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Two Functionally Different Dihydroorotic Dehydrogenases in Bacteria

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ABSTRACT

TAYLOR, W. H. (Portland State College, Portland, Ore.), M. L. TAYLOR, AND D. F. EAMES. Two functionally different dihydroorotic dehydrogenases in bacteria. J. Bacteriol. 91:2251–2256. 1966.—We have investigated the relationship between the two kinds of dihydroorotic dehydrogenases produced by bacteria. A pseudomonad, capable of growth on a salts medium with glucose, aspartate, glycerol, or orotate as the carbon source, was isolated from lake bank mud. A particle-bound dihydroorotic dehydrogenase, similar to the biosynthetic enzyme in *Escherichia coli*, was formed by the pseudomonad when the carbon source was orotate, glucose, glycerol, or aspartate. A soluble, degradative nicotinamide adenine dinucleotide phosphate-linked dihydroorotic dehydrogenase, as well as the particle-bound biosynthetic enzyme, was formed when the pseudomonad was cultivated on orotate. The biosynthetic enzyme links to oxygen or ferricyanide, but not to pyridine nucleotides. *Zymobacterium oroticum*, when cultivated on glucose, contained only the biosynthetic type of dihydroorotic dehydrogenase. The presence of two functionally different dihydroorotic dehydrogenases in the pseudomonad was suggested on the basis of the following observations: (i) the two enzyme activities were separated by centrifugation; (ii) the pyridine nucleotide-linked activity was formed only when orotate was present in the growth medium; and (iii) the biosynthetic enzyme was stable to storage at −20 C for 4 months, whereas the degradative enzyme activity was destroyed by storage under these conditions.

We (9) previously reported that the dihydroorotic dehydrogenase of *Escherichia coli* was located in the particulate portion of cell-free extracts and would not interact with pyridine nucleotides as reported for the dihydroorotic dehydrogenase of organisms utilizing orotic acid as the carbon source (2, 10). Although the pyridine nucleotide-linked dihydroorotic dehydrogenases have been accepted as the enzymes involved in the biosynthesis of pyrimidines, significant levels of pyridine nucleotide-linked dehydrogenase activity have been reported only for organisms growing on pyrimidine as the carbon source.

We isolated a bacterium capable of growing in a mineral salts medium with orotate as the carbon source. This bacterium, a pseudomonad, contained a particle-bound, biosynthetic dihydroorotic dehydrogenase regardless of the carbon source used for growth. The organism responded to the introduction of orotate to the growth medium, either as a sole or supplemental carbon source, by synthesis of a new, soluble nicotinamide adenine dinucleotide phosphate (NADP)-linked dihydroorotic dehydrogenase. Thus, we were able to compare the biosynthetic and degradative dihydroorotic dehydrogenases in the same cell.

MATERIALS AND METHODS

Isolation and cultivation of organisms. An orotate-utilizing pseudomonad was isolated by the enrichment technique with the use of a mineral salts medium containing (grams per liter): KH_2PO_4, 1.1; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 2; orotic acid, 2. The salts solution was heated to dissolve the orotic acid, and the *p*H of the medium was then adjusted to 7.0 by the addition of sodium hydroxide. Mud from the shore of Upper Klamath Lake, Oregon, was used as the inoculum for enrichment cultures. Isolation of the pseudomonad was made by streaking the liquid enrichment cultures onto plates containing the above medium with 2% agar. The organism was cultivated at room temperature. Stock cultures were maintained on orotate-mineral salts-agar, stored at 4 C, and were transferred each month.

The medium described above was used for the experimental work except that the phosphate concen-

tration was increased fivefold and the orotic acid concentration was increased twofold. Where indicated, glucose (0.5%) and sodium aspartate (0.4%) were used as carbon sources to replace orotic acid. Growth of the pseudomonad on orotate or glucose yielded 1.5 g of wet cells per liter, whereas growth on sodium aspartate yielded 2.5 g of wet cells per liter. Growth of cultures was measured as an increase in optical density by use of a Klett-Summerson colorimeter with a no. 66 filter.

Zymobacterium oroticum was cultivated in an orotate-containing medium (2) or in the same medium with 2% glucose substituted for orotate as the carbon source.

Preparation of extracts. Pyridine nucleotide-linked dihydroorotic dehydrogenase activity in cells decayed rapidly as soon as orotate had disappeared from the culture medium. We therefore followed orotate disappearance and harvested the cells before the medium was depleted of orotate. Orotate disappearance was estimated by following the decrease in absorbance at 290 mμ, measured in samples of the culture which had been clarified by centrifugation. Cultures using glucose or aspartate as carbon sources were harvested while cells were in exponential growth. Cells were harvested at 4 C, and the cell pellets were washed once with 10 volumes of 0.05 M potassium phosphate buffer (*p*H 7.5). Washed cells were resuspended to approximately 0.5 g of wet cells per ml in this buffer and were disrupted with a French Press (American Instrument co., Inc., Silver Spring, Md.) with the use of 5,000 psi of pressure. Extracts were centrifuged at 10,000 × *g* for 30 min at 4 C, and the pellet and supernatant fluid were saved. The 10,000 × *g* supernatant fluid was then centrifuged at 105,000 × *g* for 2 hr in a Spinco model L ultracentrifuge. The pellet and supernatant fluid were saved.

Enzyme assays and chemical determination. The aerobic assay for dihydroorotic dehydrogenase was performed as described by Yates and Pardee (13) with the use of a Bausch & Lomb model 505 Spectrophotometer. This assay measures the rate of orotate production from dihydroorotate, in an open cuvette, as an increase in absorbance at 290 mμ.

The ferricyanide reduction assay was performed as described by Taylor and Taylor (9), except that the *p*H of the potassium phosphate buffer was 7.6.

The NADP-linked dihydroorotic dehydrogenase activity was measured as orotate-dependent reduced NADP ($NADPH_2$) disappearance at 340 mμ as described by Udaka and Vennesland (10).

One unit of enzyme is defined as the amount of enzyme which will produce 1 μmole of product (dihydroorotic acid or orotic acid) per hr under the stated conditions. Specific activity is listed as units of enzyme per milligram of protein. In all enzyme assays, corrections were made for any activity not dependent upon the addition of substrate.

Protein concentration was measured by use of the Folin phenol reagent as described by Lowry et al. (5), with bovine serum albumin as the standard.

RESULTS

The bacterium, isolated from lake mud by the enrichment technique, was assigned to the genus *Pseudomonas* on the basis of the following characteristics. It was a gram-negative, polarly flagellated, straight rod which produced no endospores. It was a strict aerobe and produced a water-soluble yellow-green fluorescent pigment.

Many kinds of bacteria were isolated on the salts-orotate medium, including bacilli and actinomycetes. We selected the pseudomonad for these studies because it grew readily on the mineral salts-orotate medium and because glucose, glycerol, or aspartate could be substituted for orotate as a carbon source in the salts medium.

As shown in Table 1, extracts of cells cultivated with aspartate as the carbon source contained no NADP-linked dihydroorotic dehydrogenase activity. Cultivation of cells in the presence of orotate resulted in the formation of a soluble NADP-linked dihydroorotic dehydrogenase. Regardless of the substrate used as carbon source, the specific activity of the biosynthetic dihydroorotic dehydrogenase, as measured by the aerobic assay, was approximately the same.

The data shown in Table 1 for aspartate and aspartate plus orotate cultures were obtained as follows. Cells were cultivated to the middle of the exponential growth phase with aspartate as the carbon source. The cells were harvested at 4 C, and one-half of the cells were broken and fractionated as described in Materials and Methods. This extract was used to obtain data for aspartate-grown cells. The other half of the cells was incubated in aspartate growth medium containing 0.2% orotate. After 4 hr, the cells were harvested, broken, and fractionated. Data obtained with this extract are shown in Table 1 under the heading of aspartate plus orotate grown cells.

The presence of orotate in the aspartate growth medium did not alter the specific activity, as measured by the aerobic or ferricyanide assay, of the particle-bound biosynthetic dihydroorotic dehydrogenase. Cells growing on aspartate responded to the addition of orotate by producing a soluble NADP-linked dihydroorotic dehydrogenase. Recovery of the NADP-linked enzyme activity of the aspartate plus orotate extract after centrifugation at 105,000 × *g* was low because of temperature rise in the rotor during the run. In later experiments with no temperature problems, all enzyme activities were recovered.

Extracts prepared from cells grown with glucose as the carbon source contained the particulate, biosynthetic dihydroorotic dehydrogenase at the same level as found in organisms cultivated

TABLE 1. *Effect of growth substrate on dihydroorotic dehydrogenase activities*

Growth substrate	Fraction	Enzyme activities					
		Aerobic assay		Ferricyanide assay		$NADPH_2$ assay	
		Total units	Specific activity	Total units	Specific activity	Total units	Specific activity
Aspartate	Crude extract	141	0.76	246	1.31	0	0*
	Supernatant fluid, 10,000 × *g*	134	0.77	171	0.99	0	0
	Pellet, 10,000 × *g*	11	0.64	19	1.05	0	0
	Supernatant fluid, 105,000 × *g*	5	0.07	34	0.28	0	0
	Pellet, 105,000 × *g*	121	3.11	118	2.47	0	0
Aspartate + orotate	Crude extract	632	0.65	949	0.97	244.0	0.25
	Supernatant fluid, 10,000 × *g*	466	0.90	700	1.02	177.0	0.25
	Pellet, 10,000 × *g*	219	0.90	290	1.12	8.5	0.03
	Supernatant fluid, 105,000 × *g*	82	0.19	199	0.62	137.0	0.31
	Pellet, 105,000 × *g*	385	2.67	404	2.32	0.8	0.01
Orotate	Crude extract	394	0.76	1215	2.32	5030.0	9.60
	Supernatant fluid, 10,000 × *g*	319	0.66	1117	2.30	4383.0	9.10
	Pellet, 10,000 × *g*	43	0.63	140	2.00	116.0	1.80
	Supernatant fluid, 105,000 × *g*	12	0.04	1046	4.24	3753.0	12.50
	Pellet, 105,000 × *g*	297	1.93	73	1.34	176.0	1.14

* A specific activity as low as 0.01 could have been measured.

on aspartate, but again contained no NADP-linked dihydroorotic dehydrogenase.

Extracts prepared from cells cultivated with orotate as the sole carbon source contained an active NADP-linked dihydroorotic dehydrogenase. This activity remained in the supernatant fluid after centrifugation at 105,000 × *g* for 2 hr (Table 1). The NADP-linked activity that did sediment at 105,000 × *g* was quantitatively recovered by washing the pellet twice with 0.05 M potassium phosphate buffer (pH 7.5), while all the aerobic activity remained in the pellet.

In contrast to the dihydroorotic dehydrogenase of *Z. oroticum* (2), the NADP-linked enzyme in the pseudomonad was unaffected by the inclusion of 20 μmoles of cysteine in the standard assay mixture, or by incubation of the enzyme with 20 μmoles of cysteine in the standard assay mixture for 10 minutes prior to initiation of the reaction with $NADPH_2$.

Measurement of $NADPH_2$ and orotate disappearance simultaneously always showed a more rapid rate of $NADPH_2$ oxidation than orotate reduction. Thus, an $NADPH_2$ oxidase was present in all extracts, and it appeared to be active even in the presence of orotate.

The NADP-linked dihydroorotic dehydrogenase was 20-fold less active with reduced nicotinamide adenine dinucleotide ($NADH_2$) than with $NADPH_2$. The specific activity of the NADP-linked enzyme obtained from the pseudomonad was almost the same as the specific activity of the enzyme obtained from a gram-negative pleomorphic rod by Udaka and Vennesland (10).

The presence of NADP-linked activity in extracts from aspartate-grown cells could have been masked by the presence of an inhibitor. This possibility was tested by a mixing experiment under standard assay conditions. The decreases in $NADPH_2$ absorbance at 340 mμ over a 2-min period were: orotate extract, 0.220 (minus orotate, 0.016); aspartate extract, 0.037 (minus orotate, 0.036); mixture of orotate plus aspartate extract, 0.255. Thus, we could have detected the pyridine nucleotide-linked dihydroorotic dehydrogenase if it had been present in extracts prepared from aspartate-grown cells.

The specific activity of the 105,000 × *g* pellet, as measured by the aerobic and ferricyanide assays, was always lower in extracts from orotate-grown cells than in extracts from glucose or aspartate-grown cells. This lowered specific activity could account for the slower growth rate observed when orotate was used as the carbon source, or may be due to the consistent finding of more particle protein in the 105,000 × *g* pellet from extracts of orotate-grown cells.

The two dihydroorotic dehydrogenases were separated by centrifugation of the extract. The biosynthetic enzyme was localized in the par-

ticulate fractions, while the NADP-linked dihydroorotic dehydrogenase remained in the 105,000 × *g* supernatant fluid. Although both the biosynthetic and NADP-linked dihydroorotic dehydrogenases react in the ferricyanide assay, it appears that the degradative, NADP-linked dihydroorotic dehydrogenase of the pseudomonad does not react in the aerobic assay, and, conversely, the biosynthetic enzyme does not link to pyridine nucleotides.

The separation of the two functionally different dihydroorotic dehydrogenases from the same extract can also be demonstrated as shown in Table 2. The fractions used for this experiment were the 105,000 × *g* supernatant fluid and pellet prepared from orotate-grown cells. The specific activity of the particulate, biosynthetic dihydroorotic dehydrogenase, as measured by the aerobic and ferricyanide assays, remained relatively stable for 1 week at 0 C or during storage for 4 months at −20 C. Under these same conditions, the NADP-linked enzyme was inactivated much more rapidly than the biosynthetic enzyme. The $NADPH_2$ activity lost during storage was not recovered by incubation of the enzyme in the presence of cysteine.

Since two functionally different dihydroorotic dehydrogenases were found in the pseudomonad, the dihydroorotic dehydrogenase of *Z. oroticum* was investigated. We found that extracts prepared from *Z. oroticum* cells cultivated on glucose contained only a soluble, biosynthetic-type dihydroorotic dehydrogenase. Differentiation between the inducible and biosynthetic dihydroorotic dehydrogenases in extracts of cells cultivated on orotate was difficult, because all dehydrogenase activity was located in the soluble portion of the extract and because it has been shown (6) that the inducible dihydroorotic dehydrogenase may act as a dihydroorotate oxidase. Thus, further work is needed to clarify the nature of this enzyme in *Z. oroticum*.

DISCUSSION

The results reported above indicate that two different dihydroorotic dehydrogenases can exist concurrently in the bacterial cell. The degradative enzyme is a soluble, pyridine nucleotide-linked enzyme formed only during growth on orotate. The biosynthetic dihydroorotic dehydrogenase, which is particle-bound in the gram-negative bacteria thus far studied (*unpublished data*), may interact with oxygen or ferricyanide, and is formed at approximately the same level regardless of the growth substrate used. It appears that the biosynthetic and degradative dihydroorotic dehydrogenases of the pseudomonad constitute an analogy to the degradative and biosynthetic threonine deaminases (11) and ornithine decarboxylases (7) of *E. coli*.

Although there have been no reports differentiating the biosynthetic dihydroorotic dehydrogenase from the pyridine nucleotide-linked enzyme, it would appear that Beckwith et al. (1) and Yates and Pardee (13) were actually measuring the biosynthetic dihydroorotic dehydrogenase. They studied the dehydrogenase in crude extracts of *E. coli* using oxygen as the terminal electron acceptor. We (9) have found that the particulate, biosynthetic, dihydroorotic dehydrogenase of *E. coli* reacts with oxygen and not with pyridine nucleotides.

Udaka and Vennesland (10) undoubtedly did not measure the biosynthetic enzyme, since they assayed the enzyme only as $NADPH_2$ oxidation in the presence of orotate and because they were working with the soluble portion of an extract from a gram-negative organism in which this enzyme probably would be particle-bound. The level of NADP-linked dihydroorotic dehydrogenase reported by Udaka and Vennesland in the gram-negative organism when grown with aspartate as the carbon source was essentially the same as the level of $NADPH_2$ oxidase activity which we found in extracts of the aspartate-grown pseudomonad. No correction was made by

TABLE 2. *Differential loss of dihydroorotic dehydrogenase activities during storage*

Time	Specific activity					
	Aerobic assay		Ferricyanide assay		$NADPH_2$ assay	
	Particles	Supernatant fluid	Particles	Supernatant fluid	Particles	Supernatant fluid
Zero	1.93	0	1.34	4.24	1.14	12.50
1 week, 0 C	1.75	0	1.10	0.89	—	5.30
4 months, −20 C	1.43	—	1.00	0.19	<0.01	0.80

Udaka and Vennesland for $NADPH_2$ oxidase activity when calculating dihydroorotic dehydrogenase activity.

Reynolds, Lieberman, and Kornberg (8) studied decomposition of orotate by two gram-positive bacteria thought to belong to the genus *Corynebacterium*, and found each to contain an NADP-linked dihydroorotic dehydrogenase. With a partially purified enzyme, they were able to show oxidation of dihydroorotate to orotate with oxygen as the electron acceptor. We have found (*unpublished data*) that the biosynthetic dihydroorotic dehydrogenase of gram-positive organisms is located in the 105,000 $\times$ *g* supernatant fluid of cell-free extracts. Thus, it is possible that Reynolds et al. had an enzyme fraction containing a mixture of the biosynthetic and degradative dihydroorotic dehydrogenases.

Yates and Pardee (12) reported that extracts of *Z. oroticum* grown on glucose contained a low level of dihydroorotic dehydrogenase when measured with oxygen as the electron acceptor. Although aerobic enzyme activity was greatly increased when this bacterium was cultivated with orotate as the carbon source, they concluded that the induced dihydroorotic dehydrogenase may have been formed only to metabolize orotic acid, while the level of dihydroorotic dehydrogenase activity measurable in glucose-grown cells could be responsible for pyrimidine synthesis. These authors did not report any pyridine nucleotide-linked activity in extracts of glucose-grown cells, but did report a dihydroorotate oxidase activity. This oxidase activity probably was a measure of the biosynthetic dihydroorotic dehydrogenase. The higher level of dihydroorotate oxidase activity found in extracts of orotate-grown cells of *Z. oroticum* was probably due to the known oxidase activity of the inducible dihydroorotic dehydrogenase (2, 6).

Although the *Z. oroticum* inducible enzyme has been accepted as the one involved in pyrimidine synthesis, many investigators have been concerned with the feasibility of the oxidation of dihydroorotate to orotate by nicotinamide adenine dinucleotide (NAD). Graves and Vennesland (3) stated that the oxidation-reduction potential of NAD was too low to be realistically involved in biological oxidation of dihydroorotate to orotate. The involvement of NAD in the oxidation of dihydroorotate to orotate was first suggested by the experiments of Lieberman and Kornberg (4), which demonstrated that the anaerobic conversion of dihydroorotate to orotate by *Z. oroticum* extracts was stimulated by the addition of pyruvate and lactic dehydrogenase to the assay system. However, Miller and Massey (6) recently showed that neither pyridine nucleotides nor flavins appear to be involved in the direct oxidation of dihydroorotate to orotate by the enzyme from *Z. oroticum*. In anaerobic bacteria, oxygen would not be expected to be the natural electron acceptor. We are currently purifying the soluble, biosynthetic dihydroorotic dehydrogenase from another anaerobe, *Lactobacillus bulgaricus*. The pathway of electron flow in this anaerobe should be interesting, since preliminary work indicates that the biosynthetic enzyme contains no flavins, and cytochromes have not been reported in *Lactobacillus*. We have also found (*unpublished data*) a particle-bound, biosynthetic dihydroorotic dehydrogenase in Novikoff ascites tumors and in hypocotyls of *Phaseolus*.

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