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RIBITOL DEHYDROGENASE IN *AEROBACTER AEROGENES* 1033

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SUMMARY

Ribitol dehydrogenase from *Aerobacter aerogenes* strain 1033 acted only on ribitol, but was induced both by ribitol and by D-arabitol. The induction of this enzyme by D-arabitol was shown to depend on its metabolism. A mutant which produced ribitol dehydrogenase constitutively was isolated, in which the formation of this enzyme was apparently no longer regulated by the inducer, although catabolite repression continued to exert its influence. Ribitol dehydrogenase was obligatory for the utilization of ribitol under both aerobic and anaerobic conditions. Under anaerobic conditions the growth rate on ribitol was severely limited by inadequate hydrogen acceptors and was stimulated by the addition of fumarate to the growth medium.

INTRODUCTION

Aerobacter aerogenes strain 1033 has been shown to grow on ribitol, glycerol, D-arabitol, D-mannitol, D-sorbitol and myo-inositol as sole sources of carbon and energy[1-7]. *A. aerogenes* strains PRL R3 and ATCC 9621 have also been shown to utilize ribitol as a sole source of carbon and energy[7]. Extracts of these cells were found to contain ribitol dehydrogenase (ribitol-DPN oxidoreductase) which converted ribitol to D-ribulose[8-10]. In the case of *A. aerogenes* strain PRL R3, high levels of the enzyme were also reported in cells grown on D-arabitol L-arabitol and xylitol[8].

Extracts of strain 1033 grown on ribitol were also found to contain a ribitol dehydrogenase which reduced DPN in the presence of ribitol. The present studies were undertaken to determine the specificity of induction of this enzyme under conditions such that the compound tested for induction was not metabolized to a significant extent. The substrate specificity of the enzyme and its role in the adaptive utilization of ribitol under aerobic and anaerobic conditions were also investigated.

MATERIALS AND METHODS

Chemicals

Crystalline pyridine nucleotides were obtained from Pabst Laboratories; ribitol, D-arabitol, L-arabitol, meso-erythritol, D-dulcitol and D-ribose from Pfanstiehl Laboratories; D-sorbitol from H. M. Chemical Company, Ltd.; glycerol from Merck and Co.;

fumaric acid from California Corporation for Biochemical Research; succinate from Matheson Coleman and Bell; myo-inositol and triphenyl tetrazolium chloride from Nutritional Biochemicals Corp.; and the Folin-Ciocalteu phenol reagent from the Hartman-Leddon Company.

Bacteria

The studies were conducted with *A. aerogenes* strain 1033, and an arginine and guanine double auxotroph of this strain, 5P14; these organisms were obtained from Dr. B. MAGASANIK. A mutant A_2^-, which could only produce 5% of the D-arabitol dehydrogenase inducible in the wild-type was derived from 5P14 by S. A. LERNER.

Three mutants were isolated from 5P14 during the present study. R_c, which produced ribitol dehydrogenase constitutively, was obtained by a procedure reported earlier[11]. R_1^- produced ribitol dehydrogenase when grown on succinate in the presence of ribitol but was unable to utilize ribitol as a carbon source, and R_2^- was unable to produce ribitol dehydrogenase or to utilize ribitol as a carbon source. The last two mutants were obtained by treating populations of cells with ethylmethane sulfonate as a mutagen[11]. The treated cells were regrown to allow genetic segregation after which they were plated for identification and isolation by a procedure similar to the one used by ZAMENHOF for the isolation of Lac$^-$ colonies[12]. The agar had the following components: 8 g tryptone, 5 g NaCl, 17.5 g Difco bacto-agar, 4.5 g ribitol, 0.15 g K_2HPO_4, 10 mg guanine, 50 mg arginine, 45 mg triphenyltetrazolium chloride and 900 ml H_2O. R^- mutants utilized tryptone as the source of carbon and energy and formed red colonies as a result of the intracellular reduction of tetrazolium to formazan. Wild-type colonies were colorless, the production of formazan being prevented by the acid produced by ribitol fermentation. All mutants which were isolated were checked for arginine and guanine requirements to insure against the inclusion of contaminants.

Preparation of the enzyme

The culture medium, conditions for growth, and procedures for harvesting and sonically disrupting the cells have been described previously[2,4]. Unless otherwise specified the cells were grown on 0.2% carbon source. Media for growing 5P14 or its derivative strains were supplemented with 40 μg guanine and 50 μg arginine/ml. The supernatant fraction of the cell extract was assayed for ribitol dehydrogenase activity. No loss of activity was detected after storage for several hours at 2° or several weeks at −10°.

Enzyme assay

The activity of ribitol dehydrogenase was measured by following the reduction of DPN at 340 mμ in a model DU Beckman spectrophotometer at 25°. The assay solution contained 0.3 ml of 0.5 M ribitol, 0.2 ml of neutralized 0.01 M DPN, 0.6 ml 0.5 M Na_2CO_3 at pH 10.9 and sufficient water to give a volume of 3.0 ml after the addition of the enzyme. The reaction blank contained all the components except the substrate. At these concentrations of reagents the reaction rate was proportional to

the concentration of enzyme used, provided that the increase in absorbancy did not exceed 0.5 in the first 60 sec. The rate was linear during this time and was employed to calculate the enzyme activity, which was expressed as μmoles of DPN reduced/min/mg protein. The protein was determined by the use of the Folin-Ciocalteu phenol reagent, according to the procedure described by LOWRY *et al.*[13].

The activity of the enzyme increased gradually with pH to a maximum at pH 11 and decreased rapidly at higher pH. The enzyme was unstable at this pH but the rate of its inactivation was not sufficient to affect the assay.

Growth studies

Media for growth studies contained carbon sources and fumarate at a concentration of 0.2%. Growth rates of bacteria were determined in 50-ml cultures in 300-ml Belleo Nephelo culture flasks. Anaerobic studies were carried out in similar flasks sealed with ground glass joints. The flasks were flushed with N_2-CO_2 (95 : 5) for 3 min at a flow rate of 3 l/min and incubated at 37° on a rotary shaker operated at 240 cycles/min. Growth was monitored by reading the absorbancy in the side arm in a Klett colorimeter with a No. 42 filter. A sufficient inoculum of bacteria was employed to yield an initial reading of 20 Klett units.

RESULTS

Substrate specificity

Extracts of cells grown on ribitol were tested for reduction of DPN in the presence of several polyhydric alcohols. Such extracts were found to reduce DPN in the presence of ribitol but not in the presence of glycerol, meso-erythritol, D-arabitol, L-arabitol, xylitol, D-galactitol, D-mannitol, D-sorbitol or myo-inositol. The specificity of ribitol dehydrogenase is elucidated in Table I which shows that the enzyme attacked

TABLE I

SUBSTRATE AND INDUCTION SPECIFICITY OF RIBITOL DEHYDROGENASE

All compounds tested as substrates were employed at 0.05 M. Compounds tested as inducers were employed at 0.1 M under gratuitous conditions.

Compound	Configuration		Reactivity as substrate	Effectiveness as inducer
Ribitol	CH_2OH	CH_2OH	100	100*
D-Arabitol	CH_2OH	CH_2OH	0	3**
Xylitol	CH_2OH	CH_2OH	< 1	< 1
L-Arabitol	CH_2OH	CH_2OH	< 1	0
Erythritol	CH_2OH	H***	< 1	0

* Tested with strain R_1 which possessed the ability to form ribitol dehydrogenase but was unable to utilize the pentitol for metabolism.

** Tested with a mutant, A_2^-, which could produce only 3% of the D-arabitol dehydrogenase inducible in the wild-type.

*** A broken bar is used to indicate that this hydroxyl group may assume either configuration.

the all-*cis* pentitol but not the other pentitols or the all-*cis* tetratol. Testing for coenzyme specificity revealed that DPN could not be replaced by TPN.

Inducer specificity

Extracts of ribitol-grown cells contained ribitol dehydrogenase but not D-arabitol dehydrogenase (EC 1.1.1.11) (see ref. 4), glycerol dehydrogenase (EC 1.1.1.6) (see refs. 2, 3) or myo-inositol dehydrogenase (EC 1.1.1.18) (see refs 5–7). A significant activity of ribitol dehydrogenase was also found in extracts of cells grown on D-arabitol. The level of this dehydrogenase in such cells was about one tenth of that found in ribitol-grown cells. Extracts from cells grown on glycerol, D-mannitol, D-sorbitol, myo-inositol, D-ribose and glucose contained negligible ribitol dehydrogenase activity .The presence of ribitol dehydrogenase in cells grown on D-arabitol was suspected to be the result of the conversion of D-arabitol to D-xylulose[4] followed by isomerization to D-ribulose; D-ribulose itself might act as an inducer or it might give rise to small amounts of the inducer.

This hypothesis was supported by study of the inducer specificity under gratuitous conditions, *i.e.*, conditions under which the test compound was not metabolized. Two mutants were employed to compare the relative effectiveness of ribitol and D-arabitol as inducers of ribitol dehydrogenase. A mutant A_2^-, blocked in D-arabitol utilization, was grown on succinate in the presence of D-arabitol. Another mutant R_1^-, which did not grow on ribitol but which could be induced to form normal levels of ribitol dehydrogenase when grown on succinate in the presence of ribitol, was used as a control. Comparison of the levels of ribitol dehydrogenase present in the extracts of these cultures showed that cells of A_2^- exposed to D-arabitol contained only 3% of the enzyme activity present in cells of R_1^- exposed to ribitol (Table I). The level of ribitol dehydrogenase induced by D-arabitol under these gratuitous conditions was one third of the value obtained with wild-type cells grown on D-arabitol. The persistence of a weak though reduced power for D-arabitol to stimulate the formation of ribitol dehydrogenase in the mutant A_2^- may be due to the imperfection of the block in D-arabitol utilization (see METHODS AND MATERIALS).

L-Arabitol, xylitol, and meso-erythritol were not metabolized by the strain of *A. aerogenes* under study. They could be tested as gratuitous inducers of ribitol dehydrogenase by growing wild-type cells on succinate in the presence of the polyol. It was found that none of the compounds induced an appreciable level of ribitol dehydrogenase even when employed at high concentrations to minimize the permeability barrier. The results of the induction of ribitol dehydrogenase under gratuitous conditions are summarized in Table I from which it may be seen that the substrate specificity and induction specificity agreed very closely.

The role of ribitol dehydrogenase

Previous studies on the adaptive utilization of glycerol in *A. aerogenes* 1033 revealed that two separate pathways could be induced: under aerobic conditions glycerol was utilized primarily through glycerol kinase (EC 2.7.1.30) and under anaerobic conditions it was utilized primarily through a glycerol dehydrogenase[8]. In the case of the adaptive utilization of D-arabitol by this organism, only one pathway

was involved in which the first enzyme was a DPN-linked D-arabitol dehydrogenase[4,14]. In the present study the adaptive utilization of ribitol was also found to be dependent upon a single pathway, in which ribitol dehydrogenase was required in the first step. This was demonstrated by the fact that a mutant lacking this enzyme, R_2^-, failed to grow on ribitol as a sole source of carbon and energy under either aerobic or anaerobic conditions.

Aerobic and anaerobic growth on ribitol

Although ribitol could serve as the sole source of carbon and energy for *A. aerogenes* 1033 under both aerobic and anaerobic conditions, the anaerobic rate of growth was poor when compared with the rates observed with several other compounds (Table II). The slow rate of anaerobic growth on ribitol was probably due to the lack of an adequate hydrogen acceptor system, since the addition of fumarate to the medium greatly stimulated growth (Table II and Fig. 1). It has previously been shown that fumarate serves anaerobically as a hydrogen acceptor and disappears from the medium at significant rates only when a fermentable compound is also present[1,15].

TABLE II

THE EFFECT OF FUMARATE ON THE GROWTH RATES ON VARIOUS CARBON SOURCES

Carbon source	Doubling time (min)		
	Aerobic	Anaerobic	Anaerobic + fumarate
Ribitol	30	325	102
D-Arabitol	35	105	72
Glycerol	40	115	87
Glucose	32	58	51

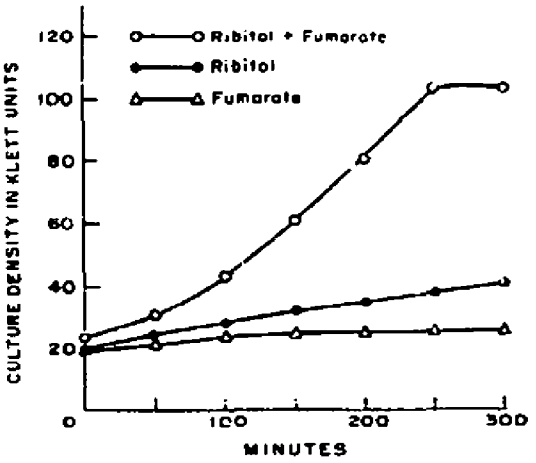

Fig. 1. Stimulation of anaerobic growth on ribitol by the presence of fumarate as a hydrogen acceptor.

TABLE III

RIBITOL DEHYDROGENASE ACTIVITIES IN CELLS GROWN ON RIBITOL UNDER VARIOUS CONDITIONS

Carbon compounds in growth medium	*Gas phase of culture*	*Dehydrogenase activity*
Ribitol	Air	2.7
Ribitol	N_2–CO_2 (95 : 5)	5.8
Ribitol + Fumarate	N_2–CO_2 (95 : 5)	5.1

The level of ribitol dehydrogenase was probably not a limiting factor in anaerobic growth on ribitol since the growth rate could be increased by a factor of three in the presence of fumarate without increasing the level of the enzyme (Tables II and III).

Levels of ribitol dehydrogenase in a constitutive mutant

A mutant R_c, which was constitutive with respect to ribitol dehydrogenase, was grown on several different carbon sources. Succinate-grown cells contained a higher level of the enzyme than cells grown on ribitol, and glucose-grown cells contained the lowest level (Table IV). These data indicate that the formation of ribitol dehydrogenase in the mutant was primarily regulated by catabolite repression, rather than by the interaction of specific induction and specific repression.

TABLE IV

LEVELS OF RIBITOL DEHYDROGENASE IN CONSTITUTIVE AND INDUCIBLE CELLS GROWN ON VARIOUS SOURCES OF CARBON AND ENERGY

Carbon source	*Ribitol dehydrogenase*	
	Inducible 5P14	*Constitutive Rc*
Glucose	0.00	0.31
Ribitol	2.70	0.64
D-Arabitol	0.31	1.34
Succinate	0.00	4.20

DISCUSSION

The studies on the inducer specificity of ribitol dehydrogenase illustrated the importance of checking the action of an apparent inducer under conditions of gratuity. In this case the inducing action of D-arabitol was shown to be a consequence of its metabolism. This raises the possibility that the non-specific induction in *A. aerogenes* strain PRL R3 of ribitol dehydrogenase by D-arabitol, L-arabitol and xylitol, and of D-arabitol dehydrogenase by ribitol[8], might also be the consequence of interconversion of metabolites.

The data on the levels of ribitol dehydrogenase in the constitutive mutant lent further support to the theory that catabolite repression operates apart from the specific repressor system[16]. In this strain the formation of ribitol dehydrogenase was apparently no longer dependent on the inducer although catabolic repression still seemed to be effective. Similar findings have been reported for β-galactosidase (EC 3. 2. 1. 23) in a constitutive strain of *Escherichia coli*[17].

It has been assumed that the mutation to constitutivity with respect to ribitol dehydrogenase involved only the specific repressor system, and not the structure of the enzyme or the catabolite repression system. In terms of this simple model it is difficult to explain the observation that the wild-type produced four times as much ribitol dehydrogenase as the constitutive mutant when grown on ribitol. Both strains should be subject to similar catabolite repression, and if the sole function of the inducer were to overcome the effect of specific repression in the wild type, then the constitutive mutant would be expected to produce at least as much of the enzyme as the wild type when grown on ribitol. Further analysis of the control of ribitol dehydrogenase in the constitutive mutant depends on knowing whether the mutation affected the o-gene or the i-gene[18], but unfortunately genetic recombination experiments are not yet possible with this organism.

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