***N*-Methylglutamate Dehydrogenase, a Flavohaemoprotein Purified from a New Pink Trimethylamine-utilizing Bacterium**

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A new strain of pink facultative methylotroph, bacterium AT2, capable of growth on methylamine, trimethylamine, methanol, formate and a range of non-C₁ substrates has been isolated. On the basis of the enzyme activities in cell-free extracts, the organism appears to have the same pathway for trimethylamine oxidation as *Pseudomonas aminovorans*, i.e. via trimethylamine *N*-oxide and *N*-methylglutamate. *N*-Methylglutamate dehydrogenase in this organism was a 'soluble' enzyme (i.e. was not sedimented at 100 000 *g* in 60 min) and reacted with the electron acceptors phenazine methosulphate, 2,6-dichlorophenolindophenol, Wurster's blue and the radical cation of 2,2'-azinodi-[3-ethylbenzthiazoline 6-sulphonate]. It was not active with NAD, ferricyanide or cytochrome *c*. The enzyme was purified to apparent homogeneity (as assessed by polyacrylamide gel electrophoresis) and was shown to contain flavin and cytochrome *c*, both of which could be reduced by *N*-methylglutamate. The flavin prosthetic group could be liberated by boiling and was probably FAD. The true *K_m* of the enzyme for *N*-methylglutamate was 0.33 mM and for 2,6-dichlorophenolindophenol, 0.20 mM.

INTRODUCTION

N-Methylglutamate has been known since 1966 as an early intermediate in the incorporation of methylamine into the cellular constituents of a number of bacteria (Shaw *et al.*, 1966; Kung, 1969; Trotsenko & Loginova, 1973; Loginova *et al.*, 1977; Trotsenko & Loginova, 1978). Hersh *et al.* (1971) demonstrated in *Pseudomonas* MA a dehydrogenase catalysing the oxidation of *N*-methylglutamate and postulated that *N*-methylglutamate plays a catalytic role in the oxidation of methylamine to formaldehyde and ammonia, thus:

Evidence supporting this concept has since been reported (Loginova & Trotsenko, 1974; Loginova *et al.*, 1977; Bamforth & Large, 1975, 1977*a*).

N-Methylglutamate dehydrogenase [*N*-methyl-L-glutamate:(acceptor) oxidoreductase (demethylating); EC 1.5.99.5] has been partially purified from *Pseudomonas* MA (Hersh *et al.*, 1972) and *P. aminovorans* (Bamforth & Large, 1977*a, b*). The enzyme in these organisms was membrane-bound, as judged by its sedimentation at 100 000 *g* and by the requirement for Triton X-100 to solubilize it, and was only active with 2,6-dichlorophenolindophenol (DCPIP), 5-methylphenazinium methylsulphate (phenazine methosulphate, PMS) or cytochrome *c* as electron acceptors. In contrast, in *P. methylica* (Loginova & Trotsenko, 1974; Netrusov, 1975), *Hyphomicrobium vulgare* 3 (Loginova *et al.*, 1977) and *H. vulgare* ZV (Loginova *et al.*, 1976), *N*-methylglutamate dehydrogenase failed to sediment at 105 000 *g* and specifically required NAD as electron acceptor.

We report here the isolation of a new pink bacterium, AT2, which when grown on

methylamine contains three to four times more *N*-methylglutamate dehydrogenase than is found in *P. aminovorans*. The enzyme in bacterium AT2 does not sediment at 50000 *g* and remains mainly in the supernatant after 60 min at 100000 *g*, but will not reduce NAD and will use only dyes as electron acceptors. The enzyme has been purified to near homogeneity and we describe the kinetic and spectral properties of the enzyme, which is a flavoprotein containing, or tightly associated with, a *c*-type cytochrome.

METHODS

Isolation, growth and maintenance of bacterium AT2. The mineral salts medium used for plates during the isolation procedure was salts medium A of Eady *et al.* (1971) with the addition of 2% (w/v) agar. The carbon sources were added as required, usually at 2 g l⁻¹. Organic acids were used as their sodium salts, amines as their hydrochlorides. For growth in liquid culture, salts medium B of Eady *et al.* (1971) was used. In some later experiments bacteria were grown on a medium containing (per litre): (NH₄)₂SO₄, 1 g; MgSO₄·7H₂O, 0.2 g; NaH₂PO₄·2H₂O, 0.57 g; K₂HPO₄, 1.55 g; trace element solution (Vishniac & Santer, 1957), 0.2 ml; with carbon source as above. Amine hydrochlorides were sterilized by autoclaving, and the remaining substrates were sterilized by filtration using Sartorius 0.45 µm membrane filters. Stock cultures of the organism were maintained on salts medium B solidified with agar, with 0.2% (w/v) trimethylamine. HCl as sole carbon source. Cultures of the organism are available from the authors.

Bacteriological tests. These were performed as described by Cruikshank (1965) or Cowan & Steel (1965).

Preparation of cell-free extracts. Bacterial pellets were suspended in 2 vol. 50 mm-potassium phosphate buffer pH 7.0, cooled to 4 °C and passed once through a French pressure cell operated at 7.58 MPa. The resulting suspension was centrifuged (20 min, 50000 *g*, 4 °C) and the supernatant was used for enzyme assays.

Enzyme assays. *N*-Methylglutamate dehydrogenase. Polystyrene cuvettes contained (total volume 3 ml) 200 µmol potassium phosphate buffer pH 7.0, 0.17 µmol DCPIP, enzyme and water. With crude extracts 3 µmol KCN was also added. After measurement of endogenous dye reduction at 600 nm, the reaction was started by addition of 2 µmol *N*-methyl-L-glutamic acid and followed at 25 °C. The reference cuvette contained buffer and enzyme only. One unit of enzyme is the amount required to catalyse the substrate-dependent reduction of 1 µmol DCPIP min⁻¹ under these conditions.

β-Galactosidase was assayed by the continuous spectrophotometric method of Wallenfels (1962). The other enzymes used as markers on the Sepharose column were assayed as described in *Biochemica Information* (1973, 1975). The enzymes of C₁ metabolism (Table 1) were assayed by methods already described (Boulton & Large, 1977; Meiberg & Harder, 1978).

Purification of N-methylglutamate dehydrogenase. Except where indicated, operations were carried out at 4 °C.

Step 1. Preparation of crude extract. Frozen bacteria (15 g) were suspended in 30 ml 50 mm-potassium phosphate buffer pH 7.0 and passed once through a French pressure cell at 7.58 MPa. The suspension was centrifuged (30 min, 50000 *g*) and the supernatant was carefully decanted. The pellet was resuspended in 15 ml 50 mm-phosphate pH 7.0 as before and once more passed through the French pressure cell and centrifuged. The supernatants were combined.

Step 2. Heat treatment. The combined supernatants were heated in a water bath at 50 °C with constant stirring for 20 min. The precipitate was removed by centrifuging (30 min, 50000 *g*).

Step 3. Streptomycin sulphate treatment. To the supernatant was added 0.1 vol. 10% (w/v) streptomycin sulphate with stirring. The mixture was centrifuged (30 min, 50000 *g*) and the precipitate was discarded.

Step 4. Ammonium sulphate precipitation. The supernatant was made 70% saturated in (NH₄)₂SO₄ by slow addition of the solid with stirring. The precipitate was separated by centrifuging (30 min, 50000 *g*) and redissolved in 5 ml 50 mm-phosphate pH 7.0.

Step 5. Gel filtration on Sepharose 4B. This material was applied to a column (100 cm × 2.5 cm diam.) of Sepharose 4B equilibrated with 10 mm-potassium phosphate pH 7.0 and washed with similar buffer. The enzyme activity appeared in the void volume and all the active fractions were combined. Triton X-100 was then added to a final concentration of 0.05% (w/v) and the mixture was stirred for 2 h.

Step 6. DEAE-cellulose chromatography. The combined fractions were applied to a column (40 cm × 2.5 cm diam.) of DEAE-cellulose (Whatman DE11) equilibrated with 10 mm-potassium phosphate pH 7.0 containing 0.05% (w/v) Triton X-100 and the column was washed with similar buffer. The enzyme was then eluted with a linear gradient (10 to 300 mM) of potassium phosphate pH 7.0 containing 0.05% (w/v) Triton X-100 (gradient volume 400 ml). The enzyme was eluted at 70 mm-phosphate. The combined active fractions were concentrated to a final volume of about 15 ml in an Amicon model 52 ultrafiltration cell with PM30 membrane. This material was then dialysed for 2 h against 10 mm-phosphate pH 7.0 containing 0.05% (w/v) Triton X-100.

Step 7. Hydroxyapatite chromatography. The enzyme was then applied to a column (20 cm × 1 cm diam.) of hydroxyapatite (Bio-Rad Bio-Gel HTP) equilibrated in 10 mM-phosphate pH 7.0 containing Triton and washed with similar buffer. It was eluted with a linear gradient (10 to 150 mM) of potassium phosphate pH 7.0 containing 0.05% (w/v) Triton X-100 (gradient volume 100 ml). The activity peak was eluted at 70 mM. The combined active fractions were concentrated by ultrafiltration as before.

Protein determinations. In the absence of Triton X-100, protein was determined by the Lowry method. In the presence of detergent, the method of Wang & Smith (1975) or the dye-binding method of Bradford (1976) was used. For the first two methods, the standard was bovine serum albumin. For the dye-binding method, the standard was bovine γ -globulin, and 0.05% (w/v) Triton X-100 was present in all solutions, since Triton decreases the colour yield (Bradford, 1976). The calibration curve in the presence of Triton was linear but 22% lower than in its absence.

Kinetic experiments. To allow use of a wide range of DCPIP concentrations, glass cuvettes of 2 mm path length were employed with a reaction volume of 0.5 ml. The range of DCPIP concentrations was from 10 to 100 μ M, and of *N*-methylglutamate concentrations from 50 to 1000 μ M. Pure enzyme (435 μ g protein) from which Triton X-100 had previously been removed was used.

Removal of Triton X-100. Before rate measurements and spectral studies were made with the purified enzyme, Triton X-100 was removed by the method of Holloway (1972) using either Bio-Beads SM-2 or Amberlite XAD-2 polystyrene beads.

Polyacrylamide gel electrophoresis. This was performed in separation gels containing 7% (w/v) polyacrylamide, without large pore gels, by the method of Davis (1964), except that the gels and running buffer contained 0.05% (w/v) Triton X-100. Activity was detected as described previously (Bamforth & Large, 1977a). Haemoproteins were detected on gels with 3,3'-diaminobenzidine (other conditions were as described by Thomas *et al.*, 1976). Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and pre-treatment of the samples were as described by Zahler (1974). In some cases, gels were pre-electrophoresed to remove persulphate. Standard molecular weight proteins for SDS gels were from the Boehringer Combithek kit (cat. no. 15661).

Spectral studies. Absorption spectra were recorded on a Cary 14 UV spectrophotometer using the 0 to 0.1 absorbance slide wire, and fluorescence spectra on a Farrand mark 1 spectrofluorimeter. Fluorescence spectra were recorded on sensitivity range 0.03 and are uncorrected.

Thin-layer chromatography. Silica gel G was used with solvents I (butan-1-ol/water/acetic acid/methanol) and IV (phosphate buffer) of Fazekas & Kokai (1971).

Molecular weight determination. The molecular weight of the partially purified enzyme (step 5) was determined by the method of Andrews (1970) on a column of Sepharose 4B (void volume 238 ml) using the following molecular weight standards: lactate dehydrogenase (rabbit muscle, BDH) molecular weight 130 000, pyruvate kinase (rabbit muscle, Sigma) 235 000, xanthine oxidase (buttermilk, Sigma) 275 000, urease (jack bean, Sigma) 490 000, β -galactosidase (*Escherichia coli*, P-L Biochemicals) 520 000, bovine thyroglobulin (Sigma) 670 000. The standards were used internally with 1 unit *N*-methylglutamate dehydrogenase. The column was eluted with 10 mM-potassium phosphate pH 7.0 containing 0.05% (w/v) Triton X-100. Proteins were detected by measurement of absorbance at 240 nm and the enzymes by quantitative assay.

Heat stability experiments. Samples (0.3 ml) of enzyme purified to step 6 were heated in a water bath at the indicated temperature in thin-walled glass tubes. Tubes were removed at the times indicated, chilled in ice and assayed for activity.

Chemicals. *N*-Methyl L-amino acids were obtained from Vega-Fox Biochemicals, Tucson, Ariz., U.S.A. The other materials were from sources described previously (Bamforth & Large, 1977a). Wurster's blue and the radical cation of 2,2'-azinodi-[3-ethylbenzthiazoline 6-sulphonate] (ABTS) were prepared as described by Duine *et al.* (1978) from precursors obtained from Sigma.

RESULTS

Isolation of bacterium AT2

A pink-pigmented organism was identified as a contaminant on agar plates of *Pseudomonas aminovorans* growing on trimethylamine as sole carbon source. By repeated picking and restreaking of single colonies on trimethylamine agar, the organism was obtained in pure culture and designated AT2.

The organism is a bacterium with the form of short, motile rods, occasionally occurring in pairs. It is Gram-negative, non-spore forming, oxidase- and catalase-positive and it decolorized litmus milk with no clotting after 1 week. Gelatin stabs were not liquefied, and anaerobic growth on either complex or synthetic media was not detected. On salts medium A,

Table 1. *Activities of enzymes oxidizing C₁ compounds in bacterium AT2 after growth on methylamine or trimethylamine*

Bacteria were disrupted with the French press and centrifuged at 50000 *g* as described in Methods. Enzyme activities were assayed in the supernatant fraction by the methods described by Boulton & Large (1977) or Meiberg & Harder (1978). Specific activities are expressed as nmol substrate transformed min⁻¹ (mg protein)⁻¹.

Enzyme	Specific activity after growth on:	
	Methylamine	Trimethylamine
<i>N</i> -Methylglutamate dehydrogenase (EC 1.5.99.5)	27	9
Dimethylamine mono-oxygenase (NADPH as electron donor) (EC 1.14.99.-)	5	58
Trimethylamine mono-oxygenase (NADPH as electron donor) (EC 1.14.13.8)	2	200
Trimethylamine- <i>N</i> -oxide aldolase (demethylase) (EC 4.1.2.32)	5	587
Methylamine dehydrogenase (EC 1.4.99.3)	0	0
Dimethylamine dehydrogenase (EC 1.5.99.-)	0	0
Trimethylamine dehydrogenase (EC 1.5.99.7)	0	0
Formaldehyde dehydrogenase (DCPIP as electron acceptor) (no EC number)	24	22
Formaldehyde dehydrogenase (NAD ⁺ as electron acceptor, glutathione required) (EC 1.2.1.1)	0	0
Methanol dehydrogenase (EC 1.1.99.8)	0	0
Formate dehydrogenase (NAD ⁺ as electron acceptor) (EC 1.2.1.2)	59	43
Hydroxypyruvate reductase (EC 1.1.1.29 or 81)	3493	3433

solidified with agar, the organism grew very well with 0.2% (w/v) concentrations of methanol, ethanol, methylamine.HCl, dimethylamine.HCl, trimethylamine.HCl, diethylamine.HCl, or sodium succinate, lactate or pyruvate. Reasonable growth (up to 50% of that on methylamine) was also detected on ethylamine, fructose and the sodium salts of fumarate, malate, formate and oxalate. No growth was observed on serine, trimethylamine *N*-oxide, tetramethylammonium chloride, glycine, glucose or the sodium salts of acetate, aspartate or citrate. Growth on nutrient agar was poor. In liquid culture, dimethylamine was only poorly utilized (as with *P. aminovorans*; den Dooren de Jong, 1928). The organism can be clearly distinguished from most pink facultative methylotrophs, since it grows well on trimethylamine, and to prevent possible contamination by such organisms, stock cultures were maintained on trimethylamine agar slopes.

Oxidative properties of intact bacteria

Washed suspensions of methylamine-grown bacteria readily oxidized methylamine, methanol, formaldehyde and formate, but not dimethylamine, trimethylamine, trimethylamine *N*-oxide, tetramethylammonium chloride or *N*-methylglutamate. Trimethylamine-grown bacteria oxidized mono-, di- and trimethylamine, methanol, formate and formaldehyde, but not tetramethylammonium salts.

Properties of cell-free extracts

The activities of a number of enzymes involved in the oxidation of C₁ compounds were determined (Table 1). The organism possesses a similar pattern to *P. aminovorans* in that in the 'soluble' cell fraction there was no detectable methanol, mono-, di- or trimethylamine dehydrogenase activity, while trimethylamine and dimethylamine mono-oxygenases, hydroxypyruvate reductase and trimethylamine-*N*-oxide aldolase (demethylase) were present.

The *N*-methylglutamate dehydrogenase activity was very largely 'soluble', i.e. most of it

Table 2. *Distribution of N-methylglutamate dehydrogenase activity in subcellular fractions of various bacteria*

Cell fraction	Bacterium AT2 (this work)		<i>Pseudomonas aminovorans</i> (Jarman, 1973)		<i>Pseudo- monas methylica</i> (Netrusov, 1975)	<i>Hypho- microbium vulgare</i> 3 (Loginova <i>et al.</i> , 1977)
	Total activity*	Specific activity*	Total activity*	Specific activity*	Specific activity†	Specific activity†
Crude extract						
(4000 <i>g</i> supernatant)	3107	17.8	925	7	14.5	—
50000 <i>g</i> supernatant	1471	9.7	285	1.6	—	76.0
50000 <i>g</i> pellet	380	19.1	425	17	—	—
100000 <i>g</i> supernatant	1327	13.4	33	0.36	17.1	103.2
100000 <i>g</i> pellet	430	43.7	260	20	0	16.1

* Activity is expressed as nmol DCPIP reduced min⁻¹ and specific activity as nmol min⁻¹ (mg protein)⁻¹.

† Specific activity is expressed as nmol NAD reduced min⁻¹ (mg protein)⁻¹.

Table 3. *Purification of N-methylglutamate dehydrogenase from bacterium AT2*

Step	Volume (ml)	Total activity (munits)	Total protein (mg)	Specific activity [munits (mg protein) ⁻¹]	Purification factor	Yield (%)
1. Crude extract	44	14960	880	17.0	1	100
2. Heat treatment	42	13770	774	17.8	1.04	92
3. Streptomycin sulphate supernatant	42	11570	777	14.9	0.88	77
4. 0 to 70% saturation ammonium sulphate precipitate	10	10730	701	15.3	0.90	72
5. Sepharose 4B eluate (combined active fractions)	103	7520	330	22.8	1.34	50
6. DEAE-cellulose eluate (combined active fractions)	22	4660	69	67.1	3.95	31
7. Hydroxyapatite eluate (combined active fractions)	15.2	2190	19	113.0	6.64	15

failed to sediment in 1 h at 100000 *g* (Table 2), and it differs in this respect from the enzyme in *P. aminovorans*. Other dehydrogenases were not assayed in the membrane fraction, so it is possible that methanol dehydrogenase was present in this fraction.

Purification of N-methylglutamate dehydrogenase

The purification of *N*-methylglutamate dehydrogenase is summarized in Table 3. The first four steps were not really purification steps, in that they did not increase the specific activity. The purpose of the heat treatment was to inactivate any more labile enzymes, the streptomycin sulphate removed nucleic acid and the ammonium sulphate precipitation concentrated the enzyme prior to gel filtration. Even the gel filtration only removed lower molecular weight material. It was found that the enzyme was excluded from Sepharose 4B because it aggregated. If Triton X-100 (which presumably disaggregated the enzyme) was added at this stage, recovery of enzyme activity from the two subsequent chromatographic columns was much improved, and its presence made polyacrylamide gel electrophoresis possible, since in the absence of Triton the enzyme failed to enter the separation gel. The two actual purification steps (6 and 7) resulted in a preparation that was nearly homogeneous on polyacrylamide gel electrophoresis at pH 8.3. Some preparations gave a single active band, which coincided with haemoprotein as detected with 3,3'-diaminobenzidine, with additionally a very minor single impurity; others gave up to three bands, each with activity.

Table 4. Activity of various compounds as electron acceptors for *N*-methylglutamate dehydrogenase

Compound	Wavelength of assay (nm)	Absorption coefficient (l mmol ⁻¹ cm ⁻¹)	$V_{\max}$ *	Apparent K_m (μ M)
DCPIP	600	20.6	54.7	65
PMS (in presence of DCPIP)	600	20.6	95.2	100
Wurster's blue	612	12.0	107.0	15.3
Radical cation of ABTS	660	9.1	151.0	63

* Expressed as nmol *N*-methylglutamate oxidized min⁻¹ (mg protein)⁻¹; this corrects for differing stoichiometry between the one-electron and two-electron acceptors.

Fig. 1. Comparative heat stability of *N*-methylglutamate dehydrogenases from *Pseudomonas aminovorans* and bacterium AT2. Samples were treated as described in Methods. Protein concentrations: *P. aminovorans* (solid symbols), 170 μ g ml⁻¹; bacterium AT2 (open symbols), 480 μ g ml⁻¹. Temperatures: ∇ , 30 °C; Δ , $\blacktriangle$, 40 °C; $\square$, 45 °C; $\circ$, $\bullet$, 50 °C.

The cause of the multiple bands is not yet known. On SDS-polyacrylamide gels the preparation gave only a single band, and a comparison of its migration distance with that of proteins of known molecular weight (from the Boehringer Combithek kit) gave a subunit molecular weight for the enzyme of 108000.

The material was thus, if not completely homogeneous, very nearly so, and hence was suitable for investigation of its spectral properties. Prior to most of the experiments using enzyme of high purity, the Triton X-100 was removed. The material at the end of step 6 was more than 90% pure and it was used for most of the substrate specificity and kinetic experiments.

Properties of the purified enzyme

pH optimum. The enzyme had a pH optimum of 7.0 when either DCPIP alone or DCPIP plus PMS were electron acceptors. This is similar to that observed with the purified enzyme from *P. aminovorans* (Bamforth & Large, 1977a).

Substrate specificity. The enzyme was active with the following *N*-methyl amino acids: *N*-methyl-L-glutamate, *N*-methyl-DL-glutamate, *N*-methyl-DL-aspartate, *N*-methyl-DL-alanine, *N*-methyl-L-alanine and sarcosine. No activity could be detected with 33 mM-*N*-methyl-D-alanine, in contrast to the *P. aminovorans* enzyme which showed activity with this substrate. When the activity of the enzyme on polyacrylamide gels was tested with the different

Fig. 2. Primary and secondary double-reciprocal plots relating velocity to substrate concentration for *N*-methylglutamate dehydrogenase. (a) Primary plot of rate and *N*-methylglutamate concentration. Concentrations of DCPIP were: $\circ$, 10 μM ; $\triangle$, 25 μM ; $\square$, 50 μM ; ∇ , 75 μM ; $\bullet$, 100 μM . (c) Primary plot of rate and DCPIP concentration. Concentrations of *N*-methylglutamate were: $\circ$, 0.05 mm; $\triangle$, 0.07 mm; $\square$, 0.1 mm; ∇ , 0.3 mm; $\bullet$, 0.5 mm; $\blacktriangle$, 1.0 mm. Secondary plots: (b) intercepts of the lines in (a) against reciprocal DCPIP concentration; (d) intercepts of the lines in (c) against reciprocal *N*-methylglutamate concentration. The intercepts of the secondary plots on the abscissa give the reciprocal of the true K_m values for the electron donor and acceptor.

substrates, once more no activity was observed with *N*-methyl-D-alanine, while the *N*-methyl L- or DL-amino acids showed a single band of activity at the same R_F on each gel. There is thus no likelihood that more than one enzyme active on *N*-methyl amino acids was present in the preparation.

Of the electron acceptors tested, no activity was observed with horse heart cytochrome *c* (in contrast to the *P. aminovorans* enzyme), NAD (in contrast to the *P. methylica* enzyme) or ferricyanide. The following compounds showed good activity as acceptors: Wurster's blue, the radical cation of ABTS, DCPIP and PMS. Comparison of their $V_{\max}$ values (Table 4) shows that the single-electron acceptors were more effective than the two-electron acceptors.

Heat stability. The enzyme was more heat stable than the enzyme from *P. aminovorans*

Fig. 3

Fig. 3. Absorption spectrum of homogeneous *N*-methylglutamate dehydrogenase. (a) Absolute spectra: 1, enzyme as prepared; 2, enzyme reduced with 1 mM-*N*-methylglutamate. (b) Difference spectrum, 2 minus 1.

Fig. 4

Fig. 4. Fluorescence spectrum of the isolated prosthetic group of *N*-methylglutamate dehydrogenase: —, excitation spectra, fluorescence measured at 520 nm; ---, fluorescence emission spectra, excitation at 450 nm. 1, Prosthetic group, prepared as described in Results. 2, Fluorescence of authentic FAD boiled in the dark for 10 min with bovine serum albumin and then treated as 1.

(Fig. 1). The half-life of the purified enzyme was 35 min at 40 °C, 10.5 min at 45 °C and about 1 min at 50 °C.

Kinetic properties. Variation of the concentration of *N*-methyl-L-glutamate and DCPIP suggested that the enzyme has Ping Pong type kinetics (Cleland, 1963) (Fig. 2). The true K_m values for the two substrates calculated from these data were 333 μM for *N*-methyl-L-glutamate and 200 μM for DCPIP. The Ping Pong nature of the reaction was confirmed by examining the effect on the rate of varying the concentration of the two substrates in a fixed ratio (1:1 in this case). A double reciprocal plot of these data also gave a linear relationship between $1/v$ and $1/s$, as is expected for a Ping Pong reaction (Plowman, 1972). The K_m values are similar to those obtained with the *P. aminovorans* enzyme (Bamforth & Large, 1977a) except that the K_m for *N*-methylglutamate is slightly higher.

Spectrum. There were changes in the absorption spectrum of the enzyme when 1 mM-*N*-methylglutamate was added (Fig. 3a). The difference spectrum (Fig. 3b) shows that the broad peaks at 440 to 470 nm and near 360 nm were bleached, the peak at 409 nm shifted to 415 nm and a new peak appeared at 552 nm. This behaviour suggests the presence of both flavin and *c*-type cytochrome in the purified enzyme. This is in contrast to *P. aminovorans* (Bamforth & Large, 1977a) where the spectrum of less pure preparations suggested the presence of a *b*-type cytochrome, but since in *P. aminovorans* the cytochrome was not reduced by substrate, it may have been an adventitious contaminant.

Molecular weight. On Sepharose 4B gel filtration in the presence of Triton X-100, the enzyme had a molecular weight of 407000. Triton is known not to affect the molecular weights of the marker proteins used (Clarke, 1975).

Properties of the prosthetic group

Attempts to resolve the enzyme by acidic ammonium sulphate fractionation (Strittmatter, 1961) resulted in an inactive preparation which could not be reactivated by FMN or FAD. The enzyme had a rather feeble fluorescence spectrum, with a very small emission maximum at 507 to 512 nm. Release of a coloured chromophore was achieved by heating the enzyme (previously dialysed against distilled water for 24 h) in a boiling water bath for 10 min, taking care to exclude light. The precipitated protein was separated by centrifuging (5 min, 5000 g). The fluorescence spectrum of the yellow supernatant (Fig. 4) clearly indicated (excitation maxima at 378 and 467 nm and a fluorescence emission maximum at 516 nm) that it contained a flavin derivative and was identical with the fluorescence spectrum of authentic FAD which had been boiled in the presence of bovine serum albumin under the same conditions. The material was taken to dryness under reduced pressure at 25 °C in the dark. The pale yellow residue was dissolved in water and chromatographed on silica gel G with authentic flavin markers (see Methods). The product had an R_F of 0.44 in solvent I and 0.37 in solvent IV. These values suggested that it was neither FMN nor FAD (R_F values, respectively, 0.20 and 0.32 in solvent I and 0.46 and 0.50 in solvent IV) nor riboflavin (R_F 0.73 in solvent I and 0.33 in solvent IV). However, material purified with a Sep-pak C₁₈ cartridge (Waters Associates) had retention times identical with FAD on liquid chromatography in two different solvent systems (J. A. Duine, J. Frank, Jzn & P. J. Large, unpublished observations).

DISCUSSION

The new bacterium differs from the pink bacteria *Pseudomonas* AM1 and *P. methylica* in being able to grow on trimethylamine, and in this respect it resembles *Pseudomonas* 3A2 of Colby & Zatman (1973). However, although whole cells can oxidize methanol, the organism differs from *Pseudomonas* 3A2 in having no detectable methanol dehydrogenase activity in the 'soluble' cell fraction. It differs from the non-pigmented *P. aminovorans* in lacking an NAD-linked glutathione-dependent formaldehyde dehydrogenase (Boulton & Large, 1977) and in this respect it resembles *Pseudomonas* AM1 (Johnson & Quayle, 1964). It also differs from *P. aminovorans* in that methylamine-grown bacteria contain only very low levels of trimethylamine-*N*-oxide aldolase and dimethylamine mono-oxygenase, both of which are present in high activity in methylamine-grown *P. aminovorans* (Jarman & Large, 1972; Boulton & Large, 1979). Nevertheless, the results in Table 1 suggest that bacterium AT2 oxidizes trimethylamine with the intermediate formation of trimethylamine *N*-oxide and *N*-methylglutamate in a similar manner to *P. aminovorans*. (For a scheme showing this pathway, see Boulton & Large, 1977. For a summary of alternative pathways, see Colby & Zatman, 1973.) It also seems probable from the high activity of hydroxypyruvate reductase in bacterium AT2 that carbon is assimilated via the serine pathway. Despite its pink pigmentation, the pattern of enzyme activities in bacterium AT2 does not resemble that of *Pseudomonas* AM1 (which contains a primary-amine dehydrogenase; Eady & Large, 1968) nor that of *P. methylica* (where an NAD-linked *N*-methylglutamate dehydrogenase is present; Loginova & Trotsenko, 1974).

Although a number of different organisms are known to contain *N*-methylglutamate dehydrogenases of various types, there appears at present to be no correlation between species or pigmentation of organism and the occurrence of *N*-methylglutamate dehydrogenase, as opposed to the direct oxidation of methylamine via a dehydrogenase or an oxidase (Loginova & Trotsenko, 1977; Trotsenko & Loginova, 1978). Nor is there at present any apparent pattern in the occurrence of the different types of *N*-methylglutamate dehydrogenase in various organisms. The resolution of this confused field must await the purification of the unstable NAD-linked enzyme (see below).

N-Methylglutamate dehydrogenase of bacterium AT2 differs from the *P. aminovorans*

enzyme in being 'soluble', i.e. it is not sedimented at 100000 *g* in 60 min, and in this respect it resembles the enzymes from *P. methylica* (Netrusov, 1975), *Hyphomicrobium vulgare* 3 (Loginova *et al.*, 1977) and *H. vulgare* ZV (Loginova *et al.*, 1976). However, unlike the latter enzymes it is quite stable and will not use NAD as electron acceptor; it is only active with PMS, DCPIP, Wurster's blue and the radical cation of ABTS. The molecular properties (molecular weight, subunit molecular weight, K_m for electron donor and acceptor, pH optimum and substrate specificity) resemble fairly closely those of the *P. aminovorans* enzyme (Bamforth & Large, 1977*a, b*), but the presence of larger amounts of the enzyme in bacterium AT2 (perhaps 15% of the soluble protein) and the lack of necessity to prepare membrane fractions has enabled us to achieve homogeneity more easily. The enzyme from bacterium AT2 seems to be more stable to heat. The exclusion of the enzyme from Sepharose 4B (exclusion limit 20×10^6 daltons) suggests that in the absence of detergent it aggregates in concentrated solution, a phenomenon characteristic of many membrane proteins (Newby & Chrambach, 1979; Homcy *et al.*, 1978). Thus it is probably not really very different from the *N*-methylglutamate dehydrogenases of *P. aminovorans* and *Pseudomonas* MA, which require high concentrations of Triton X-100 to release them into solution. Possibly the AT2 enzyme is less tightly bound, perhaps being an extrinsic rather than intrinsic membrane protein (Singer, 1974). The molecular weight of 407000 taken in conjunction with the subunit molecular weight of 108000 suggest that the enzyme may be a trimer or tetramer (Bamforth & Large, 1977*b*) probably occurring in Triton micelles as a single molecule of enzyme per micelle.

The results reported here confirm earlier suggestions that the enzyme is a flavoprotein (Bamforth & Large, 1977*a*), and the prosthetic group is not covalently bound, since it can be removed by boiling the enzyme. The flavin is probably FAD. The discovery that the preparation contains substrate-reducible *c*-type cytochrome is novel, although such a prosthetic group has been reported for the amine dehydrogenase of benzylamine-grown *P. putida* (Durham & Perry, 1978*a, b*). Well-characterized enzymes containing both haem and flavin include yeast lactate dehydrogenase (EC 1.1.2.3) (Appleby & Morton, 1969), spermidine dehydrogenase (EC 1.5.99.-) from *Serratia marcescens* (Tabor & Kellogg, 1970) and an oxygen-binding protein from *Alcaligenes eutrophus* (Probst *et al.*, 1979), although it is noteworthy that all these contain *b*-type cytochromes.

The presence of cytochrome *c* in *N*-methylglutamate dehydrogenase suggests that this enzyme, and the amine dehydrogenase of *P. putida* (Durham & Perry, 1978*a, b*), may, like methanol dehydrogenase (O'Keeffe & Anthony, 1978; Van Verseveld & Stouthamer, 1978; Netrusov *et al.*, 1977; Bamforth & Quayle, 1978), feed their electrons into the electron transport chain at the level of cytochrome *c*. It may perhaps also explain why added horse heart cytochrome *c* is not active as an electron acceptor.

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