Metabolism of D-Fructose in *Aerobacter aerogenes*: Analysis of Mutants Lacking D-Fructose 6-Phosphate Kinase and D-Fructose 1,6-Diphosphatase

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The relative significance of the pathways for the conversion of d-fructose to d-fructose 1,6-diphosphate via d-fructose 1-phosphate or d-fructose 6-phosphate in *Aerobacter aerogenes* PRL-R3 was assessed by observing growth patterns of mutants lacking either d-fructose 6-phosphate kinase or d-fructose 1,6-diphosphatase. The mutant lacking d-fructose 6-phosphate kinase grew well on d-fructose or glycerol but not on d-glucose, whereas the mutant lacking d-fructose 1,6-diphosphatase grew on d-glucose but not on d-fructose or glycerol. The data indicate that the pathway of d-fructose metabolism is primarily through d-fructose 1-phosphate rather than d-fructose 6-phosphate.

The recent discovery (2) of an inducible kinase specific for d-fructose 1-phosphate in *Aerobacter aerogenes* PRL-R3 suggested the existence of a previously unrecognized pathway of d-fructose metabolism. Subsequently, we obtained enzymatic and genetic evidence that d-fructose is phosphorylated at carbon atom 1 in this organism by a phosphoenolpyruvate-dependent phosphotransferase system (T. E. Hanson and R. L. Anderson, Federation Proc. 27:644, 1968). Thus, the pathway is believed to be: d-fructose → d-fructose 1-phosphate → d-fructose 1,6-diphosphate. In the present report, we describe further experiments which indicate that this pathway is of functional significance: a mutant lacking d-fructose 6-phosphate kinase grows readily on d-fructose or glycerol but not on d-glucose, whereas a mutant lacking d-fructose 1,6-diphosphatase grows on d-glucose but not on glycerol or d-fructose. This is in contrast to the situation in *Escherichia coli*, where d-fructose 1,6-diphosphatase-negative mutants were found to grow on both d-glucose and d-fructose but not on glycerol (1). To our knowledge, other investigations of d-fructose 6-phosphate kinase-negative mutants of bacteria have not been reported.

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Materials and Methods

**Chemicals.** Adenosine 5'-triphosphate (ATP), oxidized nicotinamide adenine dinucleotide phosphate (NADP), and reduced nicotinamide adenine dinucleotide (NADH) were from P-L Biochemicals, Milwaukee, Wis. D-Fructose 1,6-diphosphate (d-fructose-1,6-P) and crystalline α-glyceroephosphate dehydrogenase-triose phosphate isomerase were from Sigma Chemical Co., St. Louis, Mo. D-Fructose 6-phosphate (d-fructose-6-P) was from the Boehringer-Mannheim Corp., New York, N.Y. D-Fructose 1-phosphate (d-fructose-1-P), yeast glucose 6-phosphate dehydrogenase (A grade), rabbit muscle fructose diphosphate aldolase (A grade), and crystalline rabbit muscle phosphoglucose isomerase (A grade) were from Calbiochem, Los Angeles, Calif. Ethyl methanesulfonate was from Eastman Organic Chemicals, Rochester, N.Y.

**Bacteria.** The parental strains used in this investigation were *Aerobacter aerogenes* PRL-R3 and a uracil auxotroph [PRL-R3 (U-)] derived from it. The uracil auxotroph, which was obtained during the course of this investigation, was kindly given to us by Robert P. Mortlock of the University of Massachusetts. Mutant 012 was derived from strain PRL-R3, and mutant A9-1 was derived from strain PRL-R3 (U-), as described below.

**Media.** The basal mineral medium used for strain PRL-R3 and mutant 012 consisted of 0.71% Na₂HPO₄, 0.15% KH₂PO₄, 0.3% (NH₄)₂SO₄, 0.009% MgSO₄, and 0.0005% FeSO₄.7H₂O. This medium was supplemented with 0.005% uracil for the growth of strain PRL-R3 (U-) and mutant A9-1.
Sugars were autoclaved separately and were added to the basal mineral medium at a concentration of 0.5%.

**Growth of cells and preparation of extracts.** The growth curves were done in 18 × 150 mm culture tubes containing 7.0 ml of medium. The inoculum was 0.1 ml of an overnight culture (on D-glucose, except mutant A9-1, which was on D-fructose). The tubes were slanted at an angle of 55° and were agitated on a reciprocal shaker at 148 cycles/min at 30°C.

For enzyme studies, the cells were grown in 500 ml of medium in Fernbach flasks on a rotary shaker at 32°C. The cells were harvested by centrifugation during the late log phase, suspended in distilled water, and broken by treatment for 10 min in a Raytheon 10-kec sonic oscillator circulated with ice water. The cell extract was the supernatant fraction obtained after centrifugation of the broken-cell suspension for 10 min at 27,000 × g.

The growth temperature for isolation of the mutants was 32°C.

**Enzyme assays.** All assays involved the oxidation or reduction of pyridine nucleotide coenzymes and were monitored at 340 μm with a Gilford absorbance-recording spectrophotometer thermostated at 25°C. The reactions were carried out in 0.15-ml volumes in microcuvettes with a 1.0-cm light path. In all cases, the amount of extract assayed was limiting, so that the rates were proportional to enzyme concentration. Specific activities are defined as the micromoles of pyridine nucleotide oxidized or reduced per minute per milligram of protein. The assays for D-fructose-1-P kinase and D-fructose-6-P kinase contained 1.0 μmole of ATP; 2.0 μmole of MgCl2; 0.05 μmole of NADH; 1.0 μmole of D-fructose-1-P or D-fructose-6-P; excess fructose diphosphate aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase; and 10.0 μmole of buffer (glycylglycine (pH 7.5) for D-fructose-1-P kinase, and glycine (pH 8.2) for D-fructose-6-P kinase).

The assay for D-fructose 1,6-diphosphatase contained 1.0 μmole of D-fructose-1,6-P2, 1.0 μmole of MgCl2, 0.2 μmole of ethylenediaminetetraacetate, 0.1 μmole of NADP, excess phosphoglucone isomerase and glucose-6-P dehydrogenase, and 10 μmole of glycylglycine buffer (pH 7.5). The replacement of D-fructose plus ATP with 1.0 μmole of D-fructose-1-P in this assay gave no reaction, indicating the absence of phosphofructomutase activity in the extract and the coupling enzymes; thus, the product of the D-fructokinase reaction was shown to be D-fructose-6-P.

The assay for D-gluco kinase has been described (3). **Protein determination.** Protein was estimated spectrophotometrically (8).

**Selection of mutant strain A9-1 (D-fructose 6-phosphate kinase-negative).** An overnight culture of *A. aerogenes* PRL-R3 in D-glucose-mineral medium (10 ml) was harvested by centrifugation and suspended in 5 ml of basal mineral medium containing 0.2 μl ethyl methanesulfonate. After incubation for 2 hr, the cells were harvested and suspended in 100 ml of D-glucose-mineral medium. This was incubated with agitation overnight, allowing about a 25-fold increase in cell number. The cells were plated on D-fructose-mineral-agar, and the resulting colonies were replicated on D-glucose-mineral-agar, D-mannose-mineral-agar, and D-fructose-mineral-agar. Mutant A9-1 was selected as a strain which grew well on D-fructose but only slightly on D-glucose or D-mannose.

**Selection of mutant strain 012 (D-fructose 1,6-diphosphatase-negative).** An overnight culture of *A. aerogenes* PRL-R3 in D-glucose-mineral medium (10 ml) was harvested by centrifugation and suspended in 5 ml of basal mineral medium containing 0.2 μl ethyl methanesulfonate. After incubation for 2 hr, the cells were harvested and suspended in 100 ml of D-glucose-mineral medium. This was incubated with agitation overnight, allowing about a 25-fold increase in cell number. The cells were harvested, washed once in basal mineral medium, and suspended in D-fructose-mineral medium at a concentration of 2 × 108 per ml. This culture was shaken until growth doubled (measured by optical density). Penicillin G was then added at a concentration of 2,000 units per ml, and the culture was incubated for 4 hr, after which the survival was 0.1%. The penicillin-treated bacteria were plated on mineral-agar containing 0.5% D-fructose plus 0.005% D-glucose. The smallest colonies were selected and tested for growth on D-glucose, D-fructose, D-mannose, mannitol, and glycerol-mineral-agar. Mutant 012 was selected as a strain which grew well on D-glucose, D-mannose, or mannitol, but failed to grow on glycerol or D-fructose.

**RESULTS**

**Growth patterns.** Growth characteristics of the parental strain (PRL-R3) and the two mutants (A9-1 and 012) on D-glucose, D-fructose, and glycerol are shown in Fig. 1. Strain PRL-R3 grew well on all three substrates. Mutant A9-1 mimicked the parent on D-fructose and glycerol, but grew only slowly on D-glucose. Mutant 012 grew well on D-glucose but failed to grow on D-fructose or glycerol; after 24 hr, slight growth occurred occasionally on D-fructose but not on glycerol.

**Enzyme activities in cell extracts.** The data in Table 1 show that all strains contained similar levels of D-glucokinase, whereas mutant 012 was missing D-fructose 1,6-diphosphatase and mutant A9-1 was missing D-fructose-6-P kinase. D-Fructokinase activity was low in all extracts, but was consistently higher in cells grown on D-fructose than on D-glucose. It should be emphasized that this apparent D-fructokinase has not been purified, so it has not been established that the observed activity in crude cell extracts is the
Fig. 1. Growth characteristics of strain PRL-R3 and mutants on d-glucose, d-fructose, and glycerol. The growth pattern of PRL-R3 (U') (not shown) was the same as that for PRL-R3. An optical density of 0.35 was equivalent to a viable count of 8.2 x 10^6 cells per ml.

Table 1. Enzyme activities in crude cell extracts

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth substrate</th>
<th>d-Fructose-1,6-diphosphatase</th>
<th>d-Fructose-6-P kinase</th>
<th>d-Fructose-1-P kinase</th>
<th>d-Glucokinase</th>
<th>d-Fructokinase</th>
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<tr>
<td>PRL-R3</td>
<td>d-Glucose</td>
<td>0.032</td>
<td>0.120</td>
<td>&lt;0.005</td>
<td>0.099</td>
<td>0.002</td>
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<tr>
<td></td>
<td>d-Fructose</td>
<td>0.032</td>
<td>0.135</td>
<td>0.190</td>
<td>0.111</td>
<td>0.009</td>
</tr>
<tr>
<td>PRL-R3 (U')</td>
<td>d-Glucose</td>
<td>0.034</td>
<td>0.140</td>
<td>&lt;0.005</td>
<td>0.120</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>d-Fructose</td>
<td>0.033</td>
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<td>0.222</td>
<td>0.117</td>
<td>0.013</td>
</tr>
<tr>
<td>O12</td>
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<td>&lt;0.0002</td>
<td>0.120</td>
<td>&lt;0.005</td>
<td>0.110</td>
<td>0.003</td>
</tr>
<tr>
<td>A9-1</td>
<td>d-Fructose</td>
<td>0.028</td>
<td>&lt;0.005</td>
<td>0.192</td>
<td>0.112</td>
<td>0.011</td>
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</table>

*Results are expressed as micromoles of pyridine nucleotide oxidized or reduced per minute per milligram of protein in the standard assays.

result of a single enzyme possessing ATP:d-fructose 6-phosphotransferase activity.

d-Fructose-1-P kinase was found only in extracts of cells grown on d-fructose (Table 1). The inability of mutant O12 to grow on d-fructose precluded a measurement of d-fructose-1-P kinase in this strain under conditions which induced the enzyme in the other strains. However, partial induction was achieved by incubating d-glucose-grown cells in 0.25% d-glucose plus 0.25% d-fructose in mineral medium for a period of time sufficient to allow complete utilization of the d-glucose. Under these conditions, the specific activities in extracts were 0.24 in strain PRL-R3 and 0.026 in mutant O12. When the cells were harvested and extracts prepared before d-glucose utilization was complete, d-fructose-1-P kinase activity remained undetectable, indicating repression in the presence of d-glucose. The partial induction observed in mutant O12 probably occurred in the short period of growth just before or immediately after the d-glucose was exhausted and d-glucose repression was relieved. A further attempt was made to induce d-fructose-1-P kinase in mutant O12 under conditions in which d-glucose repression would be absent by exposing d-glucose-grown cells to 0.25% d-fructose in nutrient broth (0.5% Difco peptone plus 0.3% Difco beef extract, pH 7.0). d-Fructose-1-P kinase activity remained undetectable in mutant O12, although a normal level of activity was induced in strain PRL-R3. This lack of induction in mutant O12 may be attributed to catabolite repression, which is known to be enhanced during catabolism under non-growing conditions (5, 6); mutant O12 does not grow on nutrient broth, which is consistent with its lack of d-fructose 1,6-diphosphatase.

d-Fructose 1,6-diphosphatase activity as a function of assay pH is shown in Fig. 2. The extract from mutant O12 exhibited some activity at low pH values, but no activity at pH 7.5, which was the pH optimum for d-fructose 1,6-diphosphatase activity in extracts of strain PRL-R3 and mutant A9-1. The activity at low pH values is believed to be due to a nonspecific acid hexose phosphatase (1).

Discussion

The prior demonstration of an inducible d-fructose-1-P kinase (2) and an inducible phosphoenolpyruvate:d-fructose 1-phosphotransferase system (T. E. Hanson and R. L. Anderson, Federation Proc. 27:644, 1968) in A. aerogenes PRL-R3 suggested that this organism can metabolize d-fructose through the pathway, d-fructose → d-
fructose-1-P → d-fructose-1,6-P. The d-fructokinase-type activity to yield d-fructose-6-P reported in this communication is low, but its inducibility and the possibility that optimal assay conditions were not met leaves open the possibility that an alternate route, d-fructose → d-fructose-6-P → d-fructose-1,6-P may also function in this organism. The present communication serves to assess the relative importance of these two pathways during growth on d-fructose. The rationale of our approach was based on the following premises. First, the metabolism of d-fructose-6-P through the Embden-Meyerhof pathway would require d-fructose-6-P kinase, whereas the metabolism of d-fructose-1-P would not. Second, a source of d-fructose-6-P would be needed for biosynthetic pathways involving hexose monophosphates; thus, a mutant missing d-fructose-1,6-diphosphatase would not be expected to grow on glycerol or other compounds which could be converted to d-fructose 6-phosphate only through the dephosphorylation of d-fructose 1,6-diphosphate. Whether or not such a mutant would grow well on d-fructose would depend on whether the main degradative pathway were through d-fructose-6-P or d-fructose-1-P.

Mutant A9-1, missing d-fructose-6-P kinase, grows on d-fructose or glycerol as well as does the parent strain, but grows only slowly on d-glucose. This is consistent with the metabolism of d-fructose through d-fructose-1-P. If d-fructose were metabolized through d-fructose-6-P rather than d-fructose-1-P, this mutant would still be expected to grow well on glycerol, but would be expected to grow no better on d-fructose than it does on d-glucose. The residual growth on d-glucose by mutant A9-1 could indicate that the defective d-fructose-6-P kinase is partially functional in the intact cell, but more likely it is due to the metabolism of d-glucose through the hexose monophosphate pathway.

Mutant O12, missing d-fructose 1,6-diphosphatase, grows well on d-glucose but not on d-fructose or glycerol. This, too, is consistent with d-fructose being metabolized in the wild-type primarily through d-fructose-1-P rather than d-fructose-6-P. If the pathway through d-fructose-6-P were of major significance, a d-fructose 1,6-diphosphatase-negative mutant would be expected to grow on both d-glucose and d-fructose, as has been demonstrated for E. coli (1).

How widespread the pathway d-fructose → d-fructose-1-P → d-fructose-1,6-P is in nature is not yet known. However, at least one other microorganism, Bacteroides symbiosis, is known to possess d-fructose-1-P kinase (7).

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LITERATURE CITED