

Structural Aspects of the Dye-Linked Alcohol Dehydrogenase of *Rhodopseudomonas acidophila*

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1. A dye-linked alcohol dehydrogenase was purified 60-fold from extracts of *Rhodopseudomonas acidophila* 10050 grown aerobically on ethanol. 2. The properties of this enzyme were identical with those of the alcohol dehydrogenase synthesized by this organism during growth on methanol anaerobically in the light, and they are judged to be the same enzyme. 3. The enzyme gave a single protein band, coincident with alcohol dehydrogenase activity, during electrophoresis on polyacrylamide gel. 4. The amino acid composition, isoelectric point, u.v. and visible absorption spectra of the enzyme were determined and compared with those of other similar enzymes. 5. The presence of 0.7-1.0 g-atom of non-haem, acid-labile iron/mol of enzyme was shown by atomic absorption spectrophotometry and colorimetric assay. The iron could not be dissociated from the enzyme by dialysis against chelating agents. 6. E.p.r. spectroscopy of the enzyme did not indicate any redox function for the iron during alcohol dehydrogenation, but showed a signal at $g = 2.00$ consistent with the presence of a protein-bound organic free radical. 8. Antisera were raised against alcohol (methanol) dehydrogenases purified from *Rhodopseudomonas acidophila*, *Paracoccus denitrificans* and *Methylophilus methylotrophus*. 9. The antiserum to the *Rhodopseudomonas acidophila* enzyme cross-reacted with neither of the two other antisera, nor with crude extracts of methanol-grown *Hyphomicrobium* X and *Pseudomonas* AM1, thus emphasizing its singular biochemical properties.

The dye-linked alcohol dehydrogenase (EC 1.1.99.8) of *Rhodopseudomonas acidophila* strain 10050 (Sahm *et al.*, 1976) has been shown by Bamforth & Quayle (1978a) to differ in several respects from the now classical bacterial methanol dehydrogenase first isolated by Anthony & Zatman (1967a), e.g.: different specificity towards electron donors; much lower affinity for methanol; inhibition by oxygen, competitive with respect to electron donor; different specificity towards activating amino compounds; greater sensitivity to chelating agents; thermal activation. The enzyme preparation used for these studies was obtained from methanol-grown *R. acidophila*, and by the criterion of gel electrophoresis contained five other protein components (Bamforth & Quayle, 1978a). The present paper records its purification to homogeneity and a more detailed examination of some structural aspects of the enzyme.

Materials and Methods

Chemicals

All reagents were of analytical grade where possible

Abbreviation used: SDS, sodium dodecyl sulphate.

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and were obtained from sources listed previously (Bamforth & Quayle, 1978a).

Maintenance and growth of organisms

R. acidophila 10050 was maintained and grown in aerobic batch culture in the dark in basal medium (Quayle & Pfennig, 1975) containing the chelated trace-element solution SL₄ described by Sahm *et al.* (1976). Na₂CO₃ was omitted from the medium and the pH was adjusted to 7.0 by the addition of NaOH before autoclaving. For the experiments, 1-litre batches of the medium were used in 2-litre Erlenmeyer flasks shaken at 30°C on a gyrotary shaker. The carbon source was 0.2% (w/v) ethanol unless otherwise stated. Growth was followed by measurement of A₅₇₀. The mean generation time of the organism on ethanol under these conditions was 7.7h. The following organisms were grown on methanol as carbon source as previously described: *Paracoccus denitrificans* (N.C.I.B. 8944) (Bamforth & Quayle, 1978b); *Pseudomonas* AM1 (Cox & Quayle, 1976); *Hyphomicrobium* X (Harder *et al.*, 1973); *Methylophilus methylotrophus* (*Pseudomonas methylotropa*) (Byrom & Ousby, 1975; Ghosh & Quayle, 1978).

Preparation of cell-free extracts

Cells were harvested in mid-exponential phase by centrifugation at 5000g for 10 min and were washed once in 50 mM-potassium phosphate, pH 7.0, before resuspension in 5 vol. of the same buffer. Cells were disrupted by using an MSE ultrasonic disintegrator (model 150W) at full power. Exposure was in 4 × 1 min bursts interspersed with cooling in ice. Extracts were subsequently centrifuged at 38000g for 10 min and the supernatant was retained. With *R. acidophila*, the pelleted material was resuspended, resonicated and re-centrifuged as above. The supernatants, or combined supernatants from *R. acidophila*, are referred to as 'crude extract'.

Chemical determinations

Protein. This was determined routinely by the method of Kalb & Bernlohr (1977). In purified preparations of the enzyme, protein was determined both by the method of Lowry *et al.* (1951) (with dried bovine serum albumin as standard) and by the method of Warburg & Christian (1941). Samples giving a protein concentration of 480 µg/ml by the Lowry *et al.* (1951) method yielded an A_{280} of 0.475 and A_{260} of 0.295, which by the equation of Warburg & Christian (1941) gave a protein concentration of 510 µg/ml.

Iron. Acid-labile iron was released from the enzyme essentially by the method of Eady *et al.* (1971), as follows. Purified alcohol dehydrogenase was precipitated from solution by 55% saturation of the solution with $(\text{NH}_4)_2\text{SO}_4$ at 4°C for 4 days. The precipitate was collected by centrifugation at 38000g for 15 min and was redissolved in 10 mM-potassium phosphate, pH 7.0 (final concentration 7.8 mg of protein/ml). The purified, concentrated enzyme (3.9 mg of protein) was treated with 0.5 vol. of 1 M-HCl. After standing for 45 min at room temperature, 1 vol. of 1.23 M-trichloroacetic acid was added and the precipitate removed by centrifugation at 38000g for 10 min. Iron present in the supernatant was determined initially by the colorimetric method of Bard & Gunsalus (1950) with ferrous ammonium sulphate standardized by the method of Belcher & Nutten (1967) as standard. Iron was also measured by atomic-absorption spectrophotometry in a Pye Unicam SP.191 atomic-absorption spectrophotometer operating at 248.3 nm. Ferric nitrate standardized for atomic-absorption spectrophotometry was used for calibration purposes. All solutions were converted into identical concentrations in phosphate, trichloroacetate and HCl before analysis. Solutions were prepared in distilled, deionized water. All glassware was prewashed in 20% (v/v) HNO_3 , followed by distilled water. Iron was also determined in untreated samples of the protein by passage of a solution containing purified enzyme through the atomic-absorption spectrophotometer.

Sulphur. Acid-labile sulphide was measured by the method of Siegel (1965) with Na_2S as standard.

Assay of dye-linked alcohol dehydrogenase (EC 1.1.99.8)

Activity was determined spectrophotometrically by the method of Bamforth & Quayle (1978a) with 1000 µmol of methanol as substrate.

Purification of alcohol dehydrogenase

Step 1: heat treatment. Crude extracts in Pyrex boiling tubes (2.5 cm × 15 cm) were heated to 60°C by immersion in a thermostatically controlled water bath, a pear-drop condenser being used to prevent excessive evaporation. After 25 min the tubes were rapidly cooled in ice before removal of the precipitate by centrifugation at 38000g for 10 min.

Step 2: salt fractionation. The supernatant from step 1 was adjusted to 55% saturation with $(\text{NH}_4)_2\text{SO}_4$ by the slow addition of finely divided solid $(\text{NH}_4)_2\text{SO}_4$ with mixing at 4°C [the amounts required were calculated from the Table of Dawson *et al.* (1969)]. After stirring for 15 min the precipitate was removed by centrifugation at 38000g for 10 min. The supernatant was adjusted to 85% saturation in the same manner and the resultant precipitate was collected by centrifugation and redissolved in a minimum volume of 1 mM-potassium phosphate, pH 7.0.

Step 3: gel-permeation chromatography. The dissolved precipitate from step 2 was applied at 4°C to a column (2 cm × 34 cm) of Sephadex G-150 equilibrated in 1 mM-potassium phosphate, pH 7.0. The column was eluted in descending mode with the same buffer. Fractions showing alcohol dehydrogenase activity but devoid of cytochrome *c*, as located by measuring A_{416} , were pooled.

Step 4: ion-exchange chromatography. Active fractions from step 3 were applied at room temperature to a column (1 cm × 4 cm) of CM-cellulose (Whatman CM-52) equilibrated in 1 mM-potassium phosphate, pH 7.0. The column was washed with 5 column vol. of both 1 mM- and 5 mM-buffer before elution of the enzyme in 10 mM-potassium phosphate, pH 7.0.

Concentration of the enzyme. The enzyme was evaporated to dryness by freeze-drying and the resultant feathery material was redissolved in a minimum volume of distilled water and dialysed to the required ionic specifications. Full recovery of activity and protein was achieved by this procedure. Concentration with a Minicon macro-solute concentrator (Amicon Corp., Lexington, MA 02173, U.S.A.) gave 64% loss of protein. Selective thawing of samples from -15°C led to no concentration of material, the solution retaining a similar A_{280}/A_{260} ratio throughout thawing.

Polyacrylamide-gel electrophoresis

Analytical polyacrylamide-gel electrophoresis at pH 8.3 was performed at room temperature as described by Bamforth & Quayle (1978a). Protein was located by three separate methods: with Coomassie Blue as described by Bamforth & Quayle (1978a); with Naphthalene Black as described by Bamforth & Quayle (1977); and by the method of Blakesley & Boezi (1977). Alcohol dehydrogenase activity was located as described by Bamforth & Quayle (1978a). Gels were stained for haem iron by the method of Clarke (1964) and for non-haem iron by the method of Brill *et al.* (1974).

Analytical polyacrylamide-gel electrophoresis at pH 4.3 was performed at room temperature by the method of Reisfeld *et al.* (1962), protein and activity stains being performed as above.

SDS/polyacrylamide-gel electrophoresis to determine the subunit molecular weight of alcohol dehydrogenase was performed essentially as described by Bamforth & Large (1977), except that electrophoresis was at 5 mA/gel until the tracking dye Bromophenol Blue had reached the bottom of the gel. Protein was located by staining with Coomassie Blue.

Amino acid analysis

Two separate samples (0.17 mg of protein, 0.13 ml of solution) of pure alcohol dehydrogenase in 10 mM- $(\text{NH}_4)_2\text{CO}_3$ solution were evaporated to dryness in a rotary evaporator in 6-in (15 cm) Pyrex test tubes, prewashed in 20% (v/v) HNO_3 . The residues were taken up in 2 ml of 6M-HCl, frozen in liquid nitrogen and the tubes evacuated before being sealed in a flame. The samples were hydrolysed at 110°C for 48 and 72 h respectively and analysed for amino acid composition by the method of Atkin & Ferdinand (1970, 1971). Elution traces were quantified by a manual integration procedure. Tryptophan was estimated by measurement of the absorbance of an alcohol dehydrogenase solution at 280 and 294.4 nm as described by Beaven & Holiday (1952). Thiol and disulphide residues were measured in untreated and NaBH_4 -reduced portions of the enzyme by titration with 5,5'-dithiobis-(2-nitrobenzoate) as described by Cavallini *et al.* (1966). Reduction with NaBH_4 was for 30 min. In all calculations relating to amino acid composition a molecular weight of 116000 was assumed for alcohol dehydrogenase (Bamforth & Quayle, 1978a).

Isoelectric focusing

This was performed essentially as described by Bamforth & Quayle (1978b), except that the anode was arranged to be at the top of the column. The enzyme sample used was a crude extract heated at 60°C for 25 min and centrifuged at 38000g for

10 min. The sample (10 ml) was in 50 mM-potassium phosphate, pH 7.0, and consisted of 98 mg of total protein.

E.p.r. spectroscopy

Spectra were recorded at 8.972 GHz in a Varian E-line Century spectrometer (Varian Associates, Walton-on-Thames, Surrey, U.K.) with an ESR-9 cryostat (Oxford Instruments, Osney Mead, Oxford, U.K.). The microwave power was 1 mW and the modulation amplitude was nominally 4 mT. The protein concentration of the sample was 6 mg/ml.

Immunological studies

Antigens. Methanol dehydrogenases, purified to homogeneity, from the following sources were used as antigens for injection into rabbits: *R. acidophila* 10050 (this paper); *P. denitrificans* (Bamforth & Quayle, 1978b); *M. methylotrophus* (Ghosh & Quayle, 1978).

Antisera. New Zealand white rabbits (male, 2.5 kg) were inoculated intramuscularly with 1.0 ml of an emulsion which consisted of 1.0 mg of purified enzyme in aqueous solution mixed with an equal volume of Freund's Complete Adjuvant (Difco Laboratories, West Molesey, Surrey, U.K.). Then 6 weeks later 0.5 ml of aqueous solution containing 1.0 mg of enzyme was inoculated intramuscularly, and 10 days after the second inoculation 20 ml of blood was taken from the marginal ear vein, and after clotting the serum was separated and stored at -20°C.

Immunodiffusion. Ouchterlony double diffusion was conducted in Petri dishes containing 15 ml of a mixture of Tris/HCl (pH 7.3, 10 mM), NaCl (1.6%, w/v), NaN_3 (0.4%, w/v) and agarose (0.9%, w/v). When the contents of the Petri dishes had solidified, square Perspex templates (3 mm thickness and 25 mm square) were laid on top. Each template had a central hole (3 mm diameter) drilled through it, symmetrically surrounded by six similar holes, the centres of which were 5 mm apart from each other and from that of the centre hole. This system of holes formed the wells for introduction of solutions (10 μl) of antisera and antigens for immunodiffusion within the underlying agarose layer. The Petri dishes were sealed and incubated at room temperature (15-20°C) for 2-3 h and then stored at 4°C. Several dilutions of antiserum and antigen solutions were used and combinations chosen such that sharp precipitin bands appeared halfway between the wells.

Results

Purification of alcohol dehydrogenase of R. acidophila

Previous work had indicated that *R. acidophila* is capable of growth on methanol anaerobically in the

Table 1. Purification of alcohol dehydrogenase of *R. acidophila*

The crude extract was prepared from 4g (wet wt.) of cells grown aerobically on ethanol. 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	78	4.3	14.4	0.299	1	100
1. Heat treatment	68	3.8	4.3	0.884	3	78
2. 55–88%–satd.-(NH ₄) ₂ SO ₄ fraction	3.8	46.00	15.3	3.01	10	53
3. Sephadex G-150 chromatography	65	2.65	0.68	3.90	13	52
4. CM-52 chromatography	50	3.05	0.17	17.9	60	46

* Determined by the method of Kalb & Bernlohr (1977).

Table 2. Amino acid composition of alcohol dehydrogenase from *R. acidophila*

Amino acids were determined as described in the Materials and Methods section. A mol.wt. of 116000 was assumed for the enzyme. The protein concentration was determined by the methods of both Lowry *et al.* (1951) and Warburg & Christian (1941). Values of amino acid content have been rounded off to the nearest integer. Results are the average of 48h and 72h hydrolysis unless indicated otherwise.

	Amino acid content	
	(mol/mol of enzyme)	(g/100g of protein)
Aspartate	119	12
Threonine*	70	6
Serine*	59	4
Glutamate	98	11
Proline	71	6
Glycine	133	7
Alanine	87	5
Valine†	59	5
Methionine	16	2
Isoleucine	51	5
Leucine	60	6
Tyrosine*	52	7
Phenylalanine	40	5
Lysine	59	7
Histidine	21	2
Arginine	40	5
Tryptophan‡	29	5
Cysteine§	0	0
Cystine	25	2

* Values extrapolated to zero time.

† 2h hydrolysis.

‡ Assayed spectrophotometrically.

§ Estimated as total SH measurable without prior enzyme reduction.

|| Estimated as half the total SH measurable after reduction of enzyme with NaBH₄.

light or aerobically under special conditions (Pfennig & Siefert, 1977), whereas growth on ethanol occurred readily under either of these two conditions. Because of the greater convenience of growing large quantities of the organism aerobically, it was first ascertained whether the alcohol dehydrogenase obtained from cells grown aerobically on ethanol differed from that isolated from cells grown photosynthetically on methanol. It was found that the enzyme partially purified from ethanol-grown cells was unchanged in respect of the following properties: high apparent K_m for methanol (75mM), thermal activation (85% stimulation after 3min at 30°C in 100mM-Tris/HCl, pH9.0), sensitivity to KCN and $\alpha\alpha'$ -bipyridine, stability at 60°C, mobility on 7% polyacrylamide gels at pH8.3 and specific activity.

On this basis it was decided to use *R. acidophila* grown aerobically on ethanol as source of the enzyme. It may be noted that subsequent work by the purification procedure outlined in the Materials and Methods section led to a material 60-fold purified over that in the crude extract (Table 1). On analysis in 7% polyacrylamide gel at pH8.3 there was a single detectable protein band of R_F 0.1 (relative to Bromophenol Blue). This band coincided with a single band of alcohol-dependent Nitro Blue Tetrazolium reductase activity and also with a single brown band when stained for haem iron by the method of Clarke (1964). When stained for non-haem iron by the method of Brill *et al.* (1974), a faint bluish band was obtained, with R_F identical with that of alcohol dehydrogenase. The band intensified somewhat on storage in 7% (v/v) acetic acid. When gels were run at pH4.3 one major protein band was visible, of R_F 0.3 (relative to Methyl Green), which coincided with a single, slowly developing activity band. There was some indication of a very minor protein band (R_F 0.45), not associated with activity.

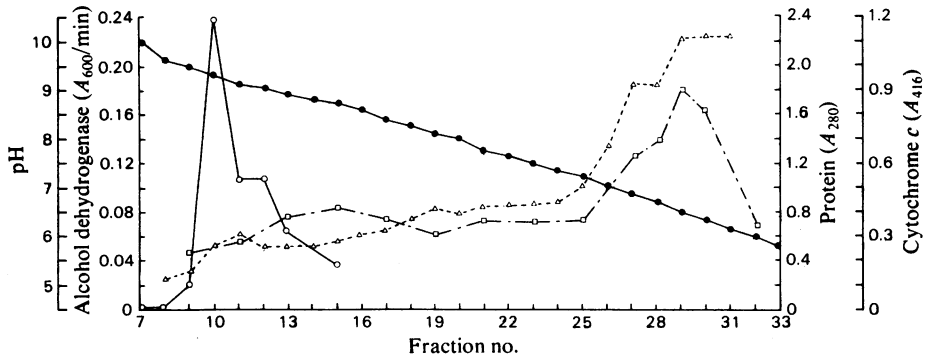


Fig. 1. Isoelectric focusing of alcohol dehydrogenase of *R. acidophila*

Focusing was performed as described in the Materials and Methods section. ●, pH; ○, alcohol dehydrogenase activity (A_{600}/min); □, cytochrome *c* (A_{416}); △, protein (A_{280}).

Subunit molecular weight of alcohol dehydrogenase

By using the protein markers of the Boehringer Combithek kit (cat. no. 15661) a subunit molecular weight of 63000 was obtained for the only band resulting from alcohol dehydrogenase. Together with a molecular weight for the holoenzyme of approx. 116000 measured by gel-permeation chromatography (Bamforth & Quayle, 1978a), this suggests that the present enzyme, in common with most enzymes of this type (Anthony & Zatman, 1967a,b), is a dimer of identical subunits.

Amino acid analysis

In amino acid content (Table 2) the enzyme is similar to previously reported methanol dehydrogenases (Anthony & Zatman, 1967a; Goldberg, 1976). Notably, this and other enzymes are deficient in free thiol groups, a property reflected in their insensitivity to inhibitors such as iodoacetic acid and *p*-chloromercuribenzoate (Goldberg, 1976; Bamforth & Quayle, 1978a). Overall, the considerable differences in kinetic properties of the alcohol dehydrogenase of *R. acidophila* compared with other dye-linked methanol dehydrogenases is not reflected in a substantially altered content of amino acid residues.

Isoelectric focusing

An isoelectric point of 9.35 for the present enzyme (Fig. 1) can be compared with values of 7.38 for an enzyme of the Anthony-Zatman type (Yamanaka & Matsumoto, 1977a) and of 3.82 and 3.7 respectively for the methanol dehydrogenase of *Pseudomonas fluorescens* S50 (Yamanaka & Matsumoto, 1977b) and *P. denitrificans* (Bamforth & Quayle, 1978b). The increased basicity of the present species is not readily predicted in terms of amino acid composition and is probably related to a differing primary structural sequence. Yamanaka & Matsumoto (1977b) noted the similarity in amino acid compo-

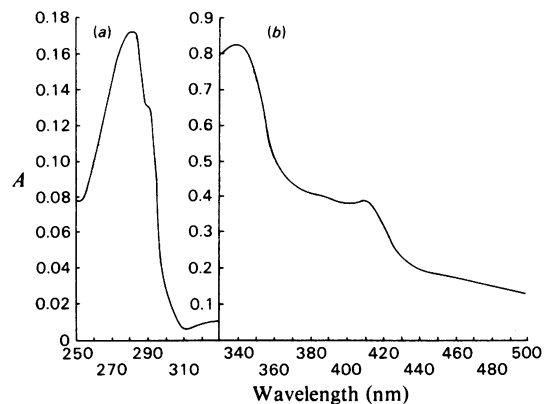


Fig. 2. Absorption spectrum of alcohol dehydrogenase of *R. acidophila*

Spectra were determined in a Unicam SP.1800 spectrophotometer against a reference cuvette containing water (light-path was 1 cm): (a) 174 µg of protein/ml; (b) 5.75 mg of protein/ml.

sition between methanol dehydrogenase of *Pseudomonas* sp. 2941 (pI 7.38) and *Pseudomonas fluorescens* S25 (pI 9.40), whereas the enzyme from *Pseudomonas fluorescens* S50 (pI 3.82) was enriched in glutamic acid.

U.v. and visible absorption spectra

Alcohol dehydrogenase shows absorption maxima at 280, 340 and 410 nm, with a shoulder at 290 nm (Fig. 2). The wavelength of the peak at 340 nm is slightly lower than that observed (345–350 nm) in all methanol dehydrogenases reported to date (e.g. Yamanaka & Matsumoto, 1977a; Patel *et al.*, 1978; Duine *et al.*, 1978), and was first tentatively interpreted in terms of a pteridine prosthetic group

(Anthony & Zatman, 1967*b*), although more recent evidence is now indicating that it is a quinonoid compound (Duine *et al.*, 1978). Methanol/methylamine mixture or dithionite were without effect on the observed spectrum.

Iron content of alcohol dehydrogenase

Iron (0.62 g-atom/mol of enzyme) was determined by atomic-absorption spectrophotometry in the intact purified alcohol dehydrogenase. Iron, released from the enzyme by acid treatment, amounted to 0.73 and 1.0 g-atom/mol of enzyme when measured by atomic-absorption spectrophotometry and colorimetric assay respectively. These values are approximate, since in all these measurements the amount of enzyme was calculated not from dry weights but from absorbance measurements by the method of Warburg & Christian (1941) (see the Materials and

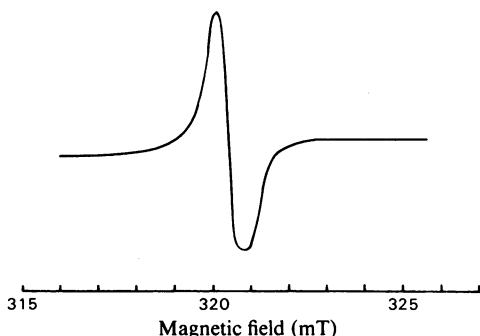


Fig. 3. *E.p.r. spectrum of alcohol dehydrogenase of R. acidophila*

The spectrum is of oxidized enzyme and was measured at 14.9 K. Other conditions were as described in the Materials and Methods section.

Methods section) and by using a molecular weight for the alcohol dehydrogenase of 116000 (Bamforth & Quayle, 1978*a*).

Acid-labile sulphur was undetectable by the method of Siegel (1965), which is sensitive to less than 2 nmol of sulphide. Assuming only one acid-labile sulphur residue per molecule of enzyme, sufficient protein was treated to give an A_{650} of at least 0.8 in the determination.

E.p.r. spectroscopy

Analysis of the untreated enzyme at 14.9 K gave a signal of $g = 2.00$ (Fig. 3). The signal was amplified greatly at 2.9 K. This is the second report of such a signal being found in a methanol dehydrogenase, Duine *et al.* (1978) having previously observed an identical signal in the enzyme from *Hyphomicrobium X*. This signal is consistent with the presence of a protein-bound organic free radical. Sodium dithionite or methanol/methylamine mixture quenched the signal from the *R. acidophila* enzyme, but methanol addition had no effect on the signal from the *Hyphomicrobium* enzyme (Duine *et al.*, 1978).

There were no signals in either enzyme preparation to indicate the presence of haem iron or iron-sulphur entities of either the ferredoxin or rubredoxin type.

Attempts to dissociate iron from alcohol dehydrogenase

We have previously reported unsuccessful attempts to inactivate alcohol dehydrogenase by dialysis of the enzyme against KCN solution (Bamforth & Quayle, 1978*a*). When the enzyme was dialysed against 250 vol. of 50 mM-EDTA (disodium salt) in 50 mM-potassium phosphate, pH 7.0, at 4°C for 16 h the total activity recovered was unaltered from that of control samples left undialysed at 4°C. Addition of 10 mM-sodium dithionite to the dialysis buffer, which was subsequently removed by dialysis for 4 h against

Table 3. *Immunological relationships between methanol dehydrogenases*

Antisera were raised against the purified enzymes from *R. acidophila*, *P. denitrificans* and *M. methylotrophus* and tested by Ouchterlony double diffusion against the same purified enzymes and also crude extracts of methanol-grown *Hyphomicrobium X* and *Pseudomonas AM1*. Experimental details are given in the Materials and Methods section. +, Positive cross-reaction; -, no cross-reaction.

Antiserum in centre well	Content of outer wells				
	Purified enzymes			Crude extracts	
	<i>R. acidophila</i>	<i>P. denitrificans</i>	<i>M. methylotrophus</i>	<i>Hyphomicrobium X</i>	<i>Pseudomonas AM1</i>
<i>R. acidophila</i>	+	-	-	-	-
<i>P. denitrificans</i>	-	+	-	+	-
<i>M. methylotrophus</i>	-	-	+	+*	+*
Serum from untreated rabbit	-	-	-	-	-

* When in adjacent wells, the precipitin bands gave confluent arcs of identity.

50mM-potassium phosphate, pH 7.0, at 4°C, made no difference, unlike the case of the ω -hydroxylase from *Pseudomonas oleovorans*, from which non-haem iron was removed by dialysis against EDTA under reducing conditions (Ruettinger *et al.*, 1977).

Immunological properties

The antisera raised against the purified enzymes from *R. acidophila*, *P. denitrificans* and *M. methylotrophus* each gave a single sharp precipitin band when diffused against its parent enzyme, but there was no cross-reaction visible between them (Table 3), thus showing the immunological non-identity of these three enzymes. The *P. denitrificans* antiserum cross-reacted with crude extract of *Hyphomicrobium* X, whereas the *M. methylotrophus* antiserum cross-reacted with crude extracts of *Hyphomicrobium* X and *Pseudomonas* AM1. The precipitin lines from the last two sources of antigen were confluent, indicating immunological identity.

Discussion

The results in the present and the previous paper (Bamforth & Quayle, 1978a) show that, whereas the dye-linked alcohol dehydrogenase of *R. acidophila* possesses similarities to other methanol dehydrogenases, it nevertheless displays many different properties. Thus, whereas the enzyme possesses a similar amino acid composition, subunit and intact protein size and similar (though not completely identical) absorption spectrum to other methanol dehydrogenases, it shows different specificities and affinities towards electron donors and amine activators; it can be thermally activated; it shows a greater sensitivity to certain chelating agents. The last-named property may be connected with a major structural singularity of the *R. acidophila* enzyme, namely the presence of acid-labile non-haem iron at 0.6–1.0g-atom/mol of intact enzyme. The only other report of significant quantities of metal in a similar enzyme is that of Duine *et al.* (1978), who found iron and manganese in preparations of methanol dehydrogenase from *Hyphomicrobium* X to the extent of 0.14g-atom of metal/mol of enzyme. The iron atom in the *R. acidophila* enzyme does not appear to be bound to either organic or inorganic sulphur residues, as neither free thiol groups nor acid-labile sulphur can be detected in the enzyme. The absence of e.p.r. signals of $g = 1.94$ or $g = 4.32$ is consistent with the present enzyme not being a ferredoxin- or rubredoxin-type protein. That an e.p.r. signal corresponding to iron is not detected in either untreated or reduced enzyme suggests that the iron atom is not involved as a redox agent during methanol dehydrogenation *in vitro*. The presence of a faint blue band on polyacrylamide gels stained by the method of Brill *et al.* (1974) probably does not reflect the presence of iron,

as similar staining was shown by methanol dehydrogenase from *P. denitrificans*, which does not contain detectable non-haem iron (Bamforth & Quayle, 1978b).

The uniqueness of the enzyme from *R. acidophila* is further emphasized by the absence of any cross-reaction between the antiserum raised against it and the purified enzymes from *P. denitrificans* and *M. methylotrophus* or crude extracts of methanol-grown *Pseudomonas* AM1 and *Hyphomicrobium* X. Similarly the antisera raised against the purified methanol dehydrogenase from *P. denitrificans* and *M. methylotrophus* showed no cross-reaction with the purified enzyme from *R. acidophila*. It may also be noted that Wolf & Hanson (1978) reported that methanol dehydrogenase purified from *Methylobacterium organophilum* was serologically related to methanol dehydrogenases from several methylotrophic bacteria, but not from *R. acidophila*.

Despite these differences, the similarity of the u.v., visible and e.p.r. spectra to those of the enzyme from *Hyphomicrobium* X leaves little doubt that the mechanism of action of these different bacterial methanol dehydrogenases must be essentially similar. Further biophysical work is needed to elucidate the precise differences at the molecular level between the enzymes from photosynthetic and non-photosynthetic organisms and the physiological rationale for these differences.

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