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Induction and Regulation of a Nicotinamide Adenine Dinucleotide-Specific 6-Phosphogluconate Dehydrogenase in *Streptococcus faecalis*

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Streptococcus faecalis grown with glucose as the primary energy source contains a single, nicotinamide adenine dinucleotide phosphate (NADP)-specific 6-phosphogluconate dehydrogenase. Extracts of gluconate-adapted cells, however, exhibited 6-phosphogluconate dehydrogenase activity with either NADP or nicotinamide adenine dinucleotide (NAD). This was shown to be due to the presence of separate enzymes in gluconate-adapted cells. Although both enzymes catalyzed the oxidative decarboxylation of 6-phosphogluconate, they differed from one another with respect to their coenzyme specificity, molecular weight, *p*H optimum, K_m values for substrate and coenzyme, and electrophoretic mobility in starch gels. The two enzymes also differed in their response to certain effector ligands. The NADP-linked enzyme was specifically inhibited by fructose-1,6-diphosphate, but was insensitive to adenosine triphosphate (ATP) and certain other nucleotides. The NAD-specific enzyme, in contrast, was insensitive to fructose-1,6-diphosphate, but was inhibited by ATP. The available data suggest that the NAD enzyme is involved primarily in the catabolism of gluconate, whereas the NADP enzyme appears to function in the production of reducing equivalents (NADPH) for use in various reductive biosynthetic reactions.

Streptococcus faecalis ferments glucose with the stoichiometric production of lactate (3, 17), and the lactate formed is derived more or less exclusively from the Embden-Meyerhof pathway (17). This is in spite of the fact that glucose-adapted cells possess relatively high constitutive levels of the enzymes of at least the oxidative portion of the hexose-monophosphate pathway (20). Preferential channelling of glucose carbon through the Embden-Meyerhof pathway appears to be accomplished in *S. faecalis* by the specific interaction of fructose-1,6-diphosphate (FDP) with a key enzyme from each of the two pathways, which results in differential alterations of their catalytic activity. The Embden-Meyerhof pathway enzyme, lactate dehydrogenase (LDH), is completely dependent upon FDP for catalytic activity (24), whereas the hexose-monophosphate pathway enzyme, 6-phosphogluconate dehydrogenase (EC 1.1.1.44), is strongly inhibited by FDP (1). Thus, under conditions where a sufficient intracellular pool of FDP is present to activate the LDH, the activity of 6-phosphogluconate dehydrogenase (6PGD) is presumably restricted.

In contrast to the glucose fermentation, the gluconate fermentation follows quite a different pattern in *S. faecalis*. The stoichiometry of the latter is very close to 1.83 moles of lactate and 0.5 mole of CO_2 per mole of gluconate degraded (21). Moreover, the specific activity of the $^{14}CO_2$ produced from gluconate-*1*-^{14}C by gluconate-adapted cells is the same as that of the substrate (21), which means that about 50% of the gluconate degraded is converted initially to ribulose-5-phosphate and CO_2 by the action of 6PGD. It is important to note that, although gluconate-adapted cells also possess the Entner-Doudoroff pathway (21)

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which yields pyruvate-*1*-^{14}C from gluconate-*1*-^{14}C, little or no $^{14}CO_2$ is produced from pyruvate-*1*-^{14}C by gluconate-adapted cell suspensions of *S. faecalis* under anaerobic conditions at *p*H 7.0 or below (C. L. Wittenberger, *unpublished data*). The studies with gluconate-*1*-^{14}C, therefore, do indeed appear to provide a fairly accurate estimation of the amount of gluconate carbon that actually passes through the 6PGD step of the hexose-monophosphate pathway. This being so, it is difficult, if not impossible, to reconcile the observed regulatory effects of FDP on the 6PGD and LDH derived from *glucose*-adapted cells with the major role played by 6PGD in gluconate catabolism by *gluconate*-adapted cells. It is to this problem that the present study was directed.

MATERIALS AND METHODS

Chemicals. The coenzymes, disodium 6-phosphogluconate, fructose-1,6-diphosphate, and streptomycin sulfate were products of Sigma Chemical Co., St. Louis, Mo. 6-Phosphogluconate-*1*-^{14}C was purchased from New England Nuclear Corp., Boston, Mass. Ultrapure ammonium sulfate was used in all enzyme fractionation procedures and was obtained from Mann Research Laboratories, New York, N.Y.

Organism and culture conditions. *S. faecalis* MR (15) was grown anaerobically in a complex medium containing gluconate (0.5%, w/v) as the primary energy source, as described previously (1). Growth substrates other than gluconate were sterilized separately and added to the medium aseptically at a final concentration of 0.5% (w/v) unless otherwise noted. Cells were harvested from all media in the stationary phase of growth. They were washed, dried by lyophilization, and stored at −20 C until used, as previously described (1).

Preparation of cell extracts. Lyophilized cell powder was suspended in cold 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.5, and the cell suspension was disrupted by treatment with a Branson model W140D Sonifier for 20 min at maximal voltage output. The disrupted cell suspension was then centrifuged in the cold at 37,000 × *g* for 30 min, and the supernatant fluid (crude extract) was collected by decantation. Crude extracts usually contained between 12 and 20 mg of protein per ml.

Enzyme assays: NADP-6PGD. Enzyme activity was measured by following the increase in absorption at 340 nm resulting from the 6-phosphogluconate-dependent reduction of nicotinamide adenine dinucleotide phosphate (NADP). Assay conditions, enzyme units, and specific activity were as previously described (1).

NAD-6PGD. Assay conditions were exactly as described for NADP-6PGD except that 3.0 mM nicotinamide adenine dinucleotide (NAD) was substituted for NADP. Enzyme units and specific activity were also as described for NADP-6PGD. Deviations from this standard assay system are noted in individual experiments.

Protein determination. Protein was determined by the biuret method (4), or, with fractions containing high concentrations of ammonium sulfate, the method of Warburg and Christian was used as described by Kalckar (6).

Starch-gel electrophoresis. Starch blocks were prepared essentially as described by Gasser (2) by dissolving 32 g of starch in 230 ml of a boiling solution containing citric acid (1.4 mM) and Na_2HPO_4 (8.6 mM). The final *p*H of the solution was 7.0. The starch solution was poured into molds, allowed to cool, and then removed for sample application and electrophoresis as described by Gasser (2).

Preliminary fractionation procedure for 6PGD. All procedures were carried out at 4 C.

Step 1: Crude extract. A crude extract was prepared from lyophilized, gluconate-grown cells as already described.

Step 2: Streptomycin sulfate treatment. A solution of streptomycin sulfate (in water) was slowly added to the crude extract with constant stirring until a final concentration of 25 mg/ml was reached. The turbid mixture was stirred for at least 5 min and then centrifuged at 37,000 × *g* for 15 min. The precipitate was discarded.

Step 3: Ammonium sulfate fractionation. Solid ammonium sulfate was added slowly to the step 2 supernatant fluid to 45% saturation. The protein precipitate was removed by centrifugation and discarded.

Step 4: Ammonium sulfate precipitation. Additional solid ammonium sulfate was slowly added to the step 3 supernatant fluid to 70% saturation. The protein precipitate was collected by centrifugation and redissolved in a minimal volume of 0.05 M Tris-hydrochloride buffer, *p*H 7.5.

A summary of the distribution of NAD- and NADP-6PGD activity in the various fractions is given in Table 1.

RESULTS

Effect of FDP on the LDH and 6PGD from glucose- or gluconate-adapted S. faecalis. One possible explanation for the active role played by 6PGD in the gluconate fermentation was that the LDH in gluconate-adapted cells, unlike the enzyme in glucose-grown cells, no longer had a requirement for FDP for catalytic activity. The intracellular pool of FDP, therefore, might be maintained at a level below that which would be inhibitory to the 6PGD. This was apparently not the case, however, for LDH activity in extracts of cells grown on gluconate, or any of several other carbon sources, was just as dependent upon FDP as was the enzyme in extracts of glucose-grown cells (Table 2).

Another possible explanation for the ability of *S. faecalis* to metabolize gluconate extensively through the 6PGD step of the hexose-monophosphate pathway was that gluconate-

TABLE 1. *Summary of preliminary fractionation procedure for 6-phosphogluconate dehydrogenase (6PGD) from gluconate-adapted S. faecalis*

Fraction[a]	Total protein (mg)	Total units[b]		Specific activity (units/mg of protein)		
		NAD	NADP	NAD	NADP	NADP/NAD
1. Crude extract	345	26.0	28.5	0.075	0.083	1.11
2. Streptomycin sulfate supernatant fluid	308	20.0	27.5	0.065	0.089	1.37
3. 0-45% $(NH_4)_2SO_4$ supernatant fluid	190	16.5	25.5	0.087	0.134	1.54
4. 45-70% $(NH_4)_2SO_4$ precipitate	120	15.0	25.0	0.125	0.208	1.66

[a] See Materials and Methods for complete description of fractions.
[b] 6PGD activity was assayed with NAD or NADP as described in Materials and Methods.

TABLE 2. *Effect of fructose-1,6-diphosphate (FDP) on lactate dehydrogenase (LDH) activity in cell-free extracts of S. faecalis grown on various carbon sources*

Carbon source[a]	LDH activity[b]	
	-FDP	+1 mM FDP
Gluconate	0.020	1.10
Glucose	0.015	1.43
Fructose	0.010	1.71
Galactose	0.010	1.11
Ribose	0.013	1.30
Sucrose	0.024	1.84
Lactose	0.011	1.49
Mannitol	0.020	1.52
Sorbitol	0.015	1.01

[a] *S. faecalis* MR was grown anaerobically in complex medium with the indicated carbon source (0.5%, w/v) as previously described (1). The inoculum for each culture had been through at least two transfers in medium containing the carbon source shown. Cells were harvested in the stationary phase of growth from 125-ml cultures and washed; cell-free extracts were prepared as described in Materials and Methods.
[b] LDH activity (units per milligram of protein) in the cell-free extracts was assayed as previously described (24). Where indicated, FDP was included in the assays.

TABLE 3. *Effect of fructose-1,6-diphosphate (FDP) on 6-phosphogluconate dehydrogenase (6PGD) activity in cell-free extracts of S. faecalis grown on various carbon sources*

Carbon source[a]	6PGD activity[b]		Inhibition (%)
	-FDP	+10 mM FDP	
Gluconate	.075	.017	77.3
Glucose	.072	.018	75.0
Fructose	.075	.014	81.3
Galactose	.060	.018	70.0
Ribose	.064	.013	79.7
Sucrose	.070	.015	78.6
Mannitol	.069	.017	75.4
Sorbitol	.080	.016	80.0

[a] *S. faecalis* MR was grown anaerobically with the carbon source shown, and cell-free extracts were prepared from cells harvested from 125 ml of medium as described for Table 2.
[b] NADP-linked 6PGD activity (units per milligram of protein) was assayed in cell-free extracts without and with FDP as described in Materials and Methods. High concentrations of FDP were used here to insure that maximal inhibition would occur in all cases. It was previously shown that the maximal inhibition with a partially purified NADP-6PGD from glucose-adapted cells was only about 80% (1).

adapted cells synthesized an altered NADP-linked 6PGD which was insensitive to inhibition by FDP. This explanation also proved unsatisfactory, for the 6PGD activity in extracts of cells grown on gluconate, glucose, or various other carbon sources was more or less equally sensitive to the negative effector (Table 3).

Coenzyme specificity of 6PGD from gluconate-adapted cells. It was established previously (1) that a partially purified 6PGD from glucose-grown cells exhibited specificity for NADP. When the enzyme from gluconate-adapted cells was tested for its coenzyme specificity, however, it was observed that the specific activity in crude extracts was about the same with either NADP or NAD (Table 4). It is important to note that 6PGD activity with NAD either was not detectable in extracts of glucose-adapted cells or, as is shown in Table 4, was occasionally observed at very low levels. Moreover, the activity with NAD observed in extracts of gluconate-adapted cells was almost completely absent in extracts of cells grown on a mixture of gluconate and glucose (Table 4). This was true regardless of whether the inoculum for the gluconate plus glucose culture was from a glucose- or a gluconate-adapted

culture. These results suggested that the 6PGD activity observed with NAD in gluconate-grown cells was due to the gluconate-induced elaboration of a separate and distinct NAD-specific enzyme whose synthesis was repressed by glucose.

Evidence for distinct NADP- and NAD-specific 6PGD activities in gluconate-adapted cells. When a cell-free extract of gluconate-adapted cells was carried through the preliminary fractionation procedure outlined in Materials and Methods, both NAD- and NADP-6PGD activities were present in all fractions. However, the ratio of specific activity with NADP to that with NAD was not constant, but actually increased from 1.11 in the crude extract to 1.66 in the step 4 fraction (Table 1). These results further indicated that gluconate-adapted cells possessed two 6PGD's which differed in their coenzyme specificity.

Partial resolution of the two activities was achieved when the step 4 fraction (Table 1) was chromatographed on a Bio-Gel P-150 column (Fig. 1), and a complete separation of the two activities was achieved by starch-gel electrophoresis of crude extracts (Fig. 2). It is to be emphasized that when cell extracts of glucose-adapted *S. faecalis* were subjected to starch-gel electrophoresis under conditions identical to those described for Fig. 2, 6PGD activity spots were seen only with NADP. It was clear, therefore, that gluconate-adapted cells did indeed possess two distinct 6PDG's: one specific for NAD and the other specific for NADP. The activity elution profile from the Bio-Gel P-150 column (Fig. 1) also shows that the two enzymes differed with respect to their molecular weights.

Effect of FDP on the NADP- and NAD-specific 6PGD from gluconate-adapted cells. The NADP-linked 6PGD was strongly inhibited by FDP, as was shown previously for the enzyme from glucose-adapted cells (1), but the NAD-specific 6PGD was totally insensitive to the FDP effect (Fig. 3). This appeared to provide an explanation for the ability of gluconate-adapted cells to metabolize gluconate extensively through the hexose-monophosphate pathway, under conditions where an intracellular pool of FDP sufficient to activate the LDH would be expected to restrict severely the activity of the NADP-linked 6PGD.

General properties of the NAD-6PGD and NADP-6PGD. As already indicated, the NAD- and NADP-linked enzymes differed from each other with respect to their electrophoretic mobility in starch gel (Fig. 2) and in terms of their relative molecular weights (Fig. 1). They also differed in their sensitivity to

TABLE 4. *6-Phosphogluconate dehydrogenase (6PGD) activity with NADP or NAD in extracts of S. faecalis grown on glucose, gluconate, or glucose plus gluconate*

Growth substrate[a]	6PGD activity[b]	
	NADP	NAD
Glucose	0.068	0.004
Gluconate	0.068	0.068
Glucose + gluconate	0.080	0.008

[a] *S. faecalis* MR was grown anaerobically with glucose (0.5%, w/v), gluconate (0.5%, w/v), or a mixture of the two (each at 0.5%, w/v) as the major energy source. A glucose-adapted culture served as the inoculum for the three cultures shown, but identical results were obtained when a gluconate-adapted inoculum was used. Growth conditions, harvesting and washing of cells, and preparation of cell-free extracts were as described for Table 2.

[b] 6PGD activity (units per milligram of protein) in the cell-free extracts was assayed with NADP or NAD as described in Materials and Methods.

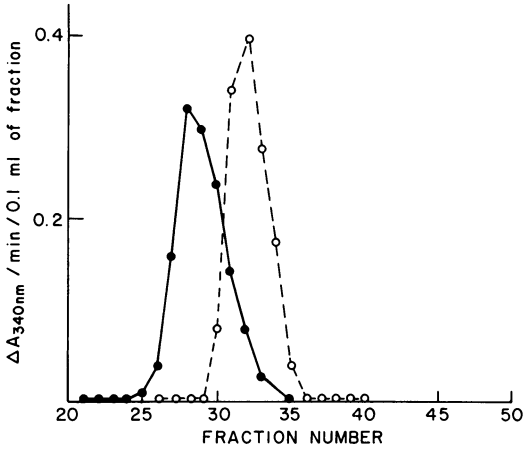

FIG. 1. *Partial resolution of NAD-linked 6PGD and NADP-linked 6PGD on a Bio-Gel P-150 column. The step 4 fraction from Table 1 was desalted by passage through a Sephadex G-25 column that was equilibrated at 4 C with 0.05 M Tris-hydrochloride buffer, pH 7.5. The Sephadex G-25 eluate (4.5 ml), which contained both NAD- and NADP-6PGD activity, was then applied to a Bio-Gel P-150 column (1.9 cm × 100 cm bed volume) that was previously equilibrated with 0.05 M Tris-hydrochloride, pH 7.5, and the sample was eluted from the column with the same buffer. The column was run at 4 C, and effluent fractions of 5 ml each were collected. The void volume of the column (140 ml) was determined with thyroglobulin as a marker. Effluent fractions were assayed for 6PGD activity with NAD (●) or NADP (○) as described in Materials and Methods.*

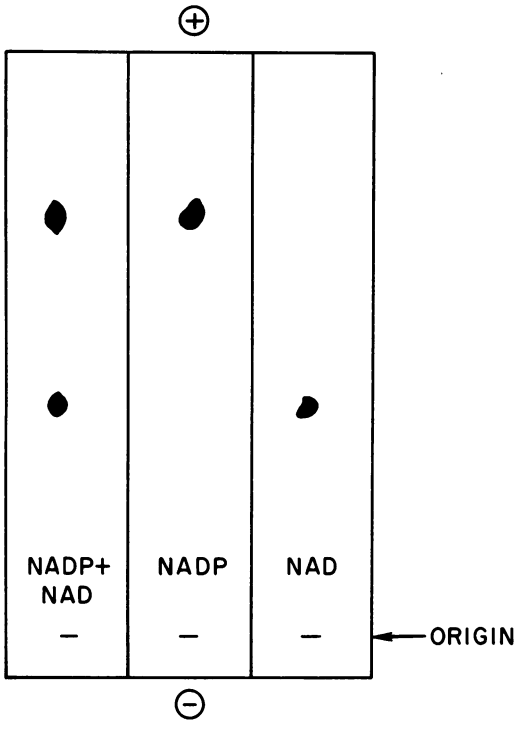

FIG. 2. *Separation of NAD-linked 6PGD and NADP-linked 6PGD by starch-gel electrophoresis. Samples of a crude extract of gluconate-adapted S. faecalis containing 50 to 70 μg of protein were applied to each of three positions on a starch block by means of filter-paper wicks. After overnight electrophoresis, under conditions described by Gasser (2), the starch block was cut longitudinally into three pieces corresponding to each of the three samples. Each piece was stained for 6PGD activity by incubation in the dark at room temperature in 25 ml of the following solution: 0.1 M Tris-hydrochloride buffer, pH 7.5; 6-phosphogluconate, 40 mg; NAD, 45 mg, or NADP, 12 mg or both, as indicated; nitro blue tetrazolium, 8.0 mg; and phenazine methosulfate, 0.6 mg. A composite of the results is represented diagrammatically.*

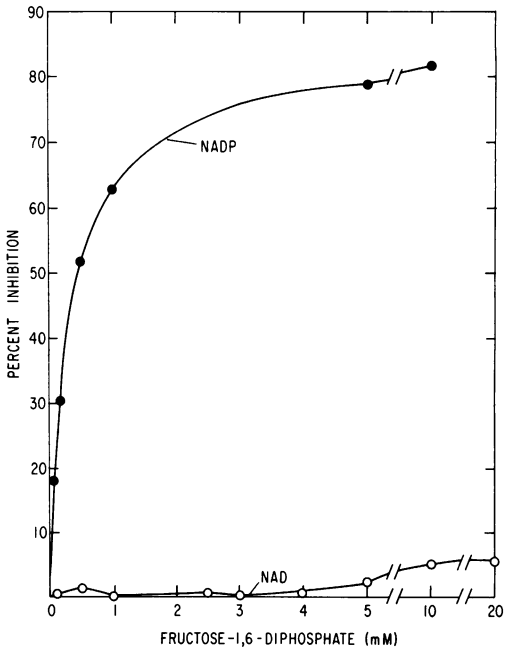

FIG. 3. *Effect of fructose-1,6-diphosphate on the NAD-linked 6PGD and NADP-linked 6PGD from gluconate-adapted S. faecalis. Assays for 6PGD with NAD (○) or NADP (●) were carried out as described in Materials and Methods except that, where indicated, various concentrations of fructose-1,6-diphosphate were included in the reaction mixtures. The source of the NAD-6PGD was pooled fractions 26 to 30 (Fig. 1), and pooled fractions 32 to 35 (Fig. 1) served as a source of the NADP-6PGD. NAD-6PGD assays each contained 80 μg of enzyme protein and NADP-6PGD assays each contained 62 μg of enzyme protein. Each reaction was initiated by adding 6-phosphogluconate immediately after mixing the other reaction components. Results for both the NAD- and NADP-linked enzymes are expressed as per cent inhibition of the initial reaction rates observed in the absence of fructose-1,6-diphosphate.*

inhibition by FDP (Fig. 3). Several other properties were studied in an attempt to characterize the two enzymes more fully.

Nature of the reaction catalyzed. A crystalline NADP-specific 6PGD from *Candida utilis* catalyzed both the oxidation and decarboxylation of 6-phosphogluconate to yield CO_2 and ribulose-5-phosphate (18). A partially purified NAD-linked 6PGD from *Leuconostoc mesenteroides* B07, however, catalyzed only the oxidation of 6-phosphogluconate to yield 2-keto-6-phosphogluconate (25). It was of interest to determine whether the NAD-linked 6PGD and the NADP-linked 6PGD from *S. faecalis* possessed decarboxylating as well as oxidative activity. Accordingly, the partially resolved enzymes obtained from the Bio-Gel P-150 column (Fig. 1) were tested for their ability to produce labeled CO_2 from 6-phosphogluconate-*1*-^{14}C. As shown in Table 5, both the NAD-6PGD and the NADP-6PGD possessed decarboxylating activity. It is considered unlikely that the decarboxylating activity observed in this experiment was due to the combined action of a dehydrogenase and a decarboxylase with the intermediate formation of 2-keto-6-phosphogluconate, as was found in *L. mesenteroides* B07 (25, 26). The addition of 10 mM semicarbazide to the complete reaction

TABLE 5. *Decarboxylating activity of partially purified NADP- and NAD-linked 6-phosphogluconate dehydrogenases (6PGD) from gluconate-adapted S. faecalis*

Reaction mixture[a]	NADP-linked 6PGD[b] $^{14}CO_2$		NAD-linked 6PGD[c] $^{14}CO_2$	
	Counts/min	Amt (μmoles)	Counts/min	Amt (μmoles)
Complete	76,149	1.42	41,105	1.15
Minus enzyme	251	0.005	291	0.008
Minus enzyme + boiled enzyme	201	0.004	101	0.003
Minus coenzyme	311	0.006	206	0.006
Coenzymes reversed	5,643	0.10	3,947	0.11

[a] Reaction vessels, gassing procedure, and method of collecting and counting $^{14}CO_2$ produced from 6-phosphogluconate-*1*-^{14}C were as described previously (1).

[b] The complete reaction mixture contained the following components in a final volume of 2.8 ml: Tris-hydrochloride buffer, *p*H 7.5, 300 μmoles; 6-phosphogluconate-*1*-^{14}C, 2.0 μmoles (107, 642 counts/min); and NADP, 2.0 μmoles. Reactions were initiated by adding NADP-6PGD (310 μg of protein) from pooled fractions 32 to 35 (Fig. 1) and were incubated at room temperature for 60 min. Reactions were then terminated by the addition of 0.2 ml of 6 N H_2SO_4. Deletions from the complete reaction mixture were as noted above. Where coenzymes were reversed, 5.0 μmoles of NAD was substituted for NADP.

[c] The complete reaction mixture was as described for the NADP-6PGD assay with the following exceptions: 3.0 μmoles of 6-phosphogluconate-*1*-^{14}C (107,642 counts/min) was added and NAD (5.0 μmoles) was substituted for NADP. Reactions were initiated by adding NAD-6PGD (421 μg of protein) from pooled fractions 26 to 30 (Fig. 1). Other conditions were as described for the NADP-6PGD assay. Where coenzymes were reversed, 2.0 μmoles of NADP was substituted for NAD.

mixtures shown in Table 5 had no effect on the decarboxylating activity of either enzyme.

A very low level of decarboxylating activity was observed with the NADP-enzyme when NAD served as the coenzyme, and the same was found with the NAD-enzyme when NADP was substituted for NAD ("coenzymes reversed," Table 5). It may be noted from the data in Fig. 1 that the pooled fractions used as a source of each enzyme in this experiment (fractions 26 to 30 for the NAD-6PGD; fractions 32 to 35 for the NADP-6PGD) were each slightly contaminated with a residual amount of the other enzyme. This probably accounts for the apparent lack of absolute coenzyme specificity observed in Table 5.

Optimal pH. The two enzymes differed in their optimal *p*H for catalytic activity. The NAD-linked enzyme exhibited a rather sharp optimum at *p*H 7.5, whereas the NADP-linked enzyme had a broader optimum, which ranged between *p*H 8.0 and 9.0 (Fig. 4).

K_m for substrate and coenzymes. The substrate and coenzyme saturation curves for both the NAD-6PGD and the NADP-6PGD followed classical Michaelis-Menten kinetics, but the apparent K_m values for coenzyme and substrate were much higher for the NAD-linked enzyme than for the NADP-linked enzyme (Table 6).

Sensitivity to inhibition by ATP. Another striking difference between the NAD-6PGD and the NADP-6PGD was in their susceptibility to inhibition by certain nucleotides. The NAD-specific enzyme was rather strongly inhibited by adenosine triphosphate (ATP), whereas the NADP-linked enzyme was not (Fig. 5). Adenosine diphosphate (ADP) and adenosine monophosphate (AMP) also inhibited the NAD-linked enzyme, but were only about 60 and 40%, respectively, as effective as ATP at equivalent concentrations. Of the other nucleoside triphosphates tested, guanosine triphosphate (GTP) was about 40% as

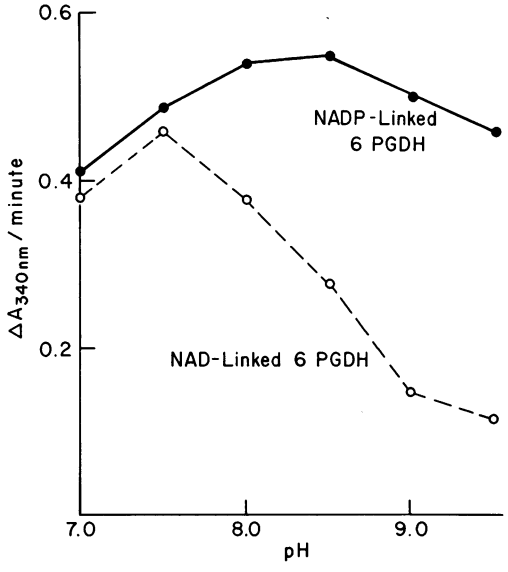

FIG. 4. *Optimal pH of NAD-linked 6PGD and NADP-linked 6PGD from gluconate-adapted S. faecalis. NAD- and NADP-6PGD assays were as described in Materials and Methods except that the pH of the Tris-hydrochloride buffer was varied as shown. The source of the NAD- and NADP-enzymes was as described for Fig. 3. Each reaction was initiated with 92 μg of NAD-6PGD protein* (○), *or with 73 μg of NADP-6PGD protein* (●) *as indicated.*

TABLE 6. *Apparent K_m values of NAD- and NADP-linked 6-phosphogluconate dehydrogenases (6PDG) from gluconate-adapted S. faecalis for their substrate and respective coenzymes*

Enzyme[a]	K_m (mM)[b]			V_{max}[c]
	NADP	NAD	6PGA	
NADP-6PGD	0.020	—	0.025	1.17
NAD-6PGD	—	0.400	0.500	0.87

[a] The source of the NAD-6PGD and NADP-6PGD was the same as described for Fig. 3.

[b] Values shown were calculated from Lineweaver-Burk plots (14) of kinetic data obtained from observed reaction rates at various concentrations of substrate or coenzyme. Substrate concentrations were varied at a fixed and saturating concentration of the appropriate coenzyme (3.0 mM NAD or 0.25 mM NADP), and coenzyme concentrations were varied at a fixed and saturating concentration of 6-phosphogluconate (6PGA, 2.0 mM). NADP-6PGD assays were initiated by adding 69 μg of enzyme protein, and NAD-6PGD reactions were initiated with 83 μg of enzyme protein.

[c] Expressed in units per milligram of protein.

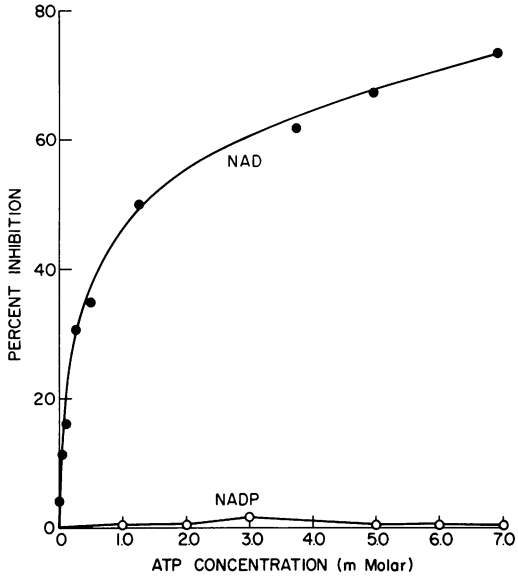

FIG. 5. *Effect of ATP on NAD-linked 6PGD and NADP-linked 6PGD from gluconate-adapted S. faecalis. NAD- and NADP-6PGD assays were as described in Materials and Methods except that, where indicated, various concentrations of ATP were included in the reaction mixtures. The source of the NAD- and NADP-6PGD was as described for Fig. 3. NAD-6PGD assays (●) were each initiated with 74 μg of enzyme protein, and NADP-6PGD assays (○) were started with 57 μg of enzyme protein. Results for both the NAD- and NADP-linked enzymes are expressed as per cent inhibition of the initial reaction rates observed in the absence of ATP.*

effective as ATP, and uridine and cytidine triphosphates were both about 15% as effective as ATP. ADP, AMP, and GTP were completely without effect on the NADP-6PGD.

ATP increased the apparent K_m for both substrate and coenzyme for the NAD-6PGD without affecting the V_{max} (Fig. 6). The inhibition by ATP, therefore, appeared to be of the competitive type with respect to both 6-phosphogluconate and NAD. Several arguments can be raised, however, against the strictly competitive nature of this inhibition. First, ATP did not inhibit the reaction completely at saturating concentrations of substrate and coenzyme (Fig. 5). This suggests that the inhibition may actually be of the partically competitive type (23). Second, the lack of structural relatedness between ATP and 6-phosphogluconate make it unlikely that these two ligands actually bind at the same site on the enzyme. Finally, the NADP-linked enzyme might reasonably be assumed to possess a similar, if not identical, binding site for 6-phosphogluconate. Yet, the NADP-enzyme was completely refractory to the inhibitory effect of ATP (Fig. 5). These arguments are, of course, inferential and indirect and cannot be taken as definitive evidence for an ATP binding site that is distinct from the substrate or coenzyme sites.

A summary of some of the general properties of the NAD-6PGD and NADP-6PGD is given in Table 7.

DISCUSSION

S. faecalis ferments gluconate by a combination of the hexose-monophosphate, Entner-Doudoroff, and Embden-Meyerhof pathways with the production of lactate and CO_2 (21). A schematic representation of these pathways is given in Fig. 7. About 50% of the gluconate degraded during growth passes through the 6PGD step of the hexose monophosphate pathway (21), and the resulting pentose phosphate enters the Embden-Meyerhof pathway at the level of fructose-6-phosphate through the action of the enzymes ribulose phosphate-3-epimerase (EC 5.1.3.1), phosphoribose isomerase (EC 5.3.1.6), transketolase (EC 2.2.1.1), and transaldolase (EC 2.2.1.2). Although transaldolase has not been found in glucose adapted cells (20), its presence in gluconate-adapted cells has been inferred from labeling studies (21). The remainder of the gluconate carbon degraded also enters the Embden-Meyerhof pathway, but at the C_3 level, by the combined action of the Entner-Doudoroff pathway enzymes, 6-phosphogluconate dehy-

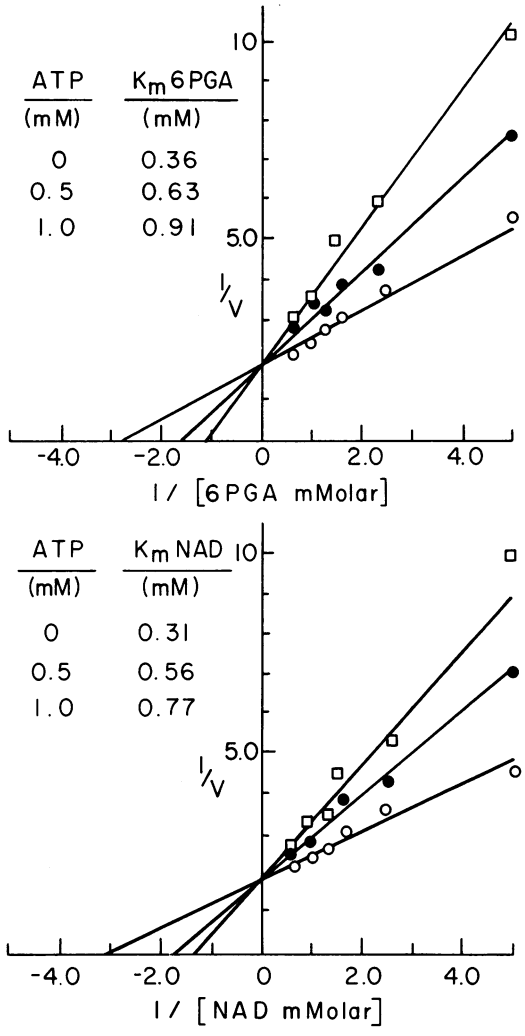

FIG. 6. *Effect of ATP on the apparent K_m of the NAD-6PGD for substrate and coenzyme. Enzyme assays were performed as described in Materials and Methods except that either the 6-phosphogluconate (upper) or NAD (lower) concentrations were varied as shown. The source of the NAD-6PGD was the same as for Fig. 3, and each reaction was initiated by the addition of 77 μg of enzyme protein. Symbols (upper and lower) are as follows: ○, no ATP; ●, 0.5 mM ATP; □, 1.0 mM ATP.*

drogenase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (21).

Studies with glucose-adapted cells established that the LDH from this organism had an absolute and specific requirement for FDP for activity (24), and that the NADP-linked 6PGD was strongly and specifically inhibited by FDP (1). These interactions are indicated in Fig. 7. This raised a question as to how gluconate-adapted cells could use the 6PGD step of the hexose-monophosphate pathway for gluconate catabolism, while at the same time producing large amounts of lactate. The answer to the question appears to reside in the observation that gluconate-adapted cells, unlike glucose-grown cells, possessed an NAD-specific 6PGD in addition to the NADP-linked enzyme (Fig. 1 and 2), and that the former enzyme was insensitive to FDP (Fig. 3).

Although this is, to our knowledge, the first report of separate NAD-6PGD and NADP-6PGD from a bacterial source, a number of other cases have been reported where organisms contain distinct NAD- and NADP-linked dehydrogenases for catalyzing a single reaction. For example, studies by Kornberg and Pricer (9) revealed the presence of distinct NAD- and NADP-linked isocitrate dehydrogenases in yeast, and LéJohn (11) found that certain *Zygomycetes* and *Chytridiomycetes* also contained these two enzymes. *Thiobacillus novellus* (10, 12, 13), yeast (5), *Neurospora* (19), and a wide variety of other fungi (11) contain separate NAD- and NADP-linked glutamate dehydrogenases, and *Escherichi coli*

TABLE 7. *General properties of the NAD- and NADP-linked 6-phosphogluconate dehydrogenases (6PGD) from S. faecalis*

Properties	NAD-6PGD	NADP-6PGD
Decarboxylating activity	+	+
Optimal *p*H	7.5	8.0–8.5
6-Phosphogluconate K_m (mM)	0.500	0.025
Coenzyme K_m (mM)	0.400	0.020
Inhibition by fructose-1,6-diphosphate	—	+
Inhibition by adenosine triphosphate	+	−

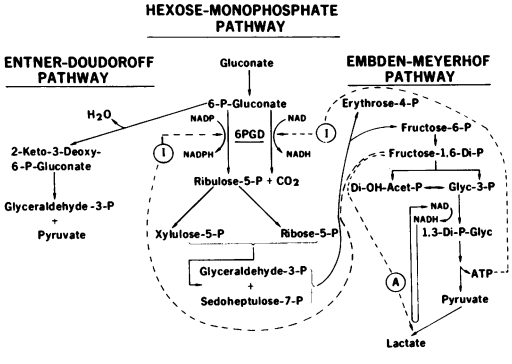

FIG. 7. *Schematic representation of the pathways involved in gluconate catabolism by S. faecalis. Sites of control are indicated by the broken lines. Ⓐ = activation; ① = inhibition.*

has been shown to have both NAD- and NADP-specific malic enzymes (8, 16, 22). In these cases, evidence was presented to support the hypothesis that the NAD-linked dehydrogenases were involved in catabolism, whereas the NADP-linked enzymes functioned in a biosynthetic capacity (see also 7). The data for the NAD-6PGD and NADP-6PGD from *S. faecalis* are in accord with this hypothesis. The NAD-linked enzyme appears to play a major role in the catabolism of gluconate. This enzyme was present at significant levels only in extracts of cells grown with gluconate as the primary energy source. Glucose-adapted cells, which are known to be unable to degrade gluconate (21), were devoid of, or possessed only very low levels of, the NAD-6PGD (Table 4). The NADP-6PGD, on the other hand, appears to be primarily involved in supplying reducing equivalents (NADPH) for the various reductive biosynthetic reactions. This enzyme was constitutive, being found at more or less equivalent levels in cells grown on glucose and a variety of other carbon sources (Table 3). Furthermore, the sensitivity of the NADP-6PGD to inhibition by FDP would be expected to limit or even preclude its functioning to supply carbon for glycolysis under anaerobic conditions. As already mentioned, the LDH from *S. faecalis* has an absolute requirement for FDP for activity (24) and, since this organism appears to lack transhydrogenase (5), the reduction of pyruvate to lactate seems to be the only mechanism available for the reoxidation of NADH formed at the glyceraldehyde-3-phosphate dehydrogenase step (Fig. 7).

The sensitivity of the NAD-6PGD to inhibition by ATP (Fig. 5) is of interest, because this may be a physiological mechanism by which *S. faecalis* can restrict the input of gluconate carbon into the Embden-Meyerhof pathway at the level of fructose-6-phosphate when the cellular energy pool (ATP) is high. It is clear that gluconate carbon could still enter the Embden-Meyerhof pathway at high ATP concentrations via the Entner-Doudoroff pathway (Fig. 7). However, substrate carbon would enter at the glyceraldehyde-3-phosphate (or pyruvate) level, which would not lead to a net increase in the ATP pool. This, of course, would still result in a depletion of substrate carbon through a non-energy-liberating process, and it seems likely that some control over the Entner-Doudoroff pathway is also operative in *S. faecalis*. This problem is currently under study.

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