REGULATION OF THE 4-HYDROXYPHENYLACETIC ACID META-CLEAVAGE PATHWAY IN AN ACINETOBACTER SP.

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Received October 10,1977

SUMMARY: Lactate-grown cultures of Acinetobacter sp. strain 3B-1 synthesize constitutively all enzymes except the 4-hydroxyphenylacetic acid-3-hydroxylase. All enzymes are further synthesized when strain 3B-1 is grown with 4-hydroxyphenylacetic acid. Induction studies with two mutant strains, one defective in the 3-hydroxylase, and the other defective in the dehydrogenase, indicate that 4-hydroxyphenylacetic acid induces the 3-hydroxylase only, and the second metabolite 3,4-dihydroxyphenylacetic acid appears to induce 3,4-dihydroxyphenylacetic acid-2,3-dioxygenase and subsequent enzymes. Thus, the enzymes of the 4-hydroxyphenylacetic acid meta-cleavage pathway are synthesized following at least two sequential inductive events.

INTRODUCTION

Acinetobacter spp. have been used extensively in studies of the modes of regulation of the β-ketoadipate pathway (1) and of the degradation of hydroaromatic compounds via the protocatechuate ortho-cleavage pathway (2). Comparisons of these modes of regulation with those of Pseudomonas spp. (3,4) have shown that the regulatory mechanisms in the two genera differ in the nature of the inducing metabolites and the degree of coordinancy. Comparisons of the mechanisms of regulation of meta-cleavage pathways in these two genera have not yet been made.

Acinetobacter sp. strain 3B-1 metabolizes 4-hydroxyphenylacetic acid by the meta-cleavage pathway (Fig. 1) elucidated by Sparnins et al (5). A previous communication from this laboratory reported studies on the regulation of the same pathway in P. putida (6). In this communication we report studies on the regulation of synthesis of some enzymes of this pathway in Acinetobacter sp. strain 3B-1.

ISSN 0006-291X

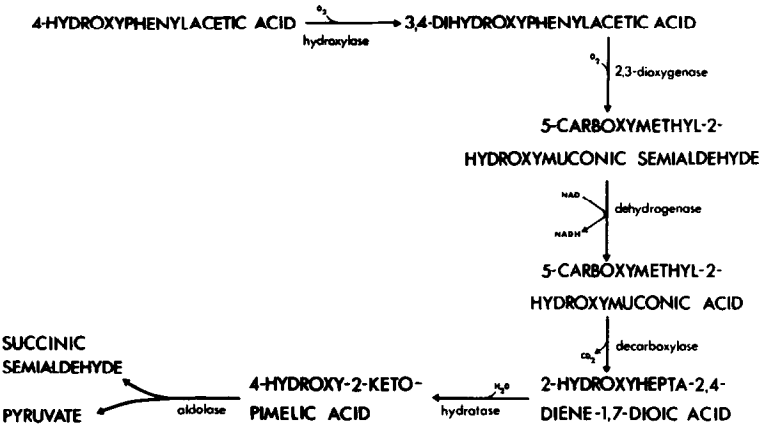

Figure 1. The meta-cleavage pathway for the degradation of 4-hydroxyphenylacetic acid.

MATERIALS AND METHODS

Organisms and conditions of growth. The wild-type strain used was Acinetobacter sp. strain 3B-1, one of the organisms used for the elucidation of the 4-hydroxyphenylacetic acid meta-cleavage pathway by Sparnins et al (5). Conditions of growth, induction of enzymes and preparation of cell suspensions were as described by Bayly and Wigmore (7) except that induction was for 4 h and the basal medium was supplemented with lactate (20 mM). The preparation of cell extracts was as described by Barbour and Bayly (6). The final concentration of inducing compounds was 2.5 mM unless otherwise stated. For experiments in which 5-carboxymethyl-2-hydroxymuconic semialdehyde was used as the inducer both the concentration and the ability of this compound to act as a substrate were monitored at each 30 min of the 4 h induction period. The concentration was calculated from the absorbance of the culture supernatant fluid at 380 nm (Eo = 30,000, pH 7.4, (5)). The ability to act as a substrate for the dehydrogenase was determined by assay with a cell-extract of 4-hydroxyphenylacetic acid-

induced strain 3B-1. To monitor the concentration of 5-carboxymethyl-2-hydroxymuconic semialdehyde and its ability to be metabolized, a flask containing the growth medium and the ring-cleavage product was incubated with a heat-killed (100°, 30 min) suspension of strain 3B-1.

Mutagenesis and selection of mutant strains. Exponential phase nutrient broth cultures of strain 3B-1 were exposed to 100 μg/ml N-methyl-N'-nitro-N-nitrosoguanidine for 30 min at 30° which resulted in a 95-99% reduction in the number of viable cells. Following overnight expression in nutrient broth, strains defective in the metabolism of 4-hydroxyphenylacetic acid were selected by procedures based on the method of Ornston et al (8). The hydroxylase-defective strain, 3B-3, was isolated from a culture cycled between the selective carbon source, quinic acid (2.5 mM) and the contraselective carbon source, 4-hydroxyphenylacetic acid (2.5 mM). The dehydrogenase-defective strain, 3B-31, was isolated in the same manner except that the contraselective carbon source was 3,4-dihydroxyphenylacetic acid (1 mM). The counter-selection of wild-type organisms was carried out in the presence of vancomycin and methicillin, each at 1 mg/ml.

Preparation of 5-carboxymethyl-2-hydroxymuconic semialdehyde. 5-Carboxymethyl-2-hydroxymuconic semialdehyde was prepared from 3,4-dihydroxyphenylacetic acid using heat-treated (50°, 3 min) extract of 4-hydroxyphenylacetic acid-induced P. putida strain P23X1 (6). This heat-treatment abolished dehydrogenase activity while not affecting 2,3-dioxygenase activity. This extract (0.5 ml aliquots) was added to 500 ml of a reaction mixture which contained 10 mM, 3,4-dihydroxyphenylacetic acid in 0.1M KH_2PO_4 buffer, pH 7.4, until all the substrate had been metabolized. The completeness of the reaction was determined in two ways: i) by

the production of 10 mM 5-carboxymethyl-2-hydroxymuconic semi-aldehyde as determined from its extinction coefficient (5); and ii) the failure to detect any 3,4-dihydroxyphenylacetic acid when the reaction mixture was used as substrate in the 2,3-dioxygenase assay. The reaction mixture was deproteinized with HCl (10M), the precipitate removed by centrifugation and the reaction mixture adjusted to pH 7.4 with 40% NaOH. The reaction mixture was reduced to 50 ml under reduced pressure and was kept at 4^{o} until required. Under these conditions it was stable for at least 7 days.

Enzyme assays. 2,3-Dioxygenase and dehydrogenase activities were determined by the method of Sparnins et al (5). Determinations of decarboxylase and hydratase activities were based on the methods of Sala-Trepat and Evans (9) and Collinsworth et al (10) respectively. The preparation of substrates for the assays of dehydrogenase, decarboxylase and hydratase have been described (5). Hydroxylase activity in cell suspensions was determined as described by Bayly and McKenzie (11).

RESULTS AND DISCUSSION

Exposure of lactate-grown strain 3B-1 to 4-hydroxyphenylacetic acid resulted in synthesis of all enzymes tested of the 4-hydroxy-phenylacetic acid meta-cleavage pathway (Table 1A). Constitutive synthesis of all enzymes except the hydroxylase was detected in lactate-grown strain 3B-1. The 2,3-dioxygenase, dehydrogenase and hydratase were synthesized constitutively to about 10% of 4-hydroxy-phenylacetic acid-induced levels whereas the decarboxylase was synthesized constitutively to about 40% of the induced level (Table 1A).

Exposure of lactate-grown strain 3B-1 to 3,4-dihydroxyphenyl-acetic acid resulted in the synthesis of all enzymes tested except

TABLE 1

A.
Specific activities of 4-hydroxyphenylacetic acid meta-cleavage pathway enzymes in lactate-grown cultures of strains 3B-1 and 3B-3, and in lactate-grown cultures exposed to either 2.5 mM 4-hydroxyphenylacetic acid or 2.5 mM 3,4-dihydroxyphenylacetic acid.

	3B-1			3B-3		
	lac[a]	4-HPA[a]	3,4-DHPA[a]	lac	4-HPA	3,4-DHPA
hydroxylase[b]	< 5	234	< 5	< 5	< 5	< 5
2,3-dioxygenase[c]	0.017	0.017	0.144	0.012	0.010	0.171
dehydrogenase[c]	0.011	0.085	0.086	0.007	0.007	0.072
decarboxylase[c]	0.018	0.043	0.042	0.011	0.009	0.051
hydratase[c,d]	0.002	0.017	0.015	0.002	0.004	0.016

B.
Specific activities of 4-hydroxyphenylacetic acid meta-cleavage pathway enzymes in lactate-grown cultures of strains 3B-1 and 3B-31, and in lactate-grown cultures exposed to either 1 mM 3,4-dihydroxyphenylacetic acid or 1 mM 5-carboxymethyl-2-hydroxymuconic semialdehyde.

	3B-1			3B-31		
	lac	3,4-DHPA	5-CM-2-HMS[a]	lac	3,4-DHPA	5-CM-2-HMS
2,3-dioxygenase[c]	0.020	0.166	0.042	0.020	0.218	0.053
dehydrogenase[c]	0.009	0.077	0.008	<0.001	<0.001	<0.001
decarboxylase[c]	0.018	0.043	0.018	0.019	0.040	0.017
hydratase[c,d]	0.002	0.017	0.003	0.002	0.020	0.002

a. Abbreviations: lac, lactate; 4-HPA, 4-hydroxyphenylacetic acid; 3,4-DHPA, 3,4-dihydroxyphenylacetic acid; 5-CM-2-HMS, 5-carboxymethyl-2-hydroxymuconic semialdehyde.
b. Values, corrected for endogenous respiration, are expressed as microlitres of oxygen taken up per hour per mg dry weight of cells.
c. Expressed as micromoles of substrate used (or product formed) per minute per mg protein.
d. Corrected for non-enzymatic breakdown of substrate.

the hydroxylase. These results suggest that 3,4-dihydroxyphenylacetic acid does not induce the hydroxylase.

When a lactate-grown culture of strain 3B-3 was exposed to

4-hydroxyphenylacetic acid none of the enzymes were synthesized above the constitutive levels (Table 1A). However, when a lactate-grown culture of 3B-3 was exposed to 3,4-dihydroxyphenylacetic acid all the enzymes except the hydroxylase were synthesized. These results indicate that strain 3B-3 does not synthesize a functional hydroxylase when exposed to 4-hydroxyphenylacetic acid, and the failure of this compound to induce any enzymes in 3B-3 suggests that it does not induce the 2,3-dioxygenase and subsequent enzymes.

Exposure of a lactate-grown culture of strain 3B-31, to 1 mM 3,4-dihydroxyphenylacetic acid resulted in all enzymes except the dehydrogenase being synthesized (Table 1B). In mutant strain 3B-31 there was no constitutive synthesis of the dehydrogenase. 3,4-Dihydroxyphenylacetic acid was used at 1 mM because it was found that the amount of ring-cleavage product which accumulated from 2.5 mM 3,4-dihydroxyphenylacetic acid completely inhibited growth. In strain 3B-1, the addition of 1 mM 3,4-dihydroxyphenylacetic acid resulted in the synthesis of the 2,3-dioxygenase and subsequent enzymes (Table 1B) to levels obtained with 2.5 mM 3,4-dihydroxy-phenylacetic acid (Table 1A).

Exposure of lactate-grown strains 3B-1 and 3B-31 to 1 mM 5-carboxymethyl-2-hydroxymuconic semialdehyde resulted in a two-fold increase in the specific activity of the 2,3-dioxygenase (Table 1A) but no increase in the specific activities of any other enzymes. These results suggest that the 2,3-dioxygenase and subsequent enzymes are subject to coincident regulation with synthesis elicited by 3,4-dihydroxyphenylacetic acid. Thus, it appears that the enzymes of the 4-hydroxyphenylacetic acid meta-cleavage pathway are synthesized following at least two sequential inductive events, each being mediated by a different effector.

In P. putida strain P23X1 the enzymes of this pathway are also synthesized following two sequential inductive events (6), 4-hydroxyphenylacetic acid inducing the hydroxylase only, while 3,4-dihydroxyphenylacetic acid induces the 2,3-dioxygenase and subsequent enzymes. However, in P. putida strain P23X1 none of the enzymes were synthesized constitutively (6), 5-carboxymethyl-2-hydroxymuconic semialdehyde did not have any inductive function and it was suggested that the genes encoding the 2,3-dioxygenase and subsequent enzymes constitute a single operon (6). However, with the induction of the 2,3-dioxygenase by the ring-cleavage product, and the high level of constitutive synthesis of the decarboxylase, it appears that in Acinetobacter strain 3B-1 the regulation of synthesis of the 2,3-dioxygenase and subsequent enzymes is more complex.

The regulation of the 4-hydroxyphenylacetic acid meta-cleavage pathway in both P. putida strain P23X1 and Acinetobacter sp. strain 3B-1 differs from the regulation of the catechol meta-cleavage pathway in P. aeruginosa strain T1 (11), P. arvilla mt-2 (12) and P. putida strain U (13,14,15,16) where all enzymes of the pathway are induced by the primary substrate. In P. putida strain U the genes encoding the enzymes of this pathway exist in two operons (17) which appear to be controlled by the same regulatory molecule (18). The nature of the controlling mechanisms regulating the synthesis of the enzymes of the 4-hydroxyphenylacetic acid meta-cleavage pathway in Acinetobacter strain 3B-1 has not been identified.

ACKNOWLEDGEMENTS

We wish to acknowledge the skilled technical assistance of Ivanka Vrkic. This work was supported in part by an A.R.G.C. Grant No. D67/16545.

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