Regulation of Pentitol Metabolism by *Aerobacter aerogenes*

I. Coordinate Control of Ribitol Dehydrogenase and D-Ribulokinase Activities

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Induction studies on *Aerobacter aerogenes* strain PRL-R3, using ribitol as the inducer-substrate, indicated that two enzymes of ribitol catabolism, ribitol dehydrogenase and D-ribulokinase, are coordinately induced. The utilization of D-arabinose as a substrate resulted in the induction of ribitol dehydrogenase as well as D-ribulokinase. Mutants which were constitutive for ribitol dehydrogenase were also constitutive for D-ribulokinase. In contrast, D-xylulokinase and D-arabitol dehydrogenase did not appear to be coordinately controlled. Induction studies and examination of D-arabitol dehydrogenase constitutive mutants indicated that the three enzymes of the converging pathways for D-arabitol and D-xylose catabolism are under separate control.

With respect to catabolic pathways of carbohydrate metabolism, it appears that coordinate control of the enzymes responsible for the degradation of only one substrate would be a selective advantage for organisms. Alternatively, if two or more substrates are degraded via a converging pathway, it would be advantageous if the enzymes required in common were controlled separately from the earlier enzymes in the pathways. Regulation of catabolic pathways has been studied in diverse systems. Hegeman (8, 9, 10) reported coordinate control of enzymes responsible for the conversion of mandelic acid to benzoate in *Pseudomonas putida*. The benzoic acid oxidase system, responsible for the conversion of benzoate to catechol, is controlled separately in this organism. Furthermore, the enzymes involved in the degradation of catechol to β-ketoacidic acid are also under coordinate control, but such control is separate from the earlier enzymes in the system. Englesberg et al. (6) studied the pathway of L-arabinose degradation in *Escherichia coli*. The four enzymes, permease, isomerase, kinase, and epimerase, required for the conversion of L-arabinose to D-xylulose-5-phosphate were found to be coordinately controlled.

In view of the above considerations, it would seem quite advantageous to study the regulation of a catabolic system which contains highly converging as well as unbranched pathways. The degradative pathways of five-carbon carbohydrates by strains of *Aerobacter aerogenes* provide such a system. *A. aerogenes* strain PRL-R3 is able to utilize, as sources of energy, seven of the eight aldopentoses and all four of the pentitols (7, 15). Of these eleven compounds, D-ribose, D-xylose, L-arabinose, D-arabitol, and ribitol are readily available to the organism in nature; on the other hand, D-arabinose, D-lyxose, L-lyxose, L-xylose, xylitol, and L-arabitol are rarely, if ever, found uncomplexed in the natural environment. Metabolism of the pentoses involves (i) the isomerization of an aldopentose to a ketopentose, (ii) phosphorylation to the corresponding pentulose-5-phosphate, and (iii) epimerization of pentulose-5-phosphate to the common intermediate, D-xylulose-5-phosphate. (In the case of D-ribose, phosphorylation to D-ribose-5-phosphate precedes isomerization and subsequent epimerization.) Pentitol utilization initially proceeds via a dehydratation to form a ketopentose; the subsequent reactions are identical to those for the pentoses.

The present investigation was undertaken to determine the pattern of regulation in the unbranched pathway of ribitol degradation and in the convergent pathways of D-arabitol and D-xylose utilization in *A. aerogenes*. In this study, the ribitol-degrading system represented the unbranched pathway, whereas the convergent route involved the utilization of D-arabitol and D-xylose. Both of these pathways are presented in
Fig. 1. The first cata
cable sequence shown here is
normally considered specific for ribitol degrada-
tion in *A. aerogenes*. However, it is also
employed in the degradation of d-arabinose, provided that
the cell possesses an isomerase for the conversion of
d-arabinose to d-ribulose. d-Arabinose is an
uncommon substrate for which a specific isomerase
enzyme does not exist. Camyre and Mortlock
(4) have indicated that strains of *A. aerogenes*
which are constitutive for L-fucose isomerase are
capable of converting d-arabinose to d-ribulose.

It seems reasonable to assume, as a working
theory, that, since d-arabinose is one of the
uncommon pentoses, there would be no selective
advantage in evolving a regulatory mechanism
for the separate control of the enzymes common to
both d-arabinose and ribitol catabolism. On the other
hand, when two naturally occurring sub-
strates, such as d-xylitol and d-arabitol, share a
common pathway, selective pressures may tend to
favor the evolution of a separate regulatory
system for the individual enzymes concerned.

**Materials and Methods**

Isolation of dehydrogenase-constitutive mutants.

Mutants of *A. aerogenes* strain PRL-R3 which are
constitutive for ribitol dehydrogenase were selected
by growth on xylitol (14). By use of wild-type PRL-R3
or a uracil-requiring auxotroph as the parental strain,
28 independent ribitol dehydrogenase constitutive
mutants were obtained. Of these 28 mutants, one
mutant was isolated after treatment with ethyl
methanesulfonate, four mutants were obtained by
Tn903 mutagenesis, whereas the remaining 23 mutants arose sponta-
neously after prolonged incubation on 0.5%
xylitol salts-agar, supplemented, when necessary,
with 0.005% uracil.

D-Arabinol dehydrogenase constitutive mutants of the
organism were isolated by the procedure of
Lin, Lerner, and Jorgensen (12).

Cultural conditions. Cells were grown aerobically
at 30 °C on a minimal medium (2) supplemented
with 0.5% carbohydrate, 1% vitamin-free casein
hydrolysate, or 2% peptone. The carbohydrates,
casein hydrolysate, peptone, and magnesium sulfate
were autoclaved separately and were added to the
medium after cooling.

To determine dehydrogenase and kinase levels in
the ribitol dehydrogenase constitutive mutants, 250-ml
Erlenmeyer flasks containing 15 ml of casein hydro-
lyse- or peptone-medium were inoculated with 5 ml
of an overnight culture of cells grown in an identical
medium. The cultures were incubated aerobically at
30 °C for 3 hr and then were harvested by centrifug-
ation.

In experiments designed to test coordinate repres-
sion and derepression of the two enzymes, overnight
casein hydrolysate-grown cultures of the constitutive
mutants were inoculated into fresh casein hydrolysate
medium, and this medium was maintained in the
logarithmic phase of growth by periodic subculture
into fresh medium. After approximately five genera-
tions of growth, cells were transferred to sterile
glucose-salts medium and were allowed to go through
approximately six generations of exponential growth
before being returned to the casein hydrolysate
medium.

In all of these experiments, growth was followed by
measuring the increase in turbidity by use of a
Klett-Sumerson colorimeter equipped with a red filter
(660 nm). The turbidity readings were converted to
mg of dry weight per ml using a standard curve. The
number of generations was determined on the basis
of these dry weight values.

The inoculum used in the induction experiments
(Fig. 2 and 3) consisted of PRL-R3 wild-type cells
grown on glucose-salts, harvested by centrifugation,
and washed once with sterile salts solution. The
washed cells were then resuspended in a sterile salts
solution to 16% of the original volume and were
shaken at 30 °C for 2 days. Induction experiments
were then begun by inoculating these starved cells
in 0.5% of the particular substrates to determine
inducer ability.

Preparation of cell-free extracts. Cells were collected
by centrifugation at 12,000 × g for 10 min at 4 °C in
an RC-2 refrigerated centrifuge (Ivan Sorvall, Inc.,
Norwalk, Conn.). The cells were then washed once
with an equal volume of cold distilled water. The
cell pellets were resuspended in 2 ml of 0.04 M tris(hy-
droxymethyl)aminomethane (Tris)-chloride buffer,
PH 7.5, containing 0.0033 M ethylenediamine
tetra-acetate and 0.0033 M diethiothreitol. The suspended
cells were placed in polyethylene tubes, and five such
tubes were placed in the cup of a 10-kr magnetostri-
tive oscillator (Raytheon Co., South Norwalk, Conn.)
containing 30 ml of distilled water. The cells were
disrupted by exposure to sonic vibration for 15 min
at 5 °C. The cell debris was removed by centrifugation
for 15 min at 27,000 × g. The supernatant fractions
were collected in chilled tubes, and these fractions
constituted the crude extracts.

**Enzymatic assays.** Assays for pentitol dehy-
drogenase activity were performed spectrophoto-
metrically by observing the rate of reduced nicotin-
amide adenine dinucleotide (NADH) oxidation in
the presence of ketopentose (18). Kinase assays were
based on continuous spectrophotometric measure-
ment of adenosine diphosphate (ADP) formation
with the pyruvate kinase-lactic acid dehydrogenase
system (3). To measure kinase activity in the presence
of large amounts of the NADH-specific pentitol
dehydrogenases, reduced nicotinamide adenine dinu-
cleotide phosphate (NADPH) was substituted for
the NADH (15). A unit of kinase or pentitol dehydrogenase activity was defined as the amount of enzyme necessary to produce an absorbancy change of 1.0 per min at 340 m\(\mu\) with a light path of 1 cm in a reaction volume of 0.15 ml. The absorbancy change was measured at 25 C in a DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) and was monitored with a model 2000 recording attachment (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Isomerase activity was determined from the rate of pentulose formation by the method of Anderson and Wood (2); at 37 C, one unit of isomerase in 2.0 ml catalyzed the formation of 1 \(\mu\) mole of pentulose per hr. Ketopentose was measured by the cysteine-carbazole test of Dische and Borenfreund (5).

The protein content of the extracts was computed by determining the ratio of absorbancies at 280 m\(\mu\) and 260 m\(\mu\) (17).

Chemicals. Vitamin-free casein hydrolysate, ribitol, D-arabinose, xyitol, p-xylene, and D-arabitol were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Peptone and Special Agar (Noble) were obtained from Difco. N-methyl-N'-nitro-N'-nitroguanidine was purchased from the Aldrich Chemical Co., Inc., Milwaukee, Wis., and ethyl methanesulfonate was purchased from Eastman Organic Chemicals, Rochester, N.Y. NADH and NADPH were obtained from P-L Laboratories, Milwaukee, Wis., and lactic acid dehydrogenase was obtained from Worthington Biochemical Corp., Freehold, N.J. Dithiiothreitol, 2-phosphoenolpyruvate, and D-ribulose ortho-nitrophenyl-hydrazone were purchased from Calbiochem, Los Angeles, Calif. Free D-ribulose was derived from the o-nitrophenyl-hydrazone derivative by the procedure of Müller, Montigel, and Reichstein (16).

RESULTS

Ribitol and D-arabinose pathways. The degradative pathways of ribitol and D-arabinose utilize a common kinase for the phosphorylation of D-ribulose (15). Although wild-type \(A\). \(aerogenes\) is incapable of isomerizing D-arabinose, the incubation of these cells with D-arabinose as the sole carbon and energy source resulted in the rapid selection of mutants possessing such isomerase activity (4). The inoculation of glucose-grown cells of wild-type PRL-R3 strain into a ribitol-salts medium resulted in the rapid induction of ribitol dehydrogenase and D-ribulokinase (Fig. 2). Similar experiments utilizing D-arabinose as the sole growth substrate resulted in a lag of 10 to 14 hr for the selection of mutants possessing isomerase activity. The induction of both ribitol dehydrogenase and D-ribulokinase was correlated in such experiments (Fig. 2) with the appearance of isomerase activity. The maximal levels of kinase and dehydrogenase induced were comparable in both cases. Since ribitol dehydrogenase was not directly involved in D-arabinose catabolism, these data suggested the possibility of the coordinate induction of ribitol dehydrogenase with D-ribulokinase.

Mutants constitutive for ribitol dehydrogenase were isolated by using xyitol as a selective substrate (14). Of the 28 isolates tested, all were also found to be constitutive for D-ribulokinase. Although the mutants showed differences in enzyme levels, a plot of constitutive dehydrogenase versus kinase activities indicated that they are coordinately controlled (Fig. 3). When incubated with ribitol these mutants could usually be induced to higher levels of dehydrogenase and kinase activity. One hypo-constitutive mutant, i.e., one which synthesized a lower level of dehydrogenase than is normally found in constitutive strains, was isolated by the procedure of Lin et al. (12), after treatment of the wild-type strain with ethyl methanesulfonate. When incubated with ribitol for an extended period of time, this mutant was not induced to higher activity for either ribitol dehydrogenase or D-ribulokinase (Table 1).

Further evidence that ribitol dehydrogenase and D-ribulokinase enzymes are regulated by the same mechanism was obtained from a study of their coordinate derepression. When cells of \(A\).
FIG. 3. Ribitol dehydrogenase versus d-ribulokinase activities in ribitol dehydrogenase constitutive mutants of Aerobacter aerogenes. Extracts of casein hydrolysate-grown cells were disrupted by sonic vibration. The straight line was determined by the method of least squares; data from the wild type were not included in the calculations.

TABLE 1. Enzyme activities of a ribitol dehydrogenase, "hypo"-constitutive mutant

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Strain</th>
<th>Specific activity (units/mg of protein)</th>
<th>Ribitol dehydrogenase</th>
<th>d-Ribulokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>Inducible</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Constitutive</td>
<td>21</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Ribitol</td>
<td>Inducible</td>
<td>117</td>
<td>19.0</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Constitutive</td>
<td>21</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

*aerogenes* strain 126 were maintained on casein hydrolysate for approximately five generations (Fig. 4A) and then were transferred to a glucose-salts medium for six generations, both enzymes were diluted out at a rate closely approximating the theoretical dilution curve (Fig. 4B). The data presented in Fig. 4C indicated a rapid and parallel increase in both enzymes when the cells were released from glucose repression by transfer to casein hydrolysate; maximal levels of activity were obtained after about 2.5 generations of growth.

**d-Arabitol and d-xylose pathways.** The induction of d-arabitol dehydrogenase, d-xylose isomerase, and d-xylulokinase upon incubation of *A. aerogenes* strain PRL-R3, with either d-arabitol or d-xylose as the sole carbon and energy sources, is shown in Fig. 5. A comparison of enzyme activities suggested that none of these three enzymes was coordinately induced. Much higher d-xylulokinase activity was obtained when d-xylose was used as the substrate-inducer. d-Xylose (→ d-xylulose) isomerase activity was detected only in the d-xylose culture, and low d-arabitol dehydrogenase activity was also found in extracts of such cells.

To further investigate regulation of the d-arabitol pathway, the procedure of Lin et al. (12) was used to isolate mutants constitutive for d-arabitol dehydrogenase. The constitutive dehydrogenase activities of extracts of such mutants after growth on casein hydrolysate are shown in Table 2. d-Xylulokinase activity could not be detected in such extracts, again indicating the lack of coordinate control for these two enzymes. All of the constitutive strains were capable of normal growth when d-arabitol was used as the substrate, and the enzyme activities of d-arabitol-grown, cell-free extracts are listed in Table 3. d-Xylulokinase activity was induced when d-arabitol was used as the substrate during incubation and growth; thus, the absence of kinase activity in the extracts of casein hydrolysate-grown cells was not due to a defect in the kinase structural gene. The induction of constitutive mutant 203 by d-arabitol is graphically represented in Fig. 6.

The enzyme activities of these four constitutive strains after growth with d-xylose as the substrate are shown in Table 4. It is of interest to note that,
constitutive mutant was incapable of growth when used as the substrate. This mutant was unable to induce D-xylulokinase in response to the use of D-xylose as an inducer-substrate. D-Xylose isomerase induction, however, was normal.

**DISCUSSION**

The convergence of the D-arabinose degradative pathway with the ribitol catabolic pathway is the result of an isomerase which converts D-arabinose to D-ribulose. In view of the data indicating that D-arabinose isomerization is catalyzed by an enzyme of the L-fucose pathway, L-fucose isomerase (4), it is not surprising that synthesis of this isomerase is under separate control from the synthesis of ribitol dehydrogenase and D-ribulokinase.

The levels of ribitol dehydrogenase and D-

**TABLE 3. Enzyme activities of D-arabitol dehydrogenase constitutive mutants grown on D-arabitol**

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>Specific activity (units/mg of protein)</th>
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<tbody>
<tr>
<td></td>
<td>D-Arabitol dehydrogenase</td>
</tr>
<tr>
<td>200</td>
<td>127.0</td>
</tr>
<tr>
<td>201</td>
<td>117.5</td>
</tr>
<tr>
<td>202</td>
<td>98.0</td>
</tr>
<tr>
<td>203</td>
<td>34.7</td>
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*All of the mutants grown on this substrate possessed D-xylose isomerase activities of less than 2.0 units per mg of protein.

Although the data support the concept of separate control for these enzymes, two of the four dehydrogenase constitutive mutants appear to have altered regulation for D-xylulokinase. Mutant strain 202 induced identical levels of D-xylulokinase in response to both D-arabitol and D-xylose as growth substrates, whereas strain 203 produced very low kinase activity during growth on D-xylose. In addition, a fifth dehydrogenase constitutive mutant displayed normal growth when D-arabitol was used as the substrate but was incapable of growth when D-xylose was used as the substrate.
ribulokinase in crude extracts of casein hydrolysate-grown cells were found to vary depending on the particular mutant examined. However, the ratio of the enzyme levels was relatively constant, reflecting their coordinate behavior. Such coordination has been noted in the case of enzymes whose structural genes are closely linked (1, 11). On the other hand, a study of the control mechanisms involved in the L-arabinose gene complex in E. coli revealed that coordination of enzyme activities alone is insufficient evidence for linkage of the genes involved (6); in this system, a definite coordination was found between the L-arabinose isomerase and the L-arabinose permease, although the structural genes corresponding to these enzymes are unlinked. Thus, in the absence of a genetic mapping system in A. aerogenes, little can be inferred from the coordinate behavior of ribitol dehydrogenase and D-ribulokinase with respect to the relative position of their structural genes on the bacterial genome. One can only state that both structural genes seem to share common regulation.

Coordinate control is not apparent in the case of D-arabitol dehydrogenase and D-xylulokinase (Table 2). The D-arabitol dehydrogenase constitutive mutants possess the genetic capacity to synthesize D-xylulokinase in response to D-arabitol as the inducer-substrate, but this kinase activity is not constitutive. Another mutant had no detectable kinase activity when grown on D-arabitol and was unable to utilize D-xylose as the sole carbon and energy source (D-xylose-negative). However, the growth of this mutant in response to D-arabitol paralleled the growth of the wild-type strain and the other mutant strains on the same substrate.

The results of the induction experiment using strain 203 demonstrated that this constitutive mutant maintained a high D-arabitol dehydrogenase level regardless of whether it was grown on casein hydrolysate, D-arabitol, or D-xylose. There was a slight decrease in dehydrogenase activity in cells grown on the sugar substrates as compared with cells grown on casein hydrolysate. This decrease can be reproduced and may reflect the repression of enzyme synthesis by products of D-arabitol catabolism.

Although the D-xylulokinase from D-xylose-grown cells was partially purified and characterized (13), the enzyme from D-arabitol-grown cells was not carefully studied. It is possible that two species of D-xylulokinase (isozymes) exist, one specifically induced by D-arabitol and the other specifically induced by D-xylose. The alternative explanation of these data would require different levels of induction of a single species of kinase, with higher activity induced by D-xylose and a lower level of activity induced by D-arabitol. Resolution of this problem is dependent upon the purification of D-xylulokinase from cells grown on D-arabitol and a comparison of its properties with the enzyme isolated from D-xylose-grown cells.

The biological advantages of sequential induction can be seen in a situation where two or more catabolic pathways converge. If isozymes are not used to control the common metabolic reactions, then selective pressures may favor segmented control at the branchpoint as the most efficient means of regulating carbohydrate catabolism. If coordinate control were to span the convergence in a metabolic pathway, the result would be the synthesis of unnecessary enzyme protein.

Examples of such expenditures of protein synthesis are found with the xylitol-utilizing and D-arabinose-utilizing strains of A. aerogenes. Growth on xylitol results from the selection of mutants constitutive for ribitol dehydrogenase and from the utilization of this nonspecific dehydrogenase to catalyze the oxidation of xylitol to D-xylulose. D-Ribulokinase (coordinate controlled with the dehydrogenase) is also synthesized constitutively by such mutants even though this enzyme has no catalytic function during xylitol catabolism (14). When D-arabinose is used as the substrate, growth is believed to result from the selection of mutants constitutive for L-fucose isomerase and from the utilization of this nonspecific enzyme for the conversion of D-arabinose to D-ribulose. As a consequence of this isomerization, the enzymes of the ribitol pathway are induced. In this case, the induction of ribitol dehydrogenase must be considered superfluous, although D-ribulokinase is essential for the further catabolism of D-arabinose. Presumably, there has been no natural selection for regulatory systems controlling xylitol or D-arabinose degradation.

ACKNOWLEDGMENTS

The authors are indebted to W. A. Wood for his helpful discussions concerning the data presented in

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>Specific activity (units/mg of protein)</th>
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<tbody>
<tr>
<td></td>
<td>D-Arabitol dehydrogenase</td>
</tr>
<tr>
<td>200</td>
<td>82.0</td>
</tr>
<tr>
<td>201</td>
<td>73.0</td>
</tr>
<tr>
<td>202</td>
<td>34.67</td>
</tr>
<tr>
<td>203</td>
<td>15.4</td>
</tr>
</tbody>
</table>

TABLE 4. Enzyme activities of D-arabitol dehydrogenase constitutive mutants grown on D-xylose
this paper and in whose laboratory this study was initiated. This investigation was supported by Public Health Service research grant AI-06848-02 from the National Institute of Allergy and Infectious Diseases and research grant GB-3864 from the National Science Foundation.

LITERATURE CITED