[33] Methanol Dehydrogenase from *Methylobacterium extorquens* AM1

By DARREN J. DAY and CHRISTOPHER ANTHONY

Methanol dehydrogenase (MDH) (EC 1.1.99.8) is the periplasmic quinoprotein which catalyzes the oxidation of methanol to formaldehyde in all gram-negative bacteria during growth on methane or methanol.[1-3] Its immediate electron acceptor is cytochrome c_L, which is oxidized by way of a conventional small cytochrome c_L and a membrane oxidase. Although the methods presented here are for *Methylobacterium extorquens* AM1, most are suitable for MDHs from other gram-negative bacteria. *Methylobacterium extorquens* AM1 is a pink facultative methylotroph that grows on methanol and methylamine and multicarbon compounds but not on methane. It has previously been called *Pseudomonas* AM1 and *Methylobacterium* AM1.

Assay of Methanol Dehydrogenase

For most work with MDH a dye-linked assay is used with phenazine ethosulfate (PES) as primary electron acceptor; reduction of this is monitored by coupling to oxygen (measured in an oxygen electrode) or by coupling to a second dye, 2,6-dichlorophenolindophenol (DCPIP) (monitored spectrophotometrically). The oxygen electrode assay is best when longer periods of reaction are required as in studies of substrate specificity. The spectrophotometric assay is best when many assays are necessary as in enzyme purification; it can also be modified for very rapid analysis of column fractions.

An important and often confusing characteristic of most MDHs is their activity in the absence of added substrate. This activity requires the presence of activator (ammonia), it is not markedly diminished by dialysis or gel filtration, and its initial rate is the same as that with added substrate. It is not subtracted from rates measured in the presence of added substrate.

[1] C. Anthony, "The Biochemistry of Methylotrophs." Academic Press, New York, 1982.
[2] C. Anthony, *Adv. Microb. Physiol.* **27,** 113 (1986).
[3] C. Anthony, *in* "Bacterial Energy Transduction" (C. Anthony, ed.), p. 293. Academic Press, London, 1988.

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Assay with Oxygen Electrode

Reagents

Tris-HCl buffer, 0.6 *M*, pH 9.0
Ammonium chloride, 0.45 *M*
Phenazine ethosulfate, 10 m*M*
Methanol, 0.2 *M*

Procedure. The reaction mixture contains, in an oxygen electrode vessel at 30°, 0.5 ml Tris buffer, 0.1 ml ammonium chloride, 0.1 ml phenazine ethosulfate, 0.1 ml methanol, and 60 units of MDH. The final volume is made to 2 ml with water. The reaction is started by addition of phenazine ethosulfate, and oxygen consumption is measured assuming that 450 nmol oxygen is dissolved in 2 ml of the buffer at 30°.

Units. One unit is defined as that amount which facilitates consumption of 1 μmol O_2 per minute. It should be noted that most preparations of MDH show endogenous dye reduction. This may be diminished, but not eradicated, by dialysis or gel filtration. The initial rate of this reaction is usually the same as that measured in the presence of added methanol, and activator (ammonia) is required. This endogenous dye reduction should be ignored (not subtracted) when calculating rates of enzyme reaction.

Spectrophotometric Assay

Reagents

Tris-HCl buffer, 0.6 *M*, pH 9.0
Ammonium chloride, 0.45 *M*
Phenazine ethosulfate, 33 m*M*
2,6-Dichlorophenolindophenol, 2.6 m*M*
Methanol, 0.3 *M*

Procedure. The reaction mixture contains, in a spectrophotometer cuvette at 30°, 0.5 ml Tris buffer, 0.1 ml ammonium chloride, 0.1 ml DCPIP, 0.1 ml methanol, and MDH. The final volume is made to 3 ml with water. The reaction is started by addition of 0.1 ml phenazine ethosulfate, and the decrease in absorption at 600 nm is measured. The reference cuvette contains water.

Units. One unit is defined as that amount which catalyzes the reduction of 1 μmol O_2 per minute between 15 and 30 sec after starting the reaction. It should be noted that most preparations of MDH show endogenous dye reduction. This may be diminished, but not eradicated, by dialysis or gel filtration. The initial rate of this reaction is usually the same as that measured in the presence of added methanol, and activator (ammonia) is

required. This endogenous dye reduction should be ignored (not subtracted) when calculating rates of enzyme reaction.

Microtiter Assay

The microtiter assay is convenient for rapid location of MDH in fractions during purification. The reagents are as listed above. They are mixed as follows: 0.5 ml Tris buffer, 0.1 ml methanol, 0.1 ml ammonium chloride, 0.1 ml phenazine ethosulfate, 0.2 ml DCPIP, and 2 ml water. The mixture is stored in a lightproof container. Small volumes (5–50 μl) are placed in wells of a microtiter plate, and the reaction is started by addition of 200 μl of the assay mixture. When MDH is present the color in the wells changes from blue-green to yellow, the time taken (1–15 min) giving an indication of the activity of the MDH present.

Methanol:Cytochrome c *Oxidoreductase Activity*

Assay of methanol:cytochrome *c* oxidoreductase activity is the natural assay for MDH, although the rates determined are usually low.[3-5] Horse heart cytochrome *c* is able to react with the specific electron acceptor for MDH, cytochrome c_L, but is unable to react directly with MDH. The MDH may thus be assayed by coupling electron transfer from methanol, through MDH, to cytochrome c_L and thence to an excess of horse heart cytochrome *c*. Similar rates are obtained in such an assay if a large amount of cytochrome c_L is the sole cytochrome present, but more cytochrome c_L is then required so that it can be detected by the spectrophotometer. The pH optimum is usually pH 7.0, but activity should be tested at other pH values. Ammonia is not usually necessary, but it may activate some preparations and so is included in the assay mixture. This assay is not suitable for use with crude extracts which may contain compounds or enzymes able to oxidize or reduce cytochrome *c*. It should be noted that increases in ionic strength markedly decrease the rate of reaction.

Stopped-flow kinetic measurements of the separate components of the MDH/cytochrome c_L reaction have been described elsewhere.[5]

Reagents

MOPS buffer, 120 m*M*, pH 7.0
Methanol, 50 m*M*
Ammonium chloride, 50 m*M*
Cytochrome *c* (equine or bovine), 0.25 m*M*
Cytochrome c_L, 50 μM

[4] M. Beardmore-Gray, D. T. O'Keeffe, and C. Anthony, *J. Gen. Microbiol.* **132,** 1553 (1983).
[5] M. Dijkstra, J. Frank, and J. A. Duine, *Biochem. J.* **257,** 87 (1989).

The cytochrome is oxidized before the reaction by addition of a grain of potassium ferricyanide, the excess reagent being removed by gel filtration on a Pharmacia (Sweden) PD10 column.

Procedure. The reaction mixture contains, in a 1-ml reaction mixture in a spectrophotometer cuvette (1-cm light path), 0.1 ml MOPS buffer, 0.1 ml ammonium chloride, 0.1 ml methanol, and 0.2 ml of cytochrome *c* and cytochrome c_L. The reaction is started by addition of MDH solution in 20 m*M* MOPS buffer, pH 7.0, and the rate of reduction of cytochrome *c* is recorded at 550 nm. The rate of reaction is constant for at least 70% of the reaction. Any reaction occurring in the absence of methanol is not subtracted from the rate measured in its presence.

Units. One unit is defined as that amount of MDH catalyzing the reduction of 1 nmol of cytochrome *c* per minute. This is calculated from the extinction coefficient for the reduced minus oxidized difference spectrum for cytochrome *c* (19 mM^{-1} cm^{-1}). Thus, one unit will increase the measured absorption by 0.019/min. An increase in absorption of 1.0/min is due to 52.6 units of enzyme.

Purification Procedure

Growth of Organism. Methylobacterium extorquens AM1 (NCIB 9133) is maintained on nutrient agar plus 0.4% (w/v) methylamine hydrochloride or in glycerol as described elsewhere in this volume.[6] Bacteria are grown aerobically at 30° on 0.5% (v/v) methanol in batch culture in mineral salts medium containing the following (g/liter): $NaH_2PO_4 \cdot 2H_2O$, 0.57; K_2HPO_4, 1.53; NH_4Cl, 3.0; $MgSO_4 \cdot 7H_2O$, 0.2; and 5 ml/liter of trace elements solution containing (mg/liter) $CaCl_2 \cdot 2H_2O$, 530; $FeSO_4 \cdot 7H_2O$, 200; $MnSO_4 \cdot 4H_2O$, 20; $ZnSO_4 \cdot 7H_2O$, 20; $CuSO_4 \cdot 5H_2O$, 4; $CoCl_2 \cdot 6H_2O$, 4; Na_2MoO_4, 4; H_3BO_3, 3; and 1 *M* HCl, 1 ml.

Bacteria are harvested by centrifugation, washed in 20 m*M* Tris-HCl buffer, pH 8.0, and resuspended in the same buffer to a density of about 3 g wet weight/10 ml. They are broken by sonication in batches of 40 ml in 20 cycles of 30 sec followed by 30 sec of cooling. Whole cells and membranes are removed by centrifugation at 50,000 *g* for 1 hr at 4°. For breakage of cells the French press is equally suitable, and, for some bacteria, periplasmic material is readily obtained and makes a better starting point.[6] The MDH of some bacteria is more labile than that from *M. extorquens;* such enzymes may be stabilized during harvesting and purification by inclusion of 25 m*M* methanol or KCN.[4,6] In some bacteria a significant proportion of MDH may be associated with membrane fractions; there is no evidence

[6] A. R. Long and C. Anthony, this volume [34].

that this is more than an artifact of preparation, and this enzyme is usually readily released by washing with 0.5 *M* KCl; the salt must then be removed by dialysis prior to ion-exchange chromatography. Alternatively, detergent may be used to release the enzyme prior to purification.[7]

Purification. The high isoelectric point of MDH allows removal of unwanted protein by passage through ion-exchange media; cytochrome c_L binds to the anion-exchange material, and cytochrome c_H passes through together with the MDH. This purification procedure can therefore also be used for the first part of the purification of the soluble cytochromes of this organism.[8] The soluble fraction from about 15 g wet weight of bacteria is passed down a column (24 × 130 mm) of DEAE-Sepharose (Fast Flow, Pharmacia) equilibrated in 20 m*M* Tris-HCl, pH 8.0. MDH and cytochrome c_H are not adsorbed; they are dialyzed against 20 liters of 10 m*M* potassium phosphate buffer (pH 7.0) and applied to a hydroxyapatite column (16 × 100 mm) equilibrated in the same buffer. MDH is eluted (at about 90 m*M* phosphate) with a linear gradient of 10–250 m*M* potassium phosphate (pH 7.0) (in 200 ml). Active fractions are pooled, concentrated on a 50-kDa cutoff membrane in an Amicon (Danvers, MA) concentrating cell, desalted by gel filtration on a Pharmacia PH10 column equilibrated in 25 m*M* MES buffer (pH 5.5), and purified by cation-exchange chromatography on a Pharmacia S-Sepharose (Fast Flow) column (24 × 85 mm) equilibrated in the same buffer. MDH is eluted (at about 100 m*M* NaCl) using a gradient of 0–250 m*M* NaCl (200 ml total volume). This produces pure methanol dehydrogenase. For smaller amounts, the final step can be replaced by cation-exchange chromatography on a 1-ml Pharmacia Mono S column equilibrated in 20 m*M* MES buffer, pH 5.5, MDH being eluted with the same buffer containing 100 m*M* NaCl. Active fractions are stored at −20°. See Table I for a summary of purification.

The procedure described above is likely to be suitable for most other methylotrophic bacteria. Many equally suitable alternative procedures may be followed, the method of choice depending on the purpose of the work and the extent to which other proteins from the same extract are required (e.g., *c*-type cytochromes[8] or modifier protein[6]). Some procedures depend on the stability of MDH at pH 4.0; this provides a method for rapid removal of nucleic acids and unwanted protein[9,10] (not suitable if modifier protein is also required). MDH is very soluble and requires about 65% saturated ammonium sulfate before any precipitation occurs; this is proba-

[7] S. Ford, M. D. Page, and C. Anthony, *J. Gen. Microbiol.* **131,** 2173 (1985).
[8] D. Day and C. Anthony, this volume [44].
[9] D. T. O'Keeffe and C. Anthony, *Biochem. J.* **192,** 411 (1980).
[10] C. Anthony, this series, Vol. 18B, p. 818.

TABLE I
PURIFICATION OF METHANOL DEHYDROGENASE[a]

Purification stage	Volume (ml)	Total units	Specific activity	Purification factor	Yield (%)
Cell-free extract	75	25.0	34	1	100
DEAE-Sepharose	110	24.2	110	3.2	96
Hydroxyapatite	21.25	10.6	303	8.8	42
S-Sepharose	6.25	8.7	364	10.6	34

[a] Units are as defined for the spectrophotometric assay, and specific activity is in milliunits per milligrams of protein as assayed by the bicinchoninic acid assay [P. K. Smith, R.I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk, *Anal. Biochem.* **150,** 76 (1985)].

bly best avoided if work on the activation of MDH by ammonia is envisaged. Gel filtration is suitable for removal of smaller proteins (e.g., the *c*-type cytochromes).

Preparation of Separate Subunits.[11] The subunits are difficult to dissociate; conditions leading to dissociation also dissociate the prosthetic group (PQQ) from the enzyme, and the process is irreversible. The following procedure separates the subunits and the PQQ from each other. Pure MDH is concentrated to about 200 μl containing about 4 mg protein. This is incubated at 80° for 20 min in 2% sodium dodecyl sulfate (SDS) in 50 m*M* Tris-HCl (pH 8.0) followed by gel filtration on a Pharmacia Superose 12 column equilibrated in 50 m*M* Tris-HCl (pH 8.0) containing 0.1 *M* NaCl and 0.2% SDS. An alternative procedure is to replace SDS with 6 *M* guanidium chloride (omitting the NaCl).

Purification of MDH from Other Methylotrophs. Methods are available for many different methylotrophic bacteria,[1] the following being especially important examples: *Methylophilus methylotrophus,*[4,12] *Hyphomicrobium* X,[5,13] *Paracoccus denitrificans,*[4,6] *Acetobacter methanolicus,*[14] and *Methylophilus glucoseoxidans.*[15]

Properties

Purity and Absorption Spectrum. The enzyme prepared as described here is pure. The faint band seen on SDS–polyacrylamide gel electrophoresis in addition to the main band is the β subunit. The absorption due to

[11] D. N. Nunn, D. Day, and C. Anthony, *Biochem. J.* **260,** 857 (1989).
[12] M. D. Page and C. Anthony, *J. Gen. Microbiol.* **132,** 1553 (1986).
[13] M. Dijkstra, J. Frank, J. E. van Wielink, and J. A. Duine, *Biochem. J.* **251,** 467 (1988).
[14] E. J. Elliott and C. Anthony, *J. Gen. Microbiol.* **134,** 369 (1988).
[15] A. P. Sokolov, N. I. Govorukhina, and Yu. A. Trotsenko, *Biokhimiya* **54,** 811 (1989).

the protein has a peak at 280 nm and a shoulder at 290 nm; that due to the prosthetic group has a peak at about 345 nm and a shoulder at about 400 nm. The green-fluorescent prosthetic group is released from the enzyme by boiling or by lowering the pH to a little below 4.

Substrate Specificity. Methanol dehydrogenase oxidizes a wide range of primary alcohols and also formaldehyde which exists as a *gem*-diol in solution.

Molecular Weight and Subunit Composition. The enzyme is a tetramer, consisting of two α subunits of about 60 kDa and two β subunits of 8.46 kDa. The primary sequence of the smaller subunit has been published as well as the N-terminal region of the α subunit.[11] Antibody raised to the whole enzyme reacts only very weakly with the small subunit.

pH Optimum, Activators, and Electron Acceptors. The pH optimum is about 9.0 when measured with artificial electron acceptors which include phenazine methosulfate, phenazine ethosulfate, or Wurster's blue. Ammonium chloride (or methylammonium chloride) is usually essential for activity with these acceptors. The physiological electron acceptor is cytochrome c_L, but the rate is usually much lower than when measured with artificial electron acceptors. With this cytochrome the pH optimum is usually 7.0 and activator is not usually required.

[34] Modifier Protein for Methanol Dehydrogenase of Methylotrophs

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Modifier protein (M protein) modifies the substrate specificity of methanol dehydrogenase (MDH). This dehydrogenase has a high affinity for a wide range of primary alcohols, which are oxidized at rates very similar to those measured with methanol.[1] It is also able to catalyze the rapid oxidation of formaldehyde to formate; the function of the M protein is probably to prevent this oxidation by decreasing the affinity of MDH for formaldehyde.[2] Some substrates, including 1,2-propanediol, 1,3-propanediol, and 1,3-butanediol, have much lower affinities for MDH, and the M protein was first discovered because of its ability to facilitate the oxidation by pure MDH of 1,2-propanediol by increasing its affinity for the dehydrogenase.[3,4]

[1] C. Anthony, *Adv. Microb. Physiol.* **27,** 113 (1986).
[2] M. D. Page and C. Anthony, *J. Gen. Microbiol.* **132,** 1553 (1986).
[3] J. A. Bolbot and C. Anthony, *J. Gen. Microbiol.* **120,** 245 (1980).
[4] S. Ford, M. D. Page, and C. Anthony, *J. Gen. Microbiol.* **131,** 2173 (1985).

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