Purification and properties of the methanol dehydrogenase from *Methylophilus methylotrophus*

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1. A dye-linked methanol dehydrogenase, resembling many others from a variety of methylotrophic bacteria, was purified to homogeneity from extracts of methanol-grown *Methylophilus methylotrophus*. 2. The enzyme was very stable in the presence of methanol; in the absence of methanol it had a half-life of 1–2 days at 4°C. 3. The value of $A_{1\text{cm}, 280}^{1\%}$ was 17.5. 4. The enzyme retained bound methanol after passage through Sephadex G-25. This tightly-bound methanol slowly exchanged with free [^{14}C]-methanol from a value of 0.27 mol of [^{14}C]methanol/mol of enzyme after 48 h incubation at 4°C to a limiting value of approx. 2.5 mol of [^{14}C]methanol/mol of enzyme after 3 weeks incubation at 4°C. 5. One mol of this enzyme reduced 89.4 mol of 2,6-dichlorophenol-indophenol (via phenazine methosulphate) in the absence of any additional methanol in the assay mixture. The source of the electrons involved in this reduction is not known.

Methanol-utilizing bacteria contain a dye-linked methanol dehydrogenase (EC 1.1.99.8) showing many characteristic physical and kinetic properties (Anthony & Zatman, 1964, 1965, 1967; Patel *et al.*, 1972, 1973, 1978; Mehta, 1973; Sperl *et al.*, 1974; Wadzinski & Ribbons, 1975; Goldberg, 1976; Michalik & Raczynska-Bojanowska, 1976; Yamanaka & Matsumoto, 1977; Bellion & Wu, 1978; Duine *et al.*, 1978; Wolf & Hanson, 1978; Bamforth & Quayle, 1978*a,b*, 1979; Colby *et al.*, 1979; Quayle, 1980; Duine & Frank, 1980*a*). The enzyme contains a novel coenzyme form of a nitrogen-containing orthoquinone referred to as methoxatin (Salisbury *et al.*, 1979) or pyrrolo-quinoline quinone (Duine & Frank, 1980*b*, 1981; Duine *et al.*, 1980). As a preliminary to a detailed kinetic analysis, the present paper reports the purification and characterization of the enzyme from methanol-grown *Methylophilus methylotrophus* (*Pseudomonas methylotrophus*) (Byrom & Ousby, 1975) and an exploratory study has been made of its so-called 'endogenous' or 'blank' reaction with electron acceptors in the absence of methanol. A preliminary report of part of this work has already been presented (Ghosh & Quayle, 1978).

Abbreviations used: DCPIP, 2,6-dichlorophenol-indophenol; SDS, sodium dodecyl sulphate; PMS, phenazine methosulphate; PES, phenazine ethosulphate.

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Materials and methods

Chemicals

PMS, PES and Nitroblue Tetrazolium were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames K2 7BH, U.K. The free radical form of *NNN'*-tetramethyl-*p*-phenylenediamine (Wurster's Blue) was prepared by the method of Michaelis & Granick (1943) from the perchlorate salt. Freshly frozen cell paste of methanol-grown *M. methylotrophus* was kindly supplied by the Agricultural Division, ICI, Billingham, Teesside, U.K., and stored at –15°C.

Preparation of cell-free extracts

For stability experiments. Cell paste (one part by weight) was suspended in 5 vol. of the appropriate buffer, cooled to 4°C and then broken by sonication for 3 × 1 min intervals in an MSE ultrasonic disintegrator (model 150 W) or two passages through a pre-cooled French pressure cell or extrusion through a Hughes press at –15°C. The extracts were then centrifuged for 20 min at 38 000 *g* and for some purposes centrifuged for a further 2 h at 100 000 *g*.

For routine purification. Cell paste was suspended as above in 50 mM-potassium phosphate, pH 7.0, cooled to 0°C in ice and then broken by two passages through a French pressure cell at 1200 lb/in² (8280 kPa). The suspension was then centrifuged at 38 000 *g* for 20 min and the pellet was discarded.

Protein determination

Protein was determined either by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, or by the method of Warburg & Christian (1941). For the experiments involving binding of [^{14}C]methanol the protein concentration was determined from its absorbance at 280nm with an experimentally determined absorption coefficient for the homogeneous protein.

Enzyme assay

Two spectrophotometric assays have been used routinely, both of which are modifications of the procedure described by Anthony & Zatman (1967).

Assay 1. Plastic cuvettes (1cm light path; W. Sarstedt, Leicester, U.K.) contained, in a final volume of 3ml: Tris/HCl, pH9.0, 375 μmol ; PMS, 3.3 μmol ; DCPIP, 0.17 μmol ; ammonium chloride, 150 μmol ; methanol, 20 μmol ; potassium cyanide, 3 μmol . The blank cuvette contained water. The assay was started by addition of enzyme to the test cuvette and followed at 30°C by the decrease in A_{600} caused by the reduction of DCPIP. A Zeiss PMQII spectrophotometer fitted with a Gilford converter and linked to a Servoscribe recorder was used.

Assay 2. This assay was performed in a similar fashion to Assay 1 but using the following complete assay mixture, in a final volume of 3ml: sodium pyrophosphate buffer, pH10.0, 375 μmol ; PES, 3.3 μmol ; DCPIP, 0.17 μmol ; ammonium chloride, 150 μmol ; methanol, 20 μmol . Potassium cyanide was omitted.

For both assays the molar absorption coefficient of DCPIP at pH9.0 and above was taken to be $21.99 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Armstrong, 1964). One unit of enzyme activity is the amount required to catalyse the reduction of 1 μmol of DCPIP/min at 30°C. For some purposes, Wurster's Blue was substituted for PES and DCPIP and its reduction followed at 560nm and with a molar absorption coefficient of $11.6 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Michaelis *et al.*, 1939).

Purification of methanol dehydrogenase

The procedure employed was that of Anthony & Zatman (1967) with the single modification that 10mM-methanol was included in all the buffers to ensure the long-term stability of the enzyme. The procedure was scaled up 10-fold to yield about 100mg of homogeneous enzyme in a single run. When working on this scale, the first $(\text{NH}_4)_2\text{SO}_4$ fraction was taken between 55 and 80% of saturation and all equilibration times were increased by a factor of three.

Polyacrylamide-gel electrophoresis

This was performed by the method of Davis (1964) with the omission of the large pore gel.

Electrophoresis was conducted in a Shandon apparatus at pH values of 4.3, 6.6, 8.5 and 9.3 using 7.5% gels. For routine screening during purification, pH6.6 was found to be the most suitable. Bromophenol Blue was used as a tracking dye above pH6.0. Gels were stained for protein by immersion in a solution of 0.025% (w/v) Coomassie Blue G250 in 3.5% (v/v) HClO_4 . Where necessary the gels were destained overnight in 7% (v/v) acetic acid.

Methanol dehydrogenase activity was detected by the immersion of unfixed gels in the assay mixture containing Tris buffer, pH9.0 (Assay 1) with DCPIP being replaced by 0.2ml of 0.8% (w/v) Nitroblue Tetrazolium (Yamanaka & Matsumoto, 1977).

SDS/polyacrylamide-gel electrophoresis was performed by the procedure of Weber & Osborn (1969) in 5% gels in the presence of 0.1% SDS. Protein samples were prepared by the method of Shapiro *et al.* (1967).

Ultracentrifugal studies

Sedimentation of homogeneous enzyme at 20°C in 0.1M-Tris/HCl, pH8.0, containing 10mM-methanol, was observed in a Spinco model E analytical ultracentrifuge at 60000rev./min, using Schlieren optics.

Determination of specific absorption coefficient $A_{1\text{cm},280}^{1\%}$

Two foil thimbles were weighed and into each was placed 2ml of a solution of homogeneous enzyme dissolved in 0.1M-Tris/HCl, pH8.0. The concentration of the enzyme solution was approx. 5 mg/ml as determined by the Lowry method. The contents of the thimbles were evaporated at 100°C to a constant dry weight and the procedure was repeated with two blank samples containing buffer alone; the dry weight of the buffer was subtracted from that of the enzyme. The absorbance of the stock enzyme solution at 280nm was determined after suitable dilution to a value of approx. 0.4. From these data a value of $A_{1\text{cm},280}^{1\%}$ was obtained.

Assay of [^{14}C]methanol

Samples of solutions containing ^{14}C were mixed with 10vol. of a scintillation fluid containing, per litre: xylene, 667ml; Triton, 333ml; 2,5-diphenyloxazole, 5g; 1,4-bis-(2,5-diphenyloxazol-2-yl)-benzene (0.5g). Samples of the mixture were then assayed in a Nuclear-Chicago liquid-scintillation counter (Isocap 300) and corrected for quenching by the channels-ratio method.

Determination of molecular weight

Gel-permeation chromatography on Sephadex G-150 was carried out by the method of Andrews (1964). Protein standards were cytochrome *c* (horse

heart, mol.wt. 12 600), ovalbumin (mol.wt. 40 000), malate dehydrogenase (ox heart mitochondria, mol.wt. 70 000), glucose 6-phosphate dehydrogenase (bovine, mol.wt. 128 000). Protein standards for SDS/polyacrylamide-gel electrophoresis were pyruvate kinase (mol.wt. 7500), bovine serum albumin monomer (mol.wt. 68 000), catalase (mol.wt. 60 000), aldolase (mol.wt. 40 000), pepsin (mol.wt. 35 000) and carbonic anhydrase (mol.wt. 29 000).

Results and discussion

Subcellular distribution of methanol dehydrogenase

After all three methods of cell breakage, approx. 50% of the total activity of the enzyme appeared in the supernatant after centrifugation of a crude extract at 100 000 g for 2 h. After incubation of the crude extract for 30 min with 1 M-KCl, about 70% of the activity appeared in the supernatant, indicating that the enzyme *in vivo* is loosely bound to the intracellular membrane.

Purification of methanol dehydrogenase

The methanol dehydrogenase from *M. methylotrophus* was purified to homogeneity by the procedure described in the Materials and methods section. The purity of the preparation was established by appearance of (i) a single protein band coinciding with enzyme activity during gel electrophoresis at four widely differing pH values and (ii) a single symmetrical peak during sedimentation at three protein concentrations (2.86, 4.14 and 5.38 mg/ml respectively). As with some other methanol dehydrogenases, the enzyme was homogeneous after 10-fold purification, indicating that it constitutes about 10% of the soluble protein of the cell. Its final specific activity was 4.34 units/mg of protein with an overall yield of 13.2%.

Stability of the enzyme

The enzyme lost activity when solutions in phosphate or Tris buffers at pH 7–8 were stored in the absence of methanol at room temperature, 4°C or –22°C, e.g., virtually all the activity was lost within 2 days at 4°C. Upon the inclusion of 10 mM-methanol, the enzyme retained over 80% of its activity in either buffer at 4°C over several days and 90% of its activity over 6 months at –22°C. In the presence of 10 mM-methanol in 0.1 M-Tris/HCl buffer, pH 7.5, the enzyme retained 95% of its activity after 10 min at 50°C, 60% of its activity after 2 min at 60°C and 10% of its activity after 2 min at 70°C.

As previously observed for the methanol dehydrogenase of *Pseudomonas* M27 (Anthony & Zatman, 1967) the enzyme was stable over a wide range of pH values. Solutions containing 50 µg of purified

enzyme dissolved in 1.0 ml of buffer containing 10 mM-methanol retained at least 80% of the initial activity after incubation for 18 h at room temperature at pH values ranging from 4.0 to 11.0. The buffers used were: acetic acid/sodium acetate, pH 4.0–5.0; potassium hydrogen phosphate, pH 6.0–7.0; Tris/HCl, pH 8.0–10.0; sodium hydrogen pyrophosphate, pH 9.0–10.0; sodium hydrogen carbonate, pH 11.0.

Molecular weight

A molecular weight of 115 000 was obtained for the enzyme in 0.1 M-Tris/HCl, pH 8.0, by using gel-permeation chromatography on Sephadex G-150. A single band was observed on SDS/polyacrylamide-gel electrophoresis corresponding to mol.wt. 62 000. This suggests that the native enzyme is a dimer consisting of two identical subunits. Molecular weights between 110 000 and 120 000 have previously been reported by many authors for dimeric methanol dehydrogenases from various methylotrophic bacteria.

Absorption spectrum

The enzyme showed absorption maxima at 280 nm and 342 nm with a broad shoulder at about 405 nm, similar to many other methanol dehydrogenases.

Specific absorption coefficient

The value for $A_{1\text{cm},280}^{1\%}$ of the purified enzyme was found to be 17.5. This may be compared with the corresponding value of 20.6 determined by Yamanaka & Matsumoto (1977) for the methanol dehydrogenase from *Pseudomonas* 2941.

Activators

Activity of the methanol dehydrogenase was absolutely dependent on the presence of NH_4^+ ions or methylamine hydrochloride. The latter activator was only 4% as effective as ammonium chloride under conditions of Assay 2.

Substrate specificity

Under the conditions of spectrophotometric assay the enzyme oxidized primary aliphatic alcohols but not secondary alcohols; there was no measurable oxidation of benzyl alcohol (Table 1). Formaldehyde was oxidized but acetaldehyde only very slowly. PMS and PES were almost equivalent as electron acceptors; Wurster's Blue, which has previously been used as an electron acceptor for the methanol dehydrogenase from *Hyphomicrobium* X (Duine *et al.*, 1978; Duine & Frank, 1980a), also accepted electrons from the methanol dehydrogenase of *M. methylotrophus*. However, the rapid rate of decomposition of this dye prevented accurate measurement of its kinetic parameters.

Table 1. *Substrate specificity of methanol dehydrogenase*

Measurements of the kinetic parameters of electron donors were carried out under conditions of Assay 2, as detailed in the Materials and methods section. The same assay conditions were used for measurement of the parameters for electron acceptors, except that 0.1 M-Tris/HCl, pH 9.0, was substituted for 0.125 M-sodium pyrophosphate buffer, pH 10.0. Apparent K_m values and maximum velocities were determined by the method of Eisenthal & Cornish-Bowden (1974) and checked by replotting by the method of Lineweaver & Burk (1934).

Substrate	Apparent K_m (mM)	Maximum velocity (units/mg)
Methanol	0.020	6.45
Ethanol	0.028	4.90
Propan-1-ol	0.280	6.05
Butan-1-ol	0.285	5.76
Formaldehyde	1.01	5.2
PES	0.395	2.50
PMS	0.44	3.55

pH Optimum

The pH optimum of the enzyme in pyrophosphate buffer was found to be 9.8.

Effect of inhibitors

The enzyme was relatively insensitive to a variety of metal-chelating agents (azide, EDTA, $\alpha\alpha'$ -bipyridine, 1,10-phenanthroline, 8-hydroxyquinoline) and thiol-binding reagents [5,5-dithiobis-(2-nitrobenzoic acid), *p*-chloromercuribenzoate, iodoacetate], as was found by Anthony & Zatman (1965) for the methanol dehydrogenase of *Pseudomonas* M27. The effect of 1 mM-sodium cyanide was 2-fold; it suppressed the large endogenous reduction of the electron acceptors by the enzyme in the absence of substrate and it stimulated the enzyme activity by 22%. It should be noted that the action of cyanide on methanol dehydrogenases is complex and apparently varies with different enzymes (Anthony & Zatman, 1965; Bamforth & Quayle, 1978a; Duine & Frank, 1980a).

Binding of methanol by methanol dehydrogenase

It has long been known that purified methanol dehydrogenases catalyse a rapid and unexpectedly large reduction of the electron acceptors in the PMS/DCPIP-coupled assay system in the absence of added methanol (Anthony & Zatman, 1964; Goldberg, 1976; Bamforth & Quayle, 1978a; Duine & Frank, 1980a). Anthony & Zatman (1964) showed that this 'endogenous reduction' persisted even after prolonged dialysis of the enzyme and concluded that the endogenous substrate was not free methanol. Duine & Frank (1980a) made the important observation that such dehydrogenation of the enzyme from *Hyphomicrobium* X in the absence

of methanol led to inactivation of the enzyme. In no case was the source traced of the electrons responsible for the endogenous reduction of the dyes. Although Anthony & Zatman (1964) had shown that free methanol could not be responsible in the case of the enzyme from *Pseudomonas* M27, there remained the possibility that the enzyme might be binding relatively large amounts of methanol, perhaps at sites other than the active site with respect to dehydrogenation. This was directly tested for in the case of the enzyme from *M. methylotrophus* in the following series of experiments. The enzyme used had been previously stored in the presence of 10 mM-methanol.

A solution was prepared of 5 mg of 5 mg of homogeneous enzyme in 0.95 ml of 0.1 M-Tris/HCl, pH 8.0, which contained 15.8 mM- ^{14}C -methanol (3.94 μCi , 145.78 kBq). The solution was incubated at 4°C and after 48 h a sample (0.2 ml) was withdrawn and applied to the top of a column (1 cm $\times$ 40 cm) of Sephadex G-25 (medium) previously equilibrated with 0.1 M-Tris/HCl buffer, pH 8.0. The column was eluted with the same buffer and fractions were collected that consisted of six drops each (fractions 1–15 inclusive) or 15 drops each for subsequent fractions (Fig. 1). Each drop was approximately 60 μl in volume. Samples (20 μl) of each fraction were tested for ^{14}C content by liquid-scintillation assay and for methanol dehydrogenase activity by Assay 1. It had previously been established that such a column would satisfactorily separate methanol dehydrogenase and free methanol on this scale. Fractions 1–5, which contained most of the enzyme activity, were pooled (1.5 ml total) and

Fig. 1. Passage of methanol dehydrogenase through a column of Sephadex G-25 after incubation with ^{14}C -methanol

Experimental details are given in the text.

three measurements were made on the pool: (i) a sample (0.1 ml) was assayed for radioactivity and found to contain 72.32 d.p.m.; (ii) a sample (0.1 ml) was diluted 10-fold and its A_{280} was found to be 0.092; (iii) a sample (50 μ l) was incubated in the mixture used for measurement of methanol dehydrogenase activity by Assay 1, but lacking methanol and cyanide, and the consequent total decrease in A_{600} was measured and found to be 0.15. From these data the following results were calculated. (i) From the measured radioactive content and the known sp. radioactivity (0.263 Ci/mol) of the [14 C]methanol, the pooled fractions contained 1.86 nmol of 14 C. (ii) From the measured A_{280} and the known $A_{1\text{cm},280}^{1\%}$ of methanol dehydrogenase (17.5), the pooled fractions contained 0.788 mg of enzyme. For a mol.wt. of 115 000 this corresponds to 6.85 nmol of methanol dehydrogenase. (iii) From the measured drop in A_{600} caused by the reduction of PMS/DCPIP by the enzyme in the absence of added methanol, and using the value $\epsilon_{600} 21.99 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for DCPIP, the pooled fractions contained 612 reducing equiv. (i.e. 612 pairs of reducing electrons).

The pooled fractions (1–15) therefore contained the proportions 1.0 nmol of 14 C:3.68 nmol of methanol dehydrogenase:329 reducing equiv.

After a further 3 weeks of incubation of the methanol dehydrogenase with [14 C]methanol at 4°C, the amount of enzyme-bound 14 C increased to a limiting value of approx. 2.5 mol of 14 C/mol of methanol dehydrogenase. This should be viewed alongside the fact that it is a dimeric enzyme and, like the enzyme from *Hyphomicrobium* X, contains 2 mol of prosthetic group/mol of enzyme (Duine *et al.*, 1980). The inference is therefore that each subunit contains one prosthetic group and can tightly bind 1 mol of methanol.

The binding experiment showed that each mol of enzyme carried 89.4 reducing equiv. Assuming that the enzyme also carried 2 mol of tightly bound methanol, it can be seen that this amount of substrate is only sufficient to account for a small percentage of the observed reducing equivalents, regardless of whether any of the bound 14 C was in the form of formaldehyde rather than methanol. We are presently unable to suggest a source of the extra reducing equivalents; this remarkable phenomenon warrants further investigation.

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