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Rhamnose-Induced Propanediol Oxidoreductase in *Escherichia coli*: Purification, Properties, and Comparison with the Fucose-Induced Enzyme

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Escherichia coli are capable of growing anaerobically on L-rhamnose as a sole source of carbon and energy and without any exogenous hydrogen acceptor. When grown under such condition, synthesis of a nicotinamide adenine dinucleotide-linked L-lactaldehyde:propanediol oxidoreductase is induced. The functioning of this enzyme results in the regeneration of nicotinamide adenine dinucleotide. The enzyme was purified to electrophoretic homogeneity. It has a molecular weight of 76,000, with two subunits that are indistinguishable by electrophoretic mobility. The enzyme reduces L-lactaldehyde to L-1,2-propanediol with reduced nicotinamide adenine dinucleotide as a cofactor. The K_m were 0.035 mM L-lactaldehyde and 1.25 mM L-1,2-propanediol, at pH 7.0 and 9.5, respectively. The enzyme acts only on the L-isomers. Strong substrate inhibition was observed with L-1,2-propanediol (above 25 mM) in the dehydrogenase reaction. The enzyme has a pH optimum of 6.5 for the reduction of L-lactaldehyde and of 9.5 for the dehydrogenation of L-1,2-propanediol. The enzyme is, according to the parameters presented in this report, indistinguishable from the propanediol oxidoreductase induced by anaerobic growth on fucose.

Cells of *Escherichia coli* growing anaerobically on fucose (6) or rhamnose (15) excrete 1,2-propanediol into the media. In the case of fucose fermentation, it has been shown that the excreted compound is formed in the metabolism of the methyl pentose (6). L-fucose is metabolized via a pathway (Fig. 1) mediated by L-fucose permease (11), L-fucose isomerase (10), L-fuculose kinase (14), and L-fuculose-1-phosphate aldolase (8). The last enzyme catalyzes the formation of dihydroxyacetone phosphate and L-lactaldehyde.

Aerobically, the lactaldehyde is converted to lactate (21) and pyruvate (6) by two consecutive, enzyme-catalyzed oxidations. Anaerobically, the lactaldehyde is reduced to L-1,2-propanediol by an oxidoreductase, thereby regenerating NAD and allowing the fermentation of fucose to proceed. The excreted propanediol is not utilizable by the cells even if molecular oxygen becomes available (13). This propanediol oxidoreductase, which is inducible anaerobically by fucose, is exploited by mutants selected for their ability to use 1,2-propanediol as a carbon and energy source (6, 13, 22). But in the mutants, this enzyme is synthesized constitutively even under aerobic conditions. Moreover, in vivo it catalyzes the reverse reaction of oxidizing propanediol to lactaldehyde, which is oxidized again to give lactate.

L-Rhamnose is utilized by a homologous or similar set of enzymes (Fig. 1), yielding also dihydroxyacetone phosphate and L-lactaldehyde as intermediates (5, 23, 24). It is noteworthy, however, that the genes of the fucose and rhamnose utilization map in two separate clusters on the *Escherichia coli* chromosome (2).

In this report we show that when the cells are grown anaerobically on rhamnose their extracts contain a propanediol oxidoreductase similar to the one induced by anaerobic growth on fucose. This protein was purified, and some of its properties are described.

MATERIALS AND METHODS

Bacteria. The parental strain used was an *E. coli* K-12, also known as E-15 (1), and here referred to as strain 1. Strain 3 was derived from strain 1 as a propanediol oxidoreductase mutant after ethyl methane sulfonate mutagenesis (22). This strain lost the ability to grow on L-fucose because of the failure to synthesize fucose permease, fucose isomerase, and fuculose kinase under inducing conditions (11). Strain 32 was a propanediol oxidoreductase-negative mutant derived from strain 3 after nitrosoguanidine mutagenesis (22). All strains were kindly provided by E. C. C. Lin, Department of Microbiology and Molecular Genetics,

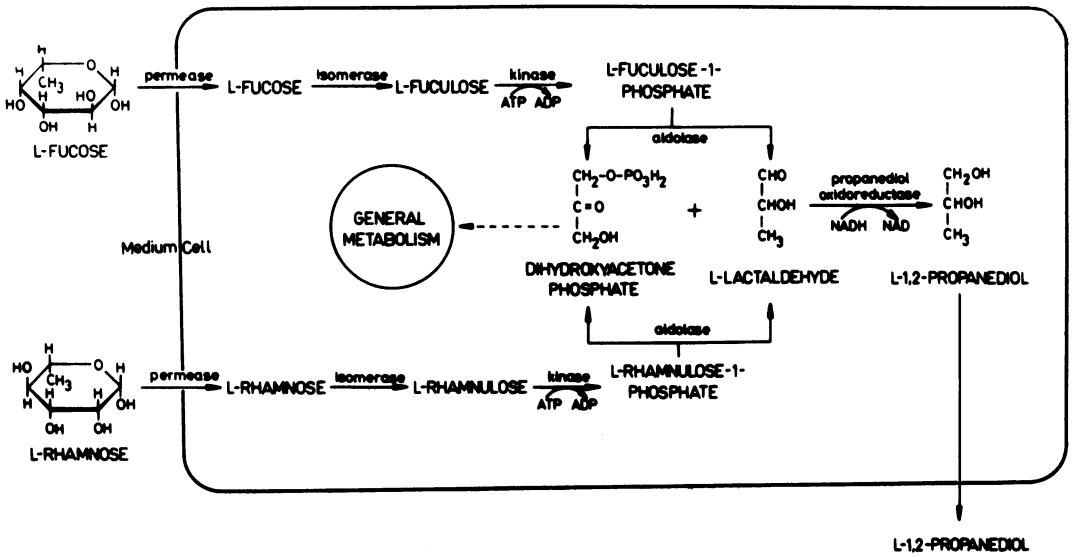

FIG. 1. *Scheme for the anaerobic catabolism of* L*-fucose and* L*-rhamnose in E. coli. The enzyme catalyzing the interconversion of propanediol and lactaldehyde is referred to as propanediol oxidoreductase, because the actual role of this protein depends upon the strain in which it is found.*

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Chemicals. DL-1,2-Propanediol was purchased from E. Merck, Darmstadt, West Germany, and purified by distillation, Casamino acids were from Difco Laboratories, Detroit, Mich., and DEAE-Sephadex was from Pharmacia Fine Chemicals, Uppsala, Sweden. Rabbit muscle phosphorylase was prepared by Manuel Pena in our laboratory. Acrylamide, *N,N'*-methylene bisacrylamide, *N,N,N',N'*-tetramethylene ethylenediamine, and sodium dodecyl sulfate (SDS) were from Bio-Rad Laboratories, Richmond, Calif.; Ultrogel AcA 44 and AcA 34 were from LKB, Bromma, Sweden. The other chemicals were of the purest grade available from commercial sources.

Preparation of compounds and their chemical analysis. L-1,2-Propanediol was synthesized from L-ethyl-lactate by the method of Golding et al. (9). The final distilled product showed an α_D^{25} of +20.1, which is in close agreement with the values of +20.7 (9) or +20.1 (3) already reported. For the preparation of D-1,2-propanediol, the procedure described by Sridhara et al. (22) was modified. Strain 3 was grown to stationary phase in 20 liters of mineral medium containing 0.1% of DL-1,2-propanediol. Grown cells were removed by centrifugation, and the supernatant fluid was concentrated by evaporation to 0.5 liters. D-1,2-Propanediol was extracted with an ether-ethyl alcohol mixture (in the ratio of 1:4 by volume) and concentrated. The final product, obtained by vacuum distillation, gave an α_D^{25} of −20.3, which is in agreement with the values of −20.5 (17) or −21.1 (22) reported previously. D- and L-1,2-propanediol concentrations were estimated by the chromotropic acid method (16), using DL-1,2-propanediol as standard.

D-, L-, and DL-lactaldehyde were synthesized, by reacting ninhydrin with L-, D-, and DL-threonine, respectively, according to the method of Zagalak et al. (27). The purified products were characterized by thin-layer chromatography (Silica Gel G) in *n*-butanol–acetone–water (4:5:1 by volume) (20). The lactaldehyde concentration was estimated by bisulfite binding (4).

Growth of cells. The inorganic components of the basal medium consisted of 34 mM NaH_2PO_4, 64 mM K_2HPO_4, 20 mM $(NH_4)_2SO_4$, 1 μM $FeSO_4$, 0.1 mM $MgSO_4$, and 10 μM $CaCl_2$. The solution was titrated with HCl to a final pH of 7.0. Carbon sources were added to the basal inorganic medium in the following concentrations: 0.01 M L-fucose and L-rhamnose for aerobic growth, and at 0.02 M for anaerobic growth; and 0.5% (wt/vol) casein acid hydrolysate for aerobic growth, and 1% (wt/vol) plus 1 mM pyruvate for anaerobic growth.

Aerobic growth was carried out at 37°C in swirled 2-liter Erlenmeyer flasks partially filled with medium. Anaerobic growth was carried out at the same temperature in 1-liter Erlenmeyer flasks completely filled with medium and gently stirred by a magnet. When larger quantities of anaerobically grown cells were needed, a 12-liter jar was used. Growth was monitored with a Klett-Summerson colorimeter with a number 42 filter.

Preparation of cell extracts. Cells were harvested at the end of logarithmic phase by centrifugation, washed in 10 mM Tris-hydrochloride buffer (pH 7.3), and suspended in four times their wet weight of the same buffer containing 2.5 mM NAD. The suspension was chilled in a tube at −20°C and sonically disrupted in an MSE sonicator set at an amplitude of 18 to 24 μm for periods of 30 s/ml of cell suspension. Extracts were clarified by centrifugation at 30,000 × *g* for 30 min at 4°C.

Enzyme assays. Spectrophotometric assays for propanediol oxidoreductase were performed at 25°C either in the direction of propanediol oxidation or in the direction of lactaldehyde reduction by following the absorbance at 340 nm (NADH formation or loss) in a Beckman DBGT recording spectrophotometer. The oxidation activity was measured in an assay mixture (1 ml) that consisted of 50 mM DL-1,2-propanediol, 100 mM sodium glycine buffer at pH 9.5, and 0.33 mM NAD. The reduction activity was measured in an assay mixture (1 ml) that consisted of 2.5 mM L-lactaldehyde, 100 mM sodium phosphate buffer at pH 7.0, and 0.125 mM NADH. In each case, the three-carbon substrate was omitted from the blank mixture. All reactions were started by the addition of the enzyme.

The concentration of protein in cell extracts and purified preparations was determined by the method of Lowry et al. (18), using bovine serum albumin as standard.

Gel electrophoresis. Acrylamide gel electrophoresis was performed in 7.5% gels at pH 7.5 by the method of Gabriel (7) and was conducted at 4°C and a current of 2.5 mA per tube for 2 h.

Sodium dodecyl sulfate (SDS) electrophoresis was performed in 7.5% acrylamide gel at pH 7.2 by the method of Weber et al. (25).

Gels were stained for propanediol oxidoreductase activity by incubation in a reaction mixture which contained (per liter): 100 mmol of glycine (brought to pH 9.5 by NaOH), 100 mmol of DL-propanediol, 1 mmol of NAD, 300 mg of nitroblue tetrazolium, and 100 mg of phenazine methosulfate. Protein was stained by treatment of the gels with 0.25% (wt/vol) Coomassie brilliant blue in 45% (vol/vol) methanol and 9% (vol/vol) acetic acid solution. After 2 h, the gels were destained by several rinsings with a 5% methanol and a 7.5% acetic acid (vol/vol) solution at 60°C.

RESULTS

Propanediol-oxidizing activity induced by rhamnose. Cell extracts of strain 1 (wild type) grown anaerobically on L-rhamnose contained a high level of propanediol oxidoreductase activity that is dependent on nicotinamide adenine dinucleotide as a coenzyme. Induction of synthesis of this enzymatic activity required both anaerobiosis and the presence of rhamnose. A similar enzyme activity was present in extracts of cells grown anaerobically on L-fucose (6) (Table 1).

To characterize this activity, extracts of cells of strain 1 grown under the four conditions were analyzed by polyacrylamide gel electrophoresis and stained for propanediol-oxidizing activity (Fig. 2). One component (c) was present in extracts of cells grown under all conditions. A second component (a) was noticeable in extracts of cells grown under all conditions except aerobically on rhamnose. A third component (b) was present in extracts of cells grown anaerobically on rhamnose or fucose, but not under the other two conditions. Band c probably represents the activity of glycerol dehydrogenase which also acts on propanediol; but the product in this case is acetol (22). Band a probably represents an enzyme produced under most anaerobic conditions. The nature of this protein was not pursued in this study. The electrophoretic mobility of the enzyme revealed as band b is the same as that of the propanediol oxidoreductase present at a high constitutive level in extracts of strain 3, which was selected for growth on propanediol as the sole source of carbon and energy. No such band was detected in extracts of strain 1 grown aerobically on fucose or in strain 32, a propanediol oxidoreductase-negative derivative of strain

TABLE 1. *Activities of propanediol oxidoreductase in crude extracts of strain 1 grown on different media*

Carbon source	Growth condition	Enzyme activity on:[a]	
		L-1,2-Propanediol	L-Lactaldehyde
Rhamnose	Aerobic	0.02	0.16
Rhamnose	Anaerobic	0.17	0.63
CAA[b]	Anaerobic	0.05	0.15
Fucose	Anaerobic	0.19	0.70

[a] Specific activities (units per milligram of protein) at 25°C.

[b] Casein acid hydrolysate.

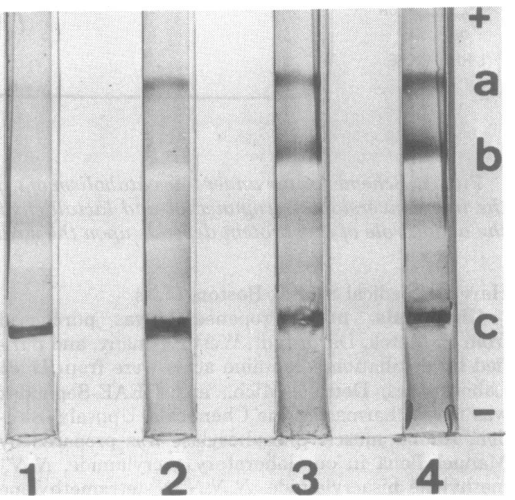

FIG. 2. *Polyacrylamide gel electrophoresis of crude extracts of E. coli stained for propanediol oxidoreductase activity. Cells were grown: (i) aerobically on L-rhamnose, (ii) anaerobically on casein hydrolysate, (iii) anaerobically on L-rhamnose and (iv) anaerobically on L-fucose. Band a probably corresponds to an unknown enzyme that oxidizes DL-1,2-propanediol. Bands b and c correspond to propanediol oxidoreductase and glycerol dehydrogenase, respectively (see text).*

3 grown on casein hydrolysate (data not shown).

Purification of the rhamnose-induced enzyme. The steps reported below were carried out at 2 to 4°C unless stated otherwise. In some purification steps, NAD was added to stabilize the enzyme (26).

The crude extract was brought to 40% saturation by the slow addition of solid ammonium sulfate. After equilibration for 20 min, the precipitate was removed by centrifugation at 30,000 × *g* for 15 min. The pellet was discarded, and the supernatant solution was brought to 55% ammonium sulfate saturation. After equilibration, the precipitate was collected by centrifugation and dissolved in 0.01 M Tris-hydrochloride buffer (pH 7.3) containing 2.5 mM NAD.

The enzyme solution was heated in a water bath at 58°C for 2.5 min and immediately cooled in an ice bath. Precipitated protein was removed by centrifugation at 30,000 × *g* for 20 min.

The supernatant fraction was applied to an Ultrogel AcA 44 column (5 by 70 cm) equilibrated and eluted (3-ml/min flux) with 0.01 M Tris-hydrochloride at pH 7.3. Seventy fractions (10 ml each) were collected and assayed for enzyme activity. Those that contained considerable levels of the activity were pooled.

NaCl was added to the pooled preparation to give a concentration of 0.18 M. The preparation was applied to a DEAE-Sephadex column (2.5 by 15 cm) equilibrated with 0.01 M Tris-hydrochloride buffer (pH 7.3) containing 0.18 M NaCl and 0.1 mM NAD. The column was washed with this same buffer. Protein was eluted (0.5-ml/min flux) with a 0.18 to 0.5 M linear gradient of NaCl in 400 ml of the same buffer. Fractions (10 ml) were collected, and those containing high enzyme activities (eluted in the vicinity of 300 mM NaCl) were combined. The enzyme solution was concentrated approximately 20 times by ultrafiltration in a Millipore chamber with a PTGC membrane (nominal molecular weight limit, 10,000).

The concentrated enzyme solution was applied to an Ultrogel AcA 44 column (1.5 by 100 cm) equilibrated with 0.01 M Tris-hydrochloride at pH 7.3 and was eluted with this same buffer (0.3-ml/min flux). Fractions (1.5 ml) were collected, and the four fractions with the highest activities were pooled and served as the source of the enzyme for the studies to be described.

Table 2 shows the results of a typical purification experiment. Since a significant level of glycerol dehydrogenase activity is present in crude cell extracts, and since this enzyme acts on carbon 2 and therefore does not reduce L-lactaldehyde, we measured the propanediol oxidoreductase by its ability to catalyze the reduction of the aldehyde with NADH as the hydrogen donor. (In the steps after the first Ultrogel chromatography, glycerol dehydrogenase was no longer detectable.)

SDS gel electrophoresis of purified propanediol oxidoreductase (about 10 μg of protein) gave a single band when stained for protein by Coomassie brilliant blue (Fig. 3A) with a mobility indicative of a molecular weight of 39,000 (Fig.

TABLE 2. *Purification of propanediol oxidoreductase*

Step	Protein (mg)	Sp act[a]	Yield (%)
Crude extract	840	0.60	100
Ammonium sulfate precipitation	330	0.97	63
Heat treatment	150	1.7	51
Ultrogel AcA 34 fractionation	28	8.0	44
DEAE-Sephadex column chromatography	10	13.0	24
Second Ultrogel AcA 34 fractionation	4	19.0	15

[a] The enzyme activity (units per milligram of protein) was assayed by the rate of NADH disappearance coupled with L-lactaldehyde reduction.

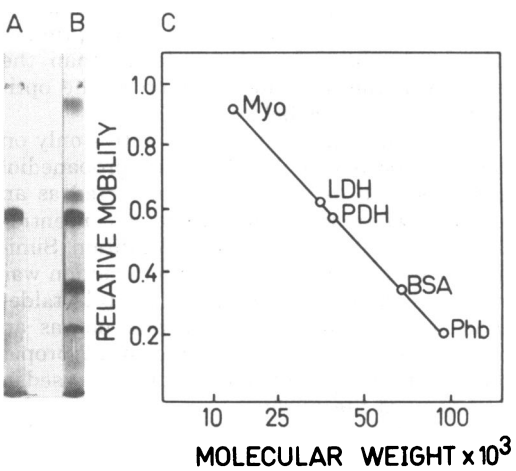

FIG. 3. *SDS-polyacrylamide gel electrophoresis of purified propanediol oxidoreductase. In gel A, a sample of purified protein (10 μg) was applied. In gel B, the same sample was applied together with the following protein markers: myoglobin (Myo), molecular weight 17,200; lactic dehydrogenase (LDH), molecular weight, 36,000; bovine serum albumin (BSA), molecular weight, 68,000; and phosphorylase b (Phb), molecular weight, 96,000. In panel C, the molecular weights of the various standard proteins have been plotted against their relative mobilities for the determination of the subunit molecular weight of propanediol oxidoreductase (PDH).*

3C). Minor traces of contaminating protein were observed if more than 30 μg of protein was applied. In the absence of SDS, even at the higher protein amounts, only a single band was visible when the gels were stained for propanediol oxidoreductase activity.

Molecular weight. The molecular weight of propanediol oxidoreductase was determined by gel permeation chromatography on a calibrated column (1.5 by 70 cm) of Ultrogel AcA 34 under nondenaturing conditions. A molecular weight of 76,000 daltons was indicated when the elution volume of the enzyme was compared with those of known protein standards: ovalbumin (molecular weight, 43,000), bovine serum albumin (molecular weight, 68,000), and yeast alcohol dehydrogenase (molecular weight, 126,000).

Catalytic properties. The enzymatic conversion of L-1,2-propanediol to L-lactaldehyde, with concomitant reduction of NAD to NADH, proceeded in a linear fashion for a longer period when the reaction mixture was buffered at pH 9.5 by glycine instead of carbonate (22). By preincubating the enzyme in each buffer for various periods of time before starting the reaction by substrate addition, we were able to show that glycine stabilized rather than activated the enzyme.

The purified enzyme catalyzed the reduction of L-lactaldehyde to propanediol at the optimum pH of 6.5, about four times faster than the reaction in the opposite direction at the optimum pH of 9.5 (see below and Fig. 5).

Propanediol oxidoreductase is active only on the L-1,2-propanediol. The D-1,2-propanediol neither served as a substrate nor acted as an inhibitor when added at equimolar concentrations under the standard assay condition. Similarly, it was shown that when the reaction was measured in the reverse direction, D-lactaldehyde acted neither as a substrate nor as an inhibitor. Consequently, racemic DL-1,2-propanediol or DL-lactaldehyde were routinely used in the assays of the enzyme.

The dehydrogenation of L-1,2-propanediol showed the substrate inhibition at concentrations higher than 25 mM (Fig. 4). (The same degree of inhibition was observed when the propanediol was added as the L-isomer in a racemic mixture.)

The kinetic parameters for the reaction catalyzed by the propanediol oxidoreductase were measured both in the direction of substrate oxidation and in the direction of substrate reduction, but in each case the measurements were carried out under the optimal conditions described in Materials and Methods. The K_m for L-1,2-propanediol was found to be 1.25 mM, and

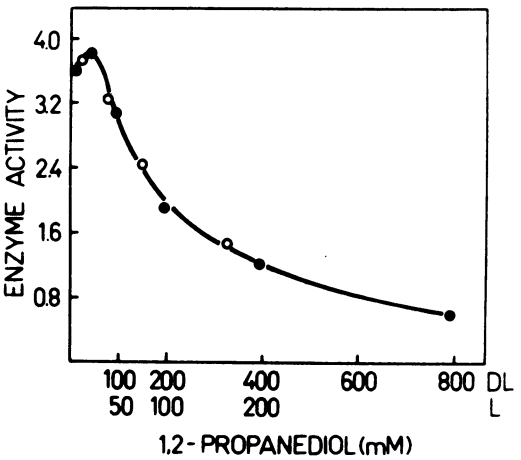

FIG. 4. *Substrate inhibition of purified propanediol oxidoreductase. Enzyme activity (units per milligram of protein) was assayed at the indicated concentrations of* DL-*1,2-propanediol* (●) *or* L-*1,2-propanediol* (○).

that for L-lactaldehyde was found to be 0.035 mM. In contrast, the K_m values for the cofactor NAD and NADH were both found to be close to 0.01 mM. The dehydrogenation of propanediol showed a V_{max} of 5.3 μmol min^{-1} mg^{-1} of protein, whereas the reduction of lactaldehyde showed a V_{max} of 20 μmol min^{-1} mg^{-1} of protein.

Effects of pH and temperature. The dehydrogenase activity of purified propanediol oxidoreductase exhibited an optimal pH of 9.5 in sodium-glycine buffer, whereas the reductase activity exhibited an optimal pH of 6.5 in sodium phosphate buffer (Fig. 5).

Arrhenius plots gave an energy of activation of 10 kcal (ca. 41.86 kJ) per mol for the dehydrogenase activity and 4.1 kcal (ca. 18.86 J) per mol for the reductase activity.

Specificity of propanediol oxidoreductase. Table 3 gives the initial rates of NAD reduction or NADH oxidation when purified propanediol oxidoreductase was tested with substrates and analogs at two different concentrations.

When tested at a concentration near the K_m of the dehydrogenase activity, only propanol and ethylene glycol showed a significant activity. Even at 50 mM, no activity was detected with methanol, isopropanol, *i*-erithritol, ribitol, xylitol, D-arabitol, sorbitol, galactitol, and mannitol (data not shown). Among the aldehydes tested at the K_m of the reductase activity, only DL-glyceraldehyde showed a significant affinity.

The enzyme did not use NADP or the analog 3-acetyl pyridine adenine dinucleotide and pyridine-3-aldehyde adenine dinucleotide as cofac-

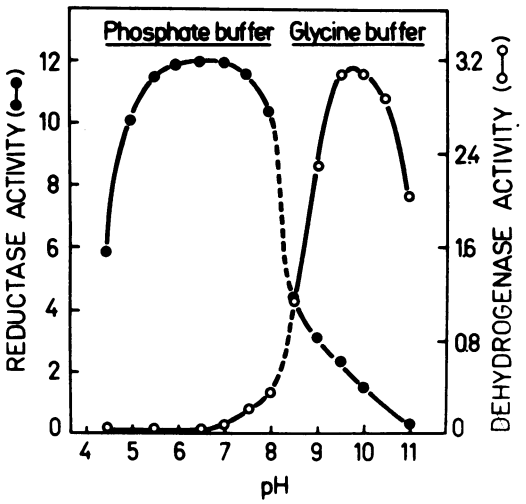

FIG. 5. *Effect of pH on the lactaldehyde reductase* (●) *and propanediol dehydrogenase* (○) *activities of purified propanediol oxidoreductase. Enzymatic activities (units per milligram of protein) were assayed in 0.1 M sodium phosphate buffer between pH 4.5 and 8.0 and in 0.1 M glycine buffer between pH 8.5 and 11.0.*

TABLE 3. *Substrate specificity of propanediol oxidoreductase*

Substrate	Relative dehydrogenase activity		Relative reductase activity	
	25 mM[a]	2.5 mM[a]	2.5 mM[a]	0.035 mM[a]
L-1,2-propanediol	100	100		
Glycerol	50	9		
Propanol	102	69		
Ethylene glycol	87	31		
Ethanol	51	12		
L-lactaldehyde			100	100
DL-glyceraldehyde			51	21
Propionaldehyde			58	8
Glycolaldehyde			74	12
Acetaldehyde			14	2

[a] Concentration of substrate in the assay mixture.

tors, but in contrast to NADP the presence of both analogs in the assay mixture inhibited the activity obtained with NAD.

Comparative study between the rhamnose- and fucose-induced enzyme activity. Propanediol oxidoreductase induced by anaerobic growth of cells of strain 1 on L-fucose was purified by the method described above for the L-rhamnose-induced enzyme. No differences in purification properties of both enzyme activities was detected along the fraction process. Electrophoretic mobilities of the final preparations in dissociating and nondissociating conditions were found to be indistinguishable for both enzymes.

A series of parallel determinations of kinetic parameters were conducted with both purified preparations. The pH profile, the V_{max} and K_m for the substrate and cofactors in both directions of catalysis were indistinguishable under our experimental conditions. Energy of activation for the dehydrogenation of propanediol by the fucose-induced enzyme was 9.2 kcal (ca. 38.5 J) per mol, very close to the value for the rhamnose-induced enzyme. Finally, no significant differences in substrate specificities were found between the two enzyme preparations.

DISCUSSION

Though L-fucose and L-rhamnose metabolism in *Escherichia coli* has been widely studied from a biochemical (5, 8, 10, 14, 23, 24) and genetic (6, 11, 13, 19) point of view, only in the case of L-fucose has the specific enzyme responsible for the fermentation of the sugar been identified and partially characterized. This enzyme is responsible for the reduction of L-lactaldehyde to propanediol, a process that regenerates NAD. We became interested in L-rhamnose fermentation because L-lactaldehyde is also an intermediate in this process. We found that, in this case too, a specific oxidoreductase is inducible only anaerobically.

The physiological role of this enzyme as lactaldehyde reductase is consistent with our observation that the K_m for the aldehyde is two orders of magnitude lower than the K_m for the alcohol. The relative magnitudes of the V_{max} and energy of activation are also consistent with this role.

In this study, no differences were noticed in the properties of the propanediol oxidoreductase purified from either rhamnose or fucose grown cells, but conclusive evidence concerning their identity cannot be drawn from the data. Small differences migh not be detected without more detailed analysis of protein fine structure, such as fingerprinting or sequencing.

Available evidence suggests that the same protein is induced when cells of *E. coli* ferment rhamnose or fucose, and this is further supported by the following facts. Strain 32, unlike strain 3, does not grow anaerobically on rhamnose, though both strains grow aerobically on this carbon source (J. Aguilar, unpublished data). The inability of strain 32 to grow anaerobically on rhamnose is presumably due to the lack of propanediol oxidoreductase activity, which besides is not induced by anaerobic growth in the presence of rhamnose (A. Boronat, unpublished data).

Nevertheless, we would like to point out that at present the question of whether *E. coli* use the same enzyme for the fermentation of both L-fucose and L-rhamnose or use two similar enzymes to accomplish the same goal remains open.

Genetic mapping experiments, as well as studies of the regulation of gene expression, are being performed to settle this problem.

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