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Purification and Characterization of the Two 6-Phosphogluconate Dehydrogenase Species from *Pseudomonas multivorans*

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The two species of 6-phosphogluconate dehydrogenase (EC 1.1.1.43) from *Pseudomonas multivorans* were resolved from extracts of gluconate-grown bacteria and purified to homogeneity. Each enzyme comprised between 0.1 and 0.2% of the total cellular protein. Separation of the two enzymes, one which is specific for nicotinamide adenine dinucleotide phosphate and the other which is active with nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate was facilitated by the marked difference in their respective isoelectric points, which were at pH 5.0 and 6.9. Comparison of the subunit compositions of the two enzymes indicated that they do not share common peptide chains. The enzyme active with nicotinamide adenine dinucleotide was composed of two subunits of about 40,000 molecular weight, and the nicotinamide adenine dinucleotide phosphate-specific enzyme was composed of two subunits of about 60,000 molecular weight. Immunological studies indicated that the two enzymes do not share common antigenic determinants. Reduced nicotinamide adenine dinucleotide phosphate strongly inhibited the 6-phosphogluconate dehydrogenase active with nicotinamide adenine dinucleotide by decreasing its affinity for 6-phosphogluconate. Guanosine-5'-triphosphate had a similar influence on the nicotinamide adenine dinucleotide phosphate-specific 6-phosphogluconate dehydrogenase. These results in conjunction with other data indicating that reduced nicotinamide adenine dinucleotide phosphate stimulates the conversion of 6-phosphogluconate to pyruvate by crude bacterial extracts suggest that in *P. multivorans*, the relative distribution of 6-phosphogluconate into the pentose phosphate and Entner-Doudoroff pathways might be determined by the intracellular concentrations of reduced nicotinamide adenine dinucleotide phosphate and purine nucleotides.

6-Phosphogluconate is a key intermediate in the dissimilation of glucose by *Pseudomonads* (4, 16, 19, 23, 26). It occurs at a branch point where the flow of material from glucose can be directed towards formation of either 5 or 3 carbon metabolites via the pentose phosphate or Entner-Doudoroff pathways. 6-Phosphogluconate dehydrogenase (6PGAD) (EC 1.1.1.43) and 6-phosphogluconate dehydratase, (EC 4.2.1.12) the first enzymes of the respective pathways, carry out reactions which represent potential control points for governing the relative rates of conversion of 6-phosphogluconate to pentose phosphate or to triose phosphate and pyruvate. In *Pseudomonas multivorans*, which differs from most pseudomonads in possessing high levels of both 6PGAD and enzymes of the Entner-Doudoroff pathway (9), the distribution of 6-phosphogluconate into the two pathways appears to be regulated by reduced nicotinamide adenine dinucleotide phosphate (NADPH) and guanosine-5'-triphosphate (GTP)-linked control of two different 6PGAD species. The formation of 6-phosphogluconate from glucose-6-phosphate appears to be regulated by similar control of a corresponding pair of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) species (22). The present report describes the isolation and characterization of the two *P. multivorans* 6PGAD species and their interactions with NADPH and GTP.

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MATERIALS AND METHODS

Growth of bacteria. *P. multivorans* strain 249 (ATCC 17616) was grown in 24-liter batch cultures containing 5×10^{-2} M phosphate buffer, pH 6.5, 10^{-3}

M $MgSO_4$, 10^{-4} $CaCl_2$, 10^{-5} $FeSO_4$, and 0.2% $(NH_4)_2SO_4$, with 0.4% sodium gluconate as sole carbon source as described elsewhere (22). The bacteria were collected by centrifugation using an RC-2B centrifuge equipped with an SZ-14 continuous flow rotor (Ivan Sorvall Inc., Norwalk, Conn.), and the cell pellets were frozen and maintained at −5 C until used for purposes of enzyme purification.

Determination of 6PGAD activity. The activity of 6PGAD (EC 1.1.1.43) was determined spectrophotometrically by measuring the formation of NAD(P)H produced in the oxidation of 6-phosphogluconate (9). The assay mixtures contained 2×10^{-1} M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.5, 10^{-2} M 2-mercaptoethanol, 5×10^{-3} M 6-phosphogluconate, 5×10^{-4} M nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP), and appropriately diluted enzyme preparation in a final volume of 1 ml. The *P. multivorans* 6PGAD active with NAD is subject to inhibition by reduced pyridine nucleotides. Its activity was measured with dilute preparations over an absorbance range of 0 to 0.2 at 340 nm. Under these conditions the rate of the reaction was linear with time, since insufficient NADH or NADPH accumulated to affect enzyme activity. The NADP-specific 6PGAD was measured over a wider absorbance range of 0 to 2.0 at 340 nm, since its activity was not influenced by NADH or NADPH. Enzyme activities are expressed in terms of units (micromoles of NADH or NADPH produced per min at 25 C) per mg of protein. Protein was measured by the Folin phenol method (11)

Determination of enzymes of the Entner-Doudoroff pathway. Bacteria from 50-ml cultures which had been grown with 0.4% gluconate as sole carbon source were washed and suspended in 0.02 M phosphate buffer, pH 6.8, containing 0.01 M 2-mercaptoethanol, and disrupted by sonic treatment for 1 min as reported earlier (9). The disrupted cell suspension was centrifuged for 10 min at 12,000 × *g* and the supernatant fraction was examined as follows: the overall conversion of 6-phosphogluconate to pyruvate (mediated by 6-phosphogluconate dehydratase (EC 4.2.1.12) and 6-phospho-2-keto-3-deoxy-D-gluconate (KDPG) aldolase (EC 4.1.2.14) or the conversion of 2-keto-3-deoxy-6-phospho-gluconate to pyruvate (mediated by KDPG aldolase) was measured at 30 C in 0.5-ml assay mixtures containing 0.2 M Tris-hydrochloride buffer, pH 8.5., 0.01 M 2-mercaptoethanol, cell-free extract, and 0.01 M 6-phosphogluconate or 0.001 M 2-keto-3-deoxy-6-phosphogluconate, which was kindly provided by H. P. Meloche. Pyruvate was determined as the dintrophenylhydrazone as described earlier (9).

Acrylamide gel electrophoresis. The general procedures and buffers for gel electrophoresis developed by Davis (3) were used in the experiments examining the homogeneity of the purified enzyme preparations and the molecular weights (MW) of the two 6PGAD species. For the later purpose the enzymes were resolved on gels of different acrylamide concentration and the MW of the enzymes were estimated by comparing the electrophoretic mobilities of the enzymes with those of several reference proteins as described by Hedrick and Smith (6). The positions of the protein bands were determined after staining the gels with amido black or specifically staining the gels for zones containing 6PGAD by incubating the gels in assay mixtures containing phenazine methosulfate and tetrazolium chloride as described earlier (9).

Determination of the subunit compositions of the two 6PGAD species. Preparations of the two 6PGAD species and reference proteins of known MW and subunit composition, which had been denatured by treatment with sodium dodecyl sulfate (SDS) in the presence of 2-mercaptoethanol, were resolved electrophoretically on 7.5% (wt/vol) acrylamide gels containing 0.2% (wt/vol) SDS, essentially as described by Weber and Osborn (25). The gels were immersed in 7% (vol/vol) acetic acid containing 5% (vol/vol) methanol in a Canalco Quick Gel Destainer (Canalco Instruments, Inc., Rockville, Md.) and SDS was removed from the gels electrophoretically. The gels were stained with 0.25% coomassie blue, and destained electrophoretically in the same acetic acid-methanol mixture to remove excess dye.

Isoelectric-focusing experiments. Isoelectric focusing of the two 6PGAD species was carried out at 0 C using a Metaloglass gel electrofocusing apparatus (Metaloglass, Inc., Boston, Mass.) according to the procedures described by Righetti and Drysdale (18). Enzyme samples containing between 50 and 80 μg of protein were layered onto 6% (wt/vol) acrylamide gels containing 4% (vol/vol) carrier ampholytes (LKB Instruments, Rockville, Md.) appropriate for a pH gradient of pH 3 to 10. The gels were supplemented with 20% (vol/vol) glycerol to prevent gel shrinkage during the course of the experiment. Glycerol and carrier ampholytes were also added to the enzyme sample to increase its density and facilitate application to the gels and to buffer against the 0.02 M NaOH contained in the upper reservoir of the electrofocusing apparatus. The lower reservoir contained 0.01 M H_3PO_4. After the enzymes had been resolved electrophoretically, the gels were sliced into about 20 segments, and each gel segment was extracted with 3 ml of distilled water. The pH of each extract was determined, and the extracts were assayed for 6PGAD activity.

Sucrose gradient centrifugation. Samples of the purified 6PGAD species were applied to 12-ml linear 5 to 20% (wt/vol) sucrose gradients (containing 0.02 M phosphate buffer, pH 6.8) in polyallomer tubes appropriate to the SB-283 rotor of a B60 ultracentrifuge (International Equipment Co., Inc., Needham, Mass.). The gradients were centrifuged at 38,000 rpm for 20 h at 4 C. After centrifugation the bottoms of the polyallomer tubes were punctured and approximately 25 fractions of equal volume were collected. Each fraction was assayed for 6PGAD activity, and the apparent MW of each enzyme was estimated by comparing its position in the gradient with that of hemoglobin, which was included in the enzyme sample as an internal marker.

Preparation of antisera. Samples of purified enzyme containing 1 mg of protein were emulsified with Freund incomplete adjuvant and injected subcutaneously into male New Zealand rabbits. Two additional

injections of similarly prepared samples were made 6 and 10 weeks after the initial injection. Four weeks after the third injection blood samples were obtained by cardiac puncture, and the serum fraction was supplemented with 0.01 M NaN_3, and stored at 5 C until used in enzyme inhibition and immunodiffusion experiments.

Immunodiffusion experiments. Agarose gels containing 0.8% (wt/vol) agarose and 0.5% (wt/vol) NaCl were prepared in plexiglass templates (5 by 5 cm). The sample wells were filled with 0.04 ml of the appropriate antiserum or purified enzyme preparation, and precipitin bands were allowed to develop during a 48-h incubation period at 25 C. The gels were washed with 0.5% (wt/vol) NaCl and the precipitin bands were stained with 0.1% (wt/vol) amido black. Excess dye was removed from the gels by washing them with 7% acetic acid.

Chemicals. All chemicals were of reagent grade. The reference proteins used in MW determinations as well as NAD and NADP were obtained from Sigma Chemical Co., St. Louis, Mo. 6-Phosphogluconate was obtained from General Biochemicals Co., Chagrin Falls, Ohio. Hydroxylapatite was purchased from Bio-Rad Laboratories, Richmond, Calif. The carrier ampholytes used in the isoelectric focusing experiments were obtained from LKB Instruments, Inc., Rockville, Md.

RESULTS

Purification of the two 6PGAD species from P. multivorans. A procedure using the same initial steps as used for purification of *P. multivorans* glucose-6-phosphate dehydrogenase (22) has been used to purify the two *P. multivorans* 6PGAD species to homogeneity. This makes it possible to obtain preparations of the four *P. multivorans* glucose-6-phosphate and 6PGAD species from the same bacterial extracts. First the bulk glucose-6-phosphate and 6PGAD activities were separated by fractionation of the disrupted cell suspensions with ammonium sulfate, and then the two 6PGAD species were resolved by diethylaminoethyl (DEAE)-cellulose column chromatography. The two enzymes were purified to homogeneity by additional chromatographic steps using cellulose phosphate and hydroxylapatite. The individual steps of the purification procedure are outlined in Tables 1 and 2, and are described in the following section. Unless indicated otherwise, the procedures were carried out between 0 and 5 C.

Preparation and fractionation of disrupted cell suspensions. Sixty grams (wet weight) of bacterial cell paste was suspended in 300 ml of standard phosphate buffer (0.02 M phosphate buffer, pH 6.8, containing 0.01 M NaN_3 and 0.01 M 2-mercaptoethanol) which had been supplemented with 500 μg of lysozyme and 10 μg of deoxyribonuclease (DNAase) per ml, and the preparation was incubated for 15 to 20 min at 30 C to allow lysozyme to act on the cells. The suspension was then exposed to high pressure (1,000 psi of N_2) in a Parr bomb (Parr Instrument Co., Moline, Ill.) and after 5 min of equilibration, the suspension was discharged from the bomb under pressure. The bacteria were disrupted as a result of the rapid decompression to atmospheric pressure. Unbroken cells and cell debris were removed by centrifugation.

An equal volume of 2% (wt/vol) streptomycin sulfate in standard phosphate buffer was added with stirring to the supernatant fraction, and the resulting precipitate was removed by centrifugation and discarded. Successive additions of solid ammonium sulfate (22, 6, and 9 g/100 ml) were made to the supernatant fraction obtained after the treatment with streptomycin sulfate. The precipitates formed after each addition of ammonium sulfate were collected by centrifugation and dissolved in standard phosphate buffer. The fraction obtained after the third addition of ammonium sulfate (ASF III) contained the bulk of the NAD- and NADP-linked 6PGAD activity from the crude bacterial extract. The fraction obtained after the second addition of ammonium sulfate contained the bulk of the glucose-6-phosphate dehydrogenase

TABLE 1. *Purification of the P. multivorans 6PGAD active with NAD*

Fraction	Vol (ml)	Protein (mg/ml)	Sp act[a]		Initial NAD-linked activity (%)
			NAD as cofactor	NADP as cofactor	
1. Cell-free extract	330	20	0.053	0.101	100
2. Ammonium sulfate precipitate III	50	21	0.193	0.34	58
3. DEAE-cellulose column eluate	40	2.2	1.77	1.362	47
4. Ammonium sulfate precipitate IV	10	4	4.05	2.835	47
5. Cellulose-PO_4 column eluate (concentrated)	3	0.23	42.3	34.0	8.4

[a] Micromoles of NADH or NADPH formed per min per milligram of protein.

TABLE 2. *Purification of the NADP-specific 6PGAD from P. multivorans*[a]

Fraction[a]	Vol (ml)	Protein (mg/ml)	Sp Act (μmol of NADPH/min per mg of protein)	Initial activity (%)
1. Cell-free extract	330	20	0.059[b]	100
2. Ammonium sulfate Fraction III	50	21	0.186[b]	48
3. DEAE-cellulose column eluate (concentrated)	20	3.0	3.8	34
4. Cellulose-PO_4 column eluate (concentrated)	10	1.7	9.0	23
5. Hydroxylapatite column eluate (concentrated)	9	0.14	46.0	8.8

[a] The purification was carried out using the same initial preparations as in the purification cited in Table 1.

[b] Corrected to reflect only NADP-linked activity of the NADP-specific enzyme. To correct for the contribution due to the enzyme active with NAD, the NADP-linked activity of this form was calculated as 80% of its NAD-linked activity.

activity, and was saved for studies of the two glucose-6-phosphate dehydrogenase species.

Chromatographic separation of the two species of 6PGAD on DEAE-cellulose columns. The ASF III fraction was dialyzed against a 50-fold greater volume of standard phosphate buffer, and the dialyzed preparation was then applied to a DEAE-cellulose column (2.5 by 45 cm) which had been equilibrated with the same buffer. The column was washed with approximately 300 ml of buffer to recover the 6PGAD active with NAD (or NADP), which did not bind to the DEAE-cellulose. Fractions of about 10-ml were collected, and those containing the enzyme active with NAD (fractions 2 to 5) were combined and dialyzed against standard phosphate buffer. After the enzyme active with NAD had been recovered, the NADP-specific enzyme was eluted from the column by increasing the phosphate concentration of the eluting buffer. A 600-ml linear gradient of 0.02 to 0.2 M phosphate buffer, pH 6.8, containing 0.01 M 2-mercaptoethanol and 0.01 M sodium azide was pumped through the column, and fractions of about 10 ml were collected and assayed for 6PGAD activity. The NADP-specific enzyme eluted at a phosphate concentration of about 0.09 M (fractions 22 to 24) as shown in the elution pattern of Fig. 1. The specific activities of the two preparations of 6PGAD obtained from the DEAE-cellulose column (step 3 of Table 1 and step 3 of Table 2) were, respectively, 35- and 64-fold higher for the enzyme active with NAD and for the NADP-specific enzyme than the corresponding activities of the crude bacterial extract (step 1 of Table 1 and step 1 of Table 2).

Further purification of the 6PGAD active with NAD or NADP. The preparation of the 6PGAD active with NAD (or NADP) obtained from the DEAE-cellulose column was treated with solid ammonium sulfate (36 g/100 ml) to precipitate the enzyme, and the precipitate was collected by centrifugation and dissolved in standard phosphate buffer. This fraction was dialyzed against standard phosphate buffer and applied to a cellulose phosphate column (2.5 by 45 cm). The enzyme was eluted from the column by progressively increasing the phosphate concentration of the elution buffer (600 ml of a linear gradient of 0.02 to 0.2 M phosphate buffer, pH 6.8, were pumped through the column and fractions of 10 ml were collected). The 6PGAD activity eluted in approximately 0.1 M phosphate buffer (fractions 24 through 27). The fractions containing enzyme activity were pooled, concentrated by ultrafiltration and stored at 0 C. The final preparation (step 5 of Table 1) had specific activities of 42 and 32 with

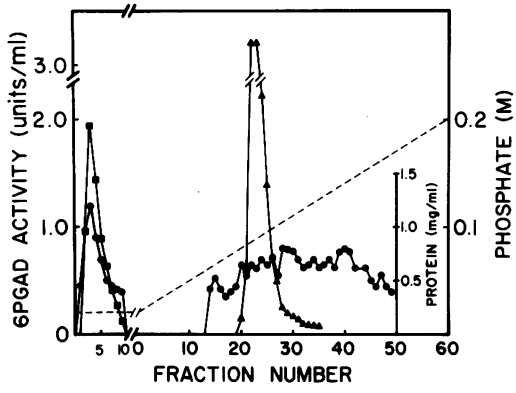

FIG. 1. *Separation of the two 6PGAD species by DEAE-cellulose column chromatography. Twenty milliliters of dialyzed ASF III fraction containing about 450 mg of protein was applied to a column (2.5 by 45 cm) (diameter × length) of DEAE-cellulose equilibrated with standard phosphate buffer. First, 320 ml of the same buffer was pumped through the column and 10-ml fractions were collected and assayed for the enzyme active with NAD. The NADP-specific enzyme was eluted from the column by applying 600 ml of a linear gradient of 2×10^{-2} to 2×10^{-1} M phosphate buffer, pH 6.8, containing 10^{-2} M 2-mercapthoethanol and 10^{-2} M sodium azide. Ten-milliliter fractions were collected and each fraction was assayed for 6PGAD activity and protein. (■) NAD-linked activity; (▲) NADP-linked activity; (●) protein; (- - -), phosphate concentration.*

NAD as cofactor and with NADP as cofactor. The overall purification of the enzyme was about 800-fold. Approximately 10% of the initial NAD-linked activity of the crude bacterial extracts was recovered in the final preparation.

Further purification of the NADP-specific 6PGAD. The preparation of NADP-specific enzyme which had been chromatographically resolved from the enzyme active with NAD (step 3 of Table 2) was dialyzed against standard phosphate buffer and applied to a cellulose phosphate column equilibrated with the same buffer. Additional buffer was pumped through the column and fractions of 10 ml were collected and assayed for 6PGAD activity. The NADP-specific enzyme did not bind to the cellulose phosphate, and eluted immediately after the void volume (fractions 2 to 4). The fractions containing enzyme activity were pooled and concentrated by ultrafiltration. The concentrated preparation of enzyme obtained from the cellulose phosphate column was applied to a hydroxylapatite column (2 by 20 cm) (diameter × length). Standard phosphate buffer (ca. 80 ml) was pumped through the column, and 4-ml fractions were collected. Each fraction was assayed for enzyme activity. The fractions possessing the greatest activity (fractions 21 through 24) were pooled and concentrated to 9 ml by ultrafiltration. The concentrated preparation had a specific activity of about 45 U/mg of protein, representing a 750-fold purification of the enzyme from the crude bacterial extract. The total recovery of NADP-linked activity due to the NADP-specific enzyme was about 10% of that present in the crude bacterial extract.

Homogeneity of the purified preparations of 6PGAD. The preparations of both the NADP-specific enzyme (step 5 of Table 2) and of the enzyme active with NAD (step 5 of Table 1) resolved on gels of different acrylamide concentration to give a single band of protein indicating that each enzyme was homogeneous both with respect to charge and size. The single band of protein obtained in each case coincided with the zone of enzyme activity when enzyme activity was visualized on the gels by coupling 6-phosphogluconate-dependent formation of NADH or NADPH to formation of insoluble tetrazolium pigment (see Fig. 2).

Stability of the preparations of the two 6PGAD species. When stored at 0 C the purified preparations of the NADP-specific enzyme (step 5 of Table 2) lost approximately 50% of their activity after 3 weeks. Those of the enzyme active with NAD were more stable but lost 50% of their activity after 5 weeks. Supplementing the preparations with 5×10^{-3} M 6-phosphogluconate did not significantly affect their stability. Freezing the preparations resulted in complete loss of enzyme activity. The purified enzymes were considerably less stable than partially purified preparations of the two enzymes. For example, the fractions containing both 6PGAD species (equivalent to step 2 of Table 1) which were obtained by treatment of bacterial extracts with ammonium sulfate retained 100% of their activity when stored for several months at 0 C. Likewise, partially purified preparations of both enzymes equivalent to steps 3 and 4 of Table 1 and steps 3 and 4 of Table 2 were completely stable when stored for more than 2 months at 0 C.

Kinetic properties of the two 6PGAD species. Saturation kinetics were determined for the different ligands using the highly purified preparations of the two 6PGAD species described in the previous section. The data were used to compare the apparent affinities of the two enzymes for 6-phosphogluconate, NAD, and NADP. The results of several experiments are summarized in Table 3. For both the NADP-specific enzyme and the enzyme active with NAD, the saturation curves for 6-phosphogluconate and for NAD and NADP were hyperbolic, and obeyed Michaelis-Menten kinetics. There was no evidence of homotropic interactions with any of the ligands. This was confirmed by Hill plots of the data which gave the expected value of 1 for Hill n.

As can be seen from the results summarized in Table 3, the NADP-specific 6PGAD and the enzyme active with NAD (as well as NADP) appeared to have similar affinities for NADP ($K_m = 2 \times 10^{-5}$ M) and for 6-phosphogluconate (with NADP as cofactor). The K_m values for 6-phosphogluconate were 2×10^{-4} and 3×10^{-4} M, respectively, for the NADP-specific enzyme and for the enzyme active with NAD or NADP.

The enzyme active with NAD or NADP had an apparent affinity for NAD ($K_m = 2 \times 10^{-4}$ M) which was 10-fold lower than that for NADP. Its apparent affinity for 6-phosphogluconate was more than twofold lower with NAD as cofactor ($K_m = 7 \times 10^{-4}$ M) than with NADP.

The results indicate that at low concentrations of substrate and pyridine nucleotides, the enzyme is more active with NADP than with NAD, suggesting that the combined activities of the two *P. multivorans* 6-phosphogluconate dehydrogenase species tend to assure preferential formation of NADPH relative to that of NADH.

Influence of NADPH, ATP, and GTP on

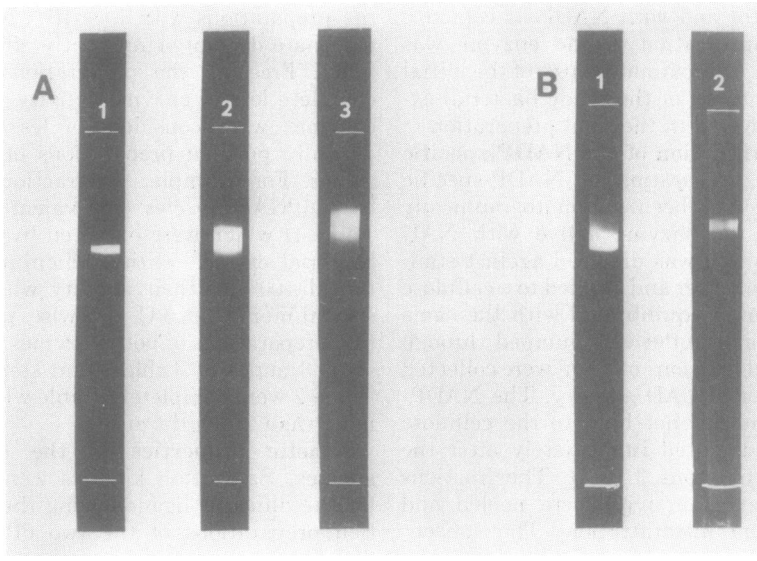

FIG. 2. *Acrylamide gel electrophoresis of purified preparations of the two P. multivorans 6PGAD species. Samples (0.1 ml) containing 60 μg of protein from a preparation equivalent to step 5 of Table 1 or 70 μg of protein from a preparation equivalent to step 5 of Table 2, 5% sucrose, and 0.02% bromphenol blue were applied to 7.5% acrylamide gels (5 by 100 mm, diameter × length), and resolved electrophoretically with a constant current of 2.5 mA per gel. Protein bands were fixed with 7% (vol/vol) acetic acid and stained with buffalo black dye. Zones of 6PGAD activity were visualized by incubating the gels at 30 C in 5 ml of standard assay mixtures containing 50 μg of phenazine methosulfate and 300 μg of 2,3,5-triphenyltetrazolium chloride per ml. (A) Gel 1 was stained for protein, gels 2 and 3 were stained respectively for NAD-linked activity and NADP-linked activity of the enzyme active with NAD or NADP. (B) Gel 1 was stained for protein and gel 2 was stained for activity of the NADP-specific 6PGAD.*

TABLE 3. *Comparison of the K_m values of the two 6PGAD species for 6-phosphogluconate, NAD, and NADP*

6PGAD species	Test ligand		NADP
	6-Phosphogluconate	NAD	
Enzyme active with NAD[a]	7×10^{-4} M (NAD as cofactor) 3×10^{-4} M (NADP as cofactor)	2×10^{-4} M	2×10^{-5} M
NADP-specific enzyme[b]	2×10^{-4} M		2×10^{-5} M

[a] The reaction mixtures contained 0.0012 U of enzyme from a preparation equivalent to step 5 of Table 1. Determinations were carried out with between 10^{-4} and 10^{-2} M 6-phosphogluconate, between 2×10^{-5} and 5×10^{-3} M NAD and between 10^{-5} and 2×10^{-3} M NADP.

[b] The reaction mixtures contained 0.01 U of enzyme from a preparation equivalent to step 5 of Table 2. The K_m values were determined over concentrations ranging between 6×10^{-5} and 10^{-2} M 6-phosphogluconate and 10^{-5} and 10^{-3} M NADP.

the activities of the two 6PGAD species. Several purine and pyridine nucleotides were found to inhibit preferentially one or the other of the two species of *P. multivorans* 6PGAD. GTP inhibited the NADP-specific enzyme, whereas adenosine 5'-triphosphate (ATP) and NADPH inhibited the enzyme active with NAD. The different effects of these inhibitors on the activities of the two enzyme species in standard assay mixtures can be seen from the data of Table 4. The mechanisms of inhibition of the enzymes by these compounds are described in the following sections.

Inhibition of the enzyme active with NAD. NADPH inhibited the enzyme active with NAD by decreasing its affinity for 6-phosphogluconate. NADPH (10^{-5} M) caused a significant increase in the K_m for 6-phosphogluconate without significantly affecting V_{max}, the activity of the enzyme at saturating concentrations of 6-phosphogluconate (Fig. 3). A similar but weaker effect of NADPH was noted when

TABLE 4. *Activities of the two 6PGAD species in the presence of different nucleotides*[a]

Additions	Enzyme active with NAD[b]		NADP-specific enzyme[c]
	NAD-linked activity	NADP-linked activity	
None	100	100	100
ATP 5×10^{-3} M	75	84	88
10^{-2} M	50	72	93
GTP 5×10^{-3} M	98	107	58
10^{-2} M	97	100	42
NADH 5×10^{-5} M	99	94	100
NADPH 10^{-5} M	45	94	100
5×10^{-5} M	28	53	102

[a] (Activity with inhibitor/activity without inhibitor) $\times$ 100.

[b] The standard assay mixtures contained 0.001 U of enzyme from a preparation equivalent to step 5 of Table 1.

[c] The standard assay mixtures contained 0.008 U of enzyme from a preparation equivalent to step 5 of Table 2.

NADP was the cofactor. In the presence of 10^{-5} M NADPH, the respective K_m values for 6-phosphogluconate with NAD and NADP as cofactor were increased from 7×10^{-4} to 3×10^{-3} M and from 3×10^{-4} to 5×10^{-4} M. NADPH did not affect the apparent affinities of the enzyme for NAD or NADP (data not shown).

Adenosine 5'-triphosphate (ATP) also influenced the apparent affinity of the enzyme active with NAD for 6-phosphogluconate, but 500-fold greater concentrations of ATP (compared to those of NADPH) were required for an effect similar to that observed with NADPH. The data of Table 5 show the influence of 5×10^{-3} M ATP and of 10^{-5} M NADPH on the apparent affinity of the enzyme for 6-phosphogluconate. As in the case of inhibition of the enzyme by NADPH, ATP had no significant influence on the apparent affinities of the enzyme for NAD or NADP and did not affect the catalytic activity (V_{max}) of the enzyme (data not shown).

GTP-linked inhibition of the NADP-specific enzyme. GTP inhibited the NADP-specific enzyme by decreasing its apparent affinity for 6-phosphogluconate. The presence of 5×10^{-3} M GTP in the assay mixtures resulted in a fivefold increase in the apparent K_m of the enzyme for 6-phosphogluconate from 2×10^{-4} to 10^{-3} M. When the data from the saturation experiments carried out in the presence of and absence of GTP were used to construct double reciprocal plots of enzyme activity versus 6-phosphogluconate concentration (Fig. 4), the same estimate of V_{max} was obtained with or without GTP, i.e., 5×10^{-3} M GTP affected neither the catalytic activity of the enzyme nor its apparent affinity for NADP. The data are summarized in Table 6.

Influence of other nucleotides on the activities of the two enzymes. A number of different nucleotides were tested to determine whether they influenced the activities of the two *P. multivorans* 6PGAD species. All the nucleotides were tested at a final concentration of 10^{-2} M in standard assay mixtures containing 0.008 U of the NADP-specific enzyme or 0.001 U of the enzyme active with NAD or NADP (prepara-

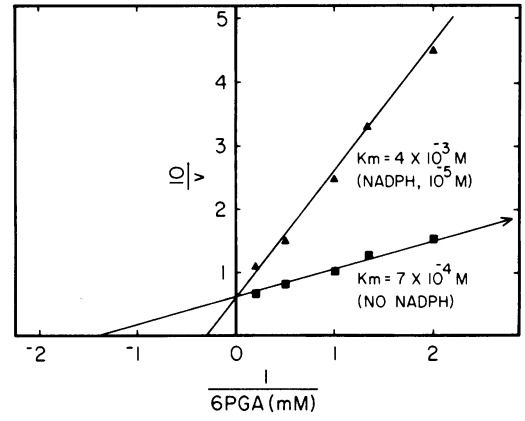

FIG. 3. *Influence of NADPH on the enzyme active with NAD as shown by double reciprocal plots of NAD-linked activity versus 6-phosphogluconate concentration. The reaction mixtures contained 0.0012 U of enzyme from a preparation equivalent to step 5 of Table 1, between 10^{-4} and 10^{-2} M 6-phosphogluconate, and 5×10^{-4} M NAD in otherwise standard assay mixtures. (▲) With 10^{-5} M NADPH, (■) with no NADPH.*

TABLE 5. *Influence of ATP and NADPH on the apparent K_m values of the 6PGAD active with NAD for 6-phosphogluconate*[a, b]

Cofactor	Modifying ligand		
	No add[b]	ATP (5×10^{-3} M)	NADPH (10^{-5} M)
NAD	7×10^{-4} M	2×10^{-3} M	4×10^{-3} M
NADP	3×10^{-4} M	5×10^{-4} M	5×10^{-4} M

[a] The reaction mixtures contained 0.0012 U of enzyme from a preparation equivalent to step 5 of Table 1.

[b] Enzyme activity was determined at concentrations of 6-phosphogluconate between 10^{-4} and 10^{-2} M.

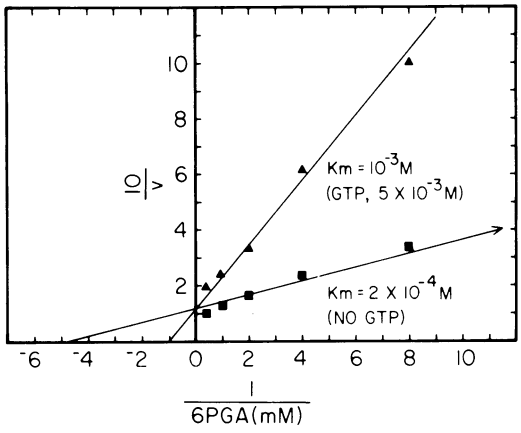

FIG. 4. *Influence of GTP on the activity of the NADP-specific enzyme as shown by double reciprocal plots of 6PGAD activity versus 6-phosphogluconate concentration. The assay mixtures contained 0.01 U of enzyme from a preparation equivalent to step 5 of Table 2, between 6 × 10^{-5} and 10^{-2} M 6-phosphogluconate and standard concentrations of other assay mixture components. (▲) With 5 × 10^{-3} M, GTP; (■) with no GTP.*

TABLE 6. *Influence of GTP on the apparent K_m values of the NADP-specific 6PGAD for 6-phosphogluconate and NADP*[a]

Ligand	Apparent K_m	
	No GTP	GTP (5×10^{-3} M)
6PGA[b]	2×10^{-4} M	10^{-3} M
NADP[c]	2×10^{-5} M	2×10^{-5} M

[a] The reaction mixtures contained 0.01 U of enzyme from a preparation equivalent to step 5 of Table 2.

[b] Enzyme activity was determined at 6-phosphogluconate concentrations between 6×10^{-5} and 10^{-2} M.

[c] Enzyme activity was determined at NADP concentrations between 10^{-5} and 10^{-3} M.

tions equivalent to step 5 of Table 2 and step 5 of Table 1). The 5′-nucleotides tested included adenosine monophosphate, adenosine diphosphate, ATP, guanosine monophosphate, guanosine diphosphate, GTP, deoxyadenosine triphosphate, deoxyguanosine triphosphate, cytidine triphosphate, and uridine triphosphate.

Except for deoxyguanosine triphosphate, which was nearly as potent an inhibitor of the NADP-specific enzyme as GTP, none of the other nucleotides significantly influenced the activity of the NADP-specific 6PGAD.

The enzyme active with NAD was inhibited by ADP and GTP as well as by ATP. However these compounds were less effective inhibitors than ATP. For example, 10^{-2} M GTP, ADP, and ATP inhibited the NAD-linked activity of the enzyme to 80, 67, and 50%, respectively, of its activity without nucleotides. None of the other nucleotides tested had any significant inhibitory effect.

Influence of different nucleotides on enzymes of the Entner-Doudoroff pathway. In view of the strong inhibition of 6PGAD by NADPH, we were interested whether key metabolites might influence the activity of 6-phosphogluconate dehydratase, the first enzyme of the Entner-Doudoroff pathway and a second potential control point affecting the metabolism of 6-phosphogluconate. As reported earlier (9), extracts of gluconate-grown *P. multivorans* have the capacity to convert 6-phosphogluconate to pyruvate, presumably via the Entner-Doudoroff pathway. When the influence of various metabolites on pyruvate formation was examined, it was found that citrate as well as a variety of nucleotides inhibited pyruvate formation, whereas magnesium and NADPH stimulated this process (see Table 7). The activity of 6-phosphogluconate dehydratase, the first enzyme of the Entner-Doudoroff pathway, is known to depend upon divalent cations (14). This probably explains the inhibition by citrate, which is an effective chelating agent, and the stimulation by magnesium, which also reversed the effect of citrate. Nucleotides are also known to form metal complexes, however magnesium did not reverse the inhibition by the nucleotides, suggesting that they might interact directly with one of the enzymes of the Entner-Doudoroff pathway. To pursue this possibility, we compared the influence of nucleotides on pyruvate formation from 6-phosphogluconate with that on KDPG aldolase, the second enzyme of the Entner-Doudoroff pathway. The *P. multivorans* extracts carried out a rapid conversion of KDPG to pyruvate, presumably through the action of KDPG aldolase. The apparent K_m for KDPG was 1.2×10^{-3} M, which is about 10-fold higher than the K_m of the corresponding enzyme from *P. fluorescens* (15). None of the compounds which inhibited or stimulated the overall conversion of 6-phosphogluconate to pyruvate (see Table 7) affected the conversion of KDPG to pyruvate. The results suggest that the nucleotides inhibit 6-phosphogluconate dehydratase activity, and that NADPH stimulates this enzyme. Such activation of 6-phosphogluconate dehydratase activity by NADPH might serve to complement the effect of NADPH on 6PGAD and increase the flow of 6-phosphogluconate into the Entner-Doudoroff pathway when the cellular levels of NADPH are

high. It is unlikely that the inhibition of 6-phosphogluconate dehydratase by nucleotides is physiologically important since high concentrations were required and nucleoside di- and monophosphates as well as the triphosphates inhibited the conversion of 6-phosphogluconate to pyruvate.

Although no data are available for intracellular levels of ATP, GTP, or NADPH in *P. multivorans*, it seems reasonable that the values are close to those reported for other gram-negative bacteria, which range between 10^{-3} and 2×10^{-3} for ATP and GTP, and between 10^{-4} and 2×10^{-4} for NADPH (1, 7, 10, 12). If these values are representative of the levels of nucleotides in *P. multivorans*, then it is unlikely that ATP or GTP would exert more than a minor control on the activities of the Entner-Doudoroff or pentose phosphate pathways, since 10^{-2} M concentrations were required for a significant effect on enzyme activity. However, at least in the case of 6PGAD, the amounts of ATP or GTP required for enzyme inhibition depended upon the levels of 6-phosphogluconate suggesting that inhibition by ATP or GTP might be important in regulating 6PGAD should the intracellular concentration of 6-phosphogluconate fall below some threshold level. The concentrations of NADPH required to inhibit 6PGAD are below the values reported for pool concentrations in gram-negative bacteria, and are consistent with the inhibition being physiologically significant. It would appear that inhibition 6PGAD by NADPH is more important in determining the relative distribution of 6-phosphogluconate into the Entner-Doudoroff and pentose phosphate pathways than interactions of 6PGAD or 6-phosphogluconate dehydratase with any other of the other ligands examined.

Substrate specificity of the two 6PGAD species. The substrate specificites of the two 6-phosphogluconate dehydrogenase species were studied by substituting different compounds for 6-phosphogluconate in otherwise standard assay mixtures containing 0.034 U of NADP-specific enzyme from a preparation equivalent to step 5 of Table 2 or 0.013 U of the enzyme active with NAD from a preparation equivalent to step 5 of Table 1. The compounds tested included glucose, 2-deoxy-glucose, gluconate, glucose-6-phosphate, fructose-6-phosphate, glucose-1-phosphate, fructose 1,6-diphosphate, ribulose-5-phosphate and 1,3-diphosphoglycerate all at a final concentration of 10^{-2} M. Under the conditions used, 1% of the activity normally observed with 6-phosphogluconate would be readily detected. Both 6PGAD species exhibited significant activity with glucose-6-phosphate as substrate. In the case of the NADP-specific enzyme, the activity observed was approximately 4% of the activity with 6-phosphogluconate. With the enzyme active with NAD, the activity with glucose-6-phosphate was approximately 2% of the activity with 6-phosphogluconate when NAD was cofactor. No activity was detected with glucose-6-phosphate when NADP was cofactor. None of the other compounds tested gave any detectable activity when substituted for 6-phosphogluconate.

TABLE 7. *Influence of nucleotides on the conversion of 6-phosphogluconate and 2-keto-3-deoxy-6-phosphogluconate to pyruvate by extracts of P. multivorans*[a]

Additions to the assay mixture	Relative rate of pyruvate formation[b] from:	
	6-Phosphogluconate	2-Keto-3-deoxy-6-phosphogluconate
None	100	100
Citrate (10^{-2} M)	47	101
$MgCl_2$ (10^{-2} M)	205	98
NAD (5×10^{-4} M)	96	105
NADH (5×10^{-4} M)	110	110
NADP (5×10^{-4} M)	120	98
NADPH (5×10^{-4} M)	195	94
ATP (10^{-2} M)	52	110
ADP[c] (10^{-2} M)	48	—
AMP[d] (10^{-2} M)	75	—
GTP (10^{-2} M)	59	118
UTP[e] (10^{-2} M)	38	—
CTP[f] (10^{-2} M)	49	—

[a] The assay mixtures contained 0.2 M Tris-hydrochloride buffer, pH 8.5, 0.01 M 2-mercaptoethanol, and between 102 and 200 μg of protein from an extract of gluconate grown bacteria, the indicated ligands, and either 0.01 M 6-phosphogluconate or 0.001 M 2-keto-3-deoxy-6-phosphogluconate. Pyruvate was determined as the dinitrophenylhydrazone.

[b] The rates of pyruvate formation in assay mixtures containing no added ligands were 56 nmol/min per mg of protein with 6-phosphogluconate and 47 nmol/min per mg of protein with 2-keto-3-deoxy-6-phosphogluconate.

[c] Adenosine 5'-diphosphate.

[d] Adenosine 5'-monophosphate.

[e] Uridine 5'-triphosphate.

[f] Cytidine 5'-triphosphate.

Activities of the two 6PGAD species in assay mixtures of different pH. The activities of the two 6PGAD species were determind in assay mixtures containing 2×10^{-1} M Tris-hydrochloride buffer at pH values between 7.2 and 9.5 (see Fig. 5). The activity of the NADP-specific enzyme did not change significantly

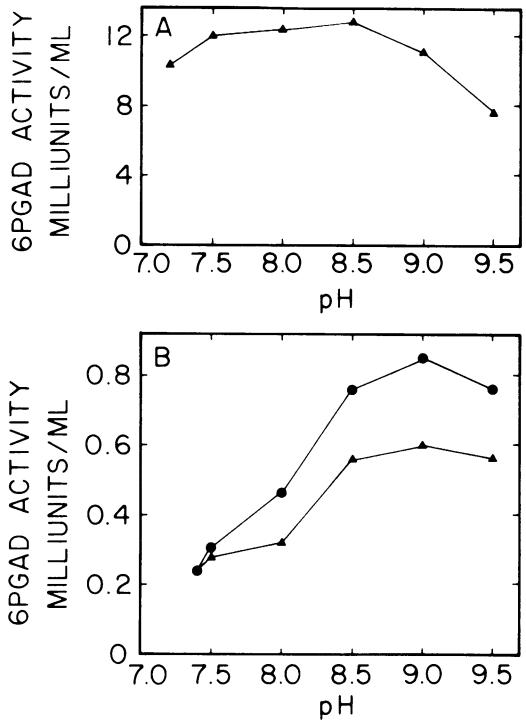

FIG. 5. *Determination of the activities of the two species of 6PGAD at different pH values. (A) NADP-specific enzyme: the assay mixture contained 1.2 μg of protein from a preparation equivalent to step 4 of Table 2. (B) Enzyme active with NAD (or NADP): the assay mixture contained 0.5 μg of protein from a preparation equivalent to step 5 of Table 1. (▲) NADP-linked activity, (●) NAD-linked activity.*

between pH 7.5 and 9.0 and showed optimal activity at about pH 8.5. The 6PGAD active with NAD progressively increased in activity as the pH was raised from 7.5 to 9.0 and was most active between pH 8.5 and 9.5. The standard assay buffer (2×10^{-1} M, Tris-hydrochloride, pH 8.5), therefore gave near optimal activity for both enzyme species.

Determination of isoelectric points of the two 6PGAD species. The procedure of Righetti and Drysdale (18) was used to determine the isoelectric point of each 6PGAD species. Samples of the two species of 6PGAD and of hemoglobin were resolved separately on 6% acrylamide gels containing the appropriate ampholyte solution to generate a gradient of pH 3 to 10. The gels were sliced into segments which were extracted with distilled water and the pH of each extract as well as its enzyme activity was determined (see Fig. 6). The pH of the gel extract containing the enzyme active with NAD was 6.9. That of the extract containing the NADP-specific enzyme was 5.0. These values are consistent with the different behavior of the two enzyme species on DEAE-cellulose and cellulose phosphate columns.

Determination of the MW of the two 6PGAD species. The MW of the two 6PGAD species were estimated by comparing their electrophoretic migration in acrylamide gels of different porosity with the migration of proteins of known MW as described by Hedrick and Smith (6). Samples of each preparation of purified 6PGAD were resolved electrophoretically on gels of 4.5, 6.0, and 7.5% (wt/vol) acrylamide, and the slopes of the lines obtained by plotting the relative mobilities of each enzyme as a function of acrylamide concentration of the gels were fit to a standard curve relating similar data obtained for several reference proteins and their MW (see Fig. 7). The MW estimates obtained by this procedure were 84,000 for the 6-phosphogluconate dehydrogen-

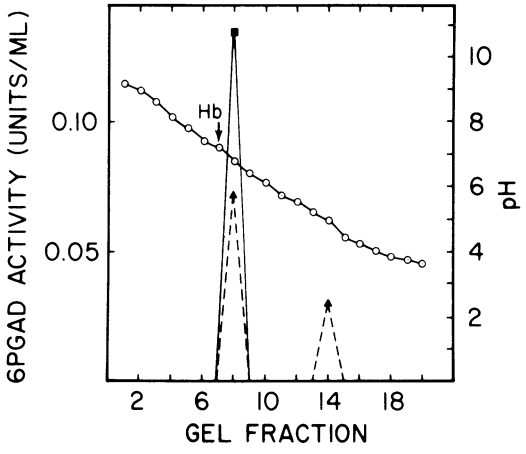

FIG. 6. *Resolution of the two P. multivorans 6PGAD species by isoelectric focusing. Samples (0.2 ml) of the enzyme active with NAD (70 μg of a preparation equivalent to step 5 of Table 2) and of the NADP-specific enzyme (70 μg of a preparation equivalent to step 5 of Table 2) were applied to separate acrylamide gels containing ampholytes appropriate for a pH 3 to 10 gradient (see Materials and Methods). A sample containing 140 μg of hemoglobin was applied to an identical gel. The proteins were resolved electrophoretically for 7 h (see Materials and Methods). The gels were sliced into 20 equal segments, and each segment was extracted with 3 ml of distilled water. A portion of each extract was assayed for enzyme activity, and the pH of the remaining fraction was determined. The position of hemoglobin was determined by measuring the absorbance of the extracts at 405 nm. Symbols: (■) NAD-linked 6PGAD activity; (▲) NADP-linked 6PGAD activity; (··) pH.*

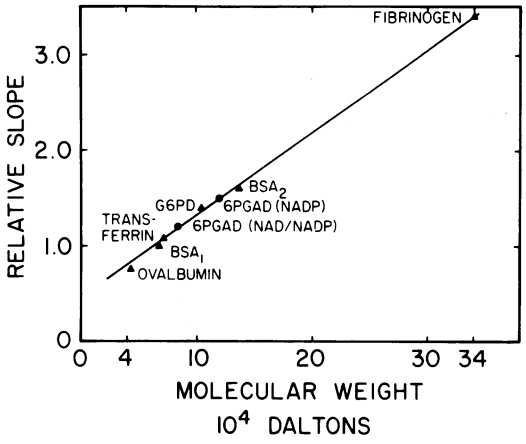

FIG. 7. *Standard curve relating the electrophoretic mobilities and MW of the two P. multivorans 6PGAD species and reference proteins of known MW. Samples containing between 20 and 50 μg of protein were applied to 4.5, 6.0, and 7.5% (wt/vol) acrylamide gels and resolved electrophoretically as described. Ovalbumin, bovine serum albumin (BSA), rabbit muscle lactic dehydrogenase (LDH) fructose-1,6-diphosphate aldolase (from rabbit), catalase (from beef liver), and yeast glucose-6-phosphate dehydrogenase (G6PD) were used as the reference proteins.*

ase active with NAD and 120,000 MW for the NADP-specific 6PGAD.

These values were in general agreement with those obtained by comparing the sedimentation behavior of the enzymes in sucrose gradients with that of bovine hemoglobin (MW 64,500), which gave values of 82,000 and 123,000, respectively, for the MW of the enzyme active with NAD and the NADP-specific enzyme. In these experiments 0.2-ml samples containing 500 μg of bovine hemoglobin and 0.3 U of purified 6PGAD from preparations equivalent to step 5 of Table 1 or step 5 of Table 2 were centrifuged through linear 5 to 20% sucrose gradients containing standard phosphate buffer. The apparent MW of the enzymes were determined by comparing the distribution of enzyme activity and hemoglobin in the gradients as described by Martin and Ames (13).

Determination of the subunit compositions of the two 6PGAD species. The number and size of subunits comprising the two 6PGAD species were determined by resolving dissociated preparations of the purified enzymes electrophoretically in acrylamide gels containing SDS. The preparations of purified enzyme were dissociated by treatment with SDS in the presence of 2-mercaptoethanol and resolved electrophoretically essentially as described by Weber and Osborn (25).

Only one band of protein was detected on the gels when dissociated preparations of either the enzyme active with NAD or the NADP-specific enzyme were examined, indicating that each enzyme was composed of subunits of identical or very similar MW. The subunits from the enzyme active with NAD migrated more rapidly than those from the NADP-specific enzyme, indicating that they were smaller than those of the NADP-specific enzyme. The electrophoretic mobilities of the subunits of the two 6PGAD species were compared to those of reference proteins of known MW and subunit composition.

When the data for the two 6PGAD subunits were fit to a standard curve relating the electrophoretic mobilities and MW of subunits from the reference proteins, respective MW values of 39,000 and 58,000 were obtained for the subunits of the enzyme active with NAD and for the NADP-specific enzyme (see Fig. 8). The data indicate that the enzyme active with NAD is composed of two subunits of approximately 40,000 MW and that the NADP-specific enzyme is composed of two subunits of approximately 60,000 MW.

The subunit composition of the NADP-specific 6PGAD is similar to that of the 6PGAD from *Neurospora crassa*, which is also specific for NADP. This enzyme has a MW between 110,000 and 120,000 and is composed of two subunits of 57,000 MW (20). It is interesting to note that the two polypeptide chains comprising the *Neurospora* enzyme may not be identical since they are specified by different genes (20). The subunits from the NADP-specific 6PGAD from *P. multivorans* have not been further characterized, and it remains possible that this enzyme is composed of two different subunits of similar size.

Immunological properties of the two 6PGAD species. Antiserum specific for each 6PGAD was prepared as described and used in enzyme inhibition and immunodiffusion studies to explore the possibility that the two enzymes might share a common evolutionary origin.

When the activities of purified preparations of the two enzymes were examined in the presence and absence of antiserum, a marked inhibition of 6PGAD activity was noted in the assay mixtures containing homologous combinations of enzyme and antiserum (see Table 8) but not in the assay mixtures containing heterologous combinations of enzyme and antiserum. At an overall dilution of tenfold, the antisera almost completely inhibited the activity of the homologous enzyme, but failed to influence the activity of the other 6PGAD.

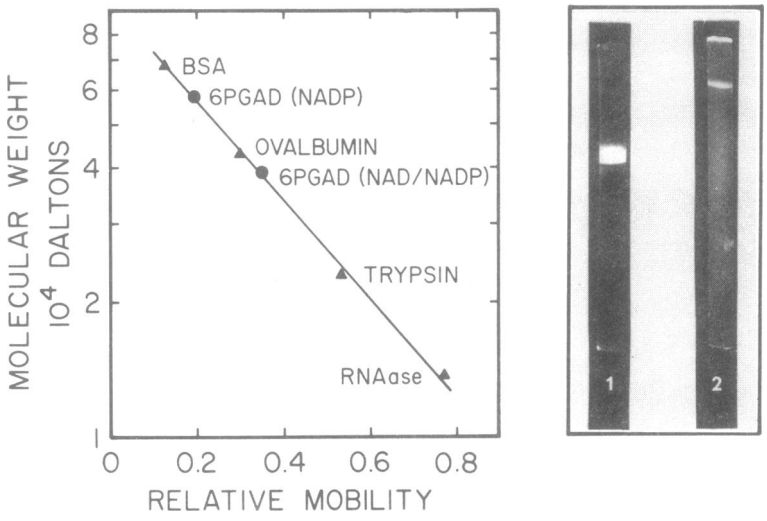

FIG. 8. *Standard curve relating the electrophoretic mobilities and molecular weights of subunits of known molecular weight in SDS gels. A. Samples of dissociated preparations of each purified 6PGAD species (containing 20 μg of the enzyme active with NAD or 10 μg of the NADP-specific enzyme) and of the standard proteins (150 μg) were resolved separately on 7.5% acrylamide gels, 4 by 100 mm) containing 0.2% SDS. The proteins were stained with coomassie brilliant blue. The MW of the subunits from each 6PGAD species were estimated by fitting the mobilities of the two enzymes to the curve. BSA, bovine serum albumin (68, 000 MW); ovalbumin (43,000 MW); trypsin (23,000 MW); RNase (13,700 MW); 6PGAD (NADP), NADP-specific 6PGAD; 6PGAD (NAD/NADP). 6PGAD active with NAD or NADP. (B) Resolution of the subunits from the two species of 6PGAD, gel 1. Subunits of the enzyme active with NAD or NADP, gel 2. Subunits of the NADP-specific enzyme.*

TABLE 8. *Inhibition of the activity of the two 6PGAD species by homologous antisera*[a]

Antiserum	Overall dilution of antiserum	6PGAD active with NAD[b]		6 PGAD specific for NADP[c]
		NAD-linked activity[c]	NADP-linked activity[c]	
Anti-6PGAD I	1:8	3	5	100
	1:50	27	46	100
(enzyme active with NAD)	1:100	54	55	100
Anti-6PGAD II	1:10	100	100	0
	1:50	92	114	40
(NADP-specific enzyme)	1:100	100	102	50

[a] (Activity with antiserum/activity without antiserum) × 100.

[b] The assay mixtures contained 0.001 U of enzyme from a preparation equivalent to step 5 of Table 1.

[c] The assay mixture contained 0.008 U of enzyme from a preparation equivalent to step 5 of Table 2.

The same antiserum and enzyme preparations were used in conventional immunodiffusion experiments in 0.8% agarose gels in an attempt to extend the results of the enzyme inhibition studies. Between 20 and 30 μg of each 6PGAD were added to different sample wells. Samples of each antiserum (0.4 ml) were added to different wells opposite the enzyme samples. Within 24 to 48 h, precipitin bands developed between the homologous pairs of enzyme and antiserum, but not between either enzyme and the heterolous serum (see Fig. 9). In each case there were in addition to the main precipitin band several minor bands. These may represent interactions with enzyme aggregates or subunits which formed in the unbuffered gels.

The inhibitory effects of the antisera on the homologous enzymes and the results of the immunodiffusion experiments indicate that the two species of 6PGAD do not share common antigenic determinants, and lead us to conclude that the genes specifying the two enzymes do not share a common origin.

DISCUSSION

The purpose of the present study was to purify and characterize the two 6PGAD species from *P. multivorans* with the aim of defining aspects of their structure and function. We were interested primarily in (i) examining interactions between the enzymes and ligands which might affect the relative distribution of 6-phosphogluconate into the Entner-Doudoroff and

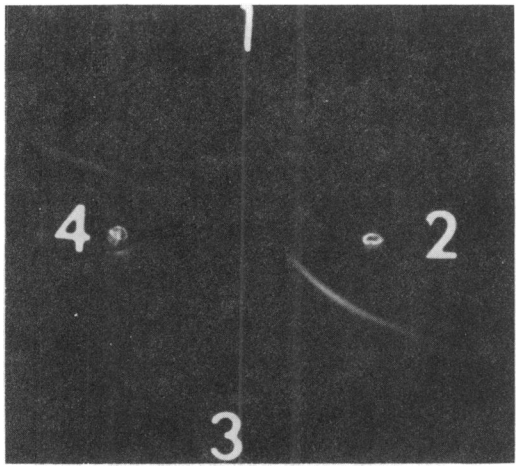

FIG. 9. *Reaction between antisera prepared against the two 6PGAD species and the homologous enzymes. Well 1 contained 30 μg of protein from a preparation of the enzyme active with NAD (equivalent to step 5 of Table 1). Well 3 contained the same amount of NADP-specific enzyme from a preparation equivalent to step 5 of Table 2. Well 2 contained 0.04 ml of antiserum prepared against the NADP-specific enzyme and well 4 0.04 ml of antiserum prepared against the enzyme active with NAD. The agarose gel was incubated for 3 days at room temperature, washed several times with 0.5% NaCl solution, and stained with 0.1% buffalo black and washed with 7% (vol/vol) acetic acid.*

pentose phosphate pathways in this bacterium (see Fig. 10) and (ii) determining whether the common loss of both 6PGAD (as well as the two glucose-6-phosphate dehydrogenase species found in this organism) in mutant strains defective in glucose catabolism (T. G. Lessie and H. A. Shuman. Annu. Meet. Amer. Soc. Microbiol. 1974, P123, p. 165) might indicate that the enzymes share a common subunit.

As reported earlier (9), the levels of the four *P. multivorans* 6-phosphogluconate and glucose-6-phosphate dehydrogenase species are regulated independently. This suggests that the genes encoding the four enzymes are not organized into a single operon, and that the joint loss of the enzymes in the mutant strains cannot be explained on the basis of such a genetic relationship. The results of our studies of the subunit structures and antigenic properties of the two 6PGAD species rule out the possibility that all four enzymes share a common subunit. At present we can offer no explanation for the apparent interdependence between the glucose-6-phosphate and 6PGAD species in this bacterium.

In *P. multivorans*, the levels of enzymes which convert 6-phosphogluconate to C_3 intermediates (via the Entner-Doudoroff pathway) are roughly equivalent to those of 6PGAD, the first enzyme of the pentose phosphate pathway. We have examined several end products of the Entner-Doudoroff and pentose phosphate pathways to determine whether thay might influence the activities of 6PGAD or 6-phosphogluconate dehydratase and thereby regulate the flow of 6-phosphogluconate into the two pathways (see Fig. 10). There are precedents for regulation of the activities of the Entner-Doudoroff enzymes and of 6PGAD in other microorganisms. Senior and Dawes have shown that in Azotobacter citrate and isocitrate as well as ATP inhibit the conversion of 6-phosphogluconate to pyruvate and that NADPH inhibits the activity of 6PGAD (21). Studies of *Streptococcus faecalis* 6PGAD by A. T. Brown and C. L. Wittenberger have revealed that this bacterium possesses two 6PGAD species: an NAD-specific enzyme which is inhibited by ATP and an NADP-specific enzyme which is inhibited by fructose diphosphate (2). In the case of *P. multivorans* our in vitro studies suggest that the most important regulatory interactions determining the distribution of 6-phosphogluconate into the branches of glucose or gluconate catabolism leading from 6-phosphogluconate (see Fig. 10) are inhibition of the 6PGAD active with NAD by NADPH and a complementary stimulation by this ligand of the rate of conversion of 6-phosphogluconate to pyruvate apparently through an effect on 6-phosphogluconate dehydratase activity.

The overall pattern of induction of enzymes of the Entner-Doudoroff and pentose phosphate pathways as well as the interactions of the first enzymes of the two pathways with different

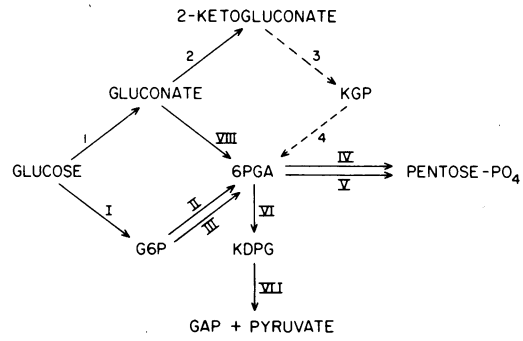

FIG. 10. *Pathways of glucose degradation in P. multivorans. The steps indicated by solid lines have been demonstrated using extracts of bacteria grown with glucose or gluconate as sole carbon source. Steps indicated by broken lines have not been examined in this bacterium, but have been demonstrated in other pseudomonas species.*

ligands suggest that a key role of the pentose phosphate pathway in addition to that of supplying five carbon intermediates is to provide for an increase in NADPH and NADH formation when the bacteria are grown on glucose or gluconate. Since the NADP-specific 6PGAD from *P. multivorans* is formed constitutively, it presumably is the primary enzyme supplying pentose phosphate for nucleotide biosynthesis. The inhibition of the enzyme by GTP is consistent with such a role. In this regard *P. multivorans* differs from *P. aeruginosa*, which appears to lack 6PGAD except during growth on glucose or gluconate, and presumably has an alternate means of synthesizing pentose phosphate when it grows on other carbon sources (8). The observation that the *P. multivorans* 6PGAD active with NAD is induced during growth with glucose or gluconate as sole carbon source, but not with citrate or pyruvate, and the fact that the enzyme exhibits activity with NAD suggests that it serves a role in the catabolism of glucose and gluconate. However, the enzyme has a greater affinity for pyridine nucleotide and 6-phosphogluconate with NADP as cofactor than with NAD. This suggests that a major function of this species is to provide the cell with additional NADPH. If the demand for NADPH is greater during growth on glucose or gluconate than with alternate carbon sources it might be because significant formation of 6-phosphogluconate occurs through direct oxidation of glucose and gluconate to 2-ketogluconate (see Fig. 10) as proposed by a number of investigators for other *Pseudomonads* (17, 23, 24, 26). The subsequent metabolism of KG via the proposed route involves its conversion to 2-keto-6-phosphogluconate which is in turn reduced to 6-phosphogluconate in a step requiring NADH or NADPH (19). We have found that extracts of *P. multivorans* possess glucose and gluconate oxidase activity when the bacteria are grown on glucose or gluconate, but not when they are grown with pyruvate or citrate ([5] and unpublished data). We have also found that 2-ketogluconate will serve as sole carbon source for growth of *P. multivorans*, and that the 6PGAD active with NAD is induced under such conditions (unpublished data). The results are consistent with formation of 6-phosphogluconate via the non-phosphorylative or direct oxidative conversion of glucose to and KG. Thus the induction of the 6PGAD active with NAD or NADP (as well as of the glucose-6-phosphate dehydrogenase active with NAD or NADP) might serve to provide additional NADH or NADPH for conversion of KG phosphate to 6-phosphogluconate as suggested by Quay et al. (17). This could explain why both the 6-phosphogluconate and glucose-6-phosphate dehydrogenases which are active with NAD or NADP are subject to strong inhibition by NADPH. Clearly, further definition of the roles of the multiple forms of glucose-6-phosphate and 6PGAD in *P. multivorans* and of alternate pathways of 6-phosphogluconate formation must await studies of the appropriate mutants. The degradation of glucose and regulation of this process in *P. multivorans* and in other *Pseudomonas* species now appears more complex than was apparent in earlier studies and is likely to show significant variations between different major groups of *Pseudomonads*. (16, 23, 26).

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LITERATURE CITED

1. Bagnara, A. S., and L. R. Finch. 1973. Relationships between intracellular contents of nucleotides and 5-pyrophosphoryl 1-pyrophosphate in *Escherichia coli*. Eur. J. Biochem. **36**:422-427.
2. Brown, A. T., and C. L. Wittenberger. 1972. Induction and regulation of a nicotinamide adenine dinucleotide specific 6-phosphogluconate dehydrogenase in *Streptococcus faecalis*. J. Bacteriol. **109**:106-115.
3. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. **121**:404-427.
4. Entner, N., and M. Doudoroff. 1952. Glucose and gluconic acid oxidation of *Pseudomonas saccharophila*. J. Biol. Chem. **196**:853-862.
5. Hauge, J. G. 1966. Glucose dehydrogenases-particulate. I. *Pseudomonas* species and *Bacterium anitratum*, p. 92-98. *In* W. A. Wood (ed.), Methods in enzymology, vol. 9. Academic Press Inc. New York.
6. Hedrick, J. L., and A. J. Smith. 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys. **126**:155-164.
7. Holms, W. H., I. D. Hamilton, and A. G. Robertson. 1972. The rate of turnover of the adenosine triphosphate pool of *Escherichia coli* growing aerobically in simple defined media. Arch. Mikrobiol. **83**:95-109.
8. Lessie, T. G., and F. C. Neidhardt. 1967. Adenosine triphosphate-linked control of *Pseudomonas aeruginosa* glucose-6-phosphate dehydrogenase. J. Bacteriol. **93**:1337-1345.
9. Lessie, T. G., and J. C. Vander Wyk. 1972. Multiple forms of *Pseudomonas multivorans* glucose-6-phosphate and 6-phosphogluconate dehydrogenases: differences in size, pyridine nucleotide specificity, and susceptibility to inhibition by adenosine-5'-triphosphate. J. Bacteriol. **110**:1107-1117.
10. London, J., and M. Knight. 1966. Concentrations of nicotinamide nucleotide coenzymes in micro-organisms. J. Gen. Microbiol. **44**:241-254.
11. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.

12. Lundquist, R., and B. M. Olivera. 1971. Pyridine nucleotide metabolism in *Escherichia coli*. J. Biol. Chem. **246**:1107-1116.
13. Martin, R. C., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372-1379.
14. Meloche, H. P., J. M. Ingram, and W. A. Wood. 1966. 2-Keto-3-deoxy-6-phosphogluconic aldolase (crystalline), p. 520-524. *In* W. A. Wood (ed.), Methods in enzymology, vol. 9. Academic Press Inc., New York.
15. Meloche, H. P., and W. A. Wood. 1966. 6-Phosphogluconic dehydrase, p. 653-656. *In* W. A. Wood (ed.), Methods in enzymology, vol. 9. Academic Press Inc., New York.
16. Ornston, L. N. 1971. Regulation of catabolic pathways in *Pseudomonas*. Bacteriol. Rev. **35**:87-116.
17. Quay, S. C., S. B. Friedman, and R. C. Eisenberg. 1972. Gluconate regulation of glucose catabolism in *Pseudomonas fluorescens*. J. Bacteriol. **112**:291-298.
18. Righetti, P., and J. W. Drysdale. 1971. Isoelectric focusing in polyacrylamide gels. Biochim. Biophys. Acta **236**:17-28.
19. Roberts, B., M. Midgley, and E. A. Dawes. 1973. The metabolism of 2-oxogluconate by *Pseudomonas aeruginosa*. J. Gen. Microbiol. **78**:319-329.
20. Scott, W. A., and T. Abramsky. 1973. Neurospora 6-phosphogluconate dehydrogenase. II. Properties of the two purified mutant enzymes. J. Biol. Chem. **248**:3542-3545.
21. Senior, P. J., and E. A. Dawes. 1971. Poly-beta-hydroxybutyrate biosynthesis and regulation of glucose metabolism in *Azotobacter beijerinkii*. Biochem. J. **125**:55-66.
22. Vander Wyk, J. C., and T. G. Lessie. 1974. Purification and characterization of the *Pseudomonas multivorans* glucose 6-phosphate dehydrogenase active with nicotinamide adenine dinucleotide. J. Bacteriol. **120**: 1033-1042.
23. Vicente, M., and J. L. Cánovas. 1973. Glucolysis in *Pseudomonas putida*: physiological roles of alternative routes from the analysis of defective mutants. J. Bacteriol. **116**:908-914.
24. Vicente, M., and J. L. Cánovas. 1973. Regulation of glucolytic enzymes in *Pseudomonas putida*. Arch. Mikrobiol. **93**:53-64.
25. Weber, K., and M. J. Osborn. 1969. The reliability of molecular weight determinations by dodecyl-sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406-4412.
26. Wood, W. A. 1955. Pathways of carbohydrate degradation in *Pseudomonas fluorescens*. Bacteriol. Rev. **19**:222-233.