

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).

The assay systems for M protein thus depend on its ability to facilitate oxidation of a "poor" substrate or to prevent oxidation of formaldehyde.

Assay Methods

Principle. The main assay determines the effect of M protein on the oxidation of 1,3-butanediol by MDH in the dye-linked MDH assay system using phenazine ethosulfate in an oxygen electrode.

Reagents

Tris-HCl buffer, 0.5 M, pH 9.0

Ammonium chloride, 0.5 M

Phenazine ethosulfate, 10 mM

1,3-Butanediol, 0.2 M

Methanol dehydrogenase, 60 units

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 of 1,3-butanediol, 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°. To confirm that M protein is present in fractions during purification it is necessary to confirm that the oxidation of formaldehyde is diminished by addition of putative M protein. This is done by repeating the assay described here with 0.1 ml of 0.2 M formaldehyde. An amount of M protein that gives a good rate with butanediol will give about 50% inhibition of the rate of formaldehyde oxidation. It is essential to use this method of confirmation because some bacteria (e.g., *Paracoccus denitrificans*) contain other compounds that have been found to stimulate oxidation of 1,3-butanediol in crude extracts.

Units. One unit is defined as that amount which facilitates consumption of 1 nmol O₂ per minute. The unit for methanol dehydrogenase is the same when it is measured in this assay system with 0.1 ml of 0.2 M methanol instead of 1,3-butanediol. 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. It should also be noted that there is usually a rapid, transient oxygen consumption recorded with 1,3-butanediol and other low-affinity substrates. This is ignored when recording stimulations in

activity on addition of M protein; the subsequent continuous (linear) oxygen consumption is used for all calculations.

Purification Procedures

Two procedures are given; one is for *Methylophilus methylotrophus*, an obligate methylotroph in which the properties of M protein were first described, and the other is for *Paracoccus denitrificans*, a facultative methylotroph (and autotroph) which has the merit of ease of formation of periplasmic fractions.

Growth of Methylophilus methylotrophus (NCIB 10515). The growth conditions do not markedly affect production of M protein. Stock cultures are maintained in 30% glycerol at -15° . Growth is on methanol either in batch culture or in oxygen-limited or carbon-limited continuous culture as previously described.^{2,5,6} After harvesting by centrifugation, cells may be stored at -20° after rapid freezing in liquid nitrogen.

Growth of Paracoccus denitrificans (NCIB 8944) and Preparation of Spheroplasts and Periplasmic Fractions. Growth conditions do not markedly affect production of M protein. Stock cultures are maintained on nutrient agar plus succinate (0.2%), and starter cultures are grown from these in nutrient broth (8 g/liter) plus succinate (0.2%). For production of M protein, growth is at 30° on methanol in aerated vessels. The culture medium contains the following (per liter): K_2HPO_4 , 6 g; KH_2PO_4 , 4 g; NH_4Cl , 1.6 g; $NaHCO_3$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 40 mg; ferrous EDTA, 0.12 g; oxoid yeast extract, 0.1 g; Hoagland's trace elements, 0.1 ml; and methanol, 0.5% (v/v). After growth to the end of the log phase, bacteria are harvested and washed in 25% of the growth volume of ice-cold 10 mM HEPES buffer (pH 7.3) containing 150 mM NaCl. The bacteria should be used immediately for preparation of periplasmic fractions.

The following method for preparation of spheroplasts is suitable for cells harvested from 20 liters of growth medium. The cells are suspended at room temperature in 800 ml of spheroplast buffer containing 200 mM HEPES (pH 7.3), 500 mM sucrose, 0.5 mM disodium EDTA, and 25 mM methanol (to stabilize the MDH). Lysozyme (1 g), dissolved in 15 ml cold water, is added, followed by 800 ml of cold methanol solution (25 mM). The suspension is divided into 4 volumes of 400 ml, incubated for 1 hr at 30° with gentle swirling, and chilled on ice-water for 5 min, and the spheroplasts are harvested by centrifugation at 5000 g for 20 min at 4° .

⁵ A. R. Cross and C. Anthony, *Biochem. J.* **192**, 421 (1980).

⁶ A. R. Cross and C. Anthony, *Biochem. J.* **192**, 429 (1980).

The periplasmic fraction (supernatant) is decanted and protease inhibitors added (benzamidine hydrochloride, 5 mM; phenylmethylsulfonyl fluoride, 0.5 mM). This process usually yields about 1600 ml of periplasmic fraction containing 0.4 mg protein/ml.

Purification of M Protein from Methylophilus methylotrophus. The bacteria are suspended (0.25 g wet weight/ml) in 20 mM HEPES-HCl buffer (pH 7.5) containing 25 mM methanol and are disrupted in an ultrasonic disintegrator in batches of 50 ml in 10 cycles of 30 sec followed by 30 sec of cooling. Whole bacteria and debris are removed by centrifugation at 40,000 g for 20 min, and membranes are removed from the supernatant by centrifugation at 150,000 g for 1 hr at 4°. The supernatant extract is applied to a column of DEAE-cellulose (8 × 6 cm) equilibrated with 12.5 mM HEPES-HCl (pH 7.5) at 4°. The column is then washed with 100 ml of the same buffer; the MDH, which does not adsorb to the column, is collected during this washing step.

The column is washed with 500 ml of the same buffer containing 50 mM KCl. The M protein is eluted with 200 ml of the same buffer containing 100 mM KCl. After concentration under pressure over an Amicon XM50 membrane, further purification is achieved by gel filtration on Sephadex G-150 (fine grade, 75 × 3.5 cm; upward flow, 16 ml/hr in 12.5 mM HEPES-HCl buffer, pH 7.5). Pooled active fractions may then be passed through the same column. The M protein is purified by anion-exchange chromatography on a Pharmacia Mono Q column or its equivalent equilibrated with HEPES buffer as before with a gradient of 0–0.5 M KCl (total volume, 26 ml). Further purification is achieved by desalting followed by a second run through Mono Q but using a shallower gradient (7 mM/ml). This separates the M protein from the bulk of the protein with 60 kDa subunits that was previously assumed to be the M protein itself. The purification process is summarized in Table I.

Methanol dehydrogenase from *M. methylotrophus* is prepared as described previously.²

Purification of M Protein from Paracoccus denitrificans. Bovine DNase I (10 mg) is added to the periplasmic fraction (5 liters, containing 2 g protein, derived from 60 liters of bacteria) which is then applied to a column of DEAE-Sepharose (12 × 5 cm) equilibrated in 20 mM HEPES buffer (pH 7.5). Proteins are eluted with the same buffer containing NaCl. The first fraction, eluting with 100 mM NaCl, just before cytochrome *c*-550, contains a nonprotein component that shows some activity in increasing the oxidation of 1,3-butanediol. This component also stimulates formaldehyde oxidation and so explains why formaldehyde is oxidized by crude extracts of *P. denitrificans* even in the presence of the inhibitory M protein. The nonprotein activator may be purified by passage through

TABLE I
PURIFICATION OF M PROTEIN FROM *Methylophilus methylotrophus*^a

Purification stage	Volume (ml)	Total units	Specific activity	Purification factor	Yield (%)
Cell-free extract	200	107	4.4	1	100
DEAE-cellulose	220	49	16.4	3.8	50
Sephadex G-150	204	20	40.5	9.3	19
Mono Q (1)	202	18	77.3	17.7	17
Mono Q (2)	200	14	140.0	31.8	14

^a In this example M protein was purified from 50 g bacteria grown in oxygen-limited continuous culture.² Units are those described in the text; specific activities are units per milligram protein.

an Amicon PM10 membrane (MW cutoff 10,000) which retains all protein. The activating component may then be purified on a Pharmacia Mono Q column equilibrated in 20 mM HEPES buffer (pH 7.5), elution occurring with 150 mM NaCl.

After complete elution of the cytochrome *c*-550 the M protein is eluted with buffer containing 200 mM NaCl, concentrated to 10 ml on an Amicon YM100 membrane, and purified further by gel filtration on a column of Sephacryl 200 (85 × 3.5 cm) equilibrated in 20 mM HEPES buffer (pH 7.5) containing 100 mM NaCl (upward flow, 10 ml/hr). Active fractions are purified by anion-exchange chromatography on a Pharmacia Mono Q column (1 ml) equilibrated in the same buffer and flowing at 1 ml/min. A gradient of 0–500 mM NaCl (total volume 26 ml) is used; M protein is eluted with 250 mM NaCl, giving a protein of about 85% purity. The purification process is summarized in Table II.

Purification of Methanol Dehydrogenase from Paracoccus denitrificans. The MDH from *P. denitrificans* has a low isoelectric point, in contrast to most MDHs which are basic proteins.¹ MDH is purified from the same periplasmic extract that is used for the purification of its M protein. It is eluted from the DEAE-Sepharose column after the M protein by increasing the NaCl concentration to 300 mM. Pooled active fractions are concentrated to 10 ml over an Amicon XM50 membrane and applied to a gel filtration column of Sephacryl S-200 (85 × 3.5 cm) equilibrated in 20 mM HEPES buffer (pH 7.5) containing 200 mM NaCl and 25 mM methanol at 4°. The purification is completed by anion-exchange chromatography on a column of Q Sepharose (10 × 2.5 cm) equilibrated in 20 mM HEPES buffer (pH 7.5) containing 25 mM methanol. After applying the protein, the column is washed in the same buffer containing 300 mM NaCl, then MDH is eluted in the same buffer containing 400

TABLE II
PURIFICATION OF M PROTEIN FROM *Paracoccus denitrificans*^a

Purification stage	Volume (ml)	Total units	Specific activity	Purification factor	Yield (%)
DEAE-Sepharose	120	6000	37.5	1	100
Sephacryl-200	63	2016	75	2	34
Mono Q	32	576	88	2.35	10

^a Because of the presence of a nonprotein component able to stimulate oxidation of 1,3-butanediol in the periplasmic fraction, it is not possible to express purification factors and yields in terms of the initial activity. It should be noted that the values cannot be compared directly (except for specific activities) with the values in Table I for *M. methylotrophus* because in the latter organism the starting material is a complete soluble fraction rather than the periplasmic fraction. Units are those described in the text; specific activities are units per milligram protein.

mM NaCl. For small amounts of MDH this anion-exchange step can be done on a Pharmacia Mono Q column. Salt is removed by concentration on an Amicon XM50 membrane followed by gel filtration on a PD10 column equilibrated with 20 mM HEPES buffer (pH 7.5) containing 25 mM methanol. The preparation is homogeneous on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). It is stored at -20° .

Properties

Stability of M Protein. The activity of M protein is very sensitive to extremes of pH and to the nature of the buffers used. All activity is lost when extracts are treated with protamine sulfate, ammonium sulfate, or low-pH buffers. MOPS buffer and phosphate buffer should not be used. HEPES buffer is the best for all purification steps. The M protein is stable when frozen at -20° .

Molecular Weight and Subunit Structure. The molecular weight of M proteins from *M. methylotrophus*,^{2,4} *Methylobacterium extorquens* AM1,^{2,4} and *P. denitrificans* are all similar in having a native molecular weight of about 130,000 as estimated by gel filtration. The subunit molecular weight was previously reported to be about 70,000,^{2,4} but subsequent work has shown that the predominant band seen on SDS–PAGE is not due to M protein, but is a contaminant of it. The subunit molecular weight, based on SDS–PAGE, is about 45,000, indicating that the protein is a trimer or, more likely, a tetramer.

Isoelectric Point. The isoelectric point is acidic in all cases, the *pI* for the protein from *M. methylotrophus* being 5.6 as measured by isoelectric focusing.²

Effect on Formaldehyde Oxidation by Methanol Dehydrogenase. M proteins inhibit formaldehyde oxidation; in the case of *M. methylotrophus* and *M. extorquens* this is by decreasing the $V_{\max}$ and the affinity of MDH for this substrate;² the mechanism has not been investigated for *P. denitrificans*.

Effect on Alcohol Oxidation by Methanol Dehydrogenase. Alcohols that are poor substrates for MDH, because of their low affinity for the enzyme, are oxidized in the absence of M protein, but only in a transient manner because they fail to protect MDH from the inhibitory effects of the artificial electron acceptor phenazine ethosulfate.^{2,4} In the presence of M protein they are oxidized because their affinity for MDH increases and the enzyme is thus protected. It should be noted that, because of this mode of action, in the presence of small amounts of M protein there is an initial rapid transient consumption followed by a slower linear progress curve.

Effect on Methanol Dehydrogenase in the Cytochrome-Linked System. The natural electron acceptor for MDH is a specific cytochrome *c* called cytochrome c_L .^{1,7,8} The effect of M protein in this system mirrors that in the dye-linked system.² With pure proteins from *M. extorquens* and *M. methylotrophus* the rate of oxidation of all alcohols (methanol, ethanol, and 1,3-butanediol) is increased but the rate of oxidation of formaldehyde is halved. The assay system is described elsewhere in this volume.^{9,10}

Periplasmic Location. Because of the difficulty of preparing stable spheroplasts of other methylotrophs, the location of the M protein has been determined only for *P. denitrificans*. No activity can be demonstrated in membrane or cytoplasmic fractions after disruption of spheroplasts; M protein is located exclusively, with MDH and soluble *c*-type cytochromes, in the periplasmic fraction of this organism.

⁷ D. N. Nunn and C. Anthony, *Biochem. J.* **256**, 673 (1989).

⁸ D. N. Nunn and M. E. Lidstrom, *J. Bacteriol.* **166**, 591 (1986).

⁹ D. Day and C. Anthony, this volume [33].

¹⁰ D. Day and C. Anthony, this volume [44].