Solubilization, Partial Purification and Properties of *N*-Methylglutamate Dehydrogenase from *Pseudomonas aminovorans*

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1. Extracts of amine-grown *Pseudomonas aminovorans* contained a particle-bound *N*-methylglutamate dehydrogenase (EC 1.5.99.5). The enzyme was not present in succinate-grown cells, and activity appeared before growth began in succinate-grown cells which had been transferred to methylamine growth medium. 2. Membrane-containing preparations from methylamine-grown cells catalysed an *N*-methylglutamate-dependent uptake of O₂ or reduction of cytochrome *c*, which was sensitive to inhibitors of the electron-transport chain. 3. *N*-Methylglutamate dehydrogenase activity with phenazine methosulphate or 2,6-dichlorophenol-indophenol as electron acceptor could be solubilized with 1% (w/v) Triton X-100. The solubilized enzyme was much less active with cytochrome *c* as electron acceptor and did not sediment in 1 h at 150000g. Solubilization was accompanied by a change in the pH optimum for activity. 4. The solubilized enzyme was partially purified by Sepharose 4B and hydroxyapatite chromatography to yield a preparation 22-fold increased in specific activity over the crude extract. 5. The partially-purified enzyme was active with sarcosine, *N*-methylalanine and *N*-methylaspartate as well as with *N*-methylglutamate. Evidence suggesting activity with *N*-methyl D-amino acids as well as with the L-forms was obtained. 6. The enzyme was inhibited by *p*-chloromercuribenzoate, iodoacetamide and by both ionic and non-ionic detergents. 2-Oxoglutarate and formaldehyde were also inhibitors. 7. Kinetic analysis confirmed previous workers' observations of a group transfer (Ping Pong) mechanism. 8. Spectral observations suggested that the partially purified preparation contained flavoprotein and a *b*-type cytochrome. 9. The role of the enzyme in the oxidation of methylamine is discussed.

Pseudomonas aminovorans (den Dooren de Jong, 1927) can grow on a number of different amines including mono-, di- and tri-methylamine and trimethylamine *N*-oxide, as sole carbon source. Although washed bacteria grown on all these substrates readily oxidize methylamine (Jarman & Large, 1972), there is no evidence for the presence in crude extracts of a dye-linked primary-amine dehydrogenase (EC 1.4.99.-) of the type found in methylamine-grown *Pseudomonas* AM1 by Eady & Large (1968, 1971) and which also occurs in a number of other bacteria (Dahl *et al.*, 1972; Colby & Zatman, 1973, 1975; Cerniglia & Perry, 1975). This enzyme oxidizes methylamine and other primary amines to the corresponding aldehydes and NH₃. Hersh and co-workers (Hersh *et al.*, 1971; Hersh, 1975) demonstrated in *Pseudomonas* sp. MA (Shaw *et al.*, 1966) grown on methylamine the presence of two enzymes, *N*-methylglutamate synthase (EC 2.1.1.21) and *N*-methylglutamate dehydrogenase (EC 1.5.99.5) and proposed that they are involved in

the cyclic oxidation of methylamine to formaldehyde and NH₃ via *N*-methylglutamate (eqns. 1–3).

This would achieve the same stoichiometry as direct oxidation by a primary amine dehydrogenase. *N*-Methylglutamate synthase was purified by Pollock & Hersh (1971) and *N*-methylglutamate dehydrogenase was solubilized and partially purified from a particulate fraction of *Pseudomonas* sp. MA by Hersh *et al.* (1972). Subsequently, these two enzymes have been demonstrated in methylamine-grown *Ps. methylica* (Loginova & Trotsenko, 1974) and in *Pseudomonas aminovorans* (Jarman, 1973). Netrusov (1975) found a soluble NAD-linked *N*-methylglutamate dehydrogenase in *Ps. methylica*.

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The present paper describes the solubilization, purification and properties of *N*-methylglutamate dehydrogenase [*N*-methyl-L-glutamate-(acceptor) oxidoreductase (demethylating)] (eqn. 2) from membrane fractions of methylamine- or trimethylamine-grown *Ps. aminovorans*, and presents evidence suggesting the involvement of the enzyme in bacterial growth on methylamine.

A preliminary report of part of this work has already been presented (Bamforth & Large, 1975).

Materials and Methods

Materials

N-Methyl-L-glutamic acid, *N*-methyl-DL-alanine, *N*-methyl-DL-aspartic acid, *N*-methyl-L-alanine and *N*-methyl-D-alanine were from Cyclo Chemical Corporation, Los Angeles, CA, U.S.A., and Sepharose 4B from Pharmacia (G.B.), London W5 5SS, U.K. Trimethylamine *N*-oxide and Naphthalene Black 12B were from BDH Chemicals, Poole, Dorset, U.K. DL-Quinacrine hydrochloride was from K & K Chemicals, Plainview, NY, U.S.A., and Bes [*NN*-bis-(2-hydroxyethyl)-2-aminoethanesulphonic acid] was from Hopkin and Williams, Romford, Essex, U.K. SKF 525-A (2-diethylaminoethyl 2',2'-diphenylvalerate hydrochloride) was a gift from Smith Kline and French Laboratories, Welwyn Garden City, Herts., U.K. All other chemicals were analytical grade, where available, and were from Fisons Scientific Apparatus, Loughborough, Leics., U.K., or Sigma (London) Chemical Co., Kingston-on-Thames KT2 7BH, U.K.

Maintenance and growth of the organism

Stock cultures of *Ps. aminovorans* N.C.I.B. 9039 were maintained as described by Eady *et al.* (1971). The carbon source was either 0.5% methylamine hydrochloride or 0.25% trimethylamine hydrochloride prepared by neutralization of the free base (and expressed as concentration of free base) as described by Boulton *et al.* (1974). Other conditions of growth and harvesting of cells were as described previously (Eady *et al.*, 1971). Some batches of trimethylamine-grown cells were obtained from the Microbial Products Section, M.R.E., Porton Down, Salisbury, Wilts., U.K. Cell material was stored either at -15°C or in liquid N₂.

Measurement of bacterial growth

Growth was measured as turbidity at 570nm in a Unicam SP.600 spectrophotometer (diluting if necessary) and related by a linear calibration curve to dry wt. of bacteria/ml (an *A*₅₇₀ value of 0.5 was equivalent to 250 µg dry wt./ml).

Chemical determinations

Protein was measured by the method of Lowry *et al.* (1951) with dried bovine serum albumin as standard. For samples containing Triton X-100 the modification of Wang & Smith (1975) was used. Triton X-100 was determined by the cobalthiocyanate method of Garewal (1973). Formaldehyde was measured colorimetrically as described by Chrastil & Wilson (1975). Glutamate was determined by the enzymic method of Bernt & Bergmeyer (1963).

Enzyme assays

N-Methylglutamate dehydrogenase. Disposable 1 cm-light-path, polystyrene cuvettes (Hughes and Hughes, Romford, Essex, U.K.) contained in a total volume of 3 ml: 200 µmol of potassium phosphate buffer, pH 6.5, 0.17 µmol of 2,6-dichlorophenol-indophenol, enzyme and water. With particulate preparations 3 µmol of KCN was also added to inhibit cytochrome oxidase activity. After measurement of endogenous dye reduction at 600nm, the reaction was started by adding 2 µmol of *N*-methyl-DL-glutamic acid or *N*-methyl-L-glutamic acid, and the rate of decrease in *A*₆₀₀ was followed at 25°C on a Unicam SP.1800 recording spectrophotometer with Unicam AR25 linear recorder. The reference cuvette contained all components except substrate and dye. One unit of activity is defined as the amount of enzyme required to catalyse the reduction of 1 µmol of 2,6-dichlorophenol-indophenol/min under these conditions. The molar extinction coefficient of the dye at pH 6.5 is $18.13 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Armstrong, 1964). Since Triton X-100 is an inhibitor of *N*-methylglutamate dehydrogenase (Bamforth & Large, 1975), when the detergent was present in the sample, account was taken of its concentration in the cuvette and an estimate of the uninhibited activity made from a plot of activity against inhibitor concentration.

Primary amine dehydrogenase. This was assayed as described by Eady & Large (1968).

Buffers

These were prepared as described by Dawson *et al.* (1969).

Preparation of membranes with *N*-methylglutamate oxidase activity

Cell paste (1 vol.) (methylamine- or trimethylamine-grown) was suspended in 2 vol. of 50mm-potassium phosphate, pH 7.0, and cooled in ice. The material was passed once through a precooled French pressure cell, subjected to a pressure of 35 kPa. The material was incubated with deoxyribonuclease (1 mg/20 ml of suspension) at 25°C to decrease viscosity and was then centrifuged at

10000g for 20min at 4°C to remove whole cells and debris. The supernatant was centrifuged at 150000g for 1h and the pellet resuspended in 5vol. of 50mm-potassium phosphate, pH7.0.

Calibration of oxygen electrode and standard assay of N-methylglutamate oxidase

An SEA metabolic oxygen meter (Scientific and Educational Aids, Windsor, Berks., U.K.) linked to a Servoscribe model RE 511 recorder (Smiths Industries, Wembley, Middlesex, U.K.) was used to measure O₂ uptake. It was calibrated by the method of Robinson & Cooper (1970). Standard assay mixtures contained 0.02ml of 100mm-N-methyl-DL-glutamate, membrane preparation and 200mm-potassium phosphate, pH7.0, in a total volume of 3.5ml. O₂ uptake was followed at 25°C after starting the reaction with substrate.

Difference spectra

These were determined on a Unicam SP.1800 spectrophotometer with Unicam AR25 linear recorder. Silica cuvettes (1cm light-path) contained, in a final volume of 1.5ml: membrane preparation, 200mm-potassium phosphate, pH7.0, and other contents as indicated. Sample and reference cuvettes were made up identically (with the exception of subsequent additions to sample cuvettes). To minimize minor differences in cuvette contents the initial contents of sample and reference cuvettes were made up together and subsequently divided equally between matched cuvettes. A little glycerol was added to cuvettes to minimize settling out of membrane material.

Preparation of cell-free extracts

Extracts were prepared by suspending 1vol. of methylamine- or trimethylamine-grown cells in 2vol. of 50mm-potassium phosphate, pH7.0, cooling the suspension to below 4°C in ice/NaCl and treating it in a Dawe Soniprobe ultrasonic disintegrator (Dawe Instruments, London W.3, U.K.) at full power for 0.5min periods for a total exposure time of 2min. Between exposures the suspension was cooled in the freezing mixture. The material was centrifuged at 10000g for 20min at 2°C to remove unbroken cells and debris. The supernatant was retained and the pellet resuspended in 2vol. of the same buffer. The sonication was repeated and debris removed as above. The two supernatants were combined and are referred to as the crude extract.

Solubilization of N-methylglutamate dehydrogenase

Crude extracts of *Ps. aminovorans* were centrifuged at 150000g for 1h. The pellet was resuspended in 5vol. of 50mm-potassium phosphate, pH7.0, by using a glass homogenizer and the suspension was

then stirred at 0°C with 0.1 vol. of 10% (w/v) Triton X-100 for 30min. The suspension was re-centrifuged at 150000g for 1h, and the supernatant is referred to as 'solubilized material'.

Purification of N-methylglutamate dehydrogenase

Step 1: concentration of enzyme and gel-permeation chromatography. To the solubilized material were added Bio-Beads SM-2 (Bio-Rad Laboratories, Bromley BR2 9LY, U.K.) (0.6g/2ml of solution) and the mixture stirred at 4°C for 2h (Holloway, 1973). This procedure removed Triton X-100 which prevented sedimentation of protein during the subsequent (NH₄)₂SO₄ treatment. The supernatant was removed with a Pasteur pipette and taken to 50% saturation with (NH₄)₂SO₄ by the slow addition of solid (NH₄)₂SO₄ and allowed to equilibrate at 2°C for 20min. The precipitate formed was removed by centrifuging at 30000g for 20min and redissolved in 2-3ml of 50mm-potassium phosphate, pH7.0, containing 1% (w/v) Triton X-100.

The dissolved precipitate was applied to a column (3cm×32cm) of Sepharose 4B previously washed with 1litre of 50mm-potassium phosphate, pH7.0, containing 1% (w/v) Triton X-100. Detergent was maintained in the buffer solution to prevent aggregation of the enzyme and its subsequent exclusion from the gel (Bamforth & Large, 1975). The column was eluted at 4°C with the same buffer and 6ml fractions were collected in an LKB Ultracrac Fraction Collector operating on a time basis (LKB Produkter A.B., Stockholm-Bromma 1, Sweden). The eluate was monitored for u.v.-absorbing material by measurement of A₂₅₄ in an LKB Uvicord (type 4701A) with recorder (type 6520A). Adjustment of the recorder baseline was necessary because of the high u.v. absorption caused by the Triton X-100.

Step 2: adsorption chromatography. The combined active fractions were applied to a column (2cm×6cm) of hydroxyapatite (Bio-Rad Bio-Gel HTP) equilibrated with 50mm-potassium phosphate, pH7.0, containing 1% (w/v) Triton X-100. After washing with this buffer, the enzyme was eluted with 200mm-potassium phosphate, pH7.0, containing 1% (w/v) Triton X-100. The active fractions, which were yellow in colour, were stored at -15°C. The procedure of extraction, solubilization and purification is summarized in Table 1.

Before further studies were performed with the enzyme, unless otherwise specified, detergent was removed by stirring with Bio-Beads SM-2, as described previously.

Polyacrylamide-gel electrophoresis

Electrophoresis in 7% (w/v) polyacrylamide gel was performed at pH8.3 by the method of Davis

Table 1. *Solubilization and partial purification of N-methylglutamate dehydrogenase from 50g wet wt of methylamine-grown Ps. aminovorans*

Purification step	Volume (ml)	Total activity (munits)	Total protein (mg)	Specific activity (munits/mg of protein)	Purification factor	Yield (%)
Crude sonic extract	154	6647	2664	2.5	1	100
Resuspended 150000g pellet	31	5249	581	9.0	3.6	79
Solubilized pellet (after removal of Triton X-100)	28	3440	214	16.2	6.5	52
Sephacrose 4B effluent (combined fractions)	94	2419*	94	25.7*	10.3	36
Hydroxyapatite eluate (combined fractions)	10.4	1843*	33	55.4*	22.2	28

* Values corrected for presence of 1% (w/v) Triton X-100.

(1964), but without a large-pore gel, in a Shandon apparatus (Shandon Scientific Co., London N.W.10, U.K.) at room temperature. Gels and running buffer contained Triton X-100 to a final concentration of 1% (w/v). Dialysis tubing was affixed to the base of each tube to prevent the gels sliding out owing to the presence of detergent. Marker dyes were not used, as they were invisible in the presence of Triton. After 2h running time protein was detected by staining the gel with 0.1% Naphthalene Black 12B in 7% (w/v) acetic acid for at least 1h, followed by destaining either electrophoretically or by repeated washings in 7% (w/v) acetic acid. (Use of trichloroacetic acid leads to a dense white precipitation in the gel.) Activity was detected by immersing unfixed gels in a solution containing: 1.0ml of 200mM-potassium phosphate, pH6.5, 0.2ml of 0.8% Nitro Blue Tetrazolium, 0.02ml of 0.10M-N-methyl-DL-glutamate and enough water to cover the gel. A purple band of formazan was deposited on the gel at the site of N-methylglutamate dehydrogenase activity, a separate gel being immersed in a solution lacking substrate to detect any non-substrate-dependent reductase activity.

Adaptation of succinate-grown Ps. aminovorans to growth on methylamine

Ps. aminovorans was taken through five serial subcultures at 30°C on 19mM-sodium succinate (0.5%, hexahydrate) as sole carbon source. When the last culture (400ml) had grown fully, it was divided into two equal volumes and the cells from each were centrifuged aseptically at 20°C and 4000g. After being washed in sterile 50mM-potassium phosphate, pH7.0, the pellets were resuspended in 500ml of sterile mineral salts medium containing either 19mM-sodium succinate or 74mM-methylamine hydrochloride as sole carbon source. These suspensions were used to inoculate 2litres of the same medium contained in 4-litre flasks (fitted with an outlet for aseptic removal of samples), which were then shaken at 30°C. The initial cell density

was 60µg dry wt./ml. Samples were withdrawn at intervals from the two cultures for measurement of growth and specific activity. Cells for specific activity determinations were rapidly centrifuged, resuspended in 2ml of 50mM-potassium phosphate, pH7.0, and stored at -15°C until needed. They were then disrupted by sonication as previously described except that a total exposure time of 45s (in 15s bursts) was used. Protein was determined by the method of Warburg & Christian (1941).

Oxidation of methylamine and N-methylglutamate by washed bacterial suspensions

Manometer flasks contained, in a total fluid volume of 3ml, in the main compartment 200µmol of potassium phosphate buffer, pH6.5, and freshly harvested washed whole methylamine-grown cells (10mg wet wt.), in the centre well 0.2ml of 20% (w/v) KOH, and in the side arm 10µmol of substrate. The gas phase was air and the temperature was 22.5°C. A control vessel was set up lacking substrate. O₂ uptake was followed for 2h.

Results

Oxidation of methylamine and N-methylglutamate by washed bacterial suspensions

Manometric studies on washed freshly harvested methylamine-grown whole cells indicated that 3.3mM-N-methyl-DL-glutamate was readily oxidized, and although the oxidation rate (endogenous subtracted) was lower than the rate of methylamine oxidation at the same substrate concentration (7.8nmol of O₂ consumed/min per mg dry wt. as against 25.6), the two rates were of a similar order of magnitude.

Distribution of N-methylglutamate dehydrogenase in crude extracts

N-Methylglutamate dehydrogenase activity was present in crude extracts of *Ps. aminovorans*

Fig. 1. Growth and changes in specific activity of *N*-methylglutamate dehydrogenase during adaptation of succinate-grown *Ps. aminovorans* to growth on methylamine

Cells of succinate-grown *Ps. aminovorans* were washed and resuspended in growth medium containing either 19mM-sodium succinate or 74mM-methylamine hydrochloride as sole carbon source. Subsequent growth was followed turbidimetrically, and samples were removed at intervals for determination of the specific activity of *N*-methylglutamate dehydrogenase. Methylamine medium: △, growth; ▲, specific activity of enzyme. Succinate medium: ○, growth; ●, specific activity of enzyme.

grown on methylamine, dimethylamine, trimethylamine and trimethylamine *N*-oxide. Specific activities for these four substrates were respectively 2.50, 3.64, 1.80 and 0.33munits/mg of protein. No activity was detectable in bacteria grown on succinate, glutamate, acetate, citrate, pyruvate, glucose or ethanol. In methylamine-grown *Pseudomonas* AM1, which has an alternative pathway for methylamine oxidation, the enzyme was detectable, although its specific activity was only 0.4munit/mg of protein compared with a specific activity for the primary amine dehydrogenase (Eady & Large, 1968) of 431 munits/mg of protein. The low activities of *N*-methylglutamate dehydrogenase in trimethylamine *N*-oxide-grown *Ps. aminovorans* probably reflect the poor growth usually observed with this carbon source.

Changes in the specific activity of N-methylglutamate dehydrogenase during adaptation of succinate-grown Ps. aminovorans to growth on methylamine

To investigate the role of *N*-methylglutamate dehydrogenase during growth on methylamine, the appearance of enzyme activity during adaptation of the bacteria from growth on succinate to growth on methylamine was followed. After five serial subcultures on 19mM-sodium succinate, no *N*-methylglutamate dehydrogenase activity was detectable in cell-free extracts. Such succinate-grown cells were resuspended in either succinate or methylamine

medium (approx. equal in g-atoms of C/litre) and samples were taken at intervals to measure growth and enzyme activity. The results of this experiment are shown in Fig. 1. Cells transferred to succinate grew without lag and *N*-methylglutamate dehydrogenase was not detectable at any stage in the growth cycle. Cells transferred to methylamine grew after a lag of 40h. Before growth there was a rapid biphasic increase in the specific activity of *N*-methylglutamate dehydrogenase from 0 to one of 2.2munits/mg of protein at the onset of growth. The fall in specific activity after the onset of growth may reflect the decreased proportion of enzyme in the growing cells as compared with cells in the lag phase.

N-Methylglutamate oxidase activity

Attempts to prepare protoplasts from *Ps. aminovorans* by lysozyme/EDTA treatment proved unsuccessful. Membranes prepared by French pressure cell breakage from methylamine- or trimethylamine-grown cells showed an *N*-methylglutamate-dependent consumption of O₂. The rate of O₂ uptake was proportional to the amount of membrane protein added to the oxygen electrode vessel. A specific activity of 8.3nmol of O₂ consumed/min per mg of protein was observed for preparations from freshly harvested and washed methylamine-grown bacteria, compared with a value of 3.5nmol/min per mg of protein for membranes prepared from cells stored at -15°C. Storage of fresh membrane

preparations overnight at either 4°C or -15°C led to a 60–70% decrease in specific activity. Unstored membrane preparations catalysed the reduction of horse heart cytochrome *c* with a specific activity of 4.6 nmol of cytochrome *c* reduced/min per mg of protein. Triton X-100-solubilized *N*-methylglutamate-dichlorophenol-indophenol reductase preparations did not consume O₂. Incubation of membrane preparations with *Naja naja* venom (about 3 mg/20 mg of protein) for 30 min at 0°C led to a 20% fall in the *N*-methylglutamate-dependent O₂ uptake and a 30% fall in *N*-methylglutamate-cytochrome *c* reductase activity. The dichlorophenol-indophenol reductase activity was unaffected. The rate of *N*-methylglutamate-dependent O₂ uptake showed a fairly sharp optimum at pH 7.0, and had an apparent *K_m* for *N*-methylglutamate of 0.137 mM. The O₂ uptake was inhibited by KCN, NaN₃, antimycin A, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide, sodium Amytal, rotenone, thenoyltrifluoroacetone and dicoumarol, though not by 29 μM-quinacrine hydrochloride. Sodium dithionite-reduced minus oxidized difference spectra showed absorption maxima at 432, 532 and 564 nm, with bleaching in the 442–520 nm region. CO-treated and dithionite-reduced minus dithionite-reduced difference spectra showed a strong peak at 423 nm. *N*-Methylglutamate-reduced minus oxidized spectra in the presence of 1 mM-KCN showed absorption maxima at 428 and 555 nm, and bleaching in the 440–510 nm region. In the presence of 0.17 mM-2-*n*-heptyl-4-hydroxyquinoline *N*-oxide the latter maximum shifted to 562 nm, with a shoulder appearing at 444 nm.

Particulate nature of the dehydrogenase and solubilization conditions

Of the total dye-linked *N*-methylglutamate dehydrogenase activity detected in crude ultrasonic extracts, 46% sedimented in 30 min at 50000g and of the activity remaining in the supernatant, almost all was sedimented after 60 min at 100000g. Various procedures were used in an attempt to solubilize the particle-bound enzyme. The criterion of solubility was failure to sediment after 1 h at 150000g. The following procedures were unsuccessful: treatment with *Naja naja* venom, exposure to low (5 mM-sodium-potassium phosphate buffer, pH 7.0) or high [0.25, 0.5 and 1 M-(NH₄)₂SO₄] ionic strength, treatment with 10 mM-EDTA, sonication of the resuspended particles for a total exposure time of 1 min, or treatment at 0°C with 50% (v/v) acetone, precooled to -15°C. When butan-1-ol at -15°C was added to an equal volume of particulate suspension at 0°C, no activity was solubilized in either aqueous or organic layers. Chaotropic agents, such as urea or guanidine hydrochloride, inactivated the membrane-bound enzyme. Solubilization was

achieved with 0.1% sodium deoxycholate or with the non-ionic polyoxyethylene detergent Triton X-100. The latter was selected as being more suitable for enzyme purification work, and the optimal conditions for solubilization were shown to be a detergent concentration in excess of 1% (w/v), and a protein concentration above 10 mg/ml.

Purity of N-methylglutamate dehydrogenase

The purification procedure of Table 1 gave a preparation at least 20-fold more active than the crude extract. Analytical polyacrylamide-gel electrophoresis of such preparations at pH 8.3 gave about ten protein bands, though the major band coincided with that showing substrate-dependent Nitro Blue Tetrazolium reductase activity. Further purification could be achieved by chromatography on DEAE-cellulose and aminopentyl-Sepharose 4B, giving purifications up to 145-fold, but yields of material were too poor to allow detailed kinetic experiments.

Variables in the spectrophotometric assay system

Effect of pH on enzyme activity. The pH-activity profile of the crude membrane-bound enzyme showed a sharp optimum at pH 6.5 (Fig. 2), whereas that for the solubilized and partially purified enzyme showed a much broader optimum shifted to higher pH values. The decrease in activity at acid pH is due to inactivation of the enzyme (see below). In determining the activity, the variation of the molar extinction coefficient of 2,6-dichlorophenol-indophenol at 600 nm with pH (Armstrong, 1964) has been taken into account. Tris/HCl buffer could not be used, since formaldehyde in the presence of Tris reduces 2,6-dichlorophenol-indophenol non-enzymically (Eady & Large, 1968).

Substrate concentration. The *N*-methylglutamate concentration in the standard assay system was 0.67 mM. The apparent *K_m* for *N*-methyl-L-glutamate obtained from double-reciprocal plots (Lineweaver & Burk, 1934) at 57 μM-2,6-dichlorophenol-indophenol using the partially purified enzyme was 25 μM (Table 2).

Enzyme concentration. The rate of reduction of dye remained linear with increasing enzyme concentration up to at least 176 μg of protein for the purified enzyme. This corresponded to a ΔA₆₀₀ of 0.028 min⁻¹.

Properties of the partially purified enzyme

Specificity for electron donors. The partially purified enzyme was found to oxidize all the *N*-methyl amino acids tested (Table 2), though no other *N*-methyl compounds tested were active. These included methylamine, dimethylamine, trimethylamine, methylisopropylamine (all as their

Fig. 2. Effect of pH on the stability and activity of *N*-methylglutamate dehydrogenase

(a) —, Effect on enzyme activity. The standard spectrophotometric assay was used. For the crude extract, buffers used were 0.2M-citric acid/trisodium citrate (●) and 0.2M-KH₂PO₄/Na₂HPO₄ (▲). Maximum activity was 2.14 munits/mg of protein. For the partially purified enzyme, the buffers used were 0.2M-citric acid/trisodium citrate (○), 0.2M-KH₂PO₄/Na₂HPO₄ (△) and 0.2M-glycine/NaOH (□). Maximum activity was 16.2 munits/mg of protein. (b) —, Effect on enzyme stability (■). Enzyme (17 munits/ml) was incubated in 0.1M-buffer at various pH values at 4°C for 30 min. Buffers used were citric acid/trisodium citrate (pH 4–6), KH₂PO₄/Na₂HPO₄ (pH 7) and NaHCO₃/Na₂HCO₃ (pH 9). Samples (0.5 ml) were removed and pipetted into standard assay mixtures at pH 6.5 (except with the potassium phosphate buffer concentration raised to 0.33M). Maximum activity was 25.3 munits/mg of protein.

hydrochlorides); tetramethylammonium chloride and trimethylamine *N*-oxide hydrochloride.

Specificity for electron acceptors. 2,6-Dichlorophenol-indophenol, phenazine methosulphate and horse heart cytochrome *c* were all found to be active as electron acceptors for the partially purified enzyme. Phenazine methosulphate was tested by coupling with 2,6-dichlorophenol-indophenol rather than by measuring phenazine methosulphate reduction directly (Singer & Kearney, 1958). Apparent *K_m* values for 2,6-dichlorophenol-indophenol, phenazine methosulphate and cytochrome *c* of 19, 18 and 110 μM respectively were obtained at 0.67 mM-*N*-methylglutamate and pH 6.5.

Stereospecificity. The enzyme preparation was found to be non-specific with respect to the enantiomeric configuration of *N*-methylglutamate. Limiting quantities (20 nmol) of either *N*-methyl-L-glutamate or *N*-methyl-DL-glutamate were each oxidized in the standard assay with the reduction respectively of 23 and 24 nmol of 2,6-dichlorophenol-indophenol. Moreover *N*-methyl-D-alanine, although oxidized more slowly than *N*-methyl-L-alanine (Table 2), had

Table 2. Substrate specificity of partially purified *N*-methylglutamate dehydrogenase of *Ps. aminovorans*. Maximum rates and apparent *K_m* values were obtained from double-reciprocal plots (Lineweaver & Burk, 1934). The standard spectrophotometric assay was used.

Substrate	Apparent <i>K_m</i> (mM)	Relative maximum velocity
<i>N</i> -Methyl-L-glutamate	0.025	1
<i>N</i> -Methyl-DL-aspartate	0.46	0.56
<i>N</i> -Methyl-L-alanine	25.0	1.85
<i>N</i> -Methyl-D-alanine	33.0	0.044
<i>N</i> -Methyl-DL-alanine	87.0	1.64
Sarcosine	90.9	0.56

Table 3. Stoichiometry of the reaction catalysed by *N*-methylglutamate dehydrogenase

The complete system contained in a final volume of 6 ml: 400 μmol of potassium phosphate buffer, pH 6.5; 0.68 μmol of 2,6-dichlorophenol-indophenol; 5 μmol of *N*-methyl-L-glutamic acid; 168 μg of partially purified *N*-methylglutamate dehydrogenase. After measuring the *A*₆₀₀, the reaction was started at 30°C by the addition of *N*-methylglutamate. After 2 h, the absorbance was again read and the reaction stopped by adding 1 ml of 0.78 M-trichloroacetic acid. Denatured protein and occluded dye were removed by centrifugation and the clear colourless supernatant, after neutralization with NaOH, was analysed for formaldehyde and L-glutamic acid as described in the Materials and Methods section.

	Complete system	Control without enzyme	Control without substrate
Reactant consumed (nmol)			
2,6-Dichlorophenol-indophenol	402	0	0
Products formed (nmol)			
Formaldehyde	479	0	0
L-Glutamate	530	24	6.4

a similar apparent *K_m* value. If the observed rate had been due to a small amount of the L-form as an impurity in the *N*-methyl-D-alanine, the expected apparent *K_m* value would have been much larger. It was not possible to follow the oxidation of the *N*-methylalanines to completion, because at the low concentration necessary to do this, the relatively high apparent *K_m* value made the reaction time so long as to be impracticable.

Stoichiometry of the reaction. The reduction of 1 mol of 2,6-dichlorophenol-indophenol was accompanied by the formation of stoichiometric amounts of formaldehyde and L-glutamate (Table 3).

Fig. 3. Kinetics of inhibition of *N*-methylglutamate dehydrogenase by formaldehyde

Standard assay conditions were used except that the buffer pH was 7.0 and phenazine methosulphate was the primary electron acceptor (added in addition to 2,6-dichlorophenol-indophenol). Formaldehyde was also present where indicated. (a) Double-reciprocal plot with *N*-methylglutamate as variable substrate. Phenazine methosulphate was present at 0.67 mM. ○, No inhibitor; △, 17 mM-formaldehyde; □, 33 mM-formaldehyde; ▲, 67 mM-formaldehyde. (b) Double-reciprocal plot with phenazine methosulphate as variable substrate. *N*-Methyl-L-glutamate was present at 0.17 mM. ○, No inhibitor; △, 33 mM-formaldehyde; □, 67 mM-formaldehyde.

These results are consistent with the following reaction occurring:

where DCPIP denotes 2,6-dichlorophenol-indophenol. This equation is equivalent to eqn. (2).

Stability of the enzyme. The enzyme was stable to repeated freezing and thawing and to storage at

-15°C at a protein concentration of 3 mg/ml. It also survived cooling to -196°C with subsequent thawing. Exposure of the enzyme to a range of pH values between 4 and 10 for a period of 30 min showed it to be acid-labile, though stable at neutrality and at pH values above 7 (Fig. 2). The enzyme was heat-labile, with half-lives of 35 min at 30°C and 5 min at 40°C .

Effect of cations on the partially purified enzyme.

The effect of adding metal ions to a final concentration in the assay mixture of 1 mM was determined. The standard assay system was used, except that phosphate buffer was replaced by 50 μmol of Bes, pH 6.6, to prevent precipitation of phosphates. The metal ions were added as their chlorides, with the exception of zinc, which was added as the sulphate. Zn^{2+} , Mn^{2+} , Ca^{2+} , Ni^{2+} , Mg^{2+} , Na^+ and K^+ had no effect. Co^{2+} (1 mM) stimulated the activity by 33%. Increasing the Co^{2+} concentration did not lead to any further stimulation. Cu^{2+} (1 mM) totally inhibited *N*-methylglutamate dehydrogenase activity. (Fe^{2+} could not be tested as it interfered with the assay system.) Prior dialysis of the enzyme against 10 mM-potassium phosphate, pH 7.0, with or without 1 mM-EDTA, at 4°C for 20 h led to a certain loss of activity, but addition of 1 mM- Co^{2+} did not lead to any significantly greater stimulation of activity than was observed in undialysed samples.

Effect of possible inhibitors. None of the chelating agents tested in the standard assay system (3.3 mM-EDTA and 2,2'-bipyridine; 0.33 mM-8-hydroxyquinoline, cuprizone and neocupreine; 10 mM-KCN) showed significant inhibition after 10 min preincubation. *o*-Phenanthroline (0.83 mM), 3 mM-diethyldithiocarbamate and 10 mM- NaN_3 interfered with the assay.

p-Chloromercuribenzoate (30 μM) with 10 min preincubation led to 81% inhibition of the partially purified enzyme. Similar preincubation with 16.6 mM-iodoacetamide led to 77% inhibition of the system.

No inhibition was caused by 0.33 mM-compound SKF 525-A, 1 mM-isoniazid, 2 mM-semicarbazide, 0.033 mM-rotenone, 1 mM-sodium Amytal, 3.3 mM-NaF or by 5 min bubbling with CO .

When tested at lower substrate concentration (33 μM -*N*-methylglutamate), 100 mM-L-glutamate was not significantly inhibitory. Combinations of 50 mM-L-glutamate and 17 mM-formaldehyde led to 23% inhibition of the enzyme. Formaldehyde alone was found to inhibit partially purified *N*-methylglutamate dehydrogenase in a mixed competitive-non-competitive fashion with respect to both *N*-methyl-L-glutamate (Fig. 3a) and phenazine methosulphate (Fig. 3b). Methanol, at the concentration present in analytical-grade formaldehyde solutions, was not inhibitory. Methylamine hydrochloride, L-alanine and glycine (all at 33 mM) were not inhibitory. Indeed, there was some stimulation by the amino acids

Fig. 4. Kinetics of *N*-methyl-L-glutamate oxidation with 2,6-dichlorophenol-indophenol as electron acceptor. Glass cuvettes (2mm light-path) contained 2,6-dichlorophenol-indophenol and *N*-methyl-L-glutamate as indicated, enzyme and 0.2M-potassium phosphate buffer, pH 7.0, in a final volume of 0.5 ml. After prior incubation at 25°C, the reaction was started by addition of *N*-methylglutamate and the reaction followed by the decrease in A_{600} . (a) Effect of varying *N*-methyl-L-glutamate concentration at a series of fixed concentrations of 2,6-dichlorophenol-indophenol (μM): ○, 25; ●, 50; △, 100; ▲, 150; □, 250; ■, 500. (b) Secondary plot of the intercepts on the ordinate of the lines in (a) against the reciprocal of the 2,6-dichlorophenol-indophenol concentration.

tested, including L-aspartate at 3.3 mM. L-Aspartate (33 mM), however, caused 56% inhibition of the enzyme. *N*-Methylglutamate dehydrogenase was totally inhibited by 1.3 mM-2-oxoglutarate.

At 11.3 μM-2,6-dichlorophenol-indophenol, 1 mM-quinacrine dihydrochloride was not inhibitory.

Partially purified *N*-methylglutamate dehydrogenase was inhibited by Triton X-100. Unlike the crude solubilized enzyme, however, removal of the detergent from preparations of the purified enzyme with Bio-Beads SM-2 did not lead to an increase in activity.

Mechanism of reaction. Double-reciprocal plots relating the rate of reduction of 2,6-dichlorophenol-indophenol to *N*-methyl-L-glutamate concentration at pH 7.0 over a 20-fold range of dye concentration yielded a series of parallel lines (Fig. 4a). The secondary plot of intercepts of these lines on the ordinate against the reciprocal of 2,6-dichlorophenol-indophenol concentration was linear (Fig. 4b) and gave a K_m value for the dye of 0.21 mM (see Table 4).

Similar results were obtained when phenazine methosulphate was used as the primary electron acceptor instead of 2,6-dichlorophenol-indophenol

(Fig. 5) and when sarcosine replaced *N*-methyl-L-glutamate as the electron donor (Fig. 6). There is some deviation from linearity at low concentrations of phenazine methosulphate (Fig. 5b).

When the reaction rate was measured at simultaneously varying concentrations of *N*-methyl-L-glutamate and 2,6-dichlorophenol-indophenol maintained in a concentration ratio of 1:1, the double-reciprocal plot of reaction rate against substrate concentration gave a straight line (Fig. 7). It can be shown for such a plot that the intercept on the abscissa is $-1/K'$, where $K' = K_A/x + K_B$, where K_A and K_B are the Michaelis constants for electron donor (A) and electron acceptor (B) respectively and x is the ratio of their concentrations, $[A]/[B]$ (Henson & Cleland, 1964). From Fig. 7 the value of K' is 0.62 mM and this agrees reasonably well with the value of 0.40 mM calculated from the values of K_A and K_B in Table 4.

Spectral properties of partially purified *N*-methylglutamate dehydrogenase. Partially purified preparations showed absorption maxima at 416 and 555 nm (Fig. 8), tentatively interpreted as indicating the presence of *b*-type cytochrome. Addition of *N*-

Table 4. Kinetic coefficients for the oxidation of *N*-methyl-L-glutamate and sarcosine by 2,6-dichlorophenol-indophenol and *N*-methyl-L-glutamate by phenazine methosulphate catalysed by partially purified *N*-methylglutamate dehydrogenase. The kinetic coefficients were measured at pH 7.0 and 25°C and are defined by the initial rate equation $e/v = \phi_0 + \phi_A/[A] + \phi_B/[B]$ (Dalziel, 1957) where $[A]$ and $[B]$ are the concentrations of electron donor and electron acceptor respectively. ϕ_A/ϕ_0 is the Michaelis constant for the electron donor (K_A) and ϕ_B/ϕ_0 is the Michaelis constant for the electron acceptor (K_B).

Electron donor ...		<i>N</i> -Methyl-L-glutamate	Sarcosine	<i>N</i> -Methyl-L-glutamate
Electron acceptor ...		2,6-Dichlorophenol-indophenol	2,6-Dichlorophenol-indophenol	Phenazine methosulphate
Parameter	Units			
ϕ_0	min	0.06	0.102	0.71
ϕ_A	$\mu\text{M} \cdot \text{min}$	11.60	41 700	49.0
ϕ_B	$\mu\text{M} \cdot \text{min}$	12.80	11.8	42.0
K_A	mM	0.193	409	0.069
K_B	mM	0.210	0.12	0.059

Fig. 5. Kinetics of *N*-methyl-L-glutamate oxidation with phenazine methosulphate as electron acceptor. Assay conditions were as described in the legend to Fig. 3, the final concentrations of phenazine methosulphate and *N*-methyl-L-glutamate being as indicated. (a) Effect of varying *N*-methyl-L-glutamate concentration at a series of fixed concentrations of phenazine methosulphate (μM): ▲, 33; △, 165; ●, 330; ○, 660. (b) Secondary plot of the intercepts on the ordinate of the lines in (a) against the reciprocal of the phenazine methosulphate concentration.

methylglutamate led to a bleaching of the spectrum in the 439–500 nm region (Fig. 8), which is characteristic of flavoprotein reduction.

Similar, more pronounced, changes were caused by sodium dithionite, which also caused changes characteristic of reduction of *b*-type cytochrome, namely a sharpening of the Soret peak with a shift to 428 nm and the appearance of β - and α -bands at 525 and 558 nm respectively (Fig. 8b).

Discussion

A number of particulate nicotinamide nucleotide-independent dehydrogenases have been identified in bacteria. Examples include dehydrogenases for D-amino acids (Marshall & Sokatch, 1968), sarcosine (Frisell, 1971; Hall *et al.*, 1971), D- and L-lactate (Kemp, 1972), malate (Francis *et al.*, 1963) and succinate (Pollock *et al.*, 1971). The present enzyme has many features in common with these, for example

Fig. 6. Kinetics of sarcosine oxidation by *N*-methylglutamate dehydrogenase with 2,6-dichlorophenol-indophenol as electron acceptor

Assay conditions were as described in the legend to Fig. 4. (a) Effect of varying sarcosine concentration at a series of fixed concentrations of 2,6-dichlorophenol-indophenol (μM): $\circ$, 25; Δ , 100; $\square$, 500. (b) Secondary plot of the intercepts on the ordinate of the lines in (a) against the reciprocal of the 2,6-dichlorophenol-indophenol concentration.

Fig. 7. Effect on the initial rate of the *N*-methylglutamate dehydrogenase reaction of varying electron-donor and electron-acceptor concentrations in a fixed ratio

Assay conditions were as described in the legend to Fig. 4. The ratio of *N*-methyl-L-glutamate concentration to 2,6-dichlorophenol-indophenol concentration was 1:1.

inducibility by substrates whose catabolism requires it, low specific activities in crude extracts (cf. Marshall & Sokatch, 1968), and similar spectral changes on addition of substrate.

Assessment of the role of *N*-methylglutamate dehydrogenase in methylamine oxidation is made difficult by the fact that comparison of relative oxidation rates for methylamine and *N*-methylglutamate by whole cells may be influenced by differences in permeability and of accessibility of the two substrates to their respective first enzymes, which are soluble (and presumably intracellular) in the case of methylamine and membrane-bound in the case of *N*-methylglutamate. Although rates of oxidation of either substrate by whole cells are of a similar order, the rate of *N*-methylglutamate-dependent 2,6-dichlorophenol-indophenol reduction in crude extracts is approximately 20-fold lower, assuming the consumption of 0.5 mol of O_2 /mol of *N*-methylglutamate oxidized. However, when crude membrane preparations were assayed for *N*-methylglutamate-dependent dye reduction and *N*-methylglutamate-dependent O_2 consumption under identical conditions of substrate concentration, the specific activity of the latter was only approximately 2-fold greater than that of *N*-methylglutamate-dichlorophenol-indophenol reductase. The observed enzyme activity seems inadequate to explain the rate of methylamine oxidation by whole

Fig. 8. Effect of reducing agents on the spectrum of partially purified *N*-methylglutamate dehydrogenase —, Untreated enzyme. Partially purified enzyme preparation (3.2 mg of protein/ml) from which Triton X-100 had been removed was examined against a reference cuvette containing 50 mM-phosphate buffer, pH 7.0. ---, Effect of dithionite; a few crystals of sodium dithionite were added. - · - ·, Effect of *N*-methyl-L-glutamate (2 μ mol); this was added to a sample of untreated enzyme.

cells, and also to account for the rate of carbon flux into cell material from calculations based on a mean generation time of 6 h (Fig. 1). Since, however, the observed rate of methylamine oxidation by washed suspensions is also inadequate to support the calculated rate of carbon assimilation, our measured whole-cell oxidation rates are probably suboptimal. It is also important that there are other pathways by which methylamine can be assimilated (and probably oxidized) by similar organisms (Pollock & Hersh, 1971; Lin & Wagner, 1975), and the quantitative significance of these in *Ps. aminovorans* has not yet been assessed. A similar problem was encountered by Pollock & Hersh (1971) in relation to the other postulated enzyme in this sequence, *N*-methylglutamate synthase (eqn. 1). An unambiguous establishment of the function of these enzymes in bacterial growth on methylamine would require the isolation of mutants lacking either the synthase or the dehydrogenase.

Membrane fractions of methylamine-grown *Ps. aminovorans* prepared by French pressure cell showed *N*-methylglutamate oxidase activity. *N*-Methyl-DL-glutamate-reduced minus oxidized difference spectra of such preparations in the presence of CN^- showed evidence for reduction of both flavo-

protein and *b*-type cytochrome. From the spectra, and from the effect of inhibitors of the electron-transport chain on this system, it seems likely that this is at least part of the pathway by which electrons are transferred from *N*-methylglutamate to oxygen *in vivo*, and that a *b*-type cytochrome may be the natural electron acceptor for *N*-methylglutamate dehydrogenase. These observations are essentially in agreement with those of Hersh *et al.* (1971). The spectral properties of the partially purified enzyme suggest that it may be a flavoprotein. Preliminary electron-paramagnetic-resonance studies of the partially purified enzyme do not show any evidence of iron-sulphur centres which can be reduced by *N*-methylglutamate, although a signal at $g = 1.94$ was produced by dithionite reduction (R. Cammack, C. W. Bamforth & P. J. Large, unpublished work). Solubilization of the enzyme from the particulate fraction by Triton X-100 led to a total loss of *N*-methylglutamate-dependent O_2 consumption and a fall in the activity observed with horse heart cytochrome *c* as electron acceptor to the value observed with the partially purified enzyme. This suggests that cytochrome *c* does not react with the primary dehydrogenase, but at a point further along the electron-transport chain towards oxygen (perhaps

N -Methyl-L-glutamate Glutamate Formaldehyde Acceptor Acceptor- H_2
 ↓ ↑ ↑ ↓ ↑
 ————— E ($E'S_1 \rightleftharpoons E'X$) ($E'Y$) E' ($E'S_2 \rightleftharpoons EZ$) E

The simplest mechanism which would account for most of our observations is shown in Scheme 1. The mixed inhibition patterns observed with formaldehyde may be explicable by the formation of dead-end complexes with various enzyme forms.

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