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Regulation of Formaldehyde Oxidation by the Methanol Dehydrogenase Modifier Proteins of *Methylophilus methylotrophus* and *Pseudomonas* AM1

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The modifier protein (M-protein) for methanol dehydrogenase (MDH) of the obligate methylotroph *Methylophilus methylotrophus* was purified almost to homogeneity (98% pure), characterized and shown to be similar to that previously described in the facultative methylotroph *Pseudomonas* AM1. It was a dimer of M_r 140000, its isoelectric point was 5·6 and it showed no spectral absorbance above 320 nm. In the dye-linked MDH assay system, the M-proteins facilitated oxidation of alcohols not normally oxidized (1,2-propanediol, 1,3-propanediol, 1,2-butanediol, 1,3-butanediol and 3-methylbutanol) by increasing the affinity of MDH for these alcohols. The effect of M-protein on the oxidation of formaldehyde was to decrease the affinity of MDH for formaldehyde by more than 97%, thus preventing any significant oxidation of formaldehyde. The M-protein also exerted its effect on the activity of MDH in the cytochrome-linked system of *M. methylotrophus*. The usual function of M-protein in methylotrophs is apparently to prevent formaldehyde oxidation. A dye-linked aldehyde dehydrogenase of wide specificity was partially purified and characterized; it failed to oxidize formaldehyde at a significant rate but it oxidized lactaldehyde at a rate sufficient to account for the oxidation of propanediol to lactate in whole bacteria.

INTRODUCTION

Methanol dehydrogenase (MDH) is a quinoprotein that interacts with the electron transport chain at the level of cytochrome *c* (Anthony, 1982, 1986). It oxidizes a wide range of primary alcohols but not those having a second substituent on the C-2 carbon atom. The observation that MDH and cytochrome *c* were essential for growth of the facultative methylotroph *Pseudomonas* AM1 on 1,2-propanediol was therefore unexpected (Bolbot & Anthony, 1980). Subsequently, it was shown that the oxidation of propanediol to lactaldehyde depends upon the presence of both MDH and a modifier protein (M-protein) which acts by increasing the affinity of MDH for propanediol (Ford *et al.*, 1985).

The obligate methylotroph *Methylophilus methylotrophus* oxidizes propanediol but is unable to grow on this substrate, suggesting that this organism might also contain an M-protein but that it must have some function other than facilitating growth on propanediol. One such possible function is that it might be involved in regulating methanol or formaldehyde oxidation. One of the remarkable features of most methanol dehydrogenases is their ability to oxidize formaldehyde to formate (Sperl *et al.*, 1974; Duine *et al.*, 1978; Beardmore-Gray *et al.*, 1983). The oxidation of formaldehyde to formate by MDH is relatively unfavourable in energetic terms, yielding one ATP per molecule oxidized (or less) (Netrusov & Anthony, 1979; Anthony, 1982, 1986; Patchett *et al.*, 1985). Because carbon is assimilated in most methylotrophs by cytoplasmic enzymes at the oxidation level of formaldehyde, its oxidation to formate in the periplasm by MDH would represent a loss of carbon substrate. As many alternative enzymes (or enzyme systems) catalysing formaldehyde oxidation have been described in methylotrophs a role for methanol dehydrogenase in formaldehyde oxidation is unlikely (Anthony, 1982;

Abbreviations: M-protein, modifier protein; MDH, methanol dehydrogenase; PES, phenazine ethosulphate.

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Attwood & Quayle, 1984). Furthermore, mutants lacking MDH are able to oxidize formaldehyde and grow at wild-type rates on methylamine during which formaldehyde oxidation is necessary (Dunstan *et al.*, 1972; Heptinstall & Quayle, 1970; Anthony, 1975; Marison & Attwood, 1982).

The present paper describes the effect of the M-protein of the facultative methylotroph *Pseudomonas* AM1 on the oxidation by MDH of a range of substrates. It also describes the purification and characterization of a second M-protein from the obligate methylotroph *M. methylotrophus*, and it demonstrates that these two M-proteins are both likely to function in the regulation of formaldehyde oxidation.

METHODS

Unless otherwise stated, methods were as described by Ford *et al.* (1985).

Growth, harvesting and breakage of bacteria. Pseudomonas AM1 (NCIB 9133) and *Methylophilus methylotrophus* (NCIB 10515) were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, UK. *Pseudomonas* AM1 was grown on 1,2-propanediol. *M. methylotrophus* was grown in O_2-limited continuous culture ($D = 0{\cdot}15\ h^{-1}$) as described by Cross & Anthony (1980*a*, *b*) and suspended (0·25 g wet weight ml^{-1}) in 20 mM-sodium phosphate buffer (pH 7·0), 20 mM-Tris/HCl (pH 8·0) or 20 mM-HEPES/HCl buffer (pH 7·5). They were disrupted in an ultrasonic disintegrator (MSE Soniprep 150) at full power while cooled in ice-water. Batches of 50 ml were disrupted for 10 cycles of 30 s followed by 30 s cooling.

Purification of methanol dehydrogenase and cytochrome c_L from M. methylotrophus. Cyanide-stabilized MDH was purified by a method based on that described by Beardmore-Gray *et al.* (1983). The buffer used throughout was 20 mM-Tris/HCl (pH 8·0) containing 25 mM-KCN. Crude soluble extract (5 g protein in 130 ml) was applied to a column of DEAE-cellulose (5 × 7 cm). The MDH was not absorbed and active fractions were pooled, concentrated under pressure over an Amicon XM-50 membrane, and applied to an upward flow column of Sephadex G-150 (fine grade; 2·5 × 75 cm; 10 ml h^{-1}). Fractions containing MDH were pooled, concentrated, and passed through a second column of DEAE-cellulose (5 × 7 cm). The MDH was not absorbed and was shown to be pure enzyme after passage through this column. It was stored at −17 °C and was freed of KCN immediately before use by passage through a Pharmacia PD-10 Sephadex column.

Cytochrome c_L was purified from bacteria grown in continuous culture under methanol-limitation as described by Cross & Anthony (1980*a*). Only one cytochrome component was observed after the second pass through Sephadex; it gave a single band on SDS-PAGE, corresponding to a M_r (relative molecular mass) of 17000.

Purification of methanol dehydrogenase and modifier protein (M-protein) from Pseudomonas AM1. These proteins were purified as described by O'Keeffe & Anthony (1980) and Ford *et al.* (1985). Both were stored at −17 °C.

Purification of the M-protein of M. methylotrophus. As was found with the M-protein of *Pseudomonas* AM1 (Ford *et al.*, 1985), all M-protein activity was lost when soluble extracts were treated with protamine sulphate, subjected to ammonium sulphate fractionation, or to acid treatment. Soluble extracts were therefore applied directly to a column of DEAE-cellulose (8 × 6 cm) equilibrated with 12·5 mM-HEPES/HCl buffer (pH 7·5) at 4 °C. The column was washed with 100 ml of the same buffer and then with 500 ml of the same buffer containing 0·05 M-KCl. The M-protein was eluted with 200 ml of the same buffer containing 0·1 M-KCl. Active fractions were pooled, concentrated under pressure over an Amicon XM-50 membrane, and purified further by gel filtration on Sephadex G-150 (fine grade, 75 × 3·5 cm; upward flow, 16 ml h^{-1} 12·5 mM-HEPES/HCl buffer, pH 7·5). Pooled active fractions were concentrated and passed again through the same column. The column eluate was further purified by FPLC on a Pharmacia Mono-Q anion exchange column (1 ml) equilibrated with 12·5 mM-HEPES/HCl buffer (pH 7·5). Samples (3 ml; 5 mg protein) were applied to the column which was washed with 6 ml of the equilibration buffer; protein was eluted with a linear concentration gradient of KCl (0–0·5 M in the same buffer) using a total volume of 26 ml.

Two major protein peaks, barely resolved, were eluted from the Mono-Q column; both had M-protein activity and their M_r values were identical as determined by SDS-PAGE. When collected as a single fraction from the Mono-Q column only a single band was observed on SDS-PAGE. The combined fractions were subjected to further analytical chromatography on hydroxylapatite, on TSK G 3000 SW (gel filtration) and on Mono-Q equilibrated with bistrispropane/HCl buffer (10 mM, pH 7·0). All columns were connected to the Pharmacia FPLC pumps and detector system. Pooled active fractions from Mono-Q (500 µl; 0·35 mg protein) were applied to a column of hydroxylapatite (Biogel HT; 5 × 100 mm) equilibrated in HEPES/HCl buffer (12·5 mM; pH 7·5); protein was eluted with 0·2 M-sodium phosphate (pH 7·5) at a flow rate of 0·25 ml min^{-1}.

Active fractions from Mono-Q (100 µl; 70 µg protein) were applied to an LKB TSK G 3000 SW column (0·75 × 60 cm) equilibrated in HEPES/HCl buffer (12·5 mM, pH 7·5) containing 25 mM-KCl and were eluted at a flow rate of 1·0 ml min^{-1}.

The pooled active fractions (50 µl; 75 µg protein) from the second pass through Sephadex were applied to a Pharmacia Mono-Q column equilibrated with bistrispropane/HCl (10 mM, pH 7·0). The column was washed with

6 ml of the equilibration buffer and M-protein was eluted with a linear concentration gradient of KCl (0–0·5 M in the same buffer, total volume 26 ml).

All these analytical chromatography methods revealed a single symmetrical protein elution peak. It was therefore concluded that the two peaks obtained previously were both forms of M-protein, one perhaps being a deamidated form of the other.

Partial purification of the dye-linked aldehyde dehydrogenase of M. methylotrophus. Crude soluble extracts prepared by sonication in sodium phosphate buffer (20 mM, pH 7·0) were diluted fourfold with cold distilled water and applied to a column of DEAE-cellulose (3 × 3 cm) equilibrated in 5 mM-sodium phosphate buffer (pH 7·0) at 4 °C. After washing with 60 ml of the same buffer, the aldehyde dehydrogenase (assayed with propanal) was eluted with 25 mM-sodium phosphate buffer (pH 7·0).

Measurement of M-protein. M-protein was assayed by measuring its ability to promote the oxidation of 1,3-butanediol by MDH. The assay system and definition of units was as described for MDH except that pure MDH (60 U, 0·052 mg) was included in the reaction mixture and the substrate was 1,3-butanediol. The concentration of 1,3-butanediol for routine assays of M-protein was 10 mM (for the protein from *Pseudomonas* AM1) or 20 mM (for that from *M. methylotrophus*).

Measurement of methanol and aldehyde dehydrogenases. MDH was assayed by the PES-linked method described by Ford *et al.* (1985). The aldehyde dehydrogenase was assayed in an O_2 electrode using propanal as substrate. Reaction mixtures contained (in 2 ml) 50 mM-sodium phosphate buffer (pH 7·0), 0·5 mM-PES and 10 mM-propanal. The reaction was started by addition of PES. One unit of dehydrogenase activity is defined as that amount of enzyme that catalyses the consumption of 1 nmol O_2 min^{-1}.

Effect of M-protein on the oxidation of alcohols and formaldehyde by methanol dehydrogenase with cytochrome c_L as electron acceptor. This was determined by a method based on that of Beardmore-Gray *et al.* (1983). Reaction mixtures (3 ml) contained horse heart cytochrome *c* (500 μM), methanol dehydrogenase (2 μM) and substrate (methanol, 3 mM; ethanol, 3 mM; formaldehyde, 100 μM; butanediol, 3 mM) in HEPES/HCl buffer (12·5 mM, pH 7·5) at 20 °C. When present, the M-protein concentration was 0·7 μM. The reaction was initiated by addition of cytochrome c_L (2 μM). At various times, samples (350 μl) were withdrawn from the reaction mixture and assayed for the extent of reduction of cytochrome *c*, and for formaldehyde, acetaldehyde or 3-hydroxybutyraldehyde.

Measurement and preparation of formaldehyde. Formaldehyde in samples from the dye-linked assay system was measured by the method of Chrastil & Wilson (1975). Samples were mixed with an equal volume of ice-cold HCl in ethanol (1%, v/v) and precipitated protein was removed by centrifugation (2000 *g*, 2 min). The supernatant fluid was withdrawn, mixed with Amberlite IR-120 ion-exchange resin (10 mg) to remove PES and the resulting colourless solution assayed for formaldehyde.

Formaldehyde in samples from the cytochrome-linked assay system was assayed as described by Nash (1953). Samples were mixed with an equal volume of ice-cold TCA, the precipitated protein was removed by centrifugation, and the supernatant fluid assayed for formaldehyde.

Formaldehyde solutions were prepared by refluxing a suspension of paraformaldehyde in water for 30 min immediately before use.

Determination of formate. Formate was assayed by the method of Höpner & Knappe (1974). Samples were mixed with equal volumes of ice-cold TCA (1·0 M), and precipitated protein was removed by centrifugation at 5000 *g* for 2 min. The supernatant fluid was neutralized with half its volume of $KHCO_3$ (1 M) and mixed with Amberlite IR 120 (10 mg) to remove PES. The colourless solution (0·8 ml) was basified with a drop of conc. NaOH and freeze-dried. The solid material was dissolved in 0·16 ml water and assayed for formate.

Determination of acetaldehyde and 3-hydroxybutanal. Acetaldehyde and 3-hydroxybutanal were assayed by the method of Asabe *et al.* (1977). Samples were mixed with equal volumes of 25% (w/v) TCA and precipitated protein was removed by centrifugation. Supernatant fluid was diluted (40 μl to 200 μl) with water and assayed, the fluorescence intensity being measured in an Aminco-Bowman spectrophotofluorimeter.

Measurement of M_r by gel filtration. This was done using the same column of Sephadex G-150 as was used in the purification of proteins; the following standards were used: γ-globulin (M_r 158000), MDH (115000), aldolase (80000), bovine serum albumin (67000), hexokinase (51000), ovalbumin (43000) and D-amino acid oxidase (37000).

Polyacrylamide gel electrophoresis. This was done as described by Ford *et al.* (1985). For M-protein the concentration of acrylamide was 7·5% (w/v); for MDH and cytochrome c_L it was 10%.

RESULTS

Purification and properties of M-protein from M. methylotrophus

Pure MDH of *M. methylotrophus* failed to oxidize low concentrations of 1,2-propanediol, 1,3-propanediol, 1,2-butanediol, 1,3-butanediol and 3-methylbutanol, except for a brief transient oxidation. By contrast, crude extracts of *M. methylotrophus* catalysed a continuous oxidation of

Table 1. *Purification of the M-protein from M. methylotrophus*

The M-protein was purified from 50 g (wet wt) of bacteria grown in continuous culture (O_2-limitation; $D = 0{\cdot}15\ h^{-1}$). All figures are quoted for active fractions after concentration. Activity is expressed as nmol min^{-1}, and specific activity as nmol min^{-1}(mg protein)$^{-1}$, using 1,3-butanediol as substrate. Details of the purification and assay procedures are given in Methods. For the purposes of comparison, it should be noted that the values for activity and specific activity in the Table of purification of the M-protein of *Pseudomonas* AM1 (Ford *et al.*, 1985) were erroneous; they should be divided by 10.

Purification stage	Volume (ml)	Activity	Specific activity	Purification factor	Yield (%)
Soluble cell extract	200	107	4·4	1	100
DEAE-cellulose	220	48·9	16·4	3·8	50
Sephadex G-150 (1)	244	54·7	33·7	7·7	31
Sephadex G-150 (2)	204	19·8	40·5	9·3	19
Mono-Q	202	18·0	77·3	17·7	17

1,2-propanediol and 1,3-butanediol, indicating that an M-protein, similar to that in *Pseudomonas* AM1, was present. It was less stable than that in *Pseudomonas* AM1. When assayed in the following buffers (all at 20 mM) the half-life (at 0 °C) for activity in crude extracts of *M. methylotrophus* was less than 10 h: Tris/HCl (pH 8·0); tricine/HCl (pH 8·0); TES/NaOH (pH 7·5); MOPS/NaOH (pH 7·0); bistrispropane/HCl (pH 7·0) and MES/NaOH (pH 6·0). It was about 24 h in potassium phosphate/NaOH (pH 7·0) and 96 h in HEPES/HCl (pH 7·5) which was therefore used throughout the purification and characterization of this M-protein. Inclusion of the protease inhibitor phenylmethylsulphonyl fluoride (0·1 mM) did not increase the half-life of the M-protein. The M-protein was purified by anion-exchange chromatography on DEAE-cellulose, gel filtration on Sephadex G-150, and further anion-exchange chromatography on a Pharmacia Mono-Q column (Table 1). It was more than 93% pure and contained only one protein impurity, as indicated by SDS-PAGE. It was stable for at least 3 months at −17 °C in HEPES/HCl (pH 7·5). Its M_r was 130000 as estimated by gel-filtration on Sephadex G-150, the M_r of subunits being 70000 (by SDS-PAGE). The isoelectric point of the M-protein was shown to be 5·6 by isoelectric focusing. Its absorption spectrum showed a single peak at 278 nm but no significant absorption at wavelengths greater than 320 nm. In all these properties the M-protein of *M. methylotrophus* was similar to that of *Pseudomonas* AM1 (Ford *et al.*, 1985).

The results shown in Table 1 indicate that the M-protein of *M. methylotrophus* comprises about 5% of the soluble protein of the organism. This was confirmed by showing that the amount of M-protein on gels after electrophoresis of crude soluble extracts was about 50% of the amount of MDH on the same gels.

Effects of M-proteins on the oxidation of alcohols by methanol dehydrogenase

Effects of M-protein on the kinetic parameters for methanol dehydrogenase. It was previously reported that the modifier protein of *Pseudomonas* AM1 increased the affinity of MDH for 1,2-propanediol; in the absence of M-protein there was only a transient oxidation of this substrate (Bolbot & Anthony, 1980; Ford *et al.*, 1985). In the present work, with low concentrations of substrate (1,3-propanediol, 1,3-butanediol, 3-methylbutanol and 1,2-butanediol), similar transient oxidations were observed but more prolonged oxidation occurred in the presence of M-protein. This also occurred with the first three of these alcohols when their concentrations were relatively much higher than their K_m values; this was not observed with 1,2-propanediol or 1,2-butanediol because of their extremely low affinities for MDH.

Table 2 lists the K_m values, V_{max} values, and the effects of M-protein on these kinetic parameters. It is clear that the alcohols fall into two groups. The first, which includes 1,2-propanediol, showed a markedly increased affinity for MDH, but the V_{max} values for these alcohols were not altered by M-protein. The result of this is that M-protein stimulated the oxidation of low concentrations of these alcohols. The effect of M-protein on MDH showed saturation kinetics with respect to M-protein concentration. Using 1,2-propanediol as substrate

Table 2. *Effect of M-protein on the kinetic parameters of MDH*

The values below were measured in the dye-linked assay system as described in Methods. The endogenous dye reduction was not subtracted from the rates measured in the absence of substrate in the presence or absence of M-protein. The rate of endogenous dye reduction was affected to the same extent by M-protein as was methanol oxidation. The K_m (mM) and V_{max} (nmol min^{-1}) for each substrate of MDH (60 U; 0·052 mg) measured with a range of M-protein concentrations were calculated from double reciprocal plots of the primary data. The values of K_m and V_{max} at saturating concentrations of M-protein were also calculated from double reciprocal plots as shown in Ford *et al.* (1985) for 1,2-propanediol. The maximum amounts of M-protein used with each substrate were as follows: (*a*) for *Pseudomonas* AM1, 1,2-propanediol 0·39 mg, 1,3-propanediol 0·24 mg, 1,3-butanediol 0·32 mg, methanol 0·18 mg, ethanol 0·18 mg, formaldehyde 0·36 mg; (*b*) for *M. methylotrophus*, 1,2-propanediol 0·26 mg, methanol 0·17 mg, formaldehyde 0·17 mg.

	Kinetic parameters in absence of M-protein		Kinetic parameters in presence of M-protein (max. experimental values obtained)		Values calculated for saturating concentrations of M-protein	
Substrate	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
(*a*) For *Pseudomonas* AM1						
1,2-Propanediol	18	111	18	7	18	5
1,3-Butanediol	48	11	48	1·8	48	1
1,3-Propanediol	51	0·8	51	0·23	51	0·83
Methanol	60	0·01	30	0·036	20	0·047
Ethanol	60	0·01	30	0·036	20	0·047
Formaldehyde (1st phase)	60*	0·01*	24	0·041	20	0·047
Formaldehyde (2nd phase)	60*	0·01*	42	0·465	42	0·635
(*b*) For *M. methylotrophus*						
1,2-Propanediol	18	174	18	23	18	9
Methanol	60	0·02	36	0·009	16	0·006
Formaldehyde (1st phase)	60*	0·25*	35	0·8	15	1·0
Formaldehyde (2nd phase)	60*	0·25*	44	6·25	30	8·95

* In the absence of M-protein there was only one phase, the V_{max} value for this being identical for methanol, ethanol and formaldehyde.

the K_m of M-protein for MDH was 70 nM for the proteins from *M. methylotrophus*, and 140 nM for those from *Pseudomonas* AM1.

The second group of substrate alcohols includes methanol and ethanol, both of which have a very high affinity for MDH. In the presence of M-protein these alcohols were oxidized less rapidly at saturating substrate concentrations, the V_{max} values being decreased to about 30% of that measured in its absence. The M-protein from *Pseudomonas* AM1 increased the K_m for methanol fivefold whereas that from *M. methylotrophus* decreased the K_m for methanol to about 30% of that measured in its absence. This is the most marked difference between the activities of the M-protein/MDH systems from the two bacteria. Its significance is uncertain.

Effects of M-protein on the stoichiometry of alcohol oxidation. When low concentrations of alcohol were used for kinetic studies the substrate was exhausted during the reaction and so a measure of the stoichiometry of the reaction was obtained. During the oxidation of ethanol by the MDH of *Pseudomonas* AM1 (in the presence or absence of M-protein) 1·0 mol of O_2 was consumed per mol of ethanol added to the reaction mixture. By contrast, 2·0 mol of O_2 were consumed per mol of methanol, indicating that methanol was being further oxidized, by way of formaldehyde, to formate. In the presence of M-protein the stoichiometry for methanol oxidation was halved suggesting that M-protein might prevent this further oxidation.

During the oxidation of methanol by the MDH of *M. methylotrophus*, in the presence or absence of M-protein, the stoichiometry was about 1·0. This suggests that formaldehyde is not oxidized further to formate in this system, a conclusion consistent with the observation that the K_m value for formaldehyde of the MDH of *M. methylotrophus* is 250 μM, which is 25 times that of the MDH from *Pseudomonas* AM1 (Table 2).

Table 3. *Effect of M-protein on the oxidation of methanol, ethanol and formaldehyde by MDH of Pseudomonas AM1*

Methanol or formaldehyde (0·2 μmol) were incubated with MDH (60 U, 0·052 mg) in the dye-linked assay system in the presence or absence of M-protein. When the rapid oxygen consumption due to oxidation of these substrates ceased, samples were removed for measurement of formaldehyde and formate as described in Methods.

Substrate	M-protein (mg)	O_2 consumption (μmol)	Products (μmol)	
			Formaldehyde	Formate
Methanol	0	0·40	0·055	0·185
	0·07	0·22	0·190	0·035
Formaldehyde	0	0·18	0·028	0·168
	0·07	0·045	0·148	0·043
Ethanol	0	0·2	–	–
	0·07	0·2	–	–

Effect of M-protein on formaldehyde oxidation

The results in Table 3 show that in the absence of M-protein, the methanol dehydrogenase of *Pseudomonas* AM1 oxidized formaldehyde to formate, and that the major product of methanol oxidation was also formate. In the presence of M-protein, however, formaldehyde oxidation was considerably diminished and the major product of methanol oxidation was formaldehyde instead of formate.

In the presence of M-protein the oxidation of formaldehyde was biphasic (Fig. 1). The kinetics of the first phase were affected by M-protein in exactly the same way as those of methanol and ethanol oxidation. That is, the K_m value was increased nearly fivefold and the V_{max} was decreased to 30%. As the concentration of M-protein increased, the initial phase was markedly diminished. The kinetics for the second phase were completely different from those of the first phase (Fig. 2). The V_{max} value was only slightly decreased but the K_m value in the presence of M-protein was 64-fold higher; this resulted in the very low rates of formaldehyde oxidation observed in the presence of M-protein. Formate (10 mM) had no effect on the oxidation of methanol or formaldehyde in the presence or absence of M-protein.

The kinetic results presented in this section on formaldehyde oxidation were obtained with the proteins from *Pseudomonas* AM1. Almost identical values were obtained when the MDH and M-protein of *M. methylotrophus* were used.

Effect of M-protein on the reduction of cytochrome c by methanol dehydrogenase of M. methylotrophus

The natural electron acceptor for MDH is a specific, periplasmic cytochrome *c* called cytochrome c_L (Beardmore-Gray *et al.*, 1983; Beardmore-Gray & Anthony, 1984; Anthony, 1986). Reduction of this cytochrome, although slow compared with the rate of reduction of PES, is more physiological, occurring at pH 7·0 in the absence of ammonia. The substrate specificity is the same as in the dye-linked assay and so formaldehyde and methanol are both good substrates. To determine whether or not M-protein altered the oxidation of formaldehyde in both systems the effect of M-protein of *M. methylotrophus* on the rates of reduction of cytochrome *c* and on the oxidation of alcohols, formaldehyde and endogenous reductant was tested. All preparations of MDH have a considerable amount of an unknown endogenous reductant which is bound to the enzyme. Although its presence is well-documented, its nature is not known (Anthony & Zatman, 1964; Ghosh & Quayle, 1981; Duine *et al.*, 1978; Beardmore-Gray & Anthony, 1984). The presence of this reductant complicates the interpretation of experiments in which the oxidation of small amounts of substrate concomitant with cytochrome reduction is measured. In spite of this difficulty, the results below show that the effects of M-protein on the affinity of MDH for alcohols and formaldehyde demonstrated in the dye-linked assay system are also expressed when the MDH is coupled to its physiological electron acceptor cytochrome c_L.

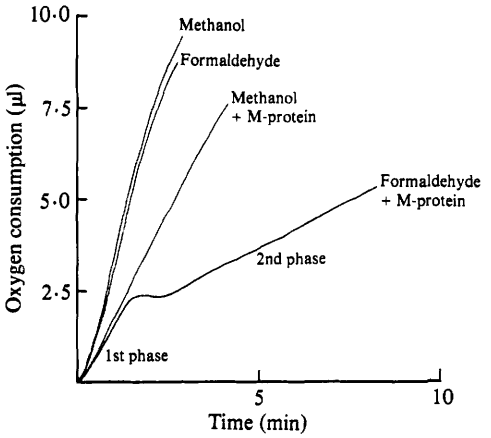


Fig. 1. Oxidation by pure MDH of *Pseudomonas* AM1 of methanol and formaldehyde in the presence and absence of M-protein. Oxygen uptake was measured in the MDH dye-linked assay system. Reaction mixtures contained pure MDH (60 U, 0·052 mg), substrate (0·1 μmol) and, where indicated, pure M-protein (0·07 mg). Reactions were started by addition of PES. Details of the assay procedure are given in Methods.

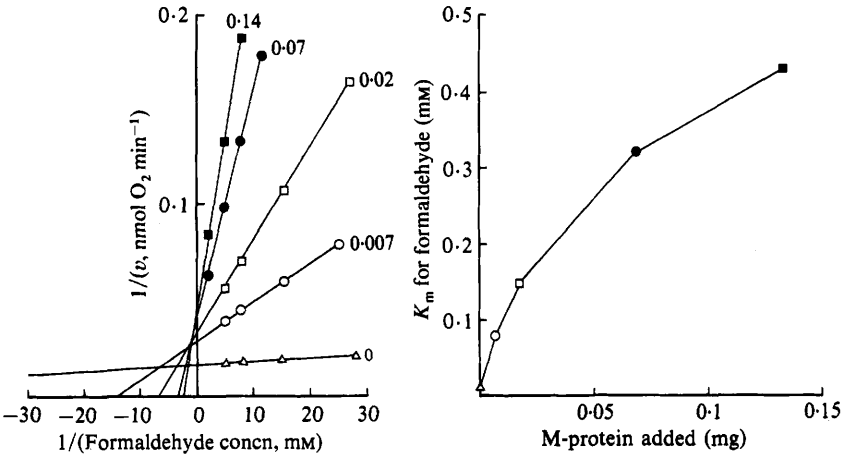


Fig. 2. Effect of M-protein on the K_m of MDH from *Pseudomonas* AM1 for formaldehyde. The K_m-lowering effect is demonstrated in a double reciprocal plot of data obtained by measuring the second phase of oxygen consumption (Fig. 1) at various concentrations of formaldehyde. Reaction mixtures contained, in the MDH dye-linked assay system, pure MDH (60 U, 0·052 mg) and various amounts of pure M-protein as indicated on the Figure. Details of the assay procedure are given in Methods.

M-protein stimulated the rate of reduction of cytochrome *c* to the same extent (about 1·5-fold) for all substrates (Table 4). Because methanol and ethanol were present in saturating concentrations this effect is likely to be due to an increase in the V_{max} of the MDH. The rate of cytochrome *c* reduction was slightly greater than the rate of oxidation of methanol or ethanol; this was probably because some of the cytochrome was being simultaneously reduced by endogenous reductant. The stoichiometry of the reaction in the presence of M-protein was almost 1 (0·94) mol of methanol oxidized per 2 mol of cytochrome reduced, indicating that little endogenous reductant was oxidized in the presence of methanol and M-protein.

1,3-Butanediol was used at a concentration below its K_m value and its rate of oxidation was markedly increased by M-protein, suggesting that M-protein was increasing the affinity of

Table 4. *Effect of M-protein on the oxidation of alcohols and formaldehyde by MDH of M. methylotrophus with cytochrome c_L as electron acceptor*

Full details of reaction mixtures and experimental procedures are given in Methods. The rates reported are initial rates derived from the progress curves for cytochrome *c* reduction and product formation (or substrate disappearance). The stoichiometries were calculated from the first data points obtained during the reaction. The oxidation of alcohols was determined by measuring aldehyde production; the oxidation of formaldehyde was determined by measuring its disappearance. The concentration of M-protein was 0·7 μM which was about 10 times the K_m value (70 nm) of MDH for this protein as measured in the dye-linked assay system.

Substrate (concn and K_m in dye-linked assay)	Presence or absence of M-protein . . .	Rate of cytochrome reduction (nmol min^{-1})*			Rate of substrate oxidation (nmol min^{-1})*			Stoichiometry (mol substrate oxidized per 2 mol cyt. reduced)*	
		(*a*) Without	(*b*) With	*b*/*a*	(*a*) Without	(*b*) With	*b*/*a*	Without	With
Endogenous reductant		8·3	12·0	1·4	–	–	–	–	–
Methanol (3 mM; 20 μM)		7·2	11·6	1·6	3·0	5·4	1·8	0·78	0·94
Ethanol (3 mM; 20 μM)		6·8	9·8	1·4	2·5	4·4	1·8	0·70	0·82
1,3-Butanediol (3 mM; 6·7 mM)		8·3	12·8	1·5	1·0	4·5	4·4	0·23	0·66
Formaldehyde (100 μM; 250 μM)		7·8	11·2	1·4	3·3	1·4	0·4	0·87	0·34

* Note that for every step in the oxidation of 1 mol of substrate (transfer of 2H) 2 mol of cytochrome are reduced.

MDH for 1,3-butanediol. This resulted in more of the alcohol being oxidized compared with endogenous reductant so that the stoichiometry of the reaction changed markedly on addition of M-protein (0·23 to 0·66 mol of substrate oxidized per 2 mol of cytochrome reduced).

The rate of oxidation of formaldehyde was similar to that of methanol oxidation in the absence of M-protein. Addition of M-protein to MDH had a very different effect, however, on the oxidation of the two substrates. Whereas the rate of oxidation of methanol was increased 1·8-fold, formaldehyde was oxidized at only 42% of the rate measured in the absence of M-protein. That the rate of reduction of cytochrome *c* increased (rather than decreased) in the presence of M-protein emphasizes that the effect on formaldehyde oxidation was due to a change in affinity for formaldehyde.

When pure M-protein from *Pseudomonas* AM1 (0·7 μM) replaced that of *M. methylotrophus* in this system, the rate of methanol oxidation was stimulated 1·4-fold whereas that of formaldehyde oxidation was reduced to 70%. The M-protein of *Pseudomonas* AM1 is thus also able to interact with the methanol dehydrogenase of *M. methylotrophus* but at the same concentration it produces relatively smaller effects.

Dye-linked aldehyde dehydrogenase of M. methylotrophus

The observation that whole cells of *M. methylotrophus* oxidized 1,2-propanediol to lactate (Bolbot & Anthony, 1980) implied not only the presence of a modifier protein able to stimulate propanediol oxidation by MDH, but also an aldehyde dehydrogenase able to oxidize lactaldehyde to lactate. This was confirmed by showing that crude extracts contained a dye-linked aldehyde dehydrogenase able to oxidize both lactaldehyde and propanal. This enzyme was partially purified by anion-exchange chromatography on DEAE-cellulose. A 12·6-fold purification was achieved, with a yield of 80%. The dehydrogenase was the only dye-linked enzyme able to oxidize propanal detectable in fractions eluted from the DEAE-cellulose column. Extracts of *M. methylotrophus* differed markedly from those of *Pseudomonas* AM1 in which at least four such dehydrogenases were present (Ford *et al.*, 1985; Weaver & Lidstrom, 1985). The relative rates (V_{max} values) for oxidation of propanal, lactaldehyde and formaldehyde in the dye-linked assay were identical (100 : 9 : 1) for the partially-pure dehydrogenase and for crude soluble extracts. This is consistent with the previous conclusion that formaldehyde is oxidized by a

Table 5. *Substrate specificity of the dye-linked aldehyde dehydrogenase of M. methylotrophus*

The values given are relative to those for propanal (propionaldehyde) which was the best substrate for the dehydrogenase. Aldehyde oxidation was measured at pH 7·0 at 30 °C (see Methods for details of assay procedure). The concentration of soluble aldehydes was 50 mM and insoluble aldehydes were added directly (10 µl) to the incubation mixture and incubated for 5 min before the reaction was started by addition of enzyme and PES. The rate measured with propanal was 190 nmol min^{-1}(mg protein)$^{-1}$. The following aldehydes were not oxidized: 2-methylpropanal, 2-ethylbutanal, methylglyoxal, glyoxylate and naphthaldehyde.

Substrate	Relative oxidation rate (%)	Substrate	Relative oxidation rate (%)
Formaldehyde	1	Cinnamaldehyde	45
Acetaldehyde	92	*p*-Methoxybenzaldehyde	35
Propanal	100	3-Hydroxybutanal (aldol)	50
n-Butanal	53	Succinic semialdehyde	39
n-Pentanal	73	Lactaldehyde	9
n-Hexanal	79		
n-Heptanal	80		
n-Octanal	76		
n-Nonanal	59		

cyclic variant of the carbon assimilation pathway in *M. methylotrophus* (Beardsmore *et al.*, 1982).

The M_r of the partially-purified aldehyde dehydrogenase was 55000 as measured by gel filtration on Sephadex G-150. The isoelectric point was estimated to be about 6·5 from its chromatographic properties. The results in Table 5 show that it oxidized a wide range of aldehydes, and that it is probably responsible for the production of lactate from the lactaldehyde produced during the oxidation of propanediol by MDH in *M. methylotrophus* previously observed by Bolbot & Anthony (1980). The specific activity in crude extracts was sufficiently great [14·5 nmol min^{-1}(mg protein)$^{-1}$] to account for the rate of lactaldehyde oxidation in whole cells.

There is a diversity of aldehyde-oxidizing enzymes in methylotrophs, the most frequently observed being the wide specificity dye-linked aldehyde dehydrogenases (Anthony, 1982; Marison & Attwood, 1982; Attwood & Quayle, 1984; Ford *et al.*, 1985; Weaver & Lidstrom, 1985). It has been suggested by Kohler & Schwarz (1982) that their function is to protect the organism from the accumulation of toxic aldehydes produced by the fortuitous oxidation of alcohols by the wide specificity MDH. Most of the enzymes oxidize formaldehyde although this is rarely the best substrate; nor are they induced in facultative methylotrophs during growth on C_1 compounds. Of those dye-linked aldehyde dehydrogenases previously described the substrate specificity of the aldehyde dehydrogenase from *M. methylotrophus* most closely resembles that of aldehyde dehydrogenase A from *Pseudomonas* AM1. The *M. methylotrophus* enzyme differs, however, in being unable to oxidize formaldehyde at a significant rate and in its M_r, which is only about half that of the dimeric enzyme from *Pseudomonas* AM1.

DISCUSSION

The observation that *M. methylotrophus* oxidized propanediol, although unable to grow on this substrate, led to the prediction that this obligate methylotroph must contain M-protein for its MDH (facilitating oxidation of propanediol to lactaldehyde), together with a non-specific aldehyde dehydrogenase able to oxidize lactaldehyde to lactate (Bolbot & Anthony, 1980; Ford *et al.*, 1985). The results in the present paper confirm this prediction and show that the M-proteins from *M. methylotrophus* and *Pseudomonas* AM1 are very similar. The results obtained using the dye-linked system indicated that the physiological role of the M-proteins might be the regulation of oxidation of formaldehyde by MDH, the V_{max} being halved (at least) and the K_m values being increased 36- to 64-fold. Preliminary experiments with the more physiological cytochrome-linked system showed that M-protein inhibited formaldehyde oxidation in both organisms.

The concentration of MDH in the periplasm of *M. methylotrophus* has been estimated to be about 0·5 mM (Beardmore-Gray *et al.*, 1983), and using similar arguments it can be calculated that the concentration of M-protein in the periplasm must be about 0·2 mM. This is so much greater than the K_m value of MDH for M-protein (70 nM) that all of the M-protein must be bound to MDH. This indicates that more than half of the MDH is not bound or that each molecule of M-protein can bind to more than one molecule of MDH at the same time. The problem of having a high concentration of unregulated (unbound) MDH might be avoided if the M-protein induces a conformational change in the MDH which is only slowly reversible after dissociation of the two proteins.

Further investigation will be required to demonstrate unequivocally that the inhibitory effect of M-protein on formaldehyde oxidation is sufficient to account for the fact that MDH is not involved in formaldehyde oxidation *in vivo* as shown by Heptinstall & Quayle (1970), Dunstan *et al.* (1972), Anthony (1975), Dawson & Jones (1981) and Groeneveld *et al.* (1984).

If prevention by M-protein of formaldehyde oxidation is essential for growth of *M. methylotrophus* then it might be expected to be synthesized constitutively. However, although the concentration of MDH was similar in O_2-limited and carbon-limited bacteria, the ratio of M-protein to MDH was much lower (1:15 compared with 1:2) in carbon-limited bacteria. In order to evaluate the significance of this it will first be necessary to know more accurately the effects of M-protein on the V_{max} and K_m values for methanol and formaldehyde in the cytochrome-linked system together with more data on the effect of growth conditions on the synthesis of M-protein.

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