

## The Role of a Methanol Dehydrogenase Modifier Protein and Aldehyde Dehydrogenase in the Growth of *Pseudomonas* AM1 on 1,2-Propanediol

By STEVEN FORD, M. DUDLEY PAGE AND CHRISTOPHER ANTHONY\*

Department of Biochemistry, University of Southampton, Southampton SO9 3TU, UK

(Received 18 February 1985; revised 2 May 1985)

---

Pure methanol dehydrogenase of *Pseudomonas* AM1 oxidizes propanediol in a transitory fashion when measured in a dye-linked assay with phenazine ethosulphate (PES) as electron acceptor. It was shown that the transitory nature of this oxidation is not because the product (lactaldehyde) is an inhibitor but because the dehydrogenase is inactivated by PES. Substrates having low affinities for the enzyme, such as propanediol, are unable to protect against this inactivation. A 'stimulatory factor', previously shown to facilitate the continuous oxidation by methanol dehydrogenase of propanediol to lactate, was shown to consist of two proteins, a modifier protein (M-protein) and an aldehyde dehydrogenase. The M-protein increased the affinity of methanol dehydrogenase for propanediol; this enabled the substrate to protect the enzyme against inactivation by PES, and it facilitated the continuous oxidation of low concentrations of propanediol to lactaldehyde. The M-protein was purified almost to homogeneity (more than 95% pure) and shown to be an acidic, dimeric protein having subunits of molecular weight 64000. The second protein component of the 'stimulatory factor' was a dye-linked aldehyde dehydrogenase which oxidized lactaldehyde to lactate but which had a greater affinity for longer-chain *normal* aliphatic aldehydes and the following characteristics: pH optimum, 7.0; isoelectric point, 4.0; molecular weight, 115000; and  $K_m$  for lactaldehyde, 16 mM.

---

### INTRODUCTION

*Pseudomonas* AM1 is a pink facultative methylotroph unable to grow on methane but able to grow on methanol and on a wide range of multicarbon compounds including 1,2-propanediol. The methanol dehydrogenase of this organism is a quinoprotein having pyrrolo-quinoline quinone (PQQ) as its prosthetic group (Duine & Frank, 1981; Anthony, 1982). The enzyme interacts with the electron transport chain at the level of cytochrome *c* but it is assayed *in vitro* using the dye phenazine ethosulphate (PES) as electron acceptor. Methanol dehydrogenase has a well-defined substrate specificity; it oxidizes a wide range of *normal* and substituted primary alcohols but it does not oxidize those having a second substituent on the C2 carbon atom. As expected, 1,2-propanediol, having two substituents on C2, is not oxidized by pure methanol dehydrogenase. In spite of this, studies on the metabolism of 1,2-propanediol by *Pseudomonas* AM1 unexpectedly indicated that methanol dehydrogenase is involved in the oxidation of this substrate; mutants lacking methanol dehydrogenase or cytochrome *c* neither grew on propanediol nor oxidized it (Bolbot & Anthony, 1980). It was subsequently shown that pure methanol dehydrogenase oxidizes 1,2-propanediol only in the presence of a 'stimulatory factor', possibly a protein(s), and that the product was not lactaldehyde but lactic acid. A complicating factor in interpreting this observation was that, although two sequential oxidation steps were involved, only propanediol and lactate (not the hypothetical intermediate lactaldehyde) were detected in reaction mixtures.

Two interpretations of the kinetics of propanediol oxidation by pure methanol dehydrogenase plus partially purified stimulatory factor were considered by Bolbot & Anthony (1980). Firstly,

---

*Abbreviations:* M-protein, modifier protein; PES, phenazine ethosulphate; PMS, phenazine methosulphate.

the stimulatory factor might act by removing (oxidizing) an initial inhibitory product (lactaldehyde); that is, that the factor might be a lactaldehyde dehydrogenase. At that time this could not be tested because of the impurity of the stimulatory factor, and because lactaldehyde was not readily available. The alternative interpretation, more consistent with the kinetic data, was that the 'stimulatory factor' binds tightly to the methanol dehydrogenase and thereby confers upon the enzyme the ability to oxidize the novel substrate 1,2-propanediol.

The present paper describes a further investigation of the oxidation of 1,2-propanediol by *Pseudomonas* AM1, in which it is shown that neither of these two interpretations is entirely correct. The 'stimulatory factor' consists of two proteins: a novel dye-linked aldehyde dehydrogenase that oxidizes lactaldehyde to lactate, and a modifier protein that binds reversibly to methanol dehydrogenase, so altering its substrate specificity.

#### METHODS

*Methanol dehydrogenase.* This enzyme was purified as described by O'Keeffe & Anthony (1980). Methanol dehydrogenase activity was assayed using a method based on that of Anthony & Zatman (1967). Modifications included the replacement of phenazine methosulphate (PMS) with phenazine ethosulphate (PES) as primary electron acceptor (Ghosh & Quayle, 1979; Bamforth & Quayle, 1978). Activity was measured at pH 9.0 in 125 mM-Tris/HCl buffer containing 22.5 mM-NH<sub>4</sub>Cl, 0.5 mM-PES and 7.5 mM-methanol in a volume of 2 ml at 30 °C; the reaction was started by addition of PES or enzyme. Oxygen utilization was followed in a Clark-type O<sub>2</sub> electrode (Rank Bros., Bottisham, Cambs., UK); rates were calculated by assuming that 450 nmol O<sub>2</sub> are dissolved in 2 ml buffer at pH 9.0 and 30 °C. One unit (U) of activity is defined as that amount which catalyses the consumption of 1 nmol O<sub>2</sub> min<sup>-1</sup>.

*Assay of aldehyde dehydrogenases.* These were assayed in the O<sub>2</sub> electrode or spectrophotometrically using various aldehydes as substrates. Reaction mixtures (2 ml) contained 135 mM-MOPS buffer, pH 7.0, and 0.5 mM-PES. For spectrophotometric assay, 85 μM-2,6-dichlorophenolindophenol was included in the reaction mixture (1 ml), its reduction being measured at 600 nm. Lactaldehyde was used at a final concentration of 50 mM, propionaldehyde at 10 mM; and insoluble aldehydes (1 μl) were added directly to the reaction mixture. One unit (U) of activity is defined as that amount which catalyses the reduction of 1 nmol O<sub>2</sub> min<sup>-1</sup> or 1 nmol 2,6-dichlorophenolindophenol min<sup>-1</sup>.

*Measurement of modifier protein (M-protein).* M-protein was assayed by measuring its ability to promote the oxidation of propanediol by methanol dehydrogenase. The assay system and unit definition were essentially the same as described for methanol dehydrogenase (above) except that pure methanol dehydrogenase (0.25 mg) was included in the reaction mixture and 60 mM-propanediol was the substrate.

*Growth, harvesting and breakage of bacteria.* *Pseudomonas* AM1 (NCIB 9133) was obtained from the National Collection of Industrial Bacteria, Aberdeen, UK. It was grown on a minimal salts medium containing the following (g l<sup>-1</sup>): (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3.0; NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 0.57; K<sub>2</sub>HPO<sub>4</sub>, 1.53. To this were added MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2 g l<sup>-1</sup>) and trace element solution (5 ml). This contained the following (mg l<sup>-1</sup>): CaCl<sub>2</sub>·2H<sub>2</sub>O, 530; FeSO<sub>4</sub>·7H<sub>2</sub>O, 200; MnSO<sub>4</sub>·4H<sub>2</sub>O, 20; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 20; CuSO<sub>4</sub>·5H<sub>2</sub>O, 4; CoCl<sub>2</sub>·6H<sub>2</sub>O, 4; Na<sub>2</sub>MoO<sub>4</sub>, 4; H<sub>3</sub>BO<sub>3</sub>, 3; 1 M-HCl, 1 ml. The growth medium was sterilized by autoclaving at 121 °C for 20 min, after which it was pH 7.0-7.2. The carbon sources 1,2-propanediol (0.5%, v/v), sodium succinate (0.2%, w/v) or methylamine hydrochloride (0.4%, w/v) were added to the medium before autoclaving. Other carbon sources (0.1%, w/v) were sterilized by passage through a Millipore filter (type Ha, pore size 0.45 μm) and added after autoclaving. Stock cultures were maintained on succinate-agar slopes containing Difco Bacto-Agar (2%, w/v) at 4 °C and were subcultured every 2 months. Cultures of up to 1 litre were grown in baffled flasks at 30 °C on a rotary shaker (90 r.p.m.) from a 5% inoculum of bacteria grown on the same carbon source. Large-scale cultures of *Pseudomonas* AM1 on 1,2-propanediol were grown in 80 litre batches in a New Brunswick fermenter having a stirring speed of 200 r.p.m. and an aeration rate of 0.1 m<sup>3</sup> min<sup>-1</sup>; the inoculum was 2 litres of propanediol-grown *Pseudomonas* AM1. Cultures were harvested at the end of the exponential phase by centrifugation at 4000 g for 15 min or by using a continuous centrifuge (Alfa-Laval). Harvested bacteria were washed twice in 20 mM-sodium phosphate buffer (pH 7.0) and resuspended (0.3 g wet weight ml<sup>-1</sup>) either in the same buffer or in 20 mM-Tris/HCl buffer (pH 8.0). They were disrupted using a programmable ultrasonic disintegrator (MSE Soniprep 150) at full power and 2 °C. Batches of 20 ml were disrupted for 20 cycles of 30 s duration followed by 30 s cooling. Whole cells and membranes were removed by centrifugation at 50000 g for 1 h at 4 °C. Protein in extracts and enzyme preparations was measured by the dye-binding method of Bradford (1976) using bovine serum albumin as standard. In column eluates, protein was estimated by absorption at 280 nm.

*Identification of lactaldehyde by gas chromatography.* A Pye-Unicam chromatograph (isothermal model) fitted with a thermal conductivity detector was used. A glass column (2.5 m × 2 mm internal diameter) packed with Chromosorb 101 (80-100 mesh) was operated at 180 °C. The injector and detection temperature was 190 °C. The

carrier gas was N<sub>2</sub>, flowing at 15–20 ml min<sup>-1</sup>. Compounds were identified by their retention times on the column. The following were used as standards: lactaldehyde, formaldehyde, acetaldehyde, propionaldehyde, lactic acid, acetol and 1,2-propanediol.

*Measurement of the product of the transitory oxidation of 1,2-propanediol by methanol dehydrogenase.* Methanol dehydrogenase (3 mg) was incubated with 1,2-propanediol (100 mM) in the dehydrogenase assay system using the O<sub>2</sub> electrode assay (above), and the oxidation allowed to go to completion. PES was removed by addition of 200 mg IR 120 Amberlite resin, which was then separated by centrifugation. The supernatant was mixed with Br<sub>2</sub> (20 μl) in a 100 ml stoppered flask and incubated in the dark at 20 °C for 120 h. The reaction mixture was diluted to 20 ml with water, which was then removed by rotary evaporation. This process was repeated until the solution was free of Br<sub>2</sub>. Water was added to the residue, the pH raised to 8.5 by addition of 0.1 M-NaOH and the solution freeze-dried. The residue was dissolved in 1 ml water and assayed enzymically for lactate by the method of Bergmeyer (1974).

*Preparation of DL-lactaldehyde.* This compound was not commercially available and so was synthesized by a method based on that of Hough & Jones (1952), the only modification being that the starting material used was methylglyoxal dimethylacetal (Fluorochem Ltd., Peakdale Road, Glossop, Derbyshire, UK) in place of methylglyoxal dibutylacetal (Nielsen & Sorenson, 1977).

Methylglyoxal dimethylacetal (20 g in 60 ml dry ether) was added dropwise to lithium aluminium hydride (5 g in 40 ml dry ether) in a 500 ml flask at 2–5 °C. When the addition was complete, excess reductant was destroyed by dropwise addition of water. After decanting the ether layer, the aqueous phase was further extracted with 100 ml ether, and the two ether extracts were pooled and evaporated under reduced pressure at 40 °C to give 14 ml of white liquid (yield, 70%). This material was added to 71 ml of a mixture of 98% formic acid, glacial acetic acid and water (1:30:40, by vol.) and heated under reflux on a boiling water bath for 30 min. After cooling, solvents were removed by evaporation under reduced pressure at 40 °C, and the residue was dissolved in 50 ml ether and dried over anhydrous sodium sulphate. The ether was then removed under reduced pressure at 40 °C, leaving a viscous yellow oil that slowly crystallized at room temperature. The white crystals were washed in ether and stored desiccated. The overall yield was 20%. Neilsen & Sorenson (1977) have shown that the oil is lactaldehyde and that the white crystals are a dimer of lactaldehyde which, on solution in water, forms a mixture of the dimer (45%) and the hydrated lactaldehyde monomer (55%). The product was identified as lactaldehyde by measurement of the melting point (109–112 °C) of the 4-nitrophenylhydrazone derivate (Huff & Rudney, 1959), by the infrared spectrum of the monomer and the <sup>1</sup>H NMR spectra of the monomer, dimer and hydrated species. The identity of lactaldehyde was confirmed by its oxidation to lactate with Br<sub>2</sub> and measurement of the L-lactate by the enzymic method of Bergmeyer (1974).

*Partial purification of aldehyde dehydrogenases A and D.* Crude soluble extracts of methylamine-grown bacteria were diluted four-fold with distilled water at 0 °C and applied to a column of DEAE-cellulose (5 × 5 cm) equilibrated with 5 mM-Tris/HCl buffer (pH 7.5). After washing with 60 ml of the same buffer, proteins were eluted with a gradient of KCl (0–0.3 M, total volume 240 ml) and 5 ml fractions collected. The aldehyde dehydrogenases were assayed using propionaldehyde as substrate. Fractions containing the two aldehyde dehydrogenases were separately pooled, concentrated under pressure over an Amicon PM30 membrane, and then fractionated by gel filtration on Sephadex G-150 (upward flow; fine grade; 3.5 × 75 cm; equilibrated in 20 mM-Tris/HCl buffer, pH 7.5). Active fractions were pooled and concentrated.

*Purification of the M-protein.* It was found that M-protein activity was lost when crude cell extracts were treated with protamine sulphate, or subjected to ammonium sulphate fractionation or to acid treatment. Therefore soluble cell extract (250 ml) was applied directly to a DEAE-cellulose column (5 × 15 cm) equilibrated with 20 mM-Tris/HCl buffer (pH 8.0) at 4 °C. The column was then washed with 400 ml of the same buffer and then with 600 ml of the same buffer containing 0.2 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, 3.5 × 75 cm; upward flow, 20 ml h<sup>-1</sup>; in MOPS buffer, 25 mM, pH 7.0). Pooled active fractions were concentrated and passed again through the same column. The concentrated eluate was further purified by FPLC on a Pharmacia Mono-Q anion exchange column (5 × 50 mm) equilibrated with MOPS buffer (20 mM, pH 7.0). The sample for purification was applied 2 ml (containing 5 mg protein) at a time; the column was washed with 6 ml of starting buffer and protein eluted with a concentration gradient (0–0.5 M-KCl) in the starting buffer (total volume, 26 ml). Three major protein peaks were observed, the last being the M-protein. Between runs the column was washed with 1 M-KCl in buffer. Fractions containing M-protein were pooled, diluted and chromatographed again on the same column. The M-protein purified by this method was shown to be almost homogeneous by PAGE.

*Estimation of molecular weights by filtration.* This was done using the same column of Sephadex G-150 as used for purification of proteins. For estimation of their molecular weights, proteins were applied to the column together with the following standards: γ-globulin, mol. wt 158 000; methanol dehydrogenase, 120 000; bovine serum albumin, 67 000; ovalbumin, 43 000; and lysozyme, 14 300.

*PAGE.* SDS-PAGE was done at pH 8.8 in Tris/HCl buffer as described by Weber & Osborn (1975); the concentration of SDS was 0.1% and that of acrylamide was 10% (w/v). Gels were stained for protein with

Coomassie Brilliant Blue (Weber & Osborn, 1975). To determine the proportion of protein in each band, peak integration was done on these gels using a Chromoscan 3 gel scanner (Joyce-Loebl, Gateshead, Tyne & Wear, UK). Molecular weights were determined in the same SDS-PAGE system using the following proteins as standards: ovotransferrin, mol. wt 76000–78000; bovine serum albumin, 68000; ovalbumin, 43000; chymotrypsinogen A, 25700; myoglobin, 17300; and cytochrome *c*, 12600.

Non-denaturing PAGE was done at pH 8.8 in 7.5% gels in Tris/glycine buffer (0.105 M with respect to glycine). Protein samples were mixed with glycerol and tracking dye (0.05% bromophenol blue in water) before application. Electrophoresis at 60 V (constant) and less than 20 mA, to avoid overheating, was run at 4 °C for 3–4 h.

Isoelectric focusing in gel rods, for the determination of isoelectric points, was done exactly as described in the Pharmacia handbook of isoelectric focusing (based on Laas & Fast-Johansson, 1979).

*Gel staining for the detection of dye-linked aldehyde dehydrogenases.* This was based on the method of Kohler & Schwartz (1982). After non-denaturing PAGE, gels were incubated in nitrobluetetrazolium (1.5 mg ml<sup>-1</sup> in 20 mM-sodium phosphate buffer, pH 7.0) for 15 min. They were then incubated for 20 min in the same buffer containing PES (1.5 mg ml<sup>-1</sup>) together with the appropriate substrate: propionaldehyde (1%, v/v), *n*-octaldehyde (1% v/v), lactaldehyde (3 mM), succinic semialdehyde (3 mM) or formaldehyde (0.5 mM). Active bands were visible as blue/black precipitates of formazan in the clear yellow gels. To obtain a rough indication of the relative activities with the different substrates, gels were scanned using a Joyce-Loebl Chromoscan 3 gel scanner.

## RESULTS

### *Production of lactaldehyde during the transitory oxidation of 1,2-propanediol by pure methanol dehydrogenase*

Lactaldehyde production during the oxidation of propanediol could not be demonstrated by Bolbot & Anthony (1980) because, when pure methanol dehydrogenase was incubated with propanediol in the dye-linked assay mixture containing PES, only a brief, transitory oxygen consumption occurred. In the present work, in an attempt to accumulate measurable amounts of lactaldehyde a very large amount of methanol dehydrogenase (3600 U) was incubated in a reaction mixture containing 100 mM-propanediol. When samples of the reaction mixture were analysed by gas chromatography a small peak corresponding to lactaldehyde was observed. This was too small for quantitative determinations and so lactaldehyde was measured enzymically (with lactate dehydrogenase) after oxidation of the reaction mixture with aqueous bromine (see Methods). It was shown that during the consumption of 4 µmol O<sub>2</sub>, 1.6 µmol L-lactaldehyde, but no lactate, were formed.

It is evident that, during propanediol oxidation in a typical 2 ml reaction volume containing initially 0.45 µmol O<sub>2</sub>, the concentration of lactaldehyde produced would never exceed 0.225 mM. This concentration of lactaldehyde, when added to a reaction mixture containing 60 mM-propanediol, was insufficient to inhibit the transitory oxidation of this substrate by methanol dehydrogenase. In order to achieve a 50% decrease in the initial rate, it was necessary to use a very high concentration of lactaldehyde (50 mM), thus demonstrating that the rapid decrease in the rate of propanediol oxidation observed during transitory oxidation cannot be accounted for by the accumulation of inhibitory concentrations of lactaldehyde.

### *The aldehyde dehydrogenases of Pseudomonas AM1*

Because pure methanol dehydrogenase is able to oxidize 1,2-propanediol to lactaldehyde (see above) those enzymes in *Pseudomonas* AM1 that were able to oxidize lactaldehyde in a dye-linked aldehyde dehydrogenase assay were investigated.

Dye-linked aldehyde dehydrogenases have been reported in a large number of methylotrophs; it has been speculated that their main function in these bacteria is to oxidize potentially toxic aldehydes produced during the oxidation of primary alcohols by the methanol dehydrogenase, and also (perhaps) to participate in the oxidation of formaldehyde (Anthony, 1982; Marison & Attwood, 1980; Kohler & Schwartz, 1982). These aldehyde dehydrogenases are usually non-specific, all use PMS or PES as electron acceptor, and they usually have a pH optimum of about 7.0.

The occurrence of four dye-linked aldehyde dehydrogenases in *Pseudomonas* AM1 was demonstrated by PAGE followed by activity staining as described by Kohler & Schwartz (1982) using formaldehyde, propionaldehyde, octaldehyde, succinic semialdehyde and lactaldehyde as

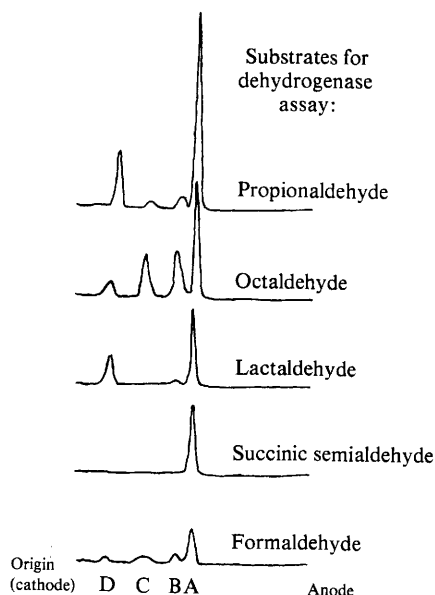


Fig. 1. The aldehyde dehydrogenases of *Pseudomonas* AM1. Soluble cell extracts of 1,2-propanediol-grown cells were electrophoresed in a non-denaturing gel system (7.5% acrylamide) and the positions in the gels of the various aldehyde dehydrogenases determined by activity staining. The dehydrogenases were labelled A–D in order of decreasing mobility towards the anode. Dehydrogenases A and D were further purified and characterized (see text).

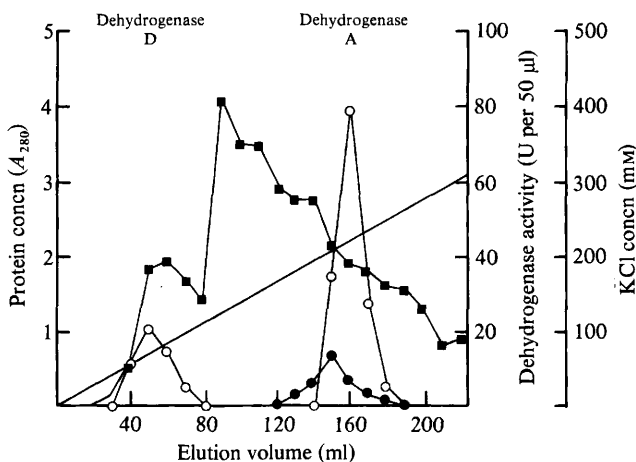


Fig. 2. Separation of aldehyde dehydrogenases A and D by anion-exchange chromatography. Soluble extract of methylamine-grown cells was applied to DEAE-cellulose and bound proteins were eluted with a KCl gradient (—). Fractions were assayed for protein (■), for aldehyde dehydrogenase activity (with propionaldehyde as substrate) (○), and for the ability to promote the oxidation of 1,2-propanediol by methanol dehydrogenase (●).

substrates. These dehydrogenases were designated A, B, C, and D in order of decreasing mobility towards the anode (Fig. 1). Two of them showed significant activity towards lactaldehyde and short-chain aldehydes such as propionaldehyde and they were further investigated by DEAE-cellulose chromatography (Fig. 2). Propionaldehyde was the best substrate for those dehydrogenases able to oxidize lactaldehyde (Fig. 1), and it was used as substrate for assaying aldehyde dehydrogenases in column eluates. Two dehydrogenases able to oxidize propionaldehyde were eluted from DEAE-cellulose; one was weakly anionic and the other strongly anionic at pH 7.5, and these were shown by PAGE and activity staining to

Table 1. *Partial purification of aldehyde dehydrogenases A and D from Pseudomonas AM1*

The crude soluble extract was prepared from 15 g (wet weight) methylamine-grown cells. Activity is expressed as  $\text{nmol min}^{-1}$  and was measured with propionaldehyde as substrate. Specific activity is expressed as  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ . All figures quoted are for active fractions after concentration. Details of purification and assay procedures are given in Methods. The first part of the table describes the separation by DEAE-cellulose chromatography of the two dehydrogenases; a total activity (A plus D) of 100% permitted the construction of individual purification tables for the two dehydrogenases. NA, Not applicable.

Fraction	Volume (ml)	Activity (U ml <sup>-1</sup> )	Specific activity	Purification factor	yield (%)
Soluble cell extract	160	696	60.7	NA	100
Aldehyde dehydrogenase A from DEAE-cellulose	28.5	2890	887	NA	74
Aldehyde dehydrogenase D from DEAE-cellulose	9.0	3720	527	NA	30
Aldehyde dehydrogenase A in soluble cell extract	160	515	44.9	1	100
in DEAE-cellulose eluate	28.5	2890	887	19.8	100
in Sephadex G-150 eluate	46.9	641	1050	23.4	36
Aldehyde dehydrogenase D in soluble cell extract	160	209	18.2	1	100
in DEAE-cellulose eluate	9.0	3720	527	28.9	100
in Sephadex G-150 eluate	2.0	7060	6080	334	42

Table 2. *Substrate specificity of aldehyde dehydrogenase A*

Aldehyde dehydrogenase A was partially purified by DEAE-cellulose chromatography and showed only one band of aldehyde dehydrogenase activity on non-denaturing polyacrylamide gels. The oxidation of the substrates listed was measured as described in Methods; apparent  $K_m$  and  $V_{max}$  values were estimated using the Lineweaver-Burk plot.  $V_{max}$  values are expressed relative to that for propionaldehyde [ $1.05 \mu\text{mol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$ ].

Substrate	$K_m$ (mM)	Relative $V_{max}$
Formaldehyde	20	5
Acetaldehyde	40	77
Propionaldehyde	6.7	100
Butyraldehyde*	0.4	25
Hexaldehyde*	0.6	33
Heptaldehyde*	0.3	39
Octaldehyde*	0.6	39
Lactaldehyde	16	30
Glyoxal	11.5	70
Methylglyoxal	—	0
Isobutyraldehyde	—	0

\* Aldehydes not miscible with water, prepared in a solution of 10% (v/v) 2-propanol in water.

correspond to aldehyde dehydrogenases A and D (Fig. 1) respectively. Aldehyde dehydrogenase A accounted for about 70% of the propionaldehyde dehydrogenase activity recovered from the DEAE-cellulose column (Fig. 2), the remaining activity being due to aldehyde dehydrogenase D. The ratios of dehydrogenase activities ( $V_{max}$  values) with propionaldehyde, formaldehyde and lactaldehyde were 1.0:0.05:0.25 respectively for aldehyde dehydrogenase A, and 1.0:0.1:0.05 for aldehyde dehydrogenase D. From these results it is evident that 92% of the lactaldehyde dehydrogenase activity in crude extracts was due to aldehyde dehydrogenase A and only 8% due to aldehyde dehydrogenase D.

*Aldehyde dehydrogenase A.* The purification procedure for this enzyme is summarized in Table 1. After chromatography on DEAE-cellulose most fractions containing aldehyde dehydrogenase A also promoted the oxidation of 1,2-propanediol by methanol dehydrogenase in

Table 3. Purification of the M-protein of *Pseudomonas AM1*

The M-protein was purified from 45 g (wet weight) 1,2-propanediol-grown cells. Activity is expressed as  $\text{nmol min}^{-1}$  and specific activity as  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ . All figures quoted are for active fractions after concentration. Details of purification and assay procedures are given in Methods. Aldehyde dehydrogenase activity was absent from the preparation after the second pass through Sephadex.

Stage	Volume (ml)	Activity (U ml <sup>-1</sup> )	Specific activity	Purification factor	Yield (%)
Soluble cell extract	255	630	34	1	100
DEAE-cellulose	22	2700	70	2.1	37
Sephadex G-150 (1)	13.4	3240	212	6.3	27
Sephadex G-150 (2)	42.4	675	278	8.2	18
Mono-Q (1)	36	378	1000	29.4	8.5
Mono-Q (2)	39.6	324	1800	52.9	8.0

its usual assay system at pH 9.0 with ammonia as activator (Fig. 2). They were pooled, concentrated and subjected to gel filtration. Fractions catalysing aldehyde dehydrogenase activity did not have the ability to promote propanediol oxidation by methanol dehydrogenase; conversely fractions having this ability were unable to oxidize propionaldehyde.

Dehydrogenase A was shown, by PAGE and activity staining, to constitute only about 5% of the protein present in the purest material produced by the procedure described in Table 1. It had a pH optimum of 7.0 and an isoelectric point of 4.0. By gel filtration, the molecular weight was shown to be 115000; the protein was insufficiently pure for the molecular weight of subunits (if any) to be determined. The best electron acceptors tested were PMS and PES; Wurster's blue was a poor acceptor, giving 6% of the rate with PMS, and the following compounds were inactive: 2,6-dichlorophenolindophenol, ferricyanide, methyl viologen, neutral red and menadione. The substrate specificity of aldehyde dehydrogenase A is summarized in Table 2. The apparent  $K_m$  values listed indicate that the dehydrogenase probably functions predominantly in the oxidation of longer-chain normal aldehydes. If it is assumed that cell material is about 50% carbon, and also that it is about 50% soluble protein, then the specific activity with respect to lactaldehyde of aldehyde dehydrogenase A in crude extracts [ $0.45 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ ] is sufficiently high to account for the specific growth rate ( $\mu = 0.1 \text{ h}^{-1}$ ) on 1,2-propanediol.

*Aldehyde dehydrogenase D.* This neutral dehydrogenase was shown by PAGE and activity staining to be identical to the dye-linked aldehyde dehydrogenase from *Pseudomonas AM1* previously described by Johnson & Quayle (1964) and considered by them to be involved in formaldehyde oxidation by this organism. This was demonstrated by using an aldehyde dehydrogenase from crude extracts of methylamine-grown *Pseudomonas AM1* purified according to their procedure. It was shown to be a monomer of molecular weight 33000. Isoelectric focusing followed by activity staining showed that this dehydrogenase has an isoelectric point of 7.0, which was also its pH optimum when measured with either formaldehyde or propionaldehyde as substrate. The ratios of activities ( $V_{\text{max}}$  values) for propionaldehyde, formaldehyde and lactaldehyde were 1.0:0.1:0.05, indicating that this neutral aldehyde dehydrogenase has little importance in oxidizing lactaldehyde in *Pseudomonas AM1*. It can be calculated that this dehydrogenase and aldehyde dehydrogenase A contribute equally to the dye-linked formaldehyde dehydrogenase activity measured at pH 7.0 in crude extracts of *Pseudomonas AM1*.

#### *The methanol dehydrogenase modifier protein (M-protein) of Pseudomonas AM1*

M-protein, purified by anion exchange chromatography on DEAE-cellulose, followed by gel filtration on Sephadex G-150 and further anion exchange chromatography on a Pharmacia Mono-Q column (Table 3), was more than 95% pure, having only one protein impurity as judged by SDS-PAGE.

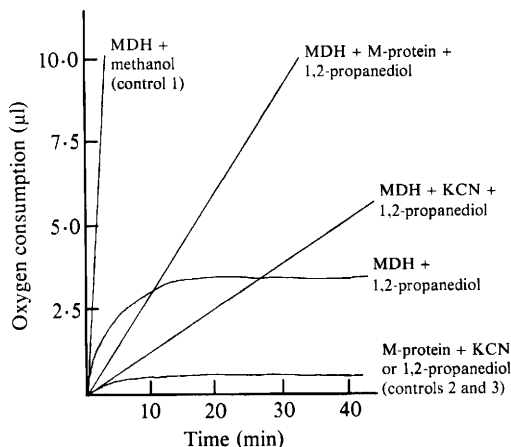


Fig. 3. Oxidation by pure methanol dehydrogenase of 1,2-propanediol in the presence and absence of pure M-protein and KCN. Oxygen uptake was measured in the methanol dehydrogenase assay system. Incubations contained pure MDH (75 U, 0.64 mg); 1,2-propanediol (30 mM); pure M-protein (0.144 mg) or KCN (25 mM). Control incubations contained (1), pure MDH plus methanol (7.5 mM); (2), 1,2-propanediol plus pure M-protein; and (3), 1,2-propanediol plus KCN plus M-protein. All reactions were started by addition of PES.

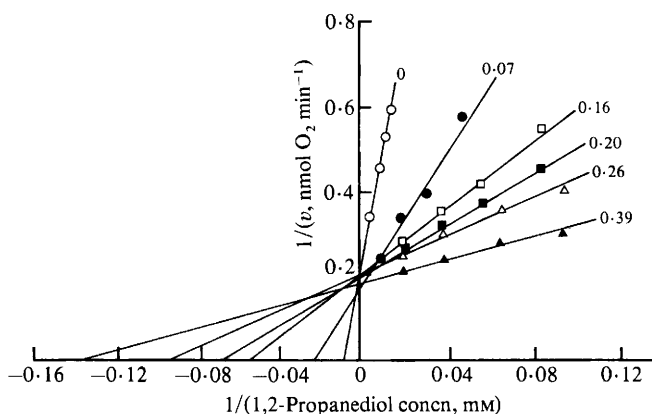


Fig. 4. Effect of pure M-protein on the  $K_m$  of methanol dehydrogenase for 1,2-propanediol. The  $K_m$ -lowering effect of the M-protein is here demonstrated in a double reciprocal plot of data obtained by measurement of the initial rates of oxygen uptake at various concentrations of 1,2-propanediol in the methanol dehydrogenase assay system. Incubations contained pure MDH (30 U, 0.26 mg) and various amounts of pure M-protein (mg) as shown on the figure.

Its molecular weight was about 140000 as estimated by gel filtration on Sephadex G-150, and 130000 as measured by SDS-PAGE carried out without prior heating of the protein in SDS. The molecular weight of subunits, produced by heating in SDS, was shown by SDS-PAGE to be 64000. The isoelectric point of M-protein was not measured directly, but was estimated to be about 4.0 because it had very similar chromatographic properties, during ion-exchange chromatography, to aldehyde dehydrogenase A (see above). The absorption spectrum of M-protein showed a single peak at about 278 nm but no significant absorption at wavelengths greater than 320 nm.

#### *Mechanism of action of M-protein*

When pure M-protein was incubated with methanol dehydrogenase in the dye-linked assay system containing propanediol as substrate, a continuous, rather than transitory,  $O_2$  consumption occurred (Fig. 3), suggesting that M-protein acts by promoting a continuous, rather than transitory, oxidation of propanediol to lactaldehyde.

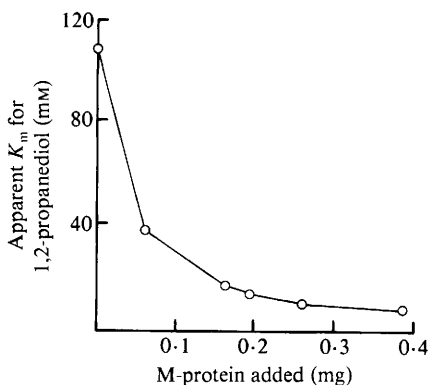


Fig. 5. Decrease in the  $K_m$  of methanol dehydrogenase for 1,2-propanediol by pure M-protein.  $K_m$  values were calculated from the data presented in Fig. 4. The greatest decrease in  $K_m$  observed was from 110 to 7 mM. The effect on methanol dehydrogenase showed saturation with respect to increasing amounts of M-protein; the ' $K_m$ ' of the M-protein for methanol dehydrogenase was 140 nM.

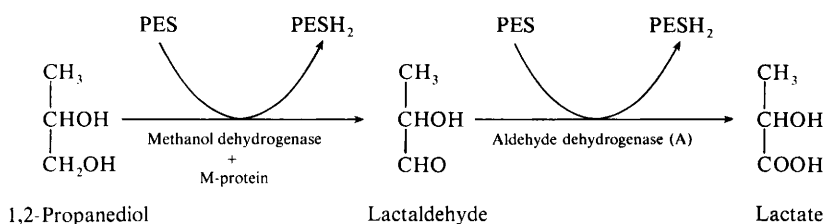


Fig. 6. *In vitro* oxidation of 1,2-propanediol to lactate in a dye-linked dehydrogenase system. Propanediol is oxidized to lactaldehyde by methanol dehydrogenase, which is inactivated by PES. M-protein increases the affinity of the enzyme for propanediol, so facilitating protection of the active site by this substrate. When also present, in less pure preparations, the aldehyde dehydrogenase (A) catalyses the further oxidation of lactaldehyde to lactate. *In vivo*, the same system operates but the M-protein is probably only essential for growth in low concentrations of propanediol.

Methanol dehydrogenase is inactivated by PMS in the absence of methanol (Anthony & Zatman, 1967), which presumably protects the active site for which it has a high affinity ( $K_m$  10–20  $\mu\text{M}$ ). This suggested that the transitory oxidation of propanediol might be due to poor protection of the active site by this low-affinity substrate. This conclusion was supported by the observation that higher propanediol concentrations led to more prolonged transitory oxidations (Bolbot & Anthony, 1980). It is also supported by the observation that KCN, a competitive inhibitor of methanol dehydrogenases (Duine & Frank, 1980; Beardmore-Gray *et al.*, 1983), is able to protect the enzyme from inactivation during the oxidation of propanediol (Fig. 3).

The results in Figs 4 and 5 show that the  $K_m$  for propanediol oxidation by methanol dehydrogenase was decreased markedly by the presence in the reaction mixture of pure M-protein. This supports the conclusion that M-protein protects the active site of the enzyme against inactivation by PES, and that it does so by increasing the affinity of methanol dehydrogenase for the relatively poor substrate propanediol.

#### DISCUSSION

The 'stimulatory factor' described by Bolbot & Anthony (1980) enabled methanol dehydrogenase to oxidize propanediol in two steps to lactate. The present work shows that this 'factor' consists of two proteins. The first is a modifier protein (M-protein) that increases the affinity of methanol dehydrogenase for propanediol, which is oxidized to lactaldehyde; and the second protein is a novel, anionic dye-linked aldehyde dehydrogenase that catalyses the subsequent oxidation of lactaldehyde to lactate (Fig. 6).

The dehydrogenase that oxidizes lactaldehyde to lactate during growth of *Pseudomonas* AM1 on propanediol (aldehyde dehydrogenase A) is unlikely specifically to function in this role because it has a higher affinity for longer-chain aldehydes than for lactaldehyde. Furthermore its specific activity did not differ markedly in bacteria from different growth substrates, and the oxidation of lactaldehyde by whole bacteria was only about 50% higher during growth on propanediol than during growth on methylamine or succinate. This aldehyde dehydrogenase was not purified sufficiently for identification of its prosthetic group although the site at which it interacts with the electron transport chain is likely to be at, or prior to, cytochrome *b* because whole cells of mutants lacking cytochrome *c* were not affected in their ability to oxidize lactaldehyde.

The action of M-protein in facilitating the protection by propanediol of methanol dehydrogenase against inactivation by PES explains the earlier observation that, whereas in whole cells methanol dehydrogenase appeared to be involved in propanediol oxidation, pure methanol dehydrogenase failed to catalyse oxidation of this substrate. This leaves open to question the role of M-protein *in vivo*, although clearly the lowering of the  $K_m$  for propanediol oxidation might be advantageous in permitting growth on relatively lower concentrations of propanediol. This explanation is probably insufficient to account for the occurrence of M-proteins in methylotrophs, however, because whole cells of the obligate methylotroph *Methylophilus methylotrophus* are able to oxidize propanediol to lactate (Bolbot & Anthony, 1980) although unable to oxidise or grow on lactate. This suggests that *M. methylotrophus* might also contain an M-protein, but that it must have some function other than facilitating growth on propanediol.

#### REFERENCES

- ANTHONY, C. (1982). *The Biochemistry of Methylotrophs*. London: Academic Press.
- ANTHONY, C. & ZATMAN, L. J. (1967). Purification and properties of the alcohol dehydrogenase of *Pseudomonas* sp. M27. *Biochemical Journal* **104**, 953-959.
- BAMFORTH, C. W. & QUAYLE, J. R. (1978). The dye-linked alcohol dehydrogenase of *Rhodospseudomonas acidophila*. *Biochemical Journal* **169**, 677-686.
- BEARDMORE-GRAY, M., O'KEEFE, D. T. & ANTHONY, C. (1983). The methanol:cytochrome *c* oxidoreductase activity of methylotrophs. *Journal of General Microbiology* **129**, 923-933.
- BERGMEYER, H. U. (1974). Assay of L-lactate using lactate dehydrogenase. In *Methods of Enzymatic Analysis*, pp. 1464-1468. Edited by H. U. Bergmeyer. London: Academic Press.
- BOLBOT, J. A. & ANTHONY, C. (1980). The metabolism of 1,2-propanediol by the facultative methylotroph *Pseudomonas* AM1. *Journal of General Microbiology* **120**, 245-254.
- BRADFORD, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.
- DUINE, J. A. & FRANK, J. (1980). Studies on methanol dehydrogenase from *Hyphomicrobium* X. Isolation of an oxidised form of the enzyme. *Biochemical Journal* **187**, 213-219.
- DUINE, J. A. & FRANK, J. (1981). Methanol dehydrogenase: a quinoprotein. In *Microbial Growth on C<sub>1</sub>-Compounds*, pp. 31-41. Edited by H. Dalton. London: Heyden.
- GHOSH, R. & QUAYLE, J. R. (1979). Phenazine ethosulphate as a preferred electron acceptor to phenazine methosulphate in dye linked enzyme assays. *Analytical Biochemistry* **99**, 112-117.
- HOUGH, L. & JONES, J. K. N. (1952). The synthesis of sugars from simpler substances. Part 4. Enzymic synthesis of 6-deoxy-D-fructose and 6-deoxy-L-sorbose. *Journal of the Chemical Society*, 4052-4055.
- HUFF, E. & RUDNEY, H. (1959). The enzymatic oxidation of 1,2-propanediol phosphate to acetol phosphate. *Journal of Biological Chemistry*, **234**, 1060-1064.
- JOHNSON, P. & QUAYLE, J. R. (1964). Oxidation of methanol, formaldehyde and formate by methanol grown *Pseudomonas* AM1. *Biochemical Journal* **93**, 281-290.
- KOHLER, J. & SCHWARTZ, A. C. (1982). Oxidation of aromatic aldehydes and aliphatic secondary alcohols by *Hyphomicrobium* spp. *Canadian Journal of Microbiology* **28**, 65-72.
- LAAS, T. & FAST-JOHANSSON, A. (1979). Isoelectric focusing with Pharmalyte in gel rods. In *Protides of the Biological Fluids* 27, pp. 693-697. Edited by H. Peeters. Oxford: Pergamon Press.
- MARISON, I. W. & ATTWOOD, M. M. (1980). Partial purification and characterization of a dye-linked formaldehyde dehydrogenase from *Hyphomicrobium* X. *Journal of General Microbiology* **117**, 305-313.
- NIELSEN, H. & SORENSEN, P. E. (1977). Kinetics and equilibria for the solvolysis of lactaldehyde in aqueous solution. *Acta chemica scandinavica* **A31**, 739-742.
- O'KEEFE, D. T. & ANTHONY, C. (1980). The interaction between methanol dehydrogenase and the autoreducible cytochromes *c* of the facultative methylotroph *Pseudomonas* AM1. *Biochemical Journal* **190**, 481-484.
- WEBER, K. & OSBORN, M. (1975). In *The Proteins* 1, pp. 179-233. Edited by H. Neurath & R. L. Hill. New York: Academic Press.