Noncarbohydrate Substrates

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Metabolism of lactate as a carbon source by Pseudomonas citronellolis occurred via a nicotinamide adenine dinucleotide (NAD)-independent L-lactate dehydrogenase, which was present in cells grown on DL-lactate but was not present in cells grown on acetate, aspartate, citrate, glucose, glutamate, or malate. The cells also possessed a constitutive, NAD-independent malate dehydrogenase instead of the conventional NAD-dependent enzyme in the tricarboxylic acid cycle. Both enzymes were particulate and used dichlororphenolindophenol or oxygen as an electron acceptor. In acetate-grown cells, the activity of pyruvate dehydrogenase and NAD phosphate-linked malate enzyme decreased, whereas NAD-independent malate dehydrogenase activity increased relative to cells grown on glucose or lactate. This was consistent with the need to maintain a supply of oxalacetate for metabolism of acetate via the tricarboxylic acid cycle. Changes in enzyme activities suggest that gluconeogenesis from noncarbohydrate carbon sources occurs via the malate enzyme (when oxalacetate decarboxylase is inhibited) or a combination of the NAD-independent malate dehydrogenase and oxalacetate decarboxylase.

In Pseudomonas citronellolis, the pathways for the interconversion of pyruvate, oxalacetate (OAA), and phosphoenolpyruvate (PEP) are more complex than the analogous pathways in other organisms (R. W. O'Brien, B. L. Taylor, and M. F. Utter, Proc. Aust. Biochem. Soc. 6:34, 1973; B. L. Taylor, Ph.D. thesis, Case Western Reserve University, Cleveland, Ohio, 1973; R. W. O'Brien, D. T. Chuang, B. L. Taylor and M. F. Utter, J. Biol. Chem., in press). This is due, in part, to the presence of both pyruvate carboxylase (EC 6.4.1.1.) and PEP carboxylase (EC 4.1.1.31) in P. citronellolis. Prior to this discovery, other organisms were found to contain either, but not both, of these anaplerotic enzymes (18). P. citronellolis also contains a very active OAA decarboxylase (EC 4.1.1.3.), working in the direction opposite that of pyruvate carboxylase, and PEP synthase (EC 2.7.1.dd), working in opposition to pyruvate kinase (EC 2.7.1.40). The net result is a complicated system of apparently futile cycles, whose complexity suggests the need for coordination in regulating the pathways for the metabolism of OAA, PEP, and pyruvate (O'Brien et al., J. Biol. Chem., in press).

The pathways of glucose catabolism to, and gluconeogenesis from, PEP and pyruvate have also been surveyed in P. citronellolis (10). The aim of the present work was to determine how P. citronellolis oxidizes noncarbohydrate carbon sources and how these oxidations may be linked to gluconeogenesis through pyruvate, OAA, and PEP.

MATERIALS AND METHODS

Growth of the organism. P. citronellolis was grown on basal medium (10) with the following carbon sources used at final concentrations of 1%: glucose, citric acid, L-aspartic acid, L-glutamic acid, and L-malic acid. DL-Lactate and potassium acetate were used at final concentrations of 0.6 and 0.5%, respectively. The free acids were neutralized to pH 7.0 with KOH prior to sterilization. Growth and harvesting of the cells were done as described previously (10).

Preparation of cell extracts. Cell extracts were prepared by sonic oscillation (10). For isolation of the particulate nicotinamide adenine dinucleotide (NAD)-independent lactate and malate dehydrogenases, the extract was centrifuged at 12,000 × g for 15 min at 5°C. The supernatant was then centrifuged at 160,000 × g for 2 h at 5°C, and the resultant pellet was suspended, in the same buffer as used for the sonic oscillation (10), to a final protein concentration of about 20 mg/ml. For measurement of other en-
zyme activities, the cell extract was centrifuged at 27,000 × g for 15 min at 5°C, and the supernatant was used for the assays.

Enzyme assays. NAD-independent malate (EC 1.1.3.3) and L-lactate dehydrogenases were assayed by two methods. The first method involved the measurement of oxygen uptake with a Clark-type oxygen electrode (Titron Industries, Victoria, Australia) at 30°C. The reaction mixture consisted of (in a final volume of 2.5 ml): 100 mM potassium phosphate buffer (pH 7.2) and cell extract (about 1.8 mg of protein). The reaction was started by adding 10 μmol of either potassium L-malate or sodium L-lactate. The second method involved the reduction of dichlorophenolindophenol (DCPIP) and was a modification of the method of Francis et al. (3). The reaction mixture consisted of (in a final volume of 1 ml): potassium phosphate buffer (pH 7.2), 100 mM; KCN (pH 8.5), 10 mM; phenazine methosulfate (PMS), 1 mM; DCPIP, 0.1 mM; potassium L-malate or sodium L-lactate, 10 mM; and cell extract (about 0.05 mg of protein). For D-lactate dehydrogenase, tris(hydroxymethyl)aminomethane-chloride (pH 7.0) was used, and the substrate was calcium D-lactate (10 mM). The rate of both reactions was determined by measuring the decrease in absorbance of DCPIP at 600 nm, and the activity was calculated by the method of Singer and Kearney (15).

The following enzymes were assayed according to the cited methods: pyruvate dehydrogenase (EC 1.2.4.1) (14); citrate synthase (EC 4.1.3.7) (19); aconitate (EC 4.2.1.3.), succinate dehydrogenase (EC 1.3.99.1), and fumarase (EC 4.2.1.2) (11); NAD phosphate (NADP)-dependent isocitrate dehydrogenase (EC 1.1.1.42) (12); α-ketoglutarate dehydrogenase (EC 1.2.4.2) (13); NADP-dependent malate enzyme (EC 1.1.1.40) (7); isocitrate lyase (EC 4.1.3.1) (1); and malate synthase (EC 4.1.3.2) (2). Protein was measured by the biuret method (4), with bovine serum albumin as a standard.

Chemicals and enzymes. Substrates, cofactors, and enzymes were obtained from Sigma Chemical Co., St. Louis, Mo., and calcium D-lactate was obtained from Mann Research Laboratories Inc., New York, N.Y.

RESULTS

Growth of P. citronellolis on D-lactate caused the induction of an NAD-independent L-lactate dehydrogenase that used DCPIP or oxygen as an electron acceptor (Table 1). The enzyme was not found in extracts of cells grown on acetate, aspartate, citrate, glucose, glutamate, or malate. No NAD-dependent lactate dehydrogenase was detected in lactate-grown cells, even in the presence of fructose 1,6-diphosphate, which activates the enzyme of Streptococcus (20). A search for an NAD-dependent lactate dehydrogenase in cells grown on acetate, aspartate, citrate, glucose, glutamate, or malate proved negative in all cases. The NAD-independent enzyme was particu- late, since after centrifugation at 160,000 × g about 90% of the activity was in the pellet (Table 1).

The reaction product was identified as pyruvate by using purified NAD-dependent lactate dehydrogenase and reduced NAD (NADH). Unlike the case for P. aeruginosa, in which both particulate D- and L-lactate dehydrogenses were induced by D-L-lactate (9), only L-lactate dehydrogenase was induced in P. citronellolis. D-Lactate did not inhibit the L-lactate dehydrogenase.

Cells of P. citronellolis grown on the substrates shown in Table 2 and on citrate, glutamate, or malate were devoid of an NAD-dependent malate dehydrogenase. Instead, the cells possessed a particulate NAD-independent malate dehydrogenase with properties similar to those of the NAD-independent lactate dehydrogenase (Table 1). The NAD-independent malate dehydrogenase appeared to be constitutive, since it was detected in cells grown on acetate, aspartate, glucose, and lactate (Table 2), and also on citrate, glutamate, and malate (data not shown). Growth of the organism on malate did not induce a higher activity of the enzyme. However, growth on acetate caused a twofold increase in activity, whereas growth on aspartate reduced the activity by about half (Table 2). The product of the enzyme reaction was identified as OAA by using purified NAD-dependent malate dehydrogenase and NADH. The properties of the NAD-independent malate dehydrogenase, namely, its constitutive particulate nature and its ability to utilize oxygen or DCPIP, but not NAD, as an electron acceptor, resemble those of the same enzyme found in

| Substrate | Electron acceptor | Sp act a
|-----------|------------------|----------------
| L-Lactate | DCPIP | 0.036 | 0.130
| | DCPIP + PMS | 0.120 | 0.450
| | Oxygen | 0.019 | 0.071
| D-Malate | DCPIP | 0.009 | 0.065
| | DCPIP + PMS | 0.069 | 0.260
| | Oxygen | 0.022 | 0.090

* Specific activities are expressed as micromoles of substrate oxidized or micromoles of oxygen consumed per minute per milligram of protein.
several species of Pseudomonas (3, 6, 16), all of which lacked the NAD-dependent enzyme.

A higher activity of both the lactate and malate dehydrogenases with DCPIP was observed when PMS was added to the assay system (Table 1). Oxidation of both lactate and malate with oxygen as the electron acceptor was completely inhibited by 1 mM KCN. Cyanide stimulated the enzyme activity by some 30% when assayed by the DCPIP method, presumably by inhibiting the transfer of electrons to oxygen.

The constitutive malate enzyme in P. citronellolis was NADP dependent (Table 2) and presumably functions in the direction of NADPH synthesis. Growth on acetate repressed the activity of the enzyme as observed in other pseudomonads (5, 8). The enzymes of the glyoxylate bypass were present in acetate-grown cells but were not measured in cells grown on the other carbon sources. The activities of the tricarboxylic acid cycle enzymes of cells grown on glutamate and citrate were similar to those of cells grown on lactate.

**DISCUSSION**

Figure 1 summarizes the pathways for the formation and dissimulation of OAA, pyruvate, and PEP that have been demonstrated in P. citronellolis in this and earlier work (10, 17; O'Brien et al., Proc. Aust. Biochem. Soc. 6:34, 1973; O'Brien et al., J. Biol. Chem., in press). Known enzyme activators and inhibitors are shown. Since the NAD-independent lactate dehydrogenase is induced by growth on DL-lactate, its function is to convert lactate to pyruvate for further oxidation via the tricarboxylic acid cycle or for conversion to PEP for gluconeogenesis, thus enabling the organism to grow on this carbon source. The NAD-independent malate dehydrogenase apparently functions in the tricarboxylic acid cycle, since it is constitutive and since we were unable to find an NAD-dependent malate dehydrogenase. All of the other enzymes of the tricarboxylic acid cycle and the malate enzyme were detected. Thus, gluconeogenesis from intermediates of the tricarboxylic acid cycle may involve either the malate enzyme or the combined action of the NAD-independent malate dehydrogenase and OAA decarboxylase (Fig. 1).

One of the requirements for efficient oxidation of the carbon source through the tricarboxylic acid cycle is a proper balance between the levels of OAA and acetyl coenzyme A (acetyl CoA). The regulation of enzymes in P. citronellolis appears to be coordinated to achieve such a balance. For example, during growth on acetate, the changes in enzyme activities suggested that there was a shift in the metabolic pattern to favor OAA synthesis. The activities of pyruvate dehydrogenase and malate enzyme were lowest in acetate-grown cells, whereas malate dehydrogenase activity was increased in these cells. Furthermore, it is likely that elevated concentrations of acetyl CoA in acetate-grown cells would inhibit OAA decarboxylase (O'Brien et al., J. Biol. Chem., in press), thus maintaining the supply of OAA necessary to metabolize the acetate via the tricarboxylic acid cycle. Under these conditions, malate enzyme would provide a bypass for generation of pyruvate for gluconeogenesis. Gluconeogenesis apparently requires NADPH (10), which could also be provided by the action of the malate enzyme or isocitrate dehydrogenase, both of which are NADP-dependent in P. citronellolis. In the case of acetate-grown cells, the malate enzyme would probably assume greater importance as a source of electrons for gluconeogenesis, since most of the isocitrate would be metab-
olized via isocitrate lyase rather than via isocitrate dehydrogenase. The repression of pyruvate dehydrogenase in acetate-grown cells would assist gluconeogenesis by preventing conversion of pyruvate to acetyl CoA. The growth of *P. citronellolis* is inhibited severely by acetate concentrations greater than 80 mM (Taylor, Ph.D. thesis). This may be due to depletion of free CoA. If so, then decreased synthesis of acetyl CoA from pyruvate in acetate-grown cells would also aid in maintaining an adequate supply of CoA for the other reactions dependent on CoA.

Growth of cells on aspartate leads to a repression of pyruvate dehydrogenase. This repression was not as great as that found in cells grown on acetate, presumably because the need to conserve OAA is not as great as in acetate-grown cells. This is also reflected in the lower activity of malate dehydrogenase in aspartate-grown cells. Malate enzyme activity is little affected, which may indicate its importance in generating NADPH for gluconeogenesis.

The recent report of the presence of pyruvate carboxylase and PEP carboxylase and the repression of the malate enzyme by acetate in *P. fluorescens* (5) may be an indication that the control mechanisms in *P. citronellolis* are of general significance in the pseudomonads.

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**LITERATURE CITED**


