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Ferrous-activated Nicotinamide Adenine Dinucleotide-linked Dehydrogenase from a Mutant of *Escherichia coli* Capable of Growth on 1,2-Propanediol

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A nicotinamide adenine dinucleotide-linked dehydrogenase has been partially purified from a mutant of *Escherichia coli* K-12 able to grow on L-1,2-propanediol as carbon and energy source. This enzyme catalyzes the dehydrogenation at carbon 1 of L-1,2-propanediol, glycerol, 1,3-propanediol, ethylene glycol, and ethyl alcohol. The purified protein requires added ferrous or manganous ions. The V_{max} and the apparent K_m for a given substrate vary with the particular metal used.

In a study of mutations that lead to the utilization of xylitol at progressively faster rates by *Aerobacter aerogenes* (22, 45), it was shown that several functions were genetically altered. The first-stage mutant became constitutive in the synthesis of ribitol dehydrogenase, an enzyme which is active on xylitol but which cannot be induced by it in wild-type cells. The second-stage mutant produced a dehydrogenase modified so that the ratio of activity on xylitol to that on ribitol was increased. The third-stage mutant became constitutive in the synthesis of the active transport system for D-arabitol, which acts on xylitol but cannot be induced by it. Together, these diverse genetic changes allowed effective capture and metabolism of the novel carbon source.

Another example of biochemical evolution of catabolic pathways has been observed in the progressive utilization of 1,2-propanediol by *Escherichia coli* (Wu, Chused, and Lin, Bacteriol. Proc., p. 52, 1967). An essential enzyme for the metabolism of this compound, found in mutant but not in wild-type cells, is a nicotinamide adenine dinucleotide (NAD)-linked propanediol dehydrogenase. The purification and some of the characteristics of this enzyme will be described.

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MATERIALS AND METHODS

Bacteria. The parental strain used was an alkaline phosphatase-negative derivative of *E. coli* K-12 Hfr-Cavalli (5, 10, 32) from the laboratory of C. Levinthal. This strain was designated in that laboratory as E15, but, for convenience, it is referred to in our studies as strain 1 (18). Strain 3, a mutant able to grow on 1,2-propanediol, was selected from strain 1 by means of the following procedure. Cells of the parental line were treated with the mutagen ethyl methanesulfonate (23, 26); a large inoculum (10^{10} cells) was transferred to 1 liter of basal medium containing 0.2% DL-1,2-propanediol as the sole potential source of carbon and energy and incubated at 37 C on a rotary shaker for 4 days. At the end of this period, the culture was visibly more turbid. A portion was withdrawn and subcultured repeatedly in a medium of DL-1,2-propanediol to allow approximately 100 mass doublings. A sample of the final culture was diluted and plated on agar containing 0.2% DL-1,2-propanediol. After 2 days of growth at 37 C, a large colony was selected. (In subsequent attempts to isolate mutants with similar growth rates on this novel carbon source, it was found that at least five mutational steps were necessary for the attainment of such a metabolic capacity.) The identity of the mutant was confirmed by phage T4. Like the parental strain, it was found to be sensitive to T4r$^+$ but resistant to T4rII (1). A spontaneous mutant, strain 25, was also selected and isolated by a similar procedure.

For the isolation of mutants no longer able to grow on 1,2-propanediol, cells of strain 3 were treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) (28), recycled on glucose, and plated on a MacConkey Agar (Difco) in which DL-1,2-propanediol was substituted for lactose. This agar contained

10 g of propanediol, 17 g of Peptone (Difco), 3 g of Proteose Peptone (Difco), 1.5 g of Bile Salts no. 3 (Difco), 5 g of sodium chloride, 0.03 g of Neutral Red (Difco), 0.001 g of Crystal Violet (Difco), 13.5 g of agar (Difco), and water to a total volume of 1 liter. Pale (nonfermenting) colonies were isolated and checked for the ability to grow on DL-1,2-propanediol as a carbon and energy source.

E. coli ML was from the Pasteur Institute collection. *E. coli* B and W were from stocks of Boris Magasanik.

Chemicals. DL-1,2-Propanediol was purchased from Fisher Scientific Co., Pittsburgh, Pa., and purified by distillation. L-1,2-Propanediol was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.; ethylene glycol and 1,3-propanediol from Eastman Kodak Co., Rochester, N.Y.; D-glyceraldehyde, L-glyceraldehyde, dihydroxyacetone, and bovine hemoglobin from Calbiochem, Los Angeles, Calif.; acetol from K & K Laboratories, Jamaica, N.Y.; L-threonine, NAD, reduced nicotinamide adenine dinucleotide (NADH), and nicotinamide adenine dinucleotide phosphate (NADP) from Sigma Chemical Co., St. Louis, Mo.; the analogues of NAD from P-L Biochemicals, Inc., Milwaukee, Wis.; *E. coli* alkaline phosphatase from Worthington Biochemical Corp., Freehold, N.J.; pig heart lactic dehydrogenase and ammonium sulfate (enzyme grade) from Mann Research Laboratories, Inc., New York, N.Y.; acid hydrolysate of casein from Nutritional Biochemicals Corp., Cleveland, Ohio; Sephadex G-200 from Pharmacia Inc., Piscataway, N.J.; and diethylaminoethyl (DEAE) anion-exchange cellulose from Bio-Rad Laboratories, Richmond, Calif. Chromotropic acid (Merck & Co., Inc., Rahway, N.J.) was recrystallized from ethyl alcohol.

Preparation of compounds and their chemical analysis. D-1,2-Propanediol was purified from a racemic mixture by growing strain 3 to stationary phase in 14 liters of medium containing 0.1% DL-1,2-propanediol as carbon source (growth limiting). After the cells were removed by centrifugation, the medium was concentrated by evaporation to 1 liter and mixed with 200 ml of ethyl ether and 800 ml of ethyl alcohol. The ether layer was collected and the aqueous phase was reextracted twice with 200 ml of ether and 800 ml of ethyl alcohol. The combined extracts were concentrated to a thick syrup and again extracted with 1 liter of the ether-ethyl alcohol mixture. After removal of the solvents from this final extract, the residue was purified by passage through a column of Dowex 1-HCO_3^- and a column of Dowex 50-H^+. The final product had a specific optical rotation $[(\alpha)_D^{25}]$ of -21.1 C, which is in close agreement with the value of -21.3 C reported for D-1,2-propanediol (46). The yield was 50% of the expected.

L-Lactaldehyde was synthesized from L-threonine (46). Presence of lactaldehyde was confirmed by bisulfite binding and by formation of the 2,4-dinitrophenylhydrazone (25). The latter was prepared by adding 10 ml of 0.1% 2,4-dinitrophenylhydrazine to 1 ml of sample containing 10 to 100 μmoles of the aldehyde. The precipitate formed was washed with water and dissolved in benzene. The benzene solution was passed through alumina and then chromatographed on paper (30) adjacent to the hydrazone of enzymatically generated L-lactaldehyde. 1,2-Propanediol, formed by enzymatic reduction of L-lactaldehyde, was verified both by the chromotropic acid method after periodate oxidation (9) and by the ninhydrin test of Jones and Riddick (16).

Growth of cells. The inorganic components of the basal medium consisted of 0.034 M NaH_2PO_4, 0.064 M K_2HPO_4, 0.02 M $(NH_4)_2SO_4$, 10^{-6} M $FeSO_4$, 3×10^{-4} M $MgSO_4$, 10^{-6} M $ZnCl_2$, and 10^{-5} M $CaCl_2$. The solution was titrated with HCl to a final *p*H of 7.0. Unless otherwise specified, sources of carbon and energy were added where indicated to give the following final concentrations: DL-1,2-propanediol, 0.2%; glycerol, 0.2%; glucose, 0.2%; fumarate, 0.1%; and casein hydrolysate, 1%.

Studies on growth rates and final cell yields were carried out at 37 C in 15 ml of medium. The cultures were incubated in 300-ml Erlenmeyer flasks fitted with side arms and swirled at about 240 cycles per min on a rotary shaker. We monitored growth by measuring the amount of turbidity in the side arm with a Klett colorimeter with a no. 42 filter. During exponential growth, one Klett unit corresponds to approximately 4×10^6 cells per ml. Cells for assay and purification of enzyme were grown in 2-liter Erlenmeyer flasks, each containing 1 liter of medium. Anaerobic growth was carried out under 95% nitrogen and 5% carbon dioxide with fumarate as exogenous hydrogen acceptor (14).

Preparation of cell-free extracts. Fully grown cultures were harvested by centrifugation at 0 C. The collected cells were washed once with ice-cold 1% NaCl, suspended in 0.01 M sodium phosphate (*p*H 7.0), and treated for 4 min in a model 60 W MSE ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London, England) while being chilled in a -10 C bath. The resulting suspension was centrifuged at 35,000 $\times$ *g* for 30 min at 0 C, and the supernatant fraction was used for enzyme assays and purification.

Enzyme assays. Dehydrogenase activities were measured at 25 C by following the appearance of NADH at 340 nm in a Gilford model 2000 recording spectrophotometer. The assay mixture for propanediol dehydrogenase, in a final volume of 3.0 ml, comprised 0.33 mM NAD, 200 mM sodium carbonate (*p*H 9.5), 100 mM DL-1,2-propanediol (unless indicated otherwise), and enzyme extract. Under usual conditions, the rate of reaction was constant for about 3 min. Glycerol dehydrogenase was assayed in 0.33 mM NAD, 33 mM ammonium sulfate, 100 mM sodium carbonate (*p*H 9.0), and 100 mM glycerol (24). Ethyl alcohol dehydrogenase was assayed in 0.67 mM NAD, 300 mM sodium carbonate (*p*H 9.0), and 1.6 M ethyl alcohohol (27). The reoxidation of NADH in the presence of various substrates was measured in a 3-ml mixture containing 0.33 mM NADH, 50 mM sodium phosphate (*p*H 7.0), 10 mM substrate, and enzyme extract. Protein concentrations were measured with the biuret reagent (12). A unit of activity represents the change of 1 μmole of NADH per min.

RESULTS

Growth properties of mutant cells. Wild-type *E. coli* K–12, B, ML, and W were each tested for their ability to utilize 1,2-propanediol as the sole source of carbon and energy. None showed any detectable growth. In contrast, strain 3, a mutant of K–12, grew readily in a medium containing this compound; the growth rate approximated that on glycerol (Fig. 1). To discover whether both optical isomers of 1,2-propanediol were utilizable, cells of the mutant were inoculated at low density in media containing limiting concentrations of the D-isomer, the L-isomer, the racemic mixture, or glycerol. Results of the growth studies are plotted in Fig. 2. On a molar basis, the maximal growth yield on L-1,2-propanediol was close to that on glycerol. The maximal growth yield on DL-1,2-propanediol was about one-half that on glycerol. No growth could be detected with D-1,2-propanediol. It was also found that with L-1,2-propanediol as carbon source, no diol-like substances remained in the media after the cessation of growth. With the racemic mixture, half of the diol content remained after growth had stopped. Evidently only the L-isomer could be utilized by the mutant.

Comparison of enzyme activities in extracts of wild-type and mutant cells. NAD-linked glycerol dehydrogenases from several bacterial sources have been reported to act also on 1,2-propanediol (11, 20, 24). An extract of strain 3 cells grown on 1,2-propanediol rapidly reduced NAD in the presence of glycerol, 1,2-propanediol, and, unexpectedly, also ethyl alcohol.

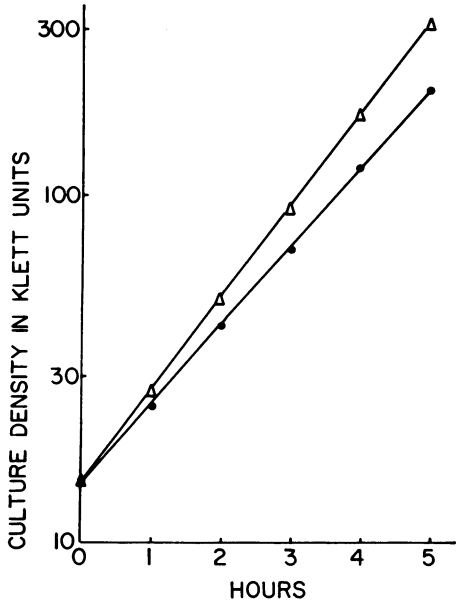

FIG. 1. *Growth rates of strain 3 on 1,2-propanediol (●) and glycerol (△).*

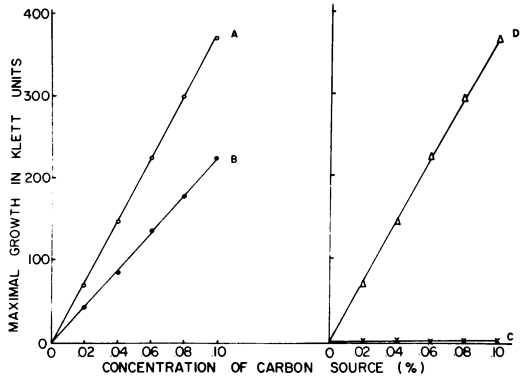

FIG. 2. *Maximal growth yields of strain 3 on L-1,2-propanediol (A), DL-1,2-propanediol (B), D-1,2-propanediol (C), and glycerol (D).*

To determine whether these activities were associated with a single protein, the crude extract was fractionated on a DEAE-cellulose column. Three peaks of enzymatic activities were found among the fractions successively eluted with a linear concentration gradient of NaCl. The first component was active only with ethyl alcohol as substrate; the second was active with glycerol, 1,2-propanediol, and ethyl alcohol; and the third was active with glycerol and 1,2-propanediol but not with ethyl alcohol.

Similar enzymic patterns were observed when the mutant was grown on glucose. For comparison, the wild-type strain 1 was grown on this sugar. Results in Fig. 3 A and 3 B clearly show that component II is novel in the mutant.

Components I and III, present in both parental and mutant cells, are probably the familiar ethyl alcohol dehydrogenase and glycerol dehydrogenase, respectively. In vivo they presumably function as reductases and are responsible for the production of ethyl alcohol and glycerol, which appear in the medium during fermentation by *E. coli* (3, 33). It may be noted that anaerobic growth elevated the levels of these two enzymes. (Compare areas under I and III for aerobic and anaerobic growth in Fig. 3. Fumarate was provided in anaerobic cultures to increase the growth yield. The increase of the two enzymes was not attributable to this supplement.) The possible role of component III as a catabolic enzyme for glycerol was excluded by the demonstration that another

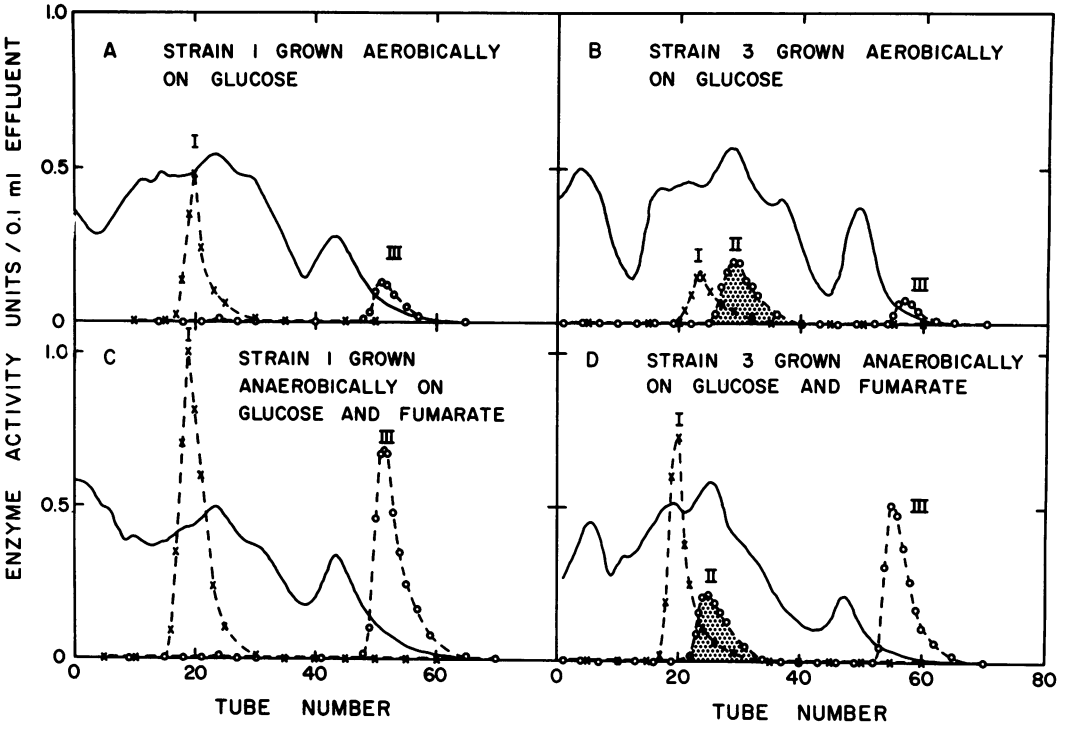

FIG. 3. *Resolution of dehydrogenase activities in extracts from parental (strain 1) and mutant (strain 3) cells by DEAE-cellulose chromatography. Solid lines represent absorbancy at 280 nm, expressed in arbitrary units. Dashed lines represent NAD-dependent enzyme activities. Component I represents activity with ethanol; components II and III with* DL-*1,2-propanediol (or glycerol). The high concentration of ethyl alcohol employed for the assay of component I inhibited the dehydrogenating activity of component II, which can also act on ethyl alcohol but at lower concentrations. Aerobic cells were grown on glucose alone, and anaerobic cells were grown on glucose with fumarate as the exogenous hydrogen acceptor.*

enzyme, glycerol kinase, was essential for glycerol utilization (18).

Purification of propanediol dehydrogenase. The new enzyme was purified from cells grown on casein hydrolysate and from cells grown on 1,2-propanediol; results from a typical experiment are given below. Unless otherwise specified, all procedures were carried out at 0 to 5 C, and the buffer used was 0.01 M sodium phosphate (*p*H 7.0).

To 60 ml of the cell-free extract containing about 850 mg of protein, 15 ml of 20% streptomycin sulfate was added slowly. The mixture was stirred for about 20 min, and the precipitate was removed by centrifugation. The supernatant volume was increased to 75 ml with buffer, and 24 g of solid ammonium sulfate was gradually added with constant stirring. After equilibration for 20 min, the precipitate was collected by centrifugation and dissolved in 35 ml of buffer. After samples had been withdrawn for protein and enzyme assay, this volume was diluted to 100 ml and introduced into a column (2.5 by 30 cm) of DEAE cellulose. The proteins were eluted with a linear gradient of NaCl (from 0 to 1 M) in 500 ml of buffer, and the effluent was collected in 5-ml fractions. The most active fractions were combined, and solid ammonium sulfate was added to 60% saturation. The precipitate collected after 30 min was dissolved in 3 ml of buffer and placed on a column (2.5 by 25 cm) of Sephadex G-200 equilibrated with buffer. A total filtrate volume of 75 ml was recovered in 2-ml fractions. Small amounts of each fraction were incubated in 3×10^{-5} M Fe^{++} at 25 C (for reasons given in the next section) and assayed for dehydrogenase activity with 1,2-propanediol as substrate. The five most active fractions were pooled, and the protein was precipitated by addition of solid ammonium sulfate to 60% saturation. The precipitate was dissolved in 2 ml of buffer. Table 1 summarizes the purification factors and yields obtained.

At all stages of purification, the activity was

proportional to protein concentration. There was no evidence that loss of activity during purification was caused by disaggregation of subunits.

Metal activation and enzyme stability. The enzyme was observed to lose most of its activity upon dialysis or upon brief storage after DEAE-cellulose chromatography. This loss was not prevented by the addition of mercaptoethanol with or without ethylenediaminetetraacetic acid (EDTA). However, as shown in Table 2, the lost activity could be restored by the addition of certain divalent cations, especially Fe^{++} and Mn^{++}. When the enzyme preparation recovered from the DEAE cellulose was filtered through the Sephadex column, virtually no activity remained. When a sample containing the enzyme protein was incubated at 25 C with 3×10^{-5} M Fe^{++} at *p*H 7.0, a gradual reactivation occurred, reaching maximal activity within 1 hr. Effective activation of the enzyme also could be achieved with 10^{-4} M Mn^{++} (Fig. 4). The enzyme, whether activated or not, was unstable below *p*H 6.5 or at temperatures above 37 C. When stored in the cold, even under favorable conditions (in the presence of Fe^{++} of Mn^{++}), activities were gradually lost and could not be restored with additional metal ions. Decay of the activity was most rapid during the first few days. Because of this instability, the enzyme was prepared fresh for each study, a procedure which accounts for the variation of specific activities in different experiments.

Substrate specificity of propanediol dehydrogenase and the influence of metal ions. Both the Fe^{++}-activated and the Mn^{++}-activated enzymes

TABLE 1. *Purification of propanediol dehydrogenase*

Step	Vol	Analysis		
		Protein	Enzyme activity	Specific activity[a]
	ml	*mg*	*units*	
Crude extract	60	840	160	0.19
Streptomycin supernatant fluid	75	840[b]	176	0.21[b]
Ammonium sulfate precipitation	35	460	147	0.32
DEAE-cellulose column chromatography	3	90	90	1.0
Sephadex G-200 fractionation	2	12	50	4.2

[a] Expressed as units per milligram of protein.
[b] Subject to error, because streptomycin sulfate interferes with the biuret reaction.

TABLE 2. *Effect of metal ions on propanediol dehydrogenase activity*[a]

Metal ion added	Enzyme activity[b]
None	25
Ba^{++}	20
Ca^{++}	23
Cd^{++}	0
Co^{++}	13
Cu^{++}	0
Fe^{++}	116
Mg^{++}	23
Mn^{++}	125
Ni^{++}	35
Zn^{++}	0

[a] Effluent fraction (40 mg of protein) from a DEAE-cellulose column was concentrated by precipitation with ammonium sulfate, redissolved in 2 ml of 0.01 M sodium phosphate at *p*H 7.0, and stored at −20 C for 12 hr. Samples (10 μliters) of this preparation were mixed with 90 μliters of the same buffer containing the divalent cation at 3×10^{-5} M and, after 1 hr at 25 C, were tested for dehydrogenase activity.
[b] Activity of a sample that was assayed (without metal addition) immediately before storage is taken as 100.

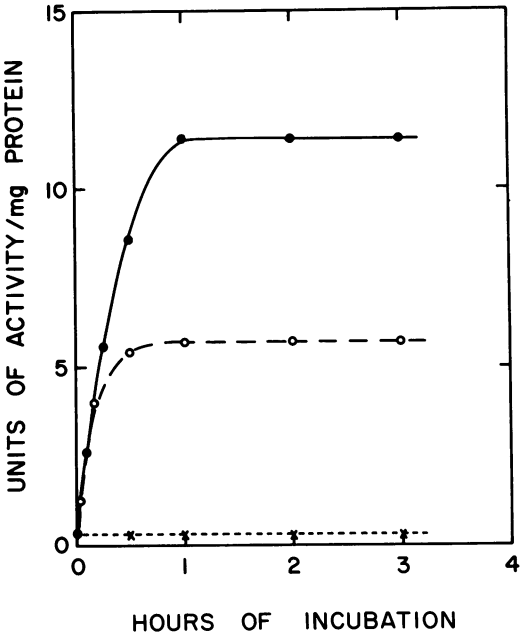

FIG. 4. *Metal-ion activation of the dehydrogenase. The fraction from the Sephadex step having the highest specific activity (5.7) was used. Reactivation was studied at 25 C by mixing 0.1 ml of the enzyme with 0.9 ml of 0.01 M sodium phosphate (pH 7.0) containing no additives (×), 3 × 10⁻⁵ M Fe⁺⁺ (○), and 10⁻⁴ M Mn⁺⁺ (●). At the intervals indicated, mixtures were sampled for propanediol dehydrogenase activity.*

acted on five compounds as substrates: L-1,2-propanediol, glycerol, 1,3-propanediol, ethylene glycol, and ethyl alcohol. Neither enzyme acted on D-1,2-propanediol, *i*-erythritol, D-arabitol, L-arabitol, ribitol, xylitol, mannitol, and sorbitol. The kinetic constants of the two metal enzymes (measured with NAD at enzyme-saturating concentrations) were different for a given substrate (Fig. 5). In Table 3, the constants for the Fe^{++}-activated dehydrogenase are compared with those of the mn^{++}-activated dehydrogenase. In all cases, the Mn^{++} enzyme exhibited a poorer apparent affinity, but the V_{max} values are equal to or higher than those observed with the Fe^{++} enzyme. Separate experiments showed that the Fe^{++}-dependent and the Mn^{++}-dependent activities were manifestations of the same protein. There was no additivity of dehydrogenase activity when both metals were tested together. Moreover, the patterns of distribution of the two types of activities during DEAE-cellulose chromatography and Sephadex fractionation were identical.

To test whether the dehydrogenase in the native state resembled either of the activated enzymes, the apparent Michaelis constants were determined with freshly prepared extracts. Although component III was also present in such a preparation, its contribution to the measured activity was relatively unimportant (Fig. 3B). The results, also given in Table 3, implicate the ferrous ion as the physiological cofactor for the enzyme.

All the compounds which served as substrates for the dehydrogenase possess a primary hydroxyl group. Thus, it seemed likely that dehydrogenation occurred at the terminal carbon giving rise to an aldehyde group. This idea was supported by the ability of the enzyme to catalyze the reoxidation of NADH in the presence of L-lactaldehyde but not of acetol.

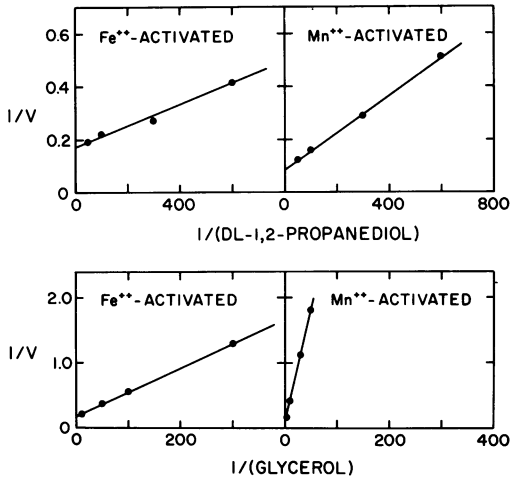

FIG. 5. *Lineweaver-Burk plots of the Fe^{++}-activated and Mn^{++}-activated dehydrogenases with DL-1,2-propanediol and glycerol as substrates. Velocities are expressed in terms of specific activity; substrate concentrations, in moles/liter.*

TABLE 3. *Comparison of kinetic constants of propanediol dehydrogenases on various substrates*[a]

Enzyme preparation	Substrate	K_m	V_{max}[b]
		M	
Fe^{++}-activated	L-1,2-Propanediol	0.002	5.7
	Glycerol	0.03	5.7
	1,3-Propanediol	0.03	5.7
	Ethylene glycol	0.007	5.7
	Ethyl alcohol	0.007	3.5
Mn^{++}-activated	L-1,2-Propanediol	0.01	11
	Glycerol	0.3	11
	1,3-Propanediol	0.3	5.7
	Ethylene glycol	0.16	11
	Ethyl alcohol	0.16	3.5
Crude extract	L-1,2-Propanediol	0.0015	0.53
	Glycerol	0.020	0.53

[a] Numerical values were derived from Lineweaver-Burk plots such as those shown in Fig. 5.
[b] Expressed as units per milligram of protein.

Stoichiometric interconversion of 1,2-propanediol and lactaldehyde. Lactaldehyde, as the product of enzymatic dehydrogenation of 1,2-propanediol, was first identified by treatment of the reaction mixture with hydrogen peroxide under a condition that would convert the aldehyde to lactic acid, which in turn could be detected with the aid of pig heart lactic dehydrogenase (15). Lactaldehyde also was quantitatively identified by means of its 2,4-dinitrophenylhydrazone. The amount of NADH formed in the reaction mixture agreed with the amount of lactaldehyde accounted for as the hydrazone (Table 4). The reverse reaction, the formation of 1,2-propanediol from lactaldehyde under the influence of the enzyme, was demonstrated by two different methods (Table 5). In this case, too, the amount of product formed agreed with the amount of NADH consumed.

Dehydrogenation at carbon 1 by the novel propanediol dehydrogenase distinguishes it functionally from the glycerol dehydrogenase present in both parental and mutant cells (component III in Fig. 3). The latter enzyme, as expected, was found to act on carbon 2; reoxidation of NADH occurred with dihydroxyacetone but not with D- or L-glyceraldehyde (data not shown).

Coenzyme specificity and pH optimum. Tests

TABLE 4. *Propanediol dehydrogenase: stoichiometry of the forward reaction*[a]

Expt	NADH formed	Lactaldehyde formed
	μmoles	*μmoles*
1	5.7	5.5
2	12.3	11.7

[a] Assay mixture, in a total volume of 3 ml, consisted of 100 mM DL-1,2-propanediol, 5 mM NAD, 200 mM sodium carbonate (*p*H 9.5), and 0.36 or 0.72 units of enzyme (specific activity 3.6). The reaction was allowed to proceed at 25 C for 1 hr, after which a sample was taken for the spectrophotometric determination of NADH, and the remainder of the mixture was brought to approximately *p*H 1 with HCl. After clarification by centrifugation, a sample was used for the estimation of lactaldehyde as the 2,4-dinitrophenylhydrazone.

TABLE 5. *Propanediol dehydrogenase: stoichiometry of the reverse reaction*[a]

Expt	NADH consumed	1,2-Propanediol formed
	μmoles	*μmoles*
1	3.0	2.8
2	5.8	5.7

[a] Assay mixture, in a total volume of 3 ml, consisted of 23 mM L-lactaldehyde, 3 mM NADH, 10 mM sodium phosphate (*p*H 7.0), and 0.18 or 0.36 units of enzyme (specific activity 3.6). The reaction was allowed to proceed at 25 C for 30 min, after which a sample was immediately taken for the spectrophotometric determination of NADH, and the remainder of the mixture was heated for 5 min in a boiling water bath. After the mixture cooled and was subjected to centrifugation, samples were taken for 1,2-propanediol estimation by the chromotropic acid method, after periodate oxidation, and by the ninhydrin method.

were carried out with DL-1,2-propanediol as substrate. Thionicotinamide-NAD (0.33 mM) gave 0.9 times the activity observed with NAD in the case of the Fe^{++}-activated enzyme and 0.4 times the activity observed with NAD in the case of the Mn^{++}-activated enzyme. NADP, 3-acetylpyridine-NAD, 3-acetylpyridine-deamino-NAD, deamino-NAD, and 3-pyridinealdehyde-NAD were inactive as coenzymes. Both the Fe^{++}- and Mn^{++}-activated enzymes displayed a broad *p*H optimum in the region of 9.5.

Distribution patterns of activated and nonactivated dehydrogenase in a density gradient. Because the activity of the enzyme disappeared upon removal of metal ions and reappeared upon incubation with either Fe^{++} or Mn^{++}, the possibility of subunit dissociation of the protein in the absence of the activators was explored. Freshly prepared enzyme (from the DEAE-cellulose step) was activated with Fe^{++} or with Mn^{++}, or not activated. In each case, a single symmetrical peak of 1,2-propanediol activity was observed when the samples were subjected to centrifugation in a sucrose density gradient (Fig. 6). With *E. coli*

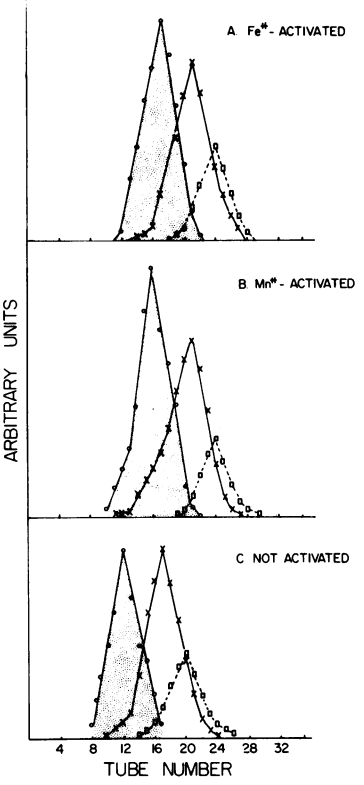

FIG. 6. *Density-gradient distribution of activated and nonactivated propanediol dehydrogenase. Freshly prepared enzyme from the DEAE-cellulose step (specific activity 2.2) was divided into three portions. One was activated with 3 × 10⁻⁵ M Fe^{++} (A), the second with 3 × 10⁻⁵ M Mn^{++} (B), and the third not activated (C). A portion of each dehydrogenase preparation (2 mg) was mixed with 0.5 mg of E. coli alkaline phosphatase and 2 mg of bovine hemoglobin in a volume of 0.15 ml and layered on 5 ml of sucrose (5 to 20% linear gradient) in 0.01 M sodium phosphate (pH 7.0). Centrifugation was in a Spinco model L centrifuge operated at 39,000 rev/min for 16 hr at 4 C. After punctures were made at the bottoms, the contents of tubes with A, B, and C were collected, respectively, in 35, 35, and 32 equal fractions. Samples of each fraction from tube C were activated with Fe^{++} before analysis. Alkaline phosphatase was assayed by the hydrolysis of p-nitrophenylphosphate (35) and hemoglobin at 540 nm. Symbols: propanediol dehydrogenase activity (○); alkaline phosphatase (×); and hemoglobin (□).*

alkaline phosphatase and bovine hemoglobin as markers, the molecular weight of the dehydrogenase under each of the three conditions was estimated, by the method of Martin and Ames (29), to be approximately 10^5.

1,2-Propanediol dehydrogenase of strain 25 Cells of strain 25, selected by recycling on DL-1,2-propanediol as a carbon source without the use of a mutagen, were grown on casein hydrolysate and examined to see whether the same solution used by strain 3 was exploited for growth on the novel carbon source. When extracts of strain 25 were assayed for an NAD-linked dehydrogenase, a similar enzyme was found. This enzyme was purified similarly. It also required Fe^{++} or Mn^{++} for activation, and its substrate specificity was essentially the same as that of the strain 3 enzyme. Several similar mutants were isolated from the wild-type strain after mutagenesis with NG (David Brandon, *unpublished results*).

Derivatives of strain 3 lacking 1,2-propanediol dehydrogenase. Four independent mutants incapable of growth on 1,2-propanediol were obtained from strain 3 after mutagenesis. Two of these mutants lacked the dehydrogenase when grown on either casein hydrolysate or glucose. The remainder exhibited normal levels of the dehydrogenase. Initial studies indicated that the latter mutants were blocked in the metabolism of lactaldehyde.

DISCUSSION

Several NAD-linked dehydrogenases have been examined for the presence of intrinsic metal ions, and, in all the cases studied, they have been found to be zinc metalloenzymes. These include alcohol dehydrogenase from yeast (40, 41) and from equine (34, 42) and human (44) liver; glutamic dehydrogenase from bovine liver (39); and lactic dehydrogenase from rabbit muscle (43). However, it has not yet been possible to dissociate the intrinsic metal reversibly from these enzymes. The spontaneous dissociation of the cofactor from 1,2-propanediol dehydrogenase not only revealed its metal dependence, but also led to the unexpected discovery that at least two different metal ions, Fe^{++} and Mn^{++}, could effectively restore catalytic activity. More significantly, the apparent affinities for several substrates depended on which metal was used for activation.

The modification of enzyme specificity by metal ions has been observed in several systems. The K_m values of myosin for hydrolysis of various ribonucleoside triphosphates depend on the types of cation present (17). Replacement of Zn^{++} at the active center of carboxypeptidase A and B by other divalent metal ions such as Cd^{++} results in a virtual curtailment of the peptidase activity and an enhancement of the esterase activity of the enzyme (6, 7, 37, 38). Zn^{++}, but not Mg^{++}, promotes the hydroxylamine phosphorylating activity of pyruvic kinase. The reverse effect holds for the fluorokinase activity of the enzyme (19). The replacement of Mg^{++} by Mn^{++} permits the incorporation of ribonucleotides into the polynucleotide synthesized by DNA polymerase (2). The propanediol dehydrogenase reported here might provide another system for the study of substrate specificity depending on the nature of the metal ion.

An intriguing question is what the original role of the structural gene for the dehydrogenase protein was in the parental strain. An NAD-linked dehydrogenase, active on 1,2-propanediol but not on glycerol, has been discovered in cells of *Pseudomonas aeruginosa* grown on the three-carbon glycol (13). However, none of the common *E. coli* strains tested in the present study is able to grow on this compound. It is improbable, therefore, that the genetic change in the mutant represents a trivial reversion of a "loss mutation" sustained by one of its predecessors. The possibility that the enzyme acts physiologically as a dehydrogenase for 1,3-propanediol, ethylene glycol, or ethyl alcohol is also unlikely because neither the mutant nor the wild-type strain can grow at an appreciable rate on any of these compounds. The same may be said of *i*-erythritol, D-arabitol, L-arabitol, ribitol, and xylitol. Nor is it likely that the protein functions as an ordinary hydrogen-disposal enzyme during fermentation, since it is absent in the parental strain grown anaerobically on glucose. The instability of the enzyme, together with the fact that several mutational steps were necessary for the derivation of the mutant strain 3, suggests that modification of substrate specificity occurred during the process. If such is the case, then the original function of the protein might not be readily revealed by closer study of the catalytic properties of the mutant enzyme.

Several examples are now available in which a mere genetic derepression of an enzyme is sufficient to permit growth on a compound which is a substrate of that enzyme but not its inducer (4, 8, 21, 22, 31, 36, 45). A systematic analysis of each of the series of 1,2-propanediol mutants is required to determine whether, and at which step, this basic strategy is employed.

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