Purification and Characterization of D(–)-Mandelate Dehydrogenase from *Rhodotorula graminis*

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A soluble, NAD⁺-dependent D(–)-mandelate dehydrogenase has been purified to homogeneity from the yeast *Rhodotorula graminis* strain KGX 39, using DEAE-Sephacel, Phenyl Sepharose and Matrex Gel Orange A affinity chromatography. The M_r of the native enzyme was 77 000 (as determined by gel filtration) and the subunit M_r was 38 000 (as determined by SDS-polyacrylamide-gel electrophoresis), indicating that the enzyme exists as a dimer. Amino acid analysis showed only one cysteine residue per subunit. There was no spectroscopic evidence for the presence of cofactors such as flavin or cytochrome. The enzyme was neither inhibited nor stimulated by a wide range of salts and metal ions, nor was it inhibited by various metal chelating agents, indicating that the enzyme has no absolute requirement for salt or metal ions. The enzyme was not inhibited by various thiol reagents. The isoelectric point was 5.9. D(–)-Mandelate dehydrogenase catalyses the oxidation of D(–)-mandelate (forward reaction) and the reduction of phenylglyoxylate (reverse reaction). Activities of the forward reaction and reverse reactions were maximal at pH 9.5 and pH 5.85, respectively. Oxidation of D(–)-mandelate produced equimolar amounts of phenylglyoxylate and NADH, and the equilibrium constant was 1.59×10^{-11} M, pH 9.2. At pH 9.5, the K_m values for D(–)-mandelate and NAD⁺ were 319 μ M and 71 μ M respectively, and the maximum velocity was 123 μ mol min⁻¹ (mg of protein)⁻¹. The apparent K_m and V values with respect to D(–)-mandelate, NAD⁺, phenylglyoxylate and NADH at pH 5.85, pH 7.0 and pH 9.5 are recorded. D(–)-Mandelate dehydrogenase uses various substituted mandelates as substrates. Several aliphatic 2-hydroxy and 2-oxocarboxylic acids did not act as substrates but were capable of inhibiting the enzyme. Comparisons are made with other mandelate dehydrogenases and NAD⁺-dependent dehydrogenases.

INTRODUCTION

Various bacteria and fungi are capable of growing on either D(–), L(+) or DL-mandelate as sole source of carbon and energy (Fewson, 1988). The first step in the dissimilation of mandelate usually involves stereospecific oxidation to phenylglyoxylate, and this is then degraded via benzaldehyde to benzoate. In several bacteria, including *Acinetobacter calcoaceticus* and probably *Pseudomonas putida*, the mandelate dehydrogenases are NAD(P)⁺-independent integral membrane proteins requiring flavin as prosthetic group (Hegeman, 1966; Allison *et al.*, 1985b; Hoey *et al.*, 1987). In the yeast *Rhodotorula graminis*, preliminary evidence indicated the presence of a dye-linked L(+)-mandelate dehydrogenase and an NAD⁺-dependent D(–)-mandelate dehydrogenase (Durham, 1984). This was the first clear example of an NAD⁺-dependent enzyme being involved in the oxidation of mandelate. More recently a 2-hydroxyisocaproate dehydrogenase from *Streptococcus faecalis* (which has marked D(–)-mandelate dehydrogenase activity) and a D(–)-mandelate dehydrogenase from *Lactobacillus curvatus* have been purified and characterized, and both are soluble NAD⁺-dependent enzymes

Abbreviation: FPLC, fast protein liquid chromatography (Pharmacia system).

(Yamazaki & Maeda, 1986; Hummel *et al.*, 1988). Clearly, the purification and characterization of the D(−)-mandelate dehydrogenase from *R. graminis* was of significant interest since no eukaryotic mandelate dehydrogenase has hitherto been characterized, and because it allows a comparison with the NAD(P)⁺-independent mandelate dehydrogenases and with the bacterial NAD⁺-linked enzymes. The present report describes the purification and characterization of the yeast enzyme, and we conclude that there are two quite different types of mandelate dehydrogenase: membrane-bound flavoproteins that catalyse essentially irreversible reactions, and soluble, NAD⁺-dependent enzymes that catalyse the reversible interconversion of mandelate and phenylglyoxylate. Preliminary accounts of part of this work have been published (Baker & Fewson, 1988).

METHODS

Materials. Chemicals were of the best quality commercially available, and most of them were obtained from the sources described by Allison *et al.* (1985*b*) and MacKintosh & Fewson (1988*a*). Matrex Gel Orange A was obtained from Amicon. Phenyl Sepharose, a pre-packed Superose 6 column and ferritin were from Pharmacia. Trifluoroacetic acid (HPLC grade) was from Rathburn Chemicals, Walkerburn, Peeblesshire, UK. Bovine serum albumin (fraction V) was from Wilfred Smith Ltd, Edgware, Middlesex, UK. Rabbit muscle pyruvate kinase, pig heart lactate dehydrogenase and cytochrome *c* were from Boehringer Mannheim, rabbit muscle aldolase from Sigma and yeast alcohol dehydrogenase from BDH.

Organism. *Rhodotorula graminis* KGX 39 was obtained from Dr D. R. Durham, Genex Corporation, Gaithersburg, MD 20877, USA.

Growth of yeast. Cells were maintained as lyophilized cultures in a filter-sterilized mixture of 3 parts heat-inactivated horse serum to 1 part 30% (w/v) glucose. Prior to growth in liquid culture, cells were rehydrated (with sterile distilled water) and subcultured onto Sabouraud-Dextrose agar (Oxoid) for 48 h at 30 °C. When large amounts of yeast were required, *R. graminis* was grown in a complex medium supplemented with 10 mM-DL-mandelate (Allison *et al.*, 1985*a*) in a 10 litre fermenter fitted with a 20 litre reservoir. An inoculum was prepared by aseptically transferring a loopful of culture from the solid medium to 200 ml of the complex medium plus 10 mM-DL-mandelate (in a 1 litre conical flask) and shaking (200 r.p.m.) at 30 °C for 19 h. The entire culture was used to inoculate the fermenter (Braun Biostat V; F. T. Scientific Instruments, Tewkesbury, Glos., UK.), which contained 10 litres of the complex medium plus 10 mM-DL-mandelate and 0.005% poly(propylene glycol) 2025 as antifoam. The fermenter was operated at 30 °C with an aeration rate of 4 litres of sterile air min^{−1} and stirring at setting 2.5 (approx. 250 r.p.m.). After growth for 24 h, 9.5 litres of culture were removed for harvesting, and the washed yeast stored at −20 °C; 9.5 litres of fresh medium were aseptically transferred from the reservoir. After a further 24 h growth, 9.5 litres of culture were removed for harvesting and stored as above; 9.5 litres of fresh medium were transferred to the fermenter and the culture grown for a final 24 h. All of the culture was then removed for harvesting, and the last of the yeast stored at −20 °C. This arrangement typically yielded 500 g wet wt of cells.

Preparation of the crude extract. All steps were carried out at a temperature of 0–4 °C. Approximately 40 g wet wt of thawed yeast cells were suspended in 3 vols of buffer A [50 mM-Tris/HCl buffer (pH 8.0) containing 1 mM-dithiothreitol], mixed with a glass homogenizer, and broken by three passages through a French pressure cell (4-3398 A; American Instrument Co., Silver Spring, MD, USA) operated at a pressure of 98 MPa. The homogenate was centrifuged at 12000 *g* for 30 min, the supernatant removed and further centrifuged at 64000 *g* for 2 h. The high-speed supernatant was filtered through Whatman GF/A glass paper and then dialysed overnight against 2 × 2 litres of buffer A. The dialysed extract was used as the starting material for enzyme purification.

Purification of D(−)-mandelate dehydrogenase. All steps were carried out at a temperature of 0–4 °C.

Chromatography on DEAE-Sephacel. Extract (approx. 97 ml) was applied at 30 ml h^{−1} to a DEAE-Sephacel column (10.2 cm × 2.6 cm) pre-equilibrated in buffer A. Once loaded, the column was washed in buffer A at 50 ml h^{−1} until the *A*₂₈₀ of the effluent decreased to approximately 0.06. Then, D(−)-mandelate dehydrogenase was eluted with a linear 0–150 mM-NaCl gradient in buffer A (total vol. 600 ml) at a flow rate of 30 ml h^{−1}. Fractions containing more than 3% of the total activity loaded were pooled.

Chromatography on Phenyl Sepharose. The DEAE-Sephacel pool (approx. 82 ml) was applied at 30 ml h^{−1} to a Phenyl Sepharose column (10.0 cm × 2.6 cm) pre-equilibrated in buffer A. Once loaded, the column was washed at 40 ml h^{−1} in buffer A until the *A*₂₈₀ of the effluent returned to the baseline. The column was then washed with 130 ml buffer A containing 25% (v/v) ethanediol, followed by buffer A containing 40% (v/v) ethanediol, which eluted the enzyme. Fractions containing more than 3% of the total activity loaded were pooled.

Chromatography on DEAE-Sephacel. The Phenyl Sepharose pool (approx. 69 ml) was applied to a DEAE-Sephacel column (3.8 cm × 1.6 cm) pre-equilibrated in buffer A. Once loaded, the column was washed with buffer A until the *A*₂₈₀ of the effluent returned to the baseline. The enzyme was then eluted with buffer A containing 100 mM-NaCl. The flow rate throughout was maintained at 30 ml h^{−1}. Active fractions were pooled (approx.

15 ml) and dialysed overnight against 2 litres of buffer B [50 mM-potassium phosphate buffer (pH 7.5) containing 1 mM-dithiothreitol].

Chromatography on Matrex Gel Orange A. The dialysed pool was applied to a Matrex Gel Orange A column (15.5 cm × 2.6 cm) pre-equilibrated in buffer B. Once the column was loaded, the pump was stopped for 30 min. The column was then washed with 200 ml buffer B followed by 300 ml buffer A. The flow was reversed and pure D(-)-mandelate dehydrogenase was eluted with buffer A containing 0.5 mM-NADH. Active fractions were pooled (approx. 44 ml) and the enzyme concentrated either by vacuum dialysis or by dialysis against dry Aquacide 2 powder (Calbiochem). The pool was further concentrated to 200 µl using a Centricon-30 microconcentrator (Amicon), and stored in buffer A containing 50% (v/v) glycerol at -20 °C.

Preparation of buffers. Buffers were prepared at the temperature at which they were to be used by adjusting the pH of ten-ninths strength solutions with NaOH or HCl and then making to volume.

Enzyme assays. The oxidation of D(-)-mandelate (forward reaction) was measured in a 1 ml reaction mixture containing 200 mM-Bicine/NaOH buffer (pH 9.5), 1 mM-NAD⁺, 5 mM-D(-)-mandelate and enzyme. During the purification procedure, column fractions were assayed as above except that 200 mM-glycine/NaOH buffer (pH 9.5) was used instead of Bicine/NaOH buffer. The reduction of phenylglyoxylate (reverse reaction) was measured in a 1 ml reaction mixture containing 200 mM-potassium phosphate buffer (pH 5.85), 200 µM-NADH, 1 mM-phenylglyoxylate and enzyme. Because of the instability of NADH at low pH, a non-enzymic rate was determined and deducted from the enzymic rate. Both assays were monitored at 340 nm, 27 °C and a ΔA_{340} of +6.3 (forward reaction) or -6.3 (reverse reaction) was taken to correspond to the turnover of 1 µmol of substrate. One unit of enzyme activity is defined as 1 µmol of substrate converted min⁻¹; specific activities are given as units (mg protein)⁻¹.

Reaction stoichiometry and equilibrium constant. D(-)-Mandelate dehydrogenase catalyses both the oxidation of D(-)-mandelate and the reduction of phenylglyoxylate. The reaction stoichiometry and equilibrium constant were determined by following the progress curve of the reaction. The concentration of substrates and products were at equilibrium when the curve reached a plateau. The concentration of NADH at equilibrium was determined from the ΔA_{340} ; the concentration of NAD⁺ at equilibrium was calculated by deducting the concentration of NADH from the concentration of NAD⁺ initially added. The initial amount of NAD⁺ added was determined enzymically using the method described by Boehringer Mannheim (1973), except that the reaction mixture was decreased to 1 ml. Both D(-)-mandelate and phenylglyoxylate were determined using reverse-phase HPLC. Samples were injected into a Bio-Rad C4 RP-304 reverse-phase column (25 cm × 0.46 cm) pre-equilibrated in 0.1% (v/v) trifluoroacetic acid. Compounds were eluted at a flow rate of 1 ml min⁻¹ and detected at 210 nm. The amount of each aromatic acid was calculated from a standard curve constructed using known amounts of compound. The apparent equilibrium constant, K'_{eq} , was determined using the equation:

$$K'_{eq} = \frac{[\text{phenylglyoxylate}].[NADH]}{[D(-)\text{-mandelate}].[NAD^+]} \times \frac{1}{\text{antilog pH}}$$

Isoelectric point. Purified D(-)-mandelate dehydrogenase (100 µg) in 25 mM-BisTris/HCl buffer (pH 6.7) was applied to an FPLC Mono P chromatofocusing column (20 cm × 0.5 cm) pre-equilibrated in the same buffer. The column was washed with 8 ml of BisTris/HCl buffer before the enzyme was eluted with Polybuffer PB 74 made 1:10 with water and adjusted to pH 3.8. Fractions (1 ml) were collected at a flow rate of 0.5 ml min⁻¹ and assayed for enzyme activity. The A_{280} of the effluent was monitored and the pH of each fraction determined.

Absorption spectrum. Purified D(-)-mandelate dehydrogenase (400 µg) in 50 mM-Tris/HCl buffer (pH 8.0) was scanned (200 nm–600 nm) in a 1 ml quartz cuvette using a Pye Unicam SP8-100 spectrophotometer at 2 nm s⁻¹.

Electrophoresis. The purity of samples during the purification procedure was monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970). Slab gels (19 cm × 9.5 cm × 0.15 cm) of 12.5% (w/v) polyacrylamide with 5.6% (w/v) stacking gels were run at 100 mA and stained for protein using 0.1% Coomassie Brilliant Blue G250 in 50% (v/v) methanol/10% (v/v) acetic acid for 45 min at 60 °C. Destaining was carried out in 10% (v/v) methanol/10% (v/v) acetic acid at the same temperature. Non-denaturing gels of 7.5% (w/v) polyacrylamide with 5.6% (w/v) stacking gels were run at 45 mA and stained either for protein as above or for D(-)-mandelate dehydrogenase activity in 66.7 mM-sodium pyrophosphate buffer (pH 9.0) containing 1 mM-NAD⁺, 1 mM-D(-)-mandelate, 550 µM-N-methylphenazonium methosulphate and 55 µM-Nitro Blue Tetrazolium in the dark for 10 min at 30 °C.

Relative molecular mass. The native M_r was estimated under non-denaturing conditions using gel filtration chromatography with an FPLC Superose 6 column (30 cm × 1 cm) pre-equilibrated in buffer A containing 100 mM-NaCl. The column was calibrated with ferritin (M_r 450 000), pyruvate kinase (M_r 237 000), aldolase (M_r 158 000), lactate dehydrogenase (M_r 140 000), ovalbumin (M_r 43 000) and cytochrome *c* (M_r 12 500). Proteins were eluted at a flow rate of 0.5 ml min⁻¹ and the A_{280} of the effluent was monitored. Fractions (250 µl) were collected and assayed for D(-)-mandelate dehydrogenase activity.

The subunit M_i under denaturing conditions was estimated by SDS-PAGE using phosphorylase *b* (M_r 94 000),

bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soya bean trypsin inhibitor (M_r 20100) and α -lactalbumin (M_r 14400) as standards. Gels were stained and then scanned with an LKB 2202 Ultrosan laser densitometer.

Amino acid analysis. Approximately 1.3 mg of purified D(-)-mandelate dehydrogenase was used to determine the amino acid composition. Samples were hydrolysed in 5.65 M-HCl at 110 °C for 24, 48, 72 and 96 h. Valine, leucine and isoleucine were determined from their respective amounts at the end of the time course; serine and threonine were determined by extrapolating back to zero time. Cysteine was detected as cysteic acid after oxidation of the original sample with performic acid for 4 h at room temperature. Hydrolysed samples were analysed with an LKB 4400 amino acid analyser. The data were normalized to alanine = 23 nmol.

Analytical methods. Protein concentrations were determined by the Lowry method, except for enzyme pools obtained during purification, where the method of Bradford (1976) was used. Bovine serum albumin was used as the standard for both methods. The pH values of solutions were usually determined with a standard laboratory pH meter, but where the volume was small (e.g. 1 ml reaction mixtures), a Radiometer type M26 pH monitor fitted with a GK 2302 micro pH electrode was used. Conductivities were determined with a Radiometer type CDM2e conductivity meter. During enzyme purification, the A_{280} of column effluents was measured with an LKB Uvicord monitor.

Kinetic parameters. The K_m and V values of D(-)-mandelate dehydrogenase with respect to its substrates and cofactors were determined by the two-substrate method described by Engel (1981). The apparent K_m and V values were determined by measuring the initial velocities in the presence of several non-saturating concentrations of the first substrate, at a fixed high concentration of the second substrate. The fixed concentration of the second substrate was at least 6.5-fold more than its apparent K_m , and in most cases was over 10-fold. The kinetic data were analysed using the Direct Linear method (Eisenthal & Cornish-Bowden, 1974) using the Enzpack computer program (Williams, 1985).

RESULTS AND DISCUSSION

Purification of D(-)-mandelate dehydrogenase

A typical purification is summarized in Table 1. The enzyme eluted from the first DEAE-Sephacel column at approximately 70 mM-NaCl, and it proved necessary to dialyse the crude extract to ensure binding of the enzyme to the gel bed. The second DEAE-Sephacel column was beneficial in removing the ethanediol from the sample and concentrating the enzyme pool. The enzyme was finally purified using the Matrex Gel Orange A column. Unlike red and blue dye-ligands which have been used extensively in the purification of NAD(P)⁺-dependent dehydrogenases, Orange A has been successful in only relatively few cases (Amicon, 1980). Changing the pH of the buffer from pH 7.5 (buffer B) to pH 8.0 (buffer A) was essential in removing contaminant proteins from the column prior to elution of D(-)-mandelate dehydrogenase with NADH. Yields were of the order of 50–60% of the original total activity and the enzyme was purified approximately 240-fold. D(-)-Mandelate dehydrogenase was purified to homogeneity as determined by denaturing and non-denaturing PAGE (Fig. 1). The enzyme was purified in good yield (1–2 mg from 40–50 g wet wt of cells) and loss of activity was negligible even after storage for several months at -20 °C. The procedure has been successfully carried out 18 times with very similar results.

Table 1. *Purification of D(-)-mandelate dehydrogenase from R. graminis strain KGX 39*

The results presented are for a typical purification starting from 40 g wet wt of cells.

Step	Volume (ml)	Concn of protein (mg ml ⁻¹)	Total protein (mg)	Activity (units ml ⁻¹)	Total activity (units)	Specific activity (units mg ⁻¹)	Purification (fold)	Yield (%)
Crude extract	97.3	13.3	1294	3.75	365	0.28	1	100
DEAE-Sephacel	82.0	0.97	79.5	4.28	351	4.41	15.7	96
Phenyl Sepharose	69.5	0.19	13.2	4.38	304	23.0	82	83
DEAE-Sephacel	15.8	0.37	5.8	15.18	240	41.3	147	65
Orange A	44.5	0.078	3.4	5.17	230	67.6	241	63

Fig. 1. (a) Electrophoresis of D(–)-mandelate dehydrogenase. The purification of D(–)-mandelate dehydrogenase was monitored using SDS-PAGE. A 12.5% (w/v) SDS-polyacrylamide gel with a 5.6% (w/v) stacking gel was run and stained as described in Methods. Lanes: A, M_r markers; B, 70 μ g of the crude extract; C, 40 μ g of the DEAE-Sepharose pool; D, 22 μ g of the Phenyl Sepharose pool; E, 22 μ g of the second DEAE-Sepharose pool; F, 12 μ g of the Matrex Gel Orange A pool. (b) The final purity of D(–)-mandelate dehydrogenase was monitored using non-denaturing PAGE. A 7.5% (w/v) polyacrylamide gel with a 5.6% (w/v) stacking gel was run and stained either for protein or for D(–)-mandelate dehydrogenase activity as described in Methods. Lanes: A, 40 μ g of crude extract stained for protein; B, 2 μ g of pure D(–)-mandelate dehydrogenase stained for protein; C, 40 μ g of crude extract stained for activity; D, 2 μ g of pure D(–)-mandelate dehydrogenase stained for activity.

Relative molecular mass

The subunit M_r was estimated to be $38\,000 \pm 1400$ SD ($n = 13$ batches of enzyme) and the native M_r was estimated to be 77 200 (76 900, 77 500). Comparison of the native and subunit M_r suggests that the enzyme exists as a dimer of identical subunits. Other D(–)-specific 2-hydroxy acid dehydrogenases have very similar M_r values: the D(–)-lactate dehydrogenases from lactic acid bacteria and staphylococci are dimers with native M_r between 70 000 and 80 000 (Garvie, 1980), the D(–)-2-hydroxyisocaproate dehydrogenase of *S. faecalis* is a dimer with a native M_r of 72 000 (Yamazaki & Maeda, 1986) and the D(–)-mandelate dehydrogenase of *L. curvatus* is also a dimer of identical subunits, although it has a somewhat lower native M_r of 60 000 (Hummel *et al.*, 1988).

Amino acid analysis

Table 2 shows the amino acid composition of D(–)-mandelate dehydrogenase (% by wt) as the mean values from two independent experiments, with an approximate number of residues per subunit. The most notable feature of the amino acid composition was the low number of cysteine residues, i.e. one per subunit. A low cysteine content has also been observed in the lactate dehydrogenases of *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus casei* (Hensel *et al.*, 1977).

Absorption spectrum, and effect of salts, metal ions and metal chelators

The absorption spectrum of D(–)-mandelate dehydrogenase (not shown) had a maximum at 280 nm but no absorbance above 340 nm, indicating that the enzyme does not contain bound flavin or cytochrome as cofactor. In contrast, the absorption spectra of the D(–) and L(+) mandelate dehydrogenases of *A. calcoaceticus* both show absorbance above 340 nm with

Table 2. *Amino acid composition of D(-)-mandelate dehydrogenase*

The mean values from two independent determinations using separate batches of enzyme are given with individual values in parentheses. The approximate number of amino acids per subunit was determined from the subunit M_r of 38000. Data were normalized to Ala = 23 nmol.

Amino acid	Mean % (by wt)	Approx. no. of residues per subunit	Amino acid	Mean % (by wt)	Approx. no. of residues per subunit
Cys*	0.32 (0.33, 0.31)	1	Met	1.21 (1.31, 1.10)	4
Asx	9.94 (10.73, 9.14)	33	Ile†	4.59 (4.41, 4.76)	16
Thr†	4.54 (4.59, 4.49)	17	Leu‡	11.36 (10.81, 11.90)	38
Ser†	4.98 (5.06, 4.90)	22	Tyr	2.83 (3.01, 2.64)	7
Glx	12.15 (12.48, 11.81)	36	Phe	6.30 (6.26, 6.33)	16
Pro	4.38 (4.22, 4.53)	18	His	4.79 (4.74, 4.83)	13
Gly	4.15 (4.06, 4.24)	28	Lys	6.28 (6.16, 6.39)	19
Ala	7.98 (7.94, 8.01)	43	Arg	8.25 (8.12, 8.38)	20
Val‡	6.00 (5.76, 6.24)	23			

* Determined as cysteic acid. † Extrapolated to zero time. ‡ Mean of 96 h values.

shoulders between 420 nm and 450 nm, and 420 nm and 460 nm respectively. FAD has been identified as the prosthetic group of the D(-)-mandelate dehydrogenase and FMN as the prosthetic group of the L(+)-mandelate dehydrogenase in this bacterium (Allison *et al.*, 1985*b*; Hoey *et al.*, 1987). Enzyme activity (after a 5 min pre-incubation at 27 °C) was neither stimulated nor inhibited by 1 mM concentrations of NaCl, Na₂SO₄, KCl, MgCl₂, NH₄Cl, CaCl₂, ZnCl₂, CoCl₂, FeCl₃, KH₂PO₄, NaH₂PO₄, MnSO₄, MnCl₂ or CuSO₄. Enzyme activity was not inhibited (after a 5 min pre-incubation) by the following metal-chelating agents each at 1 mM: EDTA, diethyldithiocarbamic acid, bathophenanthroline disulphonic acid, dihydroxybenzene disulphonic acid, pyrazole, 8-hydroxyquinoline or 2,2'-dipyridyl. This indicates that the enzyme has no absolute requirement for salts or metal ions.

Effect of thiol reagents

D(-)-Mandelate dehydrogenase was strongly inhibited by 10 mM-*N*-ethylmaleimide. However, there was little or no inhibition of enzyme activity by 10 mM-iodoacetate, 10 mM-iodoacetamide, 6.65 mM-5,5'-dithiobis(2-nitrobenzoic acid) or 5 mM-4-(chloromercuri)benzoate, even after a 6 h pre-incubation at 27 °C. This is in stark contrast to the mandelate dehydrogenases of *A. calcoaceticus*, which are susceptible to these reagents; in particular the D(-)-mandelate dehydrogenase is strongly inhibited by 4-(chloromercuri)benzoate, less than 1 µM producing 50% inhibition after only a 10 min pre-incubation at 27 °C (Allison *et al.*, 1985*b*; Hoey *et al.*, 1987). The *R. graminis* enzyme also differs from the D(-)-mandelate dehydrogenase of *L. curvatus* (94% inhibition at 100 µM-4-(chloromercuri)benzoate) the D(-)-2-hydroxyisocaproate dehydrogenase of *S. faecalis* (complete inhibition at 0.5 mM-4-(chloromercuri)benzoate) and many eukaryotic lactate dehydrogenases which are inhibited by thiol reagents (Holbrook *et al.*, 1975; Yamazaki & Maeda, 1986; Hummel *et al.*, 1988).

Effects of pH on the enzyme

Initial rates were measured in appropriate buffers over the pH range 4.6 to 11.1. Oxidation of D(-)-mandelate was maximal at pH 9.5 in either 200 mM-Bicine/NaOH buffer or 200 mM-glycine/NaOH buffer, and the maximum activity for the reduction of phenylglyoxylate was at pH 5.85 in 200 mM-potassium phosphate buffer. Enzyme activity was detectable over a wide range of pH especially when assayed using the reverse reaction. These results are very similar to other 2-hydroxyacid dehydrogenases, where the oxidation of the 2-hydroxy acid shows a higher pH optimum than the reduction of the corresponding 2-oxo acid (LéJohn, 1971; Garvie, 1980; Yamazaki & Maeda, 1986; Hummel *et al.*, 1988). The isoelectric point of purified D(-)-mandelate dehydrogenase was 5.9 (5.95, 5.85).

Table 3. Concentrations of D(-)-mandelate, NAD⁺, phenylglyoxylate and NADH at equilibrium

The forward reaction of the enzyme was measured in 200 mM-Bicine/NaOH buffer (pH 9.2) in the presence of the following starting concentrations of D(-)-mandelate and NAD⁺:

- Reaction mixture 1: 1.1 mM-D(-)-mandelate + 169 μM-NAD⁺
 Reaction mixture 2: 1.1 mM-D(-)-mandelate + 338 μM-NAD⁺
 Reaction mixture 3: 2.2 mM-D(-)-mandelate + 169 μM-NAD⁺
 Reaction mixture 4: 2.2 mM-D(-)-mandelate + 338 μM-NAD⁺

Reaction mixture	Concentration at equilibrium (μM)				Apparent K'_{eq} (M)
	D(-)-Mandelate	NAD ⁺	Phenylglyoxylate	NADH	
1	925	113	53.7	56	1.82×10^{-11}
2	920	257	75	80.4	1.43×10^{-11}
3	2150	100	76.2	69	1.53×10^{-11}
4	1980	227	102.5	110.3	1.58×10^{-11}

Reaction stoichiometry and equilibrium constant

The concentrations of D(-)-mandelate, NAD⁺, phenylglyoxylate and NADH at equilibrium (Table 3) indicate that D(-)-mandelate dehydrogenase produced approximately equimolar amounts of phenylglyoxylate and NADH and the average equilibrium constant was 1.59×10^{-11} M at pH 9.2. Therefore the reduction of phenylglyoxylate to D(-)-mandelate is thermodynamically favoured at this pH.

Substrate, cofactor and inhibitor specificity

D(-)-Mandelate dehydrogenase from *R. graminis* is an NAD⁺-dependent dehydrogenase and cannot use NADP⁺ as cofactor; neither can it use the dye 2,6-dichloroindophenol or 2,6-dichloroindophenol plus *N*-methylphenazonium methosulphate as electron acceptors, which have been used in the assay of *P. putida* and *A. calcoaceticus* mandelate dehydrogenases (Hegeman, 1966; Hills & Fewson, 1983). Similarly the D(-)-mandelate dehydrogenase of *Aspergillus niger* cannot use this dye as an electron acceptor, although it is unclear whether this enzyme is NAD⁺-dependent (Jamaluddin *et al.*, 1970).

The ability of the enzyme to use a number of substituted mandelates and mandelate analogues as substrates was tested. However, velocity values could not be determined because many of the corresponding substituted phenylglyoxylates absorb strongly at 340 nm (i.e. the monitoring wavelength), unlike phenylglyoxylate which has negligible absorbance at this wavelength. The molar absorption coefficients at 340 nm of these compounds could, in principle, be used to determine correction factors (MacKintosh & Fewson, 1988*b*) but this is not yet possible as the compounds are not commercially available. Compounds which are substrates in the oxidation reaction included: D(-)-mandelate, DL-3-hydroxymandelate, DL-4-hydroxymandelate, DL-4-hydroxy-3-methoxymandelate, DL-3-hydroxy-4-methoxymandelate, DL-2-methoxymandelate, DL-4-fluoromandelate, DL-4-chloromandelate, DL-4-bromomandelate and D(-)-hexahydromandelate. Those which are substrates in the reduction reaction included: phenylglyoxylate, phenylpyruvate and thiopheneglyoxylate. Those compounds which are not substrates for the enzyme but which inhibited activity are given in Tables 4 and 5. The enzyme was assayed in the presence of inhibitor at the calculated K_m value for D(-)-mandelate or phenylglyoxylate respectively. D(+)-Malate, citrate and glyoxylate at 10 mM were neither substrates nor inhibitors of the enzyme. Comparison of the compounds that are substrates shows them to contain some form of a ring structure (an aromatic ring, a six-carbon non-aromatic ring or a thiophene ring), whereas those which inhibited activity contained a hydrophobic side chain. In contrast to the yeast enzyme, the D(-)-2-hydroxyisocaproate dehydrogenase of *S. faecalis* and the D(-)-mandelate dehydrogenase of *L. curvatus* show catalytic activity with both aromatic and aliphatic 2-hydroxycarboxylic acids (Yamazaki & Maeda, 1986; Hummel *et al.*, 1988). It is plausible that with the yeast enzyme, the hydrophobic ring is important for the correct orientation of the substrate at the active site, and although the 2-hydroxy and 2-oxocarboxylic acids with hydrophobic side chains can bind at the active site, they lack this ring and therefore

Table 4. *Inhibition of the forward reaction of D(–)-mandelate dehydrogenase by compounds that are not substrates*

The enzyme was assayed at pH 9.5 in the presence of 1 mM-NAD⁺, 319 μ M-D(–)-mandelate and inhibitor. The initial velocity in the absence of inhibitor was taken as the 100% value. Values in parentheses represent results from two independent experiments.

Compound	Percentage inhibition
10 mM-D(–)-Lactate	17.4 (17.4, 17.4)
10 mM-DL-Phenyllactate	47.1 (46.4, 47.9)
5 mM-DL-4-Hydroxyphenyllactate	44.2 (43.5, 45)
10 mM-DL-2-Hydroxyvalerate	67.4 (68.2, 66.7)
10 mM-DL-2-Hydroxyisovalerate	31.9 (30.5, 33.4)
1 mM-DL-2-Hydroxycaproate	63.8 (63.8, 63.8)
1 mM-DL-2-Hydroxyisocaproate	55.1 (53.7, 56.6)
10 mM-Oxalate	63.1 (63.8, 62.4)
L(+)-Mandelate	L(+)-Mandelate is a competitive inhibitor with an apparent K_i of 8.2 mM

Table 5. *Inhibition of the reverse reaction of D(–)-mandelate dehydrogenase by compounds that are not substrates*

The enzyme was assayed at pH 5.85 in the presence of 200 μ M-NADH, 51.2 μ M-phenylglyoxylate and inhibitor. The initial velocity in the absence of inhibitor was taken as the 100% value. Values in parentheses represent results from two individual experiments.

Compound	Percentage inhibition
10 mM-Pyruvate	17.4 (17.4, 17.4)
10 mM-Oxaloacetate	38.5 (35.8, 41.3)
10 mM-Oxamate	77.1 (78, 76.2)
10 mM-2-Oxoglutarate	44.1 (43.2, 45)
10 mM-2-Oxovalerate	43.1 (41.3, 45)
10 mM-2-Oxoisovalerate	44.1 (43.2, 45)
1 mM-2-Oxoisocaproate	40.4 (43.2, 37.7)
2.5 mM-2-Oxo-octanoate	28.5 (30.3, 26.7)
5 mM-2-Oxo-3-methylvalerate	54.1 (54.1, 54.1)

cannot undergo catalysis. D(–)-Mandelate dehydrogenase was neither inhibited nor stimulated by 1 mM concentrations of benzaldehyde, benzoate, succinate, glucose, ATP/Mg²⁺, ADP/Mg²⁺ or acetyl-CoA. This indicates that enzyme activity is not regulated by these compounds, supporting the view that feedback inhibition is not important in the regulation of peripheral metabolic pathways (Fewson, 1988).

Kinetic properties

At pH 9.5, the K_m for D(–)-mandelate was 319 μ M (327 μ M, 310 μ M) and the K_m for NAD⁺ was 71 μ M (73 μ M, 69 μ M). The maximum velocity at pH 9.5 was 123 μ mol min^{–1} (mg protein)^{–1} (127, 118, 123). The apparent kinetic coefficients of D(–)-mandelate dehydrogenase with D(–)-mandelate, NAD⁺, phenylglyoxylate and NADH at pH 5.85 (the optimum for the reverse reaction), 7.0 and 9.5 (the optimum for the forward reaction) are given in Table 6. At all pH values tested, the apparent K_m for NADH and phenylglyoxylate were significantly lower than for NAD⁺ and D(–)-mandelate, and the apparent maximum velocities were significantly greater in the reverse direction than in the forward direction. However, D(–)-mandelate dehydrogenase is induced during the growth of *R. graminis* on mandelate (Durham *et al.*, 1984) and presumably the reaction is driven in the forward direction by the decarboxylation of the product by phenylglyoxylate decarboxylase and by the oxidation of the NADH formed.

Table 6. Apparent K_m and V values of D(–)-mandelate dehydrogenase with D(–)-mandelate, NAD^+ , phenylglyoxylate and $NADH$ at pH 5.85, pH 7.0 and pH 9.5

The numbers of individual experiments are given in parentheses, except where only two experiments were done and both values are given in parentheses. Assays were carried out in 200 mM-Bicine/NaOH buffer (pH 9.5; the optimum for the forward reaction), 200 mM-potassium phosphate buffer (pH 7.0) and 200 mM-potassium phosphate buffer (pH 5.85; the optimum for the reverse reaction).

Reaction: D(–)-mandelate + NAD^+ → phenylglyoxylate + $NADH$			
pH	K_m for D(–)-mandelate (μM)	K_m for NAD^+ (μM)	V [units (mg protein) $^{-1}$]
5.85	ND	ND	ND
7.0	3050 (3120, 2980)	309 (336, 281)	35.8 ± 5.0 (4)
9.5	325 (318, 332)	77.5 (77, 78)	86.7 ± 14.3 (4)
Reaction: Phenylglyoxylate + $NADH$ → D(–)-mandelate + NAD^+			
pH	K_m for phenylglyoxylate (μM)	K_m for $NADH$ (μM)	V [units (mg protein) $^{-1}$]
5.85	51.5 (41, 62)	38 (37, 39)	572 ± 119.3 (4)
7.0	45.5 (42, 49)	36 (35, 37)	490 ± 109.4 (4)
9.5	70.7 (65.4, 76)	26.3 (26.6, 26.0)	310 ± 41.5 (4)

ND, No detectable rate.

Conclusions

The purification and preliminary characterization of D(–)-mandelate dehydrogenase from the yeast *R. graminis* allows a comparison of its properties with other mandelate dehydrogenases which had previously been purified only from bacteria, and with various NAD^+ -dependent dehydrogenases. The enzyme shows marked similarity to both the 2-hydroxyisocaproate dehydrogenase of *S. faecalis* (which also has D(–)-mandelate dehydrogenase activity) and the D(–)-mandelate dehydrogenase of *L. curvatus* (Yamazaki & Maeda, 1986; Hummel *et al.*, 1988). All three enzymes are dimers of identical subunits and have similar properties with respect to M_r , quaternary structure, cofactor specificity, reversibility of the reaction catalysed, pH dependence of activity, effect of metal chelators and common 2-hydroxy and 2-oxocarboxylic acids which they bind. However, they do differ with respect to the effect of thiol reagents. Comparison of the yeast D(–)-mandelate dehydrogenase with both the D(–)- and L(+)-mandelate dehydrogenase of *A. calcoaceticus* shows them to be very different. Both of these bacterial enzymes are flavin-dependent integral membrane proteins that catalyse the irreversible oxidation of mandelate. They differ from the enzyme of *R. graminis* with respect to intracellular location, M_r , quaternary structure, isoelectric point, pH optimum, cofactor used and sensitivity to thiol reagents (Allison *et al.*, 1985*b*; Hoey *et al.*, 1987). Likewise the D(–)- and L(+)-mandelate dehydrogenases of *P. putida* are membrane-bound proteins presumably containing flavin as cofactor (Gunsalus *et al.*, 1953; Stanier *et al.*, 1953; Hegeman, 1966).

These results suggest that both prokaryotes and eukaryotes possess two distinct classes of mandelate dehydrogenase: integral membrane proteins requiring flavin as prosthetic group and soluble NAD^+ -dependent proteins, and it is likely that the two groups of enzymes evolved independently. This relationship between the two groups of enzymes reflects the relationships between the NAD^+ -dependent and NAD^+ -independent lactate dehydrogenases (Garvie, 1980).

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