The Dye-Linked Alcohol Dehydrogenase of *Rhodopseudomonas acidophila*

COMPARISON WITH DYE-LINKED METHANOL DEHYDROGENASES

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1. A dye-linked alcohol dehydrogenase was purified 20-fold from extracts of *Rhodopseudomonas acidophila* 10050 grown anaerobically in the light on methanol/ HCO_3^- . 2. The enzyme resembled many previously reported methanol dehydrogenases from other methylotrophic organisms in coupling to phenazine methosulphate, requiring ammonia as an activator, possessing a pH optimum of 9 and a mol.wt. of approx. 116000. In many other respects the enzyme showed singular properties. 3. The stability of the enzyme under various conditions of temperature and pH was studied. 4. Primary aliphatic amines containing up to nine carbon atoms (the longest tested) were better activators than ammonia. 5. A wide range of primary alcohols and aldehydes served as substrates, with apparent K_m values ranging from 57 mM for methanol to 6 μM for ethanol. 6. O_2 was an inhibitor competitive with respect to the alcohol substrate. In the presence of O_2 , apparent K_m values of 145 mM were recorded for methanol. 6. Cyanide and $\alpha\alpha'$ -bipyridine were inhibitors competitive with respect to the amine activator. 7. The properties of the enzyme from *Rhodopseudomonas acidophila* are compared with those of similar enzymes from other organisms, and implications of the salient differences are discussed.

Since the initial discovery of a dye-linked methanol dehydrogenase (EC 1.1.99.8) in *Pseudomonas* M27 (Anthony & Zatman, 1963) a large number of such enzymes have been described in a variety of other C_1 -utilizing bacteria. These enzymes are all very similar with respect to substrate specificity, requirement for phenazine methosulphate and NH_4^+ ions for activity, high pH optimum, mol.wt. of about 120000 and, where purified to homogeneity, they constitute about 10% of the soluble protein of the respective cells [*Pseudomonas* AM1 (Johnson & Quayle, 1964); *Pseudomonas* PP (Ladner & Zatman, 1969); *Methylococcus capsulatus* Texas (Patel *et al.*, 1972; Wadzinski & Ribbons, 1975); *Pseudomonas* RJ1 (Mehta, 1973); *Candida boidinii* (Sahm & Wagner, 1973); *Hyphomicrobium* WC, *Pseudomonas* TP1 and *Pseudomonas* W1 (Sperl *et al.*, 1974); *Pseudomonas* C (Goldberg, 1976); *Pseudomonas* ZB1J26 and *Methylomonas* ZB36P11 (Michalik & Raczynska-Bojanowska, 1976); *Methylosinus sporium* (Patel & Felix, 1976); *Pseudomonas* 2941 (Yamanaka & Matsumoto, 1977)]. Sahm *et al.* (1976) demonstrated the presence of a similar enzyme in the phototroph *Rhodopseudomonas acidophila* strain 10050 grown anaerobically in the light on methanol/ HCO_3^- . Under these conditions the organism grows autotrophically using methanol as a source of reducing power to fix CO_2 via the ribulose bisphos-

phate cycle. In several respects the methanol dehydrogenase from this organism appeared to resemble the now-classical methanol dehydrogenase, except for having a wider substrate specificity and an unusually low affinity for methanol. This affinity was apparently higher if the enzyme was isolated under anaerobic conditions.

The present paper records a more detailed study of this enzyme and compares its properties with those of the methanol dehydrogenases involved in chemoheterotrophic growth on C_1 compounds in other organisms.

A preliminary report of part of this work has been presented (Bamforth & Quayle, 1978).

Materials and Methods

Chemicals

All chemicals were from BDH Chemicals, Poole, Dorset, U.K., except for: phenazine methosulphate, bovine serum albumin and Nitro Blue Tetrazolium, which were from Sigma (London) Chemical Co., Kingston-on-Thames KT2 7BH, U.K.; 2,6-dichlorophenol-indophenol, iodoacetic acid and *N*-ethylmaleimide, which were from Koch-Light Laboratories, Colnbrook SL3 0BZ, Bucks., U.K. Coomassie Brilliant Blue was from Schwarz-Mann Biochemical (Becton, Dickinson U.K.), Wembley HA9 0PS,

Middx., U.K. KCN was obtained from Hopkin and Williams, Romford RM1 1HA, Essex, U.K. 8-Hydroxyquinoline was from J. Preston, Sheffield, U.K.

All purified enzymes were from Boehringer Corp. (London), Lewes, East Sussex, U.K.

Buffers

Buffers were prepared by the methods of Dawson *et al.* (1969).

Growth of organism

Rs. acidophila 10050 was obtained from Professor N. Pfennig (Institut für Mikrobiologie, 34 Göttingen, West Germany) and was maintained at 4°C in liquid culture in methanol medium and grown on methanol as described by Sahm *et al.* (1976). The organism was grown in batch culture at 30°C in flat-sided 500 ml bottles filled to the top and placed 30 cm from a 60 W tungsten bulb.

Cells were harvested at an A_{610} of approx. 2.5 by centrifugation at 5000g for 20 min, washed once with 50 mM-potassium phosphate, pH 7.0, and, unless required fresh, were stored at -15°C.

Rs. acidophila 7750 and *Rhodocrobium vanielii* 7255 were also obtained from Professor N. Pfennig and were grown on methanol as described for *Rs. acidophila* 10050.

Pseudomonas AM1 (N.C.I.B. 9133) was grown on methanol as described by Heptinstall & Quayle (1970).

Preparation of cell-free extracts

Cell paste (1 part by weight) was resuspended in 5 vol. of 50 mM-potassium phosphate, pH 7.0, and cooled to 0°C in ice. Cells were disrupted by sonication by using an MSE ultrasonic disintegrator (model 150 W) at full power for 4 × 0.5 min intervals interspersed by cooling in ice. Centrifugation was routinely performed at 38000g for 10 min at 4°C, although in certain experiments centrifugation was at 100000g for 1 h in a refrigerated Spinco centrifuge. In either case the supernatant obtained was referred to as the 'crude extract'.

Enzyme assays

Dye-linked alcohol dehydrogenase (EC 1.1.99.8). It has previously been observed that assay of dye-linked methanol dehydrogenase is complicated by the non-linearity of the reaction progress curves (e.g. Anthony & Zatman, 1964). The present work showed that certain modifications to the standard assay procedure of Anthony & Zatman (1967) increased the period of proportionality of A_{600} with time and enabled more accurate determination of the initial rate of reaction. The modifications entailed a decrease in the phenazine methosulphate concentration and

an increase in the 2,6-dichlorophenol-indophenol concentration.

Plastic cuvettes (1 cm light-path; W. Sarstedt, Leicester, U.K.) contained, in a final volume of 3 ml; Tris/HCl, pH 9.0, 300 μ mol; phenazine methosulphate, 1.65 μ mol; 2,6-dichlorophenol-indophenol, 0.26 μ mol; methylamine hydrochloride, 50 μ mol; methanol, 100 μ mol (or ethanol, 0.9 μ mol); enzyme; water. Reaction was started by the addition of alcohol or enzyme and followed at 30°C by measuring the decrease in A_{600} caused by the reduction of 2,6-dichlorophenol-indophenol with a Unicam SP-1800 spectrophotometer linked to a Unicam AR 25 recorder. When starting assays with alcohol, care was needed to start all assays in any one experiment after a constant period of preincubation (see below). One unit of the enzyme is that amount which catalyses the reduction of 1 μ mol of 2,6-dichlorophenol-indophenol. The molar absorption coefficient of 2,6-dichlorophenol-indophenol at 600 nm and pH 9.0 was taken to be $21.99 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Armstrong, 1964). Certain assays were performed with the oxygen electrode with O₂ as secondary electron acceptor. Standard assay mixture (3 ml) was set up as in the spectrophotometric assay, except for the omission of 2,6-dichlorophenol-indophenol, and a Rank oxygen electrode linked to a Servoscribe model RE 541 recorder was used, the system being calibrated by the method of Robinson & Cooper (1970).

Other enzyme assays. The following enzymes were assayed at 30°C by using published methods: NAD⁺-linked alcohol dehydrogenase (EC 1.1.1.1) (Dickinson, 1970); lactate dehydrogenase (EC 1.1.1.27) and hexokinase (EC 2.7.1.1) [Biochimica Information 1, Boehringer Corp. (London)]; NAD⁺-linked malate dehydrogenase (EC 1.1.1.37) (Bamforth & Quayle, 1977). A Beckman DB-GT spectrophotometer linked to a Servoscribe RE 541 recorder was used.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951). Bovine serum albumin, dried to constant weight, was used as standard.

Partial purification of alcohol dehydrogenase from *Rs. acidophila*

Step 1: heat treatment. The crude extract, centrifuged for 1 h at 100000g, was heated in a Pyrex boiling tube (2.5 cm × 15 cm) suspended in a water bath at 60°C, a pear-drop condenser being used to prevent excessive evaporation. After 25 min the extract was rapidly cooled in ice and centrifuged at 38000g for 10 min at 4°C.

Step 2: acid treatment. The stirred supernatant from step 1 was carefully adjusted to pH 4.0 by

the slow addition of 1M-HCl at room temperature (approx. 20°C). The precipitate was removed by centrifugation at 38000g for 10min at 4°C and the supernatant adjusted immediately to pH7.0 by the addition of 1M-KOH.

Step 3: salt fractionation, first phase. The supernatant from step 2 was adjusted to 55% saturation with $(\text{NH}_4)_2\text{SO}_4$ by the slow addition of solid $(\text{NH}_4)_2\text{SO}_4$ at 4°C [the amounts required were computed from the Table of Dawson *et al.* (1969)]. After stirring for 20min the precipitate was removed by centrifugation at 38000g for 10min at 4°C. The supernatant was adjusted to 85% saturation with $(\text{NH}_4)_2\text{SO}_4$ and the resultant precipitate redissolved in 2ml of 50mM-potassium phosphate, pH7.0.

Step 4: gel-permeation chromatography. The redissolved pellet from step 3 was applied to the top of a column (2cm×34cm) of Sephadex G-150 equilibrated with 50mM-potassium phosphate, pH 7.0. The column was eluted with the same buffer at 4°C and 3ml fractions were collected. Those showing alcohol dehydrogenase activity were pooled. The enzyme was separated from cytochrome *c*, whose elution was monitored by measuring the A_{416} .

Step 5: ion-exchange chromatography. The pooled active fractions from step 4 were applied at room temperature to a column (1cm×5cm) of DEAE-cellulose (Whatman DE-52) previously equilibrated with 50mM-potassium phosphate, pH7.0. Alcohol dehydrogenase activity, which passed through the column without retention, was collected.

Step 6: salt fractionation, second phase. Step 3 was repeated on the supernatant from step 5.

Polyacrylamide gel electrophoresis

This was performed by the method of Davis (1964), except for the omission of the large-pore gel. Electrophoresis was performed in a Shandon apparatus (Shandon Scientific Co., London N.W.10, U.K.) by using 7% gels at pH8.3 with Bromophenol Blue as tracking dye. Gels were fixed in 7% (v/v) acetic acid and stained for 1h with 0.1% (w/v) Coomassie Blue in 7% acetic acid. Destaining was by soaking in 7% acetic acid overnight.

Alcohol dehydrogenase activity was detected in unfixed gels by using the standard assay mixture except for replacement of 2,6-dichlorophenol-indophenol by 0.2ml of 0.8% (w/v) Nitro Blue Tetrazolium and inclusion of enough water to cover the gels. The gels were left in the dark until formazan deposition had occurred, and reaction was stopped by immersing the gel in 7% acetic acid.

Handling of enzyme under anaerobic conditions

Certain extracts of *Rs. acidophila* were prepared under anaerobic conditions as follows. Before sonication, O₂-free N₂ (B.O.C., Gases Division, Brentford, Middx., U.K.) was bubbled through the

bacterial suspension for 5min. Sonication was then performed as described above, except that N₂ was continuously bubbled through the suspension, which was held in a glass sonication vessel capped with a rubber seal through which the sonicator probe passed via an appropriately sized aperture. Gas entry and outlet ports were provided by syringe needles.

After sonication the suspension was transferred under a stream of N₂ to a centrifuge tube subsequently flushed with N₂ for 5min before sealing with a metal cap. After centrifugation at 38000g for 10min at 4°C the supernatant was decanted into a separate, capped centrifuge tube as above. The tube was warmed to 60°C, incubated for 25min and centrifuged again. The supernatant was transferred under a stream of N₂ to a glass bottle, which was capped with a Suba-Seal stopper and flushed with N₂ for 15min. Inlet and outlet gas ports were again provided by syringe needles. The material obtained was referred to as the 'anaerobic cell-free extract'. Reducing agents such as dithiothreitol and dithionite, which are frequently used in buffer solutions to assist anaerobiosis (e.g. Eady *et al.*, 1972), could not be added in this case, as they reduced the artificial electron acceptor used in the enzyme assay.

Certain assays were performed under anaerobic conditions. The standard assay system was used; all reaction components except enzyme were added to the cuvette, which was then fitted with a Suba-Seal cap through which passed two syringe needles. One was attached to the gas source. The appropriate gas mixture (100% N₂ for anaerobiosis) was bubbled through the cuvette for 3–5min at room temperature, followed by simultaneous removal of the syringes. The reaction was started by the addition of anaerobic cell-free extract by micro-syringe. In experiments where the N₂:O₂ ratio was varied, a modified form of the gas-mixing apparatus of Houslay & Tipton (1973), as described by Brook & Large (1976), was used. The gas-flow meters which were used provided a flow rate of 0–8 litres/min and were supplied by G. A. Platon (Basingstoke, Hants., U.K.). Dissolved O₂ concentrations were determined at room temperature with a Rank oxygen electrode linked to a Servoscribe model RE541 recorder. The oxygen electrode was calibrated by the method of Robinson & Cooper (1970).

Results

Subcellular distribution of alcohol dehydrogenase in Rs. acidophila

When crude extracts of *Rs. acidophila* 10050 were centrifuged at 100000g for 1h at 4°C, 79% of the recovered activity was in the supernatant fraction. The presence of 21% of the activity in the pellet fraction suggested that the enzyme may be loosely

attached to the membrane fraction of the cell and is released into the soluble fraction by sonication. However, membrane material prepared from methanol-grown *Rs. acidophila* by using the lysozyme/EDTA method of protoplast formation (Repaske, 1956) was devoid of alcohol dehydrogenase activity, which was entirely located in the soluble fraction after centrifugation at 38000g for 15min at room temperature.

Partial purification of alcohol dehydrogenase from Rs. acidophila

The purification procedure led to a 20-fold purification over that in the crude extract (Table 1). This material was not homogeneous when examined by polyacrylamide-gel electrophoresis at pH 8.3: there were at least six protein bands of approximately equal intensity, the slowest-moving band corresponded to a single band of methanol-dependent formazan deposition in unfixed gels tested for alcohol dehydrogenase activity. Single bands of formazan deposition of the same relative mobility with respect to Bromophenol Blue were obtained when either ethanol or butan-1-ol replaced methanol as electron donor.

Conditions for assay of alcohol dehydrogenase

Apart from the non-linearity of the reaction progress curve, measurement of initial rates of dye-linked methanol dehydrogenase are usually complicated by the presence of high endogenous dye reductase activity (Anthony & Zatman, 1964). By contrast, crude cell-free extracts of methanol-grown *Rs. acidophila* prepared under aerobic conditions usually showed a total lack of endogenous dye reduction. However, extracts prepared anaerobically catalysed a non-substrate-dependent reduction of 2,6-dichlorophenol-indophenol, which was nevertheless totally dependent on the simultaneous

presence of phenazine methosulphate and an amine activator. The rate of endogenous dye reduction was proportional to the amount of extract added. These results suggest the identity of the endogenous dye reductase with alcohol dehydrogenase. Both the substrate-linked and the endogenous activities were inhibited by cyanide.

On prolonged incubation it was observed that there was a slow increase in A_{600} , suggesting that the 2,6-dichlorophenol-indophenol was being re-oxidized. The final A_{600} corresponded to that before addition of extract.

Effect of protein concentration. The initial rate of substrate-dependent dye reduction in the standard assay was proportional to the enzyme concentration up to 105 μ g of protein per cuvette for the partially purified enzyme. This corresponded to a ΔA_{600} of 2.052/min. Linearity was observed in the assay of the enzyme at all stages of purification, up to the rate quoted above.

Effect of dye concentration. The enzyme followed classical Michaelis-Menten kinetics with respect to phenazine methosulphate, and an apparent K_m of 0.15 mM was obtained by the method of Eisenthal & Cornish-Bowden (1974). Substrate inhibition was found at phenazine methosulphate concentrations in excess of 1.1 mM. The greater the concentration of phenazine methosulphate, the greater was the non-linearity of the reaction progress curve.

The reaction rate was directly proportional to the concentration of 2,6-dichlorophenol-indophenol up to 120 μ M (the highest concentration tested). The greater the concentration of 2,6-dichlorophenol-indophenol the greater was the linearity of the reaction progress curve. Anthony & Zatman (1964) interpreted the rapid loss of linearity in the assay in terms of the continual decrease in the 2,6-dichlorophenol-indophenol concentration during the progress

Table 1. *Partial purification of alcohol dehydrogenase from Rs. acidophila*

The crude extract was prepared from 4 g (wet wt.) of the organism grown on methanol anaerobically in the light. Experimental details are given in the Materials and Methods section.

Step	Volume (ml)	Enzyme activity (units/ml)	Protein concn. (mg/ml)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract (100000g supernatant)	19.5	3.32	11.6	0.286	1	100
1. Heat treatment	17.5	3.52	4.6	0.765	2.7	96
2. pH 4 treatment	19.7	2.74	2.8	0.979	3.4	83
3. 55-85%-satd.-(NH ₄) ₂ SO ₄ fraction	2.3	7.3	8.7	0.839	2.9	26
4. Sephadex G-150 pooled active fractions	28.5	0.78	0.25	3.12	10.9	34
5. DE-52 effluent	25.0	0.41	0.18	2.28	8.0	16
6. 55-85%-satd.-(NH ₄) ₂ SO ₄ precipitate	2.8	1.28	0.23	5.57	19.5	6

of the reaction. The above observations support their conclusion. The 2,6-dichlorophenol-indophenol concentration in the standard assay procedure was the maximum compatible with the spectrophotometer.

Although the reaction could be followed, in the absence of 2,6-dichlorophenol-indophenol, with O₂ as the secondary electron acceptor (see Table 3), this was, of course, not feasible for anaerobic studies. It was observed that the gradients of reaction progress curves studied with the oxygen electrode increased with time. No reaction was observed under any conditions in the absence of phenazine methosulphate.

Effect of pH. An optimum pH of about 9 was obtained for the present enzyme with methanol or ethanol as electron donor.

Effect of activators. The reaction was absolutely dependent on the presence of NH₄⁺ ions or salts of primary amines (Table 2). There appears to be a correlation between the affinity of the enzyme for the amine and the hydrophobic character of the latter. Secondary and tertiary methylamines were not activators.

Effect of electron-donor concentration. When assayed under aerobic conditions with 0.55mm-phenazine methosulphate in the standard assay, aerobically extracted alcohol dehydrogenase showed apparent *K_m* values (Lineweaver & Burk, 1934) of 145mm and 17μm for methanol and ethanol respectively. Maximum velocities were, however, similar. When extracts were prepared and assayed under

anaerobic conditions an apparent *K_m* value of 42mm was obtained for methanol under the above conditions. An extract prepared without anaerobic precautions and subsequently bubbled with N₂ for 1.5h before anaerobic assay gave an apparent *K_m* value of 40mm for methanol in the above assay. When the same extract was assayed in the standard aerobic mixture an apparent *K_m* of 95mm was obtained for methanol.

Effect of temperature. Preincubation of the enzyme in the standard assay mixture (minus methanol) for 10min at 30°C in the dark led to a 69% stimulation of alcohol dehydrogenase activity. As shown in Fig. 1 this apparent activation was maximal after approx. 6min and decreased thereafter. Preincubation at room temperature led to less activation than at 30°C. Experiments showed that the activation could be achieved by incubation in buffer alone, and either Tris/HCl buffer, pH9.0, or 100mm-potassium phosphate, pH7.0, were equally effective. Preincubation at temperatures greater than 30°C led to little increase in the activation. Activation could be reversed by cooling. Enzyme assays at 30°C in which reaction was started by addition of enzyme at 4°C gave linear rates of dye reduction for at least 30s. The acceleration in rate during this period of assay was negligible.

Table 2. Activation of alcohol dehydrogenase by ammonia and primary amines

All amines were tested as their hydrochlorides in the standard assay mixture. Apparent *K_m* values and maximum velocities were determined by the method of Lineweaver & Burk (1934). Dimethylamine and trimethylamine were tested at final concentrations of 36mm and 42mm respectively.

Activator	Apparent <i>K_m</i> (mm)	Relative maximum velocity
None	—	0
Ammonia	42.0	1.9
Methylamine	2.1	1*
Ethylamine	0.6	0.29
n-Butylamine	0.26	0.17
n-Hexylamine	0.05	1*
n-Heptylamine	0.029	1*
n-Nonylamine	0.026	1.2
Dimethylamine	—	0
Trimethylamine	—	0

* The actual rate was 4.8μmol of 2,6-dichlorophenol-indophenol reduced/min per ml of enzyme solution.

Fig. 1. Effect of preincubation on the initial rate of reaction of alcohol dehydrogenase

The reaction mixture (except methanol) was incubated in the dark at the temperature and for the time indicated before starting reaction with methanol. ○, 30°C; ●, room temperature (approx. 20°C).

The methanol dehydrogenase activity of crude extracts of *Rs. acidophila* 10050 was also activated by 68% under the above conditions, whereas pre-incubation of crude extracts of *Pseudomonas* AM1 in 100mM-Tris/HCl buffer, pH9.0, for 5min before starting the reaction led to a 24% decrease in methanol dehydrogenase activity compared with the control, which was not preincubated [assay conditions for *Pseudomonas* AM1 extract were as described by Anthony & Zatman (1967)].

Stability of alcohol dehydrogenase

pH stability. Incubation of the partially purified enzyme at 0°C over a range of pH values showed that in 30min the enzyme was 50% inactivated at pH values of approx. 4.2 and 11.5 and was stable at pH 7 (Fig. 2).

Heat stability. The partially purified enzyme survived heating in 50mM-potassium phosphate, pH7.0, at 60°C for 25min without significant loss of activity, but at 78°C had a half-life of 10min. The presence of ethanol as stabilizer (Sahm *et al.*, 1976) had little influence on the heat stability.

Cold stability. The partially purified enzyme was stable in 50mM-phosphate, pH7.0, in the absence of ethanol over 2 days at -15°C, but lost 90% of its activity after 21 days storage (the protein concentration was 230 µg/ml). However, over the same period only 20% of the activity was lost at -15°C when the enzyme was stored in the presence of 10mM-ethanol (the protein concentration in this case was 180 µg/ml).

The inclusion of ethanol as stabilizer led to a very high endogenous dye reductase activity, which was not diminished by dialysis, suggesting that the alcohol was firmly bound to the enzyme.

Fig. 2. *pH stability of alcohol dehydrogenase* Partially purified enzyme (35 µg of protein; 0.1 ml) was incubated in the appropriate buffer solution (300 µmol; 1.0 ml) for 30 min in ice. Samples (0.5 ml) were then withdrawn and assayed in the standard assay mixture except that the concentration of Tris/HCl, pH9.0, was raised to 1 M. Maximum activity was 2.2 units/mg of protein.

Electron-donor specificity

The enzyme was relatively non-specific with respect to the alcohol substrate (Table 3). The specificity obtained with the oxygen-electrode assay was similar to that previously obtained with a less-pure enzyme preparation by Sahm *et al.* (1976) using the spectrophotometric assay.

Effect of inhibitors

The enzyme was sensitive to the presence of KCN (Table 4), behaviour which is unlike that reported for any other dye-linked methanol dehydrogenase. The lack of inhibition by CO suggests that cyanide does not bind to a haem group, and the lack of inhibition with semicarbazide suggests that cyanohydrin formation with a free carbonyl function is not the basis of cyanide inhibition. The inhibition by $\alpha\alpha'$ -bipyridine and EDTA would suggest metal chelation as being the most likely mode of inhibition by cyanide. However, dialysis of the enzyme at 4°C for 4h against 1000 vol. of 10mM-KCN in 50mM-potassium phosphate, pH7.0, followed by assay of 10 µl of enzyme in the standard assay mixture (i.e. a 300-fold dilution of the cyanide to a final concentration of 0.03 mM) resulted in no detectable inhibition. Inhibition was evident when the cyanide concentration in the cuvette was raised to 1 mM. This suggests that inhibition by cyanide is reversible, and hence that a metal atom has not been removed from the molecule. Similar results were observed with $\alpha\alpha'$ -bipyridine.

Cyanide and $\alpha\alpha'$ -bipyridine were shown to be competitive inhibitors of the enzyme from *Rs.*

Table 3. *Alcohol substrate specificity of alcohol dehydrogenase*

Standard assay conditions involving the oxygen electrode (see the Materials and Methods section) were used. Benzyl alcohol and 2-methoxyethanol (at 3.3 and 33 mM) were not substrates.

Substrate	Apparent K_m (mM)	Relative maximum velocity
Methanol	57	2.8
Ethanol	0.006	1.0*
Propan-1-ol	0.15	6.5
Butan-1-ol	0.4	2.3
Propan-2-ol	4.2	3.5
2-Methylpropan-1-ol	1.2	6.3
Butan-2-ol	4.7	5.9
Allyl alcohol	3.0	4.4
Formaldehyde	4.8	0.77
Acetaldehyde	1.9	2.2

* The actual rate was 1 µmol of O₂ consumed/min per ml of enzyme solution.

Table 4. Inhibition of alcohol dehydrogenase
Inhibitors were included in the standard assay mixture under aerobic conditions at the concentration indicated and were preincubated with the enzyme in the dark for the period stated, before starting reaction with methanol. Values in parentheses relate to the activity of the control preincubated without inhibitor for 10 min and hence take account of the activation of the enzyme at 30°C during the preincubation period.

Inhibitor	Final concn. (mm)	Preincubation (min)	Relative activity (%)
None	—	0	100
None	—	10	169 (100)
KCN	1.0	0	24
αα'-Bipyridine	1.0	0	65
Pyridine	10.0	0	88
EDTA	10.0	10	106 (37)
Diaminoethane	4.5	10	169 (100)
1,10-Phenanthroline	3.3	0	109
8-Hydroxyquinoline	3.3	0	117
Sodium oxalate	10.0	0	88
p-Chloromercuribenzoate	0.33	10	176 (107)
Iodoacetic acid	3.3	0	106
	16.5	0	122
N-Ethylmaleimide	10.0	0	101
	33.3	0	99
CO	3 min bubbling at room temperature		128
Semicarbazide hydrochloride	1.0	0	100
ZnSO ₄	1.0	0	108
HgCl ₂	1.0	0	96
MnCl ₂	1.0	0	0
NaCl	1.0	0	107
CuSO ₄	1.0	0	0
MgCl ₂	1.0	0	91
FeCl ₃	1.0	0	96
KNO ₃	1.0	0	98
CoCl ₂	1.0	0	0

acidophila with respect to the activating amine; inhibition by αα'-bipyridine was non-competitive with respect to electron donor and electron acceptor (Table 5).

Mechanism of reaction and the effect of O₂ on the enzyme

Under anaerobic conditions, measurement of the initial rate of the alcohol dehydrogenase reaction with respect to methanol concentration over a range of concentrations of phenazine methosulphate gave rise to a series of parallel lines when the reciprocal of the initial velocity was plotted against the reciprocal of the methanol concentration at each of the dye concentrations. The secondary plots of the slopes and intercepts of these lines against the reciprocal of the dye concentration are shown in Figs. 3(a) and 3(b).

Table 5. Inhibition of alcohol dehydrogenase by αα'-bipyridine and cyanide
The type of inhibition and K_i values (mm) were determined by the method of Dixon (1953).

Inhibitor	Varied substrate	Type of inhibition	K _i (mm)
αα'-Bipyridine	Methanol	Non-competitive	1.9
	Phenazine methosulphate	Non-competitive	1.4
	Methylamine	Competitive	1.5
KCN	Methanol	Non-competitive	0.6
	Methylamine	Competitive	0.91

Fig. 3. Kinetics of methanol oxidation by alcohol dehydrogenase under anaerobic and aerobic conditions
Reaction was performed as in the standard assay mixture at final concentrations of methanol between 1.7 and 20mm. From double-reciprocal plots of initial reaction velocity against methanol concentration at a series of phenazine methosulphate concentrations the respective slopes and intercepts were plotted against the reciprocal of the concentration. (a) Intercepts plotted against reciprocal phenazine methosulphate concentration: ○, anaerobic assays; ●, aerobic assays. (b) Slopes plotted against reciprocal phenazine methosulphate concentration: △, anaerobic assays; ▲, aerobic assays. The O₂ concentration in aerobic reaction mixtures was 0.317 mm.

From these plots a series of initial-rate parameters as defined by Dalziel (1957) were derived and are listed in Table 6.

Table 6. *Kinetic coefficients for the oxidation of methanol by alcohol dehydrogenase under aerobic and anaerobic conditions*

The kinetic coefficients were measured at pH 9.0 and 30°C and are defined by the initial-rate equation $e/v = \phi_0 + \phi_1/[S_1] + \phi_2/[S_2]$ (Dalziel, 1957) where $[S_1]$ and $[S_2]$ are the concentrations of electron donor and electron acceptor respectively, e is the enzyme concentration and v is the initial reaction velocity. ϕ_1/ϕ_0 is the Michaelis constant (K_1) for the electron donor and ϕ_2/ϕ_0 is the Michaelis constant (K_2) for the electron acceptor. $1/\phi_0$ is the maximum velocity of the reaction.

Gas phase	Kinetic constants				
	ϕ_0 (min)	ϕ_1 (mm·min)	ϕ_2 (mm·min)	K_1 (mm)	K_2 (μ M)
Air (0.317 mm-O ₂)	65	1240	2.7	19.1	41.6
N ₂	85	415	2.7	4.9	31.7

Table 7. *Effect of O₂ concentration on the initial rate of the alcohol dehydrogenase reaction*

Various O₂/N₂ mixtures were prepared as described in the Materials and Methods section and were bubbled through the reaction mixture (minus enzyme) for 5 min. Reaction was started by the addition of anaerobic cell-free extract. Dissolved O₂ concentrations were determined by oxygen electrode as described in the Materials and Methods section. N.D., not determined.

N ₂ /O ₂ composition (v/v)	Flow rate (litres/min)	Dissolved O ₂ concentration (mm)	Relative reaction rate
6:0	4.4	0	100
5:1	5.8	0.261	71
3:3	5.6	N.D.	68
2:4	5.8	N.D.	67
1:5	5.8	N.D.	47
0:6	4.4	N.D.	33

The experiment performed without taking anaerobic precautions (other than preparing the enzyme anaerobically) also yielded a series of parallel lines in the primary plot, with linear secondary plots (Figs. 3a and 3b). Comparison of the initial-rate parameters derived under aerobic and anaerobic conditions (Table 6) shows that the factor most affected by the nature of the gas phase was ϕ_1 and, hence, the true Michaelis constant for methanol.

Table 7 shows that increasing the ratios of O₂ to N₂ led to increasing inhibition of the enzyme, indicating that the constituent of air which is responsible for inhibition of the enzyme is, in fact, O₂.

O₂ was also shown to be a competitive inhibitor with respect to ethanol and butan-1-ol; apparent

K_1 values of 0.57 and 0.30 mm respectively were measured under standard assay conditions at an O₂ concentration of 0.317 mm.

Effect of O₂ and cyanide on alcohol dehydrogenases from other organisms

Anaerobic extracts were prepared from *Pseudomonas* AM 1 grown aerobically on methanol, and *Rs. acidophila* 7750 and *Rm. vannielii* 7255 both grown anaerobically in the light on methanol, and were assayed under anaerobic and aerobic conditions for their ability to oxidize methanol (or butan-1-ol in the case of *Pseudomonas* AM 1). O₂ had no effect on the affinity of the enzyme from *Pseudomonas* AM 1 for the alcohol substrate. O₂ was a competitive inhibitor with respect to alcohol of the enzyme from *Rs. acidophila* 7750, but inhibition was mixed competitive-non-competitive in *Rm. vannielii* 7255. The last two enzymes had apparent K_m values for methanol of 196 mm and 48 mm respectively under aerobic conditions.

KCN (1 mm) did not inhibit the *Pseudomonas* AM 1 enzyme. It caused 7% inhibition of the *Rm. vannielii* enzyme and 10% inhibition of the *Rs. acidophila* 7750 enzyme under conditions where the *Rs. acidophila* 10050 enzyme was inhibited by 80%.

Molecular weight of alcohol dehydrogenase from Rs. acidophila

A mol.wt. of 116000 was obtained for the enzyme in 20 mm-potassium phosphate, pH 7.0, by using gel-permeation chromatography on Sephadex G-150 by the method of Andrews (1964). Standard proteins used to calibrate the column were: malate dehydrogenase (ox heart mitochondria, mol.wt. 62000), hexokinase (yeast, mol.wt. 100000), lactate dehydrogenase (pig heart, mol.wt. 140000) and alcohol dehydrogenase (NAD⁺-linked, yeast, mol.wt. 151000). The void volume of the column was determined with Blue Dextran.

Discussion

In several respects the dye-linked alcohol dehydrogenase isolated from *Rs. acidophila* 10050 grown phototrophically on methanol resembles many previously reported methanol dehydrogenases from other methylotrophic organisms; thus it couples to phenazine methosulphate, requires ammonia as an activator, has a pH optimum of approx. 9 and has a mol.wt. of approx. 120000. In many other respects, however, the enzyme differs markedly from these classical methanol dehydrogenases.

The enzyme from *Rs. acidophila* has a different substrate specificity compared with other methanol dehydrogenases. In general, electron-donor substrates for the methanol dehydrogenases of many methylotrophic bacteria are primary alcohols, with

methanol being the best substrate (Anthony & Zatman, 1965; Sperl *et al.*, 1974; Patel & Felix, 1976; Yamanaka & Matsumoto, 1977). Secondary alcohols are not oxidized, although Goldberg (1976) reported a low activity of the enzyme from *Pseudomonas C* with propan-2-ol. Aldehydes which exist in aqueous solution substantially as hydrates mimic primary alcohols as substrates and can be oxidized. Thus formaldehyde is generally oxidized almost as well as methanol, and acetaldehyde, trifluoroacetaldehyde and trichloroacetaldehyde may serve as poor substrates (Sperl *et al.*, 1974). Against this background the enzyme from *Rs. acidophila* stands apart; it oxidizes secondary alcohols such as propan-2-ol and butan-2-ol and, in addition to formaldehyde, it oxidizes acetaldehyde (and propionaldehyde; Sahm *et al.*, 1976). The wide substrate specificity of the enzyme prompts us to refer to it as a 'dye-linked alcohol dehydrogenase'.

Differences in substrate specificity are also evident with respect to the activator. All methanol dehydrogenases are activated by ammonia and, in most cases [see Michalik & Raczynska-Bojanowska (1976) for an exception] methylamine, with ammonia being the preferred activator for *Pseudomonas* 2941 (Yamanaka & Matsumoto, 1977) and *Pseudomonas C* (Goldberg, 1976). By contrast, many aliphatic primary amines and ammonia serve as activators for the *Rs. acidophila* enzyme. The enzyme shows a greater affinity for the amines than for ammonia and the affinity increases with their increasing chain length, at least up to nonylamine (the longest tested). Since the basicity of primary amines is relatively unaffected by the length of the carbon chain, the increasing efficacy of the long-chain amines indicates that in this enzyme the binding site for the amine activator is hydrophobic in nature, and hence *in vivo* the activator might be a lipophilic membrane component, possibly a basic protein.

The enzyme from *Rs. acidophila* is inhibited by the metal-chelating agents $\alpha\alpha'$ -bipyridine and EDTA; where tested, other methanol dehydrogenases are similarly inhibited. By contrast, the enzyme from *Rs. acidophila* is the only enzyme so far reported to be inhibited by cyanide. Kinetic experiments reported in the present study have shown that the inhibition by cyanide and $\alpha\alpha'$ -bipyridine is competitive with respect to the amine activator, methylamine. This suggests that at the active centre of the enzyme, the amino group of the activator forms a ligand with an electrophilic moiety, possibly a metal atom, a site for which cyanide and $\alpha\alpha'$ -bipyridine can compete.

An interesting property of the enzyme from *Rs. acidophila*, not previously reported in other dye-linked methanol dehydrogenases, is its reversible activation on incubation at 30°C in buffer solution alone. This behaviour is reminiscent of succinate dehydrogenase from pig heart mitochondria, which

can be activated by up to 4-fold on incubation in phosphate, Tris or borate buffers at 37°C (Thorn, 1962). The activation of succinate dehydrogenase was reversible and it was postulated that the free enzyme existed in unactivated and activated forms, differing in configuration, the equilibrium between the two forms being temperature-dependent. Further work is needed to clarify the mechanism of thermal activation of the enzyme from *Rs. acidophila*.

Probably the most striking property of the enzyme from *Rs. acidophila* is its extremely low affinity for methanol (apparent K_m 0.145M), an observation first reported by Sahm *et al.* (1976) and which led to the present extended investigation. This K_m value may be compared with those of methanol dehydrogenases from non-photosynthetic methylotrophs, which have an apparent K_m for methanol of approx. 10 μ M (Sperl *et al.*, 1974; Michalik & Raczynska-Bojanowska, 1976; Yamanaka & Matsumoto, 1977). Such a low affinity is a most unexpected property for a key enzyme that initiates the attack on a growth substrate. Sahm *et al.* (1976) noted that the apparent K_m of the enzyme with respect to methanol was lowered 10-fold if the enzyme was prepared and assayed anaerobically. This observation has been confirmed in the present study by using more-purified enzyme, and kinetic analysis has shown that O₂ acts as an inhibitor, competitive with respect to the alcohol substrate. Hence the apparent 'activation' of the enzyme under anaerobic conditions is, in reality, due to the release of the enzyme from the presence of an inhibitor. No such observation has been made with previously reported methanol dehydrogenases. The enzyme from *Pseudomonas* AM1 was shown not to be affected in this manner, whereas enzymes from other photosynthetic bacteria grown anaerobically in the light on methanol, namely *Rs. acidophila* 7750 and *Rm. vannielii* 7255, were similar to that from *Rs. acidophila* 10050.

The most common explanation for O₂ sensitivity of an enzyme invokes the oxidation of essential thiol groups. This does not seem a likely explanation for the behaviour of the enzyme from *Rs. acidophila*, because it is not appreciably inhibited by the thiol reagents *p*-chloromercuribenzoate, iodoacetate and *N*-ethylmaleimide. Another reason for O₂ sensitivity can be reaction of O₂ with a substrate or reaction intermediate, as with nitroreductase where electron transfer from intermediary nitro radical anions to O₂ takes place (Mason & Holtzman, 1975). This explanation seems unlikely in the present case, with O₂ being a competitive inhibitor with respect to the alcohol substrate. The true explanation must await the elucidation of the active centre of the alcohol dehydrogenase and the reaction mechanism that it catalyses. We may suppose that there is an electrophilic site in the enzyme which binds both an amino group from the activator and certain metal-chelating

agents, and there is also an electrophilic site which binds the alcohol and can competitively bind O_2 . We might speculate that both sites are at the active centre of the enzyme which contains a metal group, this metal group being involved in binding of the above four ligands. The role of the pteridine-like cofactor, which all methanol dehydrogenases, apparently including that from *Rs. acidophila* (Sahm *et al.*, 1976), possess, is not known.

The properties of the enzyme from *Rs. acidophila* pose several fascinating evolutionary and metabolic questions. Studies in bacterial methylotrophic metabolism have revealed the ubiquity of the now-classical methanol dehydrogenase with its many highly distinctive properties. It seems likely that such enzymes, all of which are highly efficient primary alcohol dehydrogenases, must have evolved from a common ancestor. On the other hand, phototrophic organisms (as far as has been examined) synthesize a somewhat different enzyme whose kinetic properties suggest that it is a higher-alcohol dehydrogenase by design and a methanol dehydrogenase by accident. It might therefore have evolved quite independently with respect to the classical methanol dehydrogenase or, alternatively, the photosynthetic bacteria may possess a primitive form of alcohol dehydrogenase which has not fully evolved to the form found in aerobic organisms.

The very low affinity of the photosynthetic enzyme for methanol and its competitive inhibition by O_2 may be causally related to the fact that although *Rs. acidophila* grows readily under aerobic conditions in the dark on a variety of organic substrates, including ethanol (Pfennig, 1969), we have been unable to grow it appreciably in batch culture on methanol/ HCO_3^- under air/ CO_2 (19:1). Its growth physiology on methanol under conditions of lower O_2 partial pressure needs to be explored further.

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