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NADP-Dependent Glutamate Dehydrogenase from a Facultative Methylotroph, *Pseudomonas* sp. Strain AM1

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The NADP-dependent glutamate dehydrogenase (EC 1.4.1.4.) elaborated by the methylotrophic bacterium *Pseudomonas* sp. strain AM1 when growing on succinate and ammonium chloride was studied. The enzyme, which has a pH optimum of 9.0, was purified 140-fold and shown to have K_m values of 20.2 mM, 0.76 mM, 0.033 mM, and 31.6 mM for ammonia, α-ketoglutarate, NADPH, and glutamate, respectively. The native molecular weight was determined by polyacrylamide gel electrophoresis to be 190,000, and electrophoresis under denaturing conditions in the presence of sodium dodecyl sulfate revealed a minimum molecular weight of 50,000. The enzyme was highly specific; NADH was unable to replace NADPH in the reaction, various α-keto acids could not replace α-ketoglutarate, and neither methylamine nor hydroxylamine could substitute for ammonia. Glutamate dehydrogenase was synthesized by the bacteria only when ammonia was its nitrogen source and was repressed if methylamine or nitrate were provided as sources of nitrogen instead of ammonia.

Pseudomonas sp. strain AM1, a facultative methylotroph, can grow by using methylamine as the sole source of carbon, nitrogen, and energy (21) or can use methylamine as the sole nitrogen source if an alternative, more preferred carbon source is supplied (5). When methylamine acts as the nitrogen source, the nitrogen from it is assimilated via glutamine synthetase (EC 6.3.1.2) and glutamine:2-oxoglutarate aminotransferase (EC 1.4.1.13), and the cells possess only small amounts of NADP-dependent glutamate dehydrogenase (EC 1.4.1.4) (GDH) under these conditions. However, if ammonium chloride is provided as the nitrogen source with succinate as the carbon source, the organism elaborates a high level of an NADP-dependent GDH that has a pH optimum of 9.0 (4). This finding was of interest because previous studies had indicated that GDH was absent in some methylotrophs (18, 25), even after growth with ammonia as the nitrogen source. Moreover, the GDH gene from *Escherichia coli* had been cloned into *Methylophilus methylotrophus* to try to increase cell yield of this organism in industrial use (27).

Nevertheless, when ammonia is the sole nitrogen source, *Pseudomonas* sp. strain AM1 still possesses relatively high levels of biosynthetically active glutamine synthetase, thereby casting some doubt on the role of GDH in this organism (4). Although it has been briefly reported that type I methanotrophs have both GDH activities (20), no study of GDH from a methylotrophic bacterium has been described. We therefore decided to examine this enzyme in more detail, and this manuscript presents the results of those studies. NADP-dependent GDHs have been found in *E. coli* (23), *Salmonella typhimurium* (10), *Nitrosomonas europaea* (14), *Brevibacterium flavium* (24), and three strains of *Bacillus* spp. (11, 13, 22). *Coprinus cinereus* (1), *Hydrogenomas* sp. strain H16 (16), *Thiobacillus novellus* (17), and *Pseudomonas aeruginosa* (15) possess both NAD- and NADP-dependent enzymes, and *Mycoplasma laidlawii* has a GDH of dual coenzyme specificity (28).

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MATERIALS AND METHODS

Growth and maintenance of organism. *Pseudomonas* sp. strain AM1 (ATCC 14718) was grown in 500-ml batches of minimal salts medium (7) at 30°C. Fifty millimolar succinate, methylamine, or 0.5% (vol/vol) methanol were used as carbon sources; 50 mM NH_4Cl, methylamine, or 0.1% (wt/vol) KNO_3 were used as nitrogen sources. For larger amounts of cells, *Pseudomonas* sp. strain AM1 was grown in 10-liter batches of minimal salts medium containing succinate-NH_4Cl. Growth was started with a 5% inoculum from a culture previously grown on the same carbon and nitrogen sources and monitored with a Bausch & Lomb Spectronic 20, with a wave-length of 650 nm.

Cells were harvested at mid-exponential phase by centrifugation and washed twice with 20 mM phosphate buffer (pH 7.2). The cells were then quickly frozen in liquid nitrogen and stored at −20°C.

Preparation of cell extracts. Frozen cells were thawed and resuspended in 50 mM Tris-hydrochloride buffer (pH 8.0) at a ratio of 1 g of cells (wet weight) to 1 ml of buffer. The cells were disrupted by passage twice through a French pressure cell at 138 MPa. The exudate was centrifuged at 38,000 × *g* for 40 min, and the pellet was discarded.

Protein assay. Protein was determined by the method of Bradford (8). Bovine serum albumin was used in the preparation of standard curves.

Enzyme assays. GDH (EC 1.4.1.4) was assayed by following the initial rates of NADPH oxidation at 340 nm in a Gilford 222 spectrophotometer. The reaction mixture contained 2-ketoglutarate (10 μmol); NH_4Cl (100 μmol); NADPH (0.25 μmol); and Tris-hydrochloride buffer (pH 9.0) (5.0 μmol), in a total volume of 1.0 ml. For assay of the reverse (glutamate-deaminating) reaction, the mixture contained glutamate (100 μmol) and NADP (1 μmol) in 50 mM Tris buffer (pH 9.0) in a total volume of 1.0 ml.

Electrophoresis. Polyacrylamide gels are described by the following notation. T denotes the total weight of acrylamide and *N,N'*-methylenebisacrylamide(bis) in 100 ml of solvent. C denotes the amount of bisacrylamide expressed as a percentage of the total amount of monomer (19).

Routine electrophoresis was performed with gels of the

TABLE 1. Summary of purification procedure

Fraction	Vol (ml)	U/ml	Protein (mg/ml)	Sp act (U/mg of protein)	Purification
Crude extract	35.0	7.72	24.0	0.322	1
Streptomycin sulfate treatment	37.0	7.60	20.0	0.380	1
Acetone precipitation	11.5	18.00	16.8	1.07	3
Heat treatment	11.5	18.00	6.4	2.81	9
DEAE-cellulose chromatography	28.0	2.35	0.11	21.4	66
Heat treatment	28.0	2.35	0.08	29.4	91
Acetone precipitation	1.0	14.5	0.32	45.2	140

following composition: stacking gel (50 mM Tris-hydrochloride) (pH 6.7), 0.05% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine, 0.1% (wt/vol) ammonium persulfate, 3.08% T, and 2.50% C; separating gel (750 mM Tris-hydrochloride) (pH 8.9), 0.06% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine, 0.05% (wt/vol) ammonium persulfate, 6.0% T, and 3.23% C. The running buffer was 15 mM Tris-glycine (pH 8.3).

Protein samples ranged from 10 to 50 μg per tube and were detected after electrophoresis by a modification of the method of Chrambach et al. (9). Gels were fixed in 12% (wt/vol) trichloroacetic acid for 10 min and stained with a freshly prepared solution of Coomassie brilliant blue R250 (0.1% [wt/vol] in 12% trichloroacetic acid) at 60°C for 1 h. Gels were destained overnight in 7% (vol/vol) acetic acid.

Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed as described by Weber and Osborn (26), except that gels composed of 9.24% T and 3.51% C were used.

Electrophoretic mobilities (R_f) of protein bands were calculated from densitometric scans at 554 nm with a spectrophotometer equipped with a Gilford 2410-S linear transport device.

Activity staining. GDH was detected after electrophoresis on polyacrylamide gels by immersing the gels in a solution containing glutamate (500 μmol); $NADP^+$ (10 μmol); Tris-hydrochloride (pH 9.0) (250 μmol); phenazine methosulfate (0.5 mg); and nitrobluetetrazoleum (1.0 mg) in a final volume of 5.0 ml. The reaction was terminated after 10 min by replacing the staining solution with 7% acetic acid. GDH appeared as a single purple band of formazan precipitate. Controls in which glutamate was omitted did not produce any stain in the gel.

Enzyme purification. Unless otherwise indicated, all procedures were carried out at 4°C and centrifugations were at 38,000 × *g* for 10 min at 0°C. A 50 mM Tris-hydrochloride (pH 8.0) buffer was used and will be referred to simply as buffer.

(i) Streptomycin sulfate treatment. A neutralized 10% (wt/vol) streptomycin sulfate solution was slowly added to the crude cell extract (0.1 ml of streptomycin sulfate per 1 ml of extract). The solution was stirred for 5 min and centrifuged to remove precipitated nucleic acids.

(ii) Acetone precipitation. The supernatant fluid from the previous step was chilled to 0°C. Acetone at −70°C was added until its concentration was 10% by volume. The precipitate was collected by centrifugation and dissolved in a minimal amount of buffer.

(iii) Heat treatment. The protein solution from the preceding step was heated and maintained at 58°C for 5 min. Denatured proteins were removed by centrifugation and discarded.

(iv) DEAE-cellulose chromatography. The supernatant fluid from step (iv) was chromatographed on a DEAE-cellulose column (10 by 2.5 cm) previously equilibrated with buffer. A linear gradient of 150 to 350 mM Tris-hydrochloride buffer (pH 8.0) was applied, and fractions eluting between 250 and 300 mM were pooled. The enzyme eluted as a single peak from DEAE-cellulose columns.

(v) Heat treatment. The pooled fractions were heat treated as described above.

(vi) Acetone precipitation. The supernatant fluid from step (v) was chilled to 0°C. Acetone at −70°C was added to it until its concentration was 20% by volume, and the mixture was centrifuged. To the supernatant, acetone was again added until its concentration was 40% by volume. The resulting precipitate was collected by centrifugation and dissolved in 25 mM Tris-hydrochloride (pH 8.0) containing 50% (vol/vol) glycerol, 1 mM 2-ketoglutarate, and 1 mM dithiothreitol.

The procedure described resulted in the 140-fold enhancement of enzyme-specific activity with a recovery of 5%. When stored in 25 mM Tris (pH 8.0)–1 mM dithiothreitol–1 mM 2-ketoglutarate–50% glycerol at −20°C, the preparation retained >95% activity for 1 month and had 60% of its initial activity after 8 months.

Molecular weight estimation. Molecular weight was determined by a modification of the method of Hedrick and Smith (12), with polyacrylamide disk gel electrophoresis. The composition of the stacking gel was identical to that described for routine electrophoresis. The separating gel composition was also similar, but the total monomer concentration was varied from 5 to 8% T, with bisacrylamide concentration kept constant at 3.23% C.

Chemicals. 2-ketoglutarate, glutamate, $NADP^+$, NADPH, ammonium persulfate, Tris, phenazine methosulfate, nitroblue tetrazoleum, *N,N,N',N'*-tetramethylethylenediamine, pepsin, ovalbumin, lactate dehydrogenase, catalase, and urease were purchased from Sigma Chemical Co. *N,N'*-methylenebisacrylamide, SDS, and glutamine were purchased from Aldrich Chemical Co. Electrophoresis grade acrylamide was purchased from J. T. Baker Chemical Co.

RESULTS

Enzyme purification. The results of a typical enzyme purification are depicted in Table 1. A 140-fold purification was achieved with a final specific activity of 45.2 U/mg of protein. Polyacrylamide disk electrophoresis of the purified GDH preparation revealed the presence of two faint minor bands and one major band of protein which coincided with the single band obtained by activity staining of both the crude extract and the purified enzyme.

Molecular weight estimation. Figure 1 shows the molecular weight calibration obtained from electrophoresis of standard proteins and GDH at different gel compositions. From these data, the molecular weight of GDH was estimated to be 190,000 ± 10,000. Electrophoresis under denaturing condi-

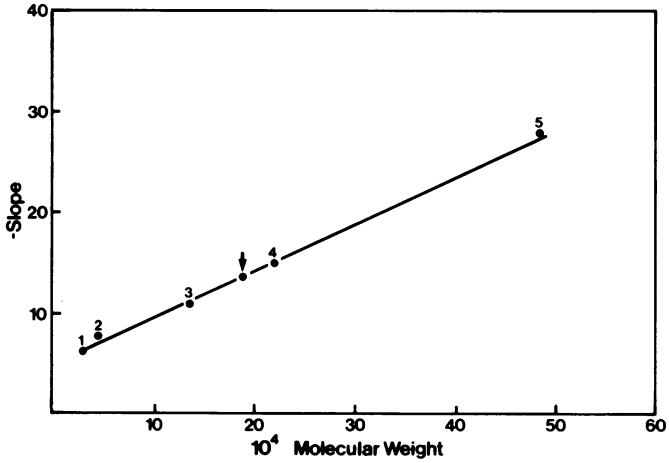

FIG. 1. Molecular weight calibration obtained from electrophoresis of standard proteins in acrylamide gels of varying monomer concentrations. Values on the ordinate were obtained from plots of mobility versus total monomer concentration for GDH and standard proteins subjected to electrophoresis in gels made of 5, 6, 7, and 8% total monomer concentration. Standard proteins used and their molecular weights were: (1) pepsin (32,700); (2) ovalbumin (45,000); (3) lactate dehydrogenase (140,000); (4) catalase (220,000); and (5) urease (483,000). Arrow indicates value obtained for GDH.

tions in the presence of SDS revealed a minimum molecular weight of 50,000 ± 4,000 (Fig. 2).

Kinetic properties. The enzyme exhibited typical Michaelis-Menten kinetics. K_m values determined with the nonvariable substrates at saturating concentrations (millimolar) were: NH_3, 20.2 (compared with 18.2 for the crude extract [3]); 2-ketoglutarate, 0.76; and NADPH, 0.033. The reaction was reversible, and the K_m for glutamate in the deaminating direction was 31.6 mM.

Substrate specificity and inhibition. GDH was highly specific for its substrates. Ammonia could not be replaced by methylamine or hydroxylamine. NADH could not replace NADPH, and 2-ketoadipate, oxaloacetate, and pyruvate were unable to substitute for 2-ketoglutarate. The effects of various substrate analogs, amino acids, and nucleotides on

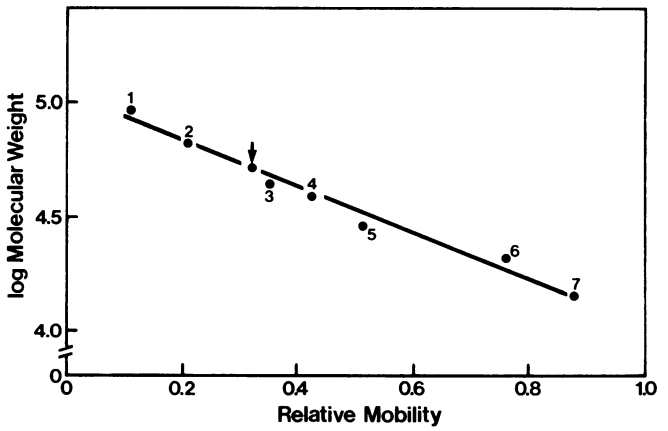

FIG. 2. Determination of minimum molecular weight by SDS-gel electrophoresis. The standard proteins used and their molecular weights were: (1) phosphorylase B (92,500); (2) bovine serum albumin (66,200); (3) ovalbumin (45,000); (4) aldolase (40,000); (5) carbonic anhydrase (31,000); (6) soybean trypsin inhibitor (21,500); and (7) lysozyme (14,400). Arrow indicates mobility of GDH.

TABLE 2. Effect of various inhibitors on GDH activity

Inhibitor	Conc (mM)	% Inhibition	
		Standard assay conditions	Nonstandard assay conditions
2-Ketoadipate	5	0	4[a]
Oxaloacetate	5	0	7[a]
Pyruvate	5	6	4[a]
Glutarate	5	15	20[a]
Glyoxylate	5	0	11[a]
Glutamate	5	0	16[a]
Glutamine	5	0	9[a]
Serine	5	11	
Glycine	5	11	
Methylamine	40	0	0[b]
Hydroxylamine	40	33	44[b]
ATP	1	6	
ADP	1	−17	
AMP	1	−17	
$MgCl_2$	1	0	
$ZnCl_2$	2	19	
$HgCl_2$	0.1	29	

[a] Concentrations of 2-ketoglutarate and inhibitors were 2 mM.
[b] Ammonium chloride concentration was 40 mM.

the aminating reaction are shown in Table 2. It can be seen that the enzyme is not markedly influenced by any of the organic compounds tested, with hydroxylamine at 40 mM showing the largest inhibitory effect. The relatively strong effect of Hg^{2+} at 0.1 mM could indicate the involvement of sulfhydryl groups in the reaction.

Regulation of GDH synthesis. The effect of the composition of the growth medium on GDH levels in the cells was investigated, and the results are shown in Table 3. For these experiments, extracts from the exponential-growth phase were used from cultures that had been started with a 5% inoculum from a culture grown under the same conditions. The data shown are the averages of at least three separate determinations. GDH seems to be produced in this organism in response to NH_4Cl in the growth medium. Glutamate in the growth medium was without affect, although the cells failed to grow on glutamate alone or with glutamate as the sole nitrogen source, perhaps indicating an inability of glutamate to enter the cell. The lowest levels of GDH were found when NH_4Cl was absent from the growth medium, e.g., when methylamine or nitrate was the nitrogen source. If methylamine is also the sole carbon source, then external

TABLE 3. Effect of various nitrogen sources on GDH activity

Growth condition[a]	Sp act (nmol of $NADP^+$ formed per min/mg of protein)
Succinate-NH_4Cl	367 ± 45
Succinate-NH_4Cl (10 mM)	80 ± 10
Succinate-NH_4Cl-$MeNH_2$	342 ± 50
Succinate-$MeNH_2$	67 ± 7
$MeNH_2$	52 ± 20
$MeNH_2$-NH_4Cl	25 ± 13
MeOH-NH_4Cl	67 ± 3
Succinate–NH_4Cl–5 mM glutamate	357 ± 38
Glutamate	—[b]
Succinate-glutamate	—[b]
Succinate-KNO_3	0

[a] NH_4Cl was used at a concentration of 50 mM except where stated.
[b] No cell growth.

NH_4Cl is without effect. The low level of GDH found in cells grown on methanol and ammonium chloride was surprising in view of the value obtained from cells grown on succinate and ammonium chloride.

DISCUSSION

The results described in this paper indicate that the GDH found in *Pseudomonas* sp. strain AM1 is involved in the assimilation of ammonia by this organism. The enzyme was present in cells in high amounts only when ammonium chloride was the nitrogen source and very little or zero enzyme was found when methylamine or nitrate was provided as the nitrogen source. However, the enzyme is not produced in the presence of ammonium chloride if methylamine is the sole carbon source. This could indicate that under these conditions the cells are using the nitrogen from methylamine and not from the exogenous ammonium chloride (since methylamine provides a large excess of nitrogen because of its carbon/nitrogen ratio of 1). This concept receives support from findings obtained from studies on methylamine uptake into these bacteria which showed that ammonia could not inhibit methylamine uptake in cells grown on methylamine alone but could cause an inhibition in cells grown with methylamine as the sole nitrogen source (3, 5, 6). However, it has been pointed out that methylotrophically growing cells are not ATP limited, but NADH limited, unlike cells growing on multicarbon compounds (2). Thus, the availability of the necessary coenzymes could influence the means of nitrogen assimilation. This could also explain the lower level of GDH found in cells grown on methanol-ammonia in comparison with those grown on succinate-ammonia.

The K_m value of GDH obtained for ammonia (20.2 mM) is relatively high compared with that for 2-ketoglutarate. However, this is not unusual as GDHs from several other sources have similarly high K_m values (11, 13, 14).

The fact that there are also high levels of glutamine-synthetase–glutamine:2-oxoglutarate aminotransferase present during growth of *Pseudomonas* sp. strain AM1 on succinate-ammonium chloride, coupled with the previous findings that other methylotrophs completely lack GDH (18, 20, 25, 27), raised the possibility that the GDH found in *Pseudomonas* sp. strain AM1 was used for glutamate degradation rather than its synthesis. However, the very high K_m value for glutamate (31.6 mM) in the deaminating reaction together with the low K_m value for 2-ketoglutarate (0.76 mM) tend to rule out this hypothesis. Although it might be expected that an enzyme in such a biosynthetic role would be repressed by an external supply of glutamate, this was not the case. However, *Pseudomonas* sp. strain AM1 was unable to utilize glutamate for growth nor as a sole nitrogen source, so it is possible that glutamate could not be transported into this organism.

The enzyme has a molecular weight of 190,000 as determined by gel electrophoresis with gels of varying pore size. This method was used because it was not possible to recover activity after passage of the enzyme preparation through gel-filtration columns. Attempts to reconstitute activity by recombining fractions from such columns were also unsuccessful. GDH enzymes from several other organisms have molecular weights in the region of 300,000 (10, 13, 14, 23, 24); but the NAD-dependent enzyme from *P. aeruginosa* has a molecular weight of 110,000 (15), and, on the other extreme, Epstein and Grossowicz reported that the enzyme from a thermophilic bacillus had a molecular weight of 2×10^6 (11). Denaturing SDS-electrophoresis revealed a minimum molecular weight of 50,000, indicating that the enzyme is composed of four subunits. GDH enzymes from *E. coli* (23) and *Bacillus megaterium* (13) have six subunits, and that from *Mycoplasma laidlawii* has five (28).

As for most other GDHs, the enzyme from *Pseudomonas* sp. strain AM1 is highly specific for all three of its substrates, and it seems to be remarkably insensitive to inhibitors. Its main means of regulation therefore appears to be through repression and derepression depending on the presence of ammonia in the growth medium. A different methylamine-utilizing organism, *Pseudomonas* sp. strain MA, shows a similar response to ammonia, except that an NAD-dependent alanine dehydrogenase is produced in addition to low levels of NADP-dependent GDH (4).

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