GLUCONATE METABOLISM IN PSEUDOMONAS: A NOVEL PATHWAY OF GLYCERALDEHYDE-3-PHOSPHATE METABOLISM

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SUMMARY

A novel sequence of reactions for converting glyceraldehyde-3-phosphate to pyruvate has been found in a Pseudomonad. This sequence, involving the intermediates methylglyoxal and D- and L-lactate, suggests that substrate-level phosphorylation may not occur in the conversion of gluconate to pyruvate. This pathway may account for the strictly aerobic characteristic of this organism.

The primary route of gluconate dissimilation in Pseudomonas is believed to be the Entner-Doudoroff (E-D) pathway (1);

Glucon. ⟶ 6-P-Glucon. ⟶ 2-Keto-3-Deoxy-6-P-Glucon. ⟶ Pyruvate + G-3-P. (1)

The conversion of glyceraldehyde-3-P (G-3-P) to pyruvate via reactions of the glycolytic scheme has never been convincingly demonstrated in Pseudomonas and related genera. Indeed, Entner and Doudoroff (1,2) could not demonstrate the presence of triosephosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating) EC 1.2.1.12) in P. saccharophila, and ascribed this to the presence of an active phosphatase which rapidly and specifically cleaved the phosphate from G-3-P. This enzyme is also found in extracts of the Pseudomonad, Pseudomonas G6, used in the work reported here (3).

We suggest that G-3-P is not metabolized to pyruvate via the glycolytic scheme in Pseudomonas, but a novel pathway may be involved. This pathway involves the enzymes glyceraldehyde-3-phosphatase (3-phospho-D-glyceraldehyde phosphohydrolase EC 3.1.3.__), glyceraldehyde dehydratase (D-glyceraldehyde hydro-lyase EC 4.2.1__), glyoxalase I [S-lactoyl-glutathione methyl-glyoxal-lyase (isomerizing) EC 4.4.1.5], glyoxalase II (S-2-hydroxyacylglutathione hydrolase EC 2.1.2.6) D- and L-lactate oxidase (D-lactate: cytochrome

c oxidoreductase EC 1.1.2.4 and L-lactate: cytochrome c oxidoreductase ED 1.1.2.3):

G-3-P $\xrightarrow{-P_i}$ Glyceraldehyde $\xrightarrow{-H_2O}$ Methylglyoxal $\xrightarrow{H_2O}$ D,L-lactate $\xrightarrow{O_2}$ Pyruvate (2).

MATERIALS AND METHODS

Pseudomonas G 6 was originally selected for the constitutive production of galactose dehydrogenase from a culture of Pseudomonas saccharophila (4). The cells were grown on gluconate salts medium as described previously (5). For labeling experiments cells were grown to mid log phase, harvested by centrifugation, washed twice in Tris-HCl buffer, pH 7.5, and incubated with either 1-C^{14} or 6-C^{14} gluconate in the presence and absence of cyanide (see legend under Fig. 1 and 3 for details of incubation mixture). Cell-free studies were carried out with crude supernatant which was prepared by rupturing washed cells with glass beads in a Gifford-Wood Minimill. After centrifugation for 30 min at 10,000 rpm, the crude supernatant solution was submitted to centrifugation at 144,000 x g for 2 hrs at 4°C. Protein concentration was estimated by the method of Lowry (6). The incubation mixture is described under Fig. 2.

Samples of 10 µl were withdrawn at various time intervals from the incubation mixture and spotted on a sheet of Whatman No. 1 filter paper. The sample was chromatographed in two dimensions, using the solvents isobutanol: pyridine:water:acetic acid (12:6:4:1) and isopropanol:pyridine:water:acetic acid(8:4:4:1). After drying, the chromatograms were placed in contact with Kodak no-screen Medical X-ray Film NS 547 for two days. The resulting radioautograms were used to locate the radioactive spots of interest, after which the spots were cut out and counted directly in a Packard Tri-Carb liquid scintillation counter equipped with punched tape output. Lactate and pyruvate were identified by chromatography with cold carrier and by the reaction catalyzed by lactate dehydrogenase.

High speed supernatant was prepared as described above. Glyoxalase I and II were measured according to the method of Racker (7). The method is

based on the absorption of light at 240 nm by the thioester bond of S-lactoyl-glutathione.

The compound S-lactoylgluthathione was prepared by incubating commercial glyoxalase I (6 enzyme units) with equimolar amounts of methylglyoxal and reduced glutathione in a total volume of 1 ml. The reaction was allowed to proceed for 30 min and stopped with 0.5 ml perchloric acid. The solution was centrifuged and the supernatant neutralized with KOH. The resulting precipitate was eliminated by centrifugation and the product formed S-lactoylglutathione ($\varepsilon_{240}^{mM} = 3.37$), was used for the enzymatic assay of glyoxalase II.

The assay for glyceraldehyde dehydratase was carried out in a total volume of 1 ml containing 40 μmoles of Tris-HCl buffer, pH 7.1, 2 μmoles of D-glyceraldehyde, commercial glyoxalase I, 0.01% reduced glutathione and 10 μl crude supernatant. Glyceraldehyde-3-phosphatase was assayed in a similar incubation mixture after substituting 2 μmoles of G-3-P for D-glyceraldehyde. All reactions were initiated with enzyme and monitored at 240 nm against a control mixture minus crude supernatant enzyme.

RESULTS AND DISCUSSION

Differentially labeled gluconate was utilized to test the functioning of the proposed pathway. This pathway predicts that if gluconate is labeled at C-1, the label should appear in pyruvate but not in lactate under either aerobic or anaerobic conditions; if gluconate is labeled at C-6, the label would appear in both lactate and pyruvate under aerobic conditions, but only in lactate under anaerobic conditions. On the other hand, if G-3-P were converted to pyruvate by the glycolytic pathway, there should be no difference between the results obtained with gluconate labeled at C-1 and C-6 since both would give rise to labeled pyruvate under both aerobic and anaerobic conditions.

Whole cells were incubated aerobically with gluconate labeled at C-6. As can be seen in Fig. 1a, radioactivity appeared in both lactate and

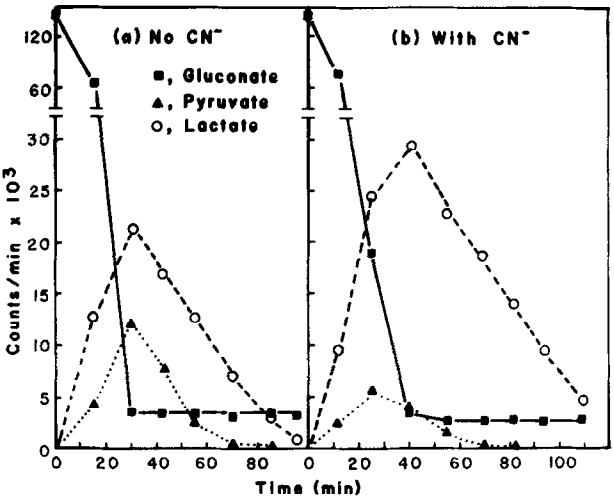

Fig. 1. The metabolism of 6-C^{14}-gluconate by whole cells of Pseudomonas G-6 in the absence (a) and presence (b) of cyanide. The incubation mixture (0.5 ml) contained 50 μmoles Tris-HCl, pH 7.5, 1.15 μmoles of labeled gluconate (sp. act. 6.18 x 10^6 cpm/μmole), 0.85 mg (dry weight) of cells and 0.14 μmoles of NaCN where required. Aeration was provided by stirring the incubation mixture with a magnetic disc, and the temperature of incubation was 30°C.

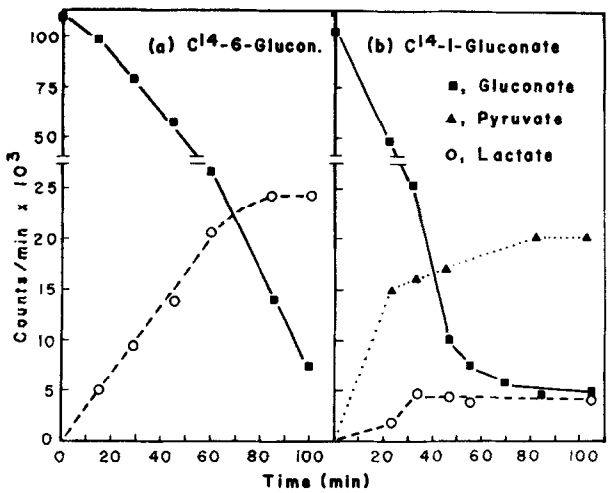

Fig. 2. Incubation of high speed supernatant of a cell extract with 6-C^{14}-(a) and 1-C^{14}-gluconate (b). There was no detectable incorporation of label into pyruvate in (a). Incubations were carried out at 30°C in a total volume of 0.57 ml. The incubation mixture contained 2.4 mg of protein, 5 μmoles ATP, 2.5 μmoles of Tris-HCl, pH 7.5 and 1.3 μmoles of gluconate.

pyruvate. The rate of incorporation of radioactivity into and maximum level of radioactivity in lactate were somewhat higher than those corresponding to pyruvate.

When gluconate reached a minimum concentration, lactate and pyruvate disappeared at approximately equal rates. These kinetics are consistent with the formation of pyruvate from lactate.

In order to minimize the conversion of lactate to pyruvate by the oxidases, the experiment was also carried out in the presence of NaCN. As shown in Fig. 1b, cyanide markedly increased the level of radioactivity in lactate. There was, moreover, a noticeable decrease in the rate and total accumulation of label in pyruvate.

The results in Fig. 1 do not, in themselves, clearly distinguish between our proposed pathway and the glycolytic pathway of lactate production. Because the production of lactate by glycolysis involves the enzyme lactate deydrogenase, we assayed for this enzyme in crude cell extracts, but were unable to detect it when either NADH or NADPH were used as coenzymes in the reduction of pyruvate. Since Fructose-1,6-diphosphate has been reported to be an activator of the lactate dehydrogenase of some bacteria (8), this compound was also added to the assay system and found to have no effect. The absence of detectable lactate dehydrogenase and the results in Fig. 1 strongly suggest that lactate is not formed *via* the glycolytic pathway in *Pseudomonas* G6.

This conclusion is further supported by Fig. 2a, which shows that labeled pyruvate was not formed from C-6 labeled gluconate during incubation of the latter with a high-speed supernatant fraction of a cell extract. This is consistent with our observation that lactate oxidation is catalyzed by a particulate fraction.

The results of incubating whole cells with C-1 labeled gluconate are shown in Fig. 3. Under aerobic conditions, relatively little label accumulated in pyruvate, an observation consistent with the rapid metabolism of

TABLE I

ACTIVITIES OF ENZYMES IN PROPOSED PATHWAY

Substrate	μmoles S-lactoyl glutathione formed per min/mg protein	μmoles S-lactoyl glutathione hydrolyzed per min/mg protein
G-3P	.093	
Glyceraldehyde	.19	
Methylglyoxal	.143	
S-lactoyl glutathione		.503

Enzyme assays were carried out with crude extracts as described in Materials and Methods.

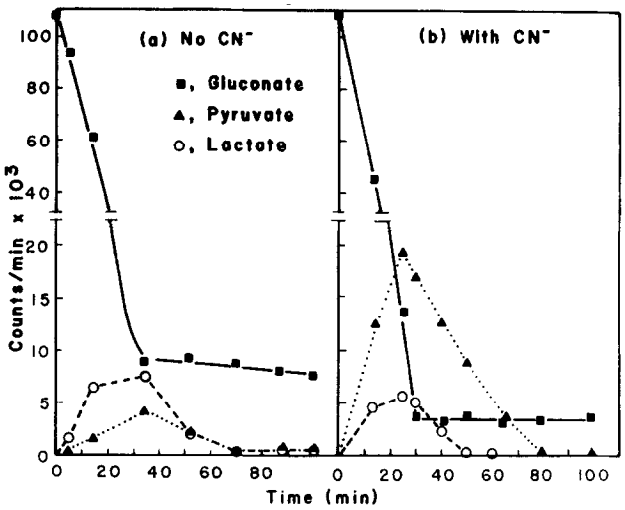

Fig. 3. Metabolism of 1-C^{14}-gluconate by whole cells of Pseudomonas G6 in the absence (a) and presence (b) of cyanide. The incubation mixture was identical to that described in Fig. 1. The specific activity of the labeled gluconate was 5.05×10^6 cpm/μmole.

pyruvate. It was surprising, however, that more label could be detected in lactate than in pyruvate. In the presence of cyanide, the level of radioactivity in pyruvate increased markedly, as would be expected from a decreased utilization of pyruvate. The incorporation of radioactivity into lactate was not significantly affected by the presence of cyanide, again

supporting the view that lactate is not formed from pyruvate. Whether the low level of incorporation into lactate was due to an ancillary pathway or to other factors such as CO_2 fixation cannot yet be determined.

Incubation of C-1 labeled gluconate with the high speed supernatant fraction of a cell extract (Fig. 2b) confirmed the results obtained with whole cells. The label accumulated in pyruvate up to a constant level, and a small but significant portion of the radioactivity also appeared in lactate.

These studies with differentially labeled gluconate clearly indicate that pyruvate arises predominantly from the carboxyl-containing half of the gluconate molecule, while lactate is predominantly formed from the last three carbon atoms of gluconate. These results, along with the presence of the necessary enzymes as shown in Table I, strongly support scheme 2 above for the conversion of G-3-P to pyruvate in Pseudomonas.

The evidence summarized in Figs. 1,2, and 3 and Table I indicates that the metabolism of gluconate in Pseudomonas G6 probably involves scheme 2. Such a scheme, involving no substrate-level phosphorylation, may explain why most Pseudomonads are obligate aerobes. These observations are consistent with a report by Kashket and Brodie (9) that no net production of ATP occurs in P. aeruginosa after destruction of quinones by irradiation with light at 360 nm or under anaerobic conditions; Kashket and Brodie concluded that ATP synthesis occurs only by means of oxidative phosphorylation in P. aeruginosa.

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