L(+)-Mandelate dehydrogenase from *Rhodotorula graminis*: purification, partial characterization and identification as a flavocytochrome *b*

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L(+)-Mandelate dehydrogenase was purified to homogeneity from the yeast *Rhodotorula graminis* KGX 39 by a combination of $(NH_4)_2SO_4$ fractionation, ion-exchange and hydrophobic-interaction chromatography and gel filtration. The amino-acid composition and the N-terminal sequence of the enzyme were determined. Comprehensive details of the sequence determinations have been deposited as Supplementary Publication SUP 50172 (4 pages) at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1993) **289**, 9. The enzyme is a tetramer as judged by comparison of its subunit M_r value of 59100 and native M_r of 239900, estimated by SDS/PAGE and gel filtration respectively. There is one molecule of haem and approx. one molecule of non-covalently bound FMN per subunit. 2,6-Dichloroindophenol, cytochrome *c* and ferricyanide can all serve as electron acceptors. L(+)-Mandelate dehydrogenase is stereospecific for its substrate. D(−)-Mandelate and L(+)-hexahydromandelate are competitive inhibitors. The enzyme has maximum activity at pH 7.9 and it has a pI value of 4.4. $HgCl_2$ and 4-chloromercuribenzoate are potent inhibitors, but there is no evidence that the enzyme is subject to feedback inhibition by potential metabolic effectors. The evidence suggests that L(+)-mandelate dehydrogenase from *R. graminis* is a flavocytochrome *b* which is very similar to, and probably (at least so far as the haem domain is concerned) homologous with, certain well-characterized yeast L(+)-lactate dehydrogenases, and that the chief difference between them is their mutually exclusive substrate specificities.

INTRODUCTION

A considerable number of micro-organisms can metabolize one or both enantiomers of mandelate and the initial attack is usually by a stereospecific dehydrogenation, although in a few organisms racemization or ring hydroxylation can occur (Fewson, 1988, 1992). The bacterial mandelate dehydrogenases that have been examined so far are membrane-bound and NAD(P)-independent and they seem to be very similar to certain bacterial membrane-bound, NAD(P)-independent lactate dehydrogenases. Thus, both D(−)-mandelate and D(−)-lactate dehydrogenases from the bacterium *Acinetobacter calcoaceticus* and D(−)-lactate dehydrogenase from *Escherichia coli* contain FAD as a non-covalently bound cofactor and have monomeric M_r values of around 60000, whereas L(+)-mandelate and L(+)-lactate dehydrogenases from *A. calcoaceticus*, *E. coli* and *Pseudomonas putida* contain FMN and have monomeric M_r values of about 40000 (Tsou et al., 1990; Fewson, 1992). *Rhodotorula graminis* is an imperfect yeast that can grow well on mandelate as sole source of carbon and energy (Durham, 1984; Durham et al., 1984). We have previously purified and characterized its D(−)-mandelate dehydrogenase which, quite unlike the equivalent bacterial enzymes, is soluble and NAD-dependent (Baker and Fewson, 1989; Baker et al., 1992). We therefore set out to characterize the L(+)-mandelate dehydrogenase from *R. graminis* and in this paper show that it represents a new type of microbial mandelate dehydrogenase which is a flavocytochrome *b* similar to, and (at least so far as the haem domain is concerned) homologous with, the L(+)-lactate dehydrogenase ('flavocytochrome b_2') which has been well characterized in the yeasts *Saccharomyces cerevisiae* and *Hansenula anomala* (Chapman et al., 1991). Parts of this work have been described in a preliminary communication (Yasin and Fewson, 1992) and a description of some kinetic properties of the enzyme has been published (Smékal et al., 1993).

EXPERIMENTAL

Materials

Chemicals were the best quality commercially available and most of them were obtained from the sources described by MacKintosh and Fewson (1988) and Chalmers and Fewson (1989).

Growth of yeast and preparation of extracts

R. graminis KGX 39 was maintained, grown, harvested and stored as described in Baker and Fewson (1989). Frozen yeast pellets were suspended in two volumes of ice-cold 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg of Triton X-100/ml, and broken by four passages through an ice-cold French pressure cell (American Instrument Co., Silver Spring, MD, U.S.A.) operated at a pressure of 98 MPa. The homogenate was centrifuged at 12000 $g_{av.}$ for 30 min to remove intact cells and debris and the supernatant fraction was then centrifuged at 113000 $g_{av.}$ for 2 h. The supernatant ('extract') was used as the source of L(+)-mandelate dehydrogenase.

Purification of L(+)-mandelate dehydrogenase

An extract was prepared from approx. 70–90 g wet wt of *R. graminis*. All steps were carried out at 0–4 °C.

Step 1: $(NH_4)_2SO_4$ fractionation

Solid $(NH_4)_2SO_4$ was added to the extract over a 15 min period to give 40% saturation. The solution was stirred for a further 1 h

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and then the precipitate was removed by centrifuging at 52000 $g_{av.}$ for 20 min. More $(NH_4)_2SO_4$ was added to the resulting supernatant fraction to give 60% saturation and the precipitate which was collected by centrifuging at 52000 $g_{av.}$ for 20 min was dissolved in 20 mM Tris/HCl buffer, pH 7.5, which contained 0.5 mg of Triton X-100/ml ('buffer A'). The solution was dialysed overnight against buffer A.

Step 2: chromatography on a DEAE-Sephacel column

The dialysed $(NH_4)_2SO_4$-fractionated sample was applied to a DEAE-Sephacel column (2.6 cm × 24 cm) which had been equilibrated with buffer A. After loading, the column was washed with 200 ml of buffer A, 700 ml of 20 mM Tris/HCl buffer, pH 6.5, containing 0.5 mg of Triton X-100/ml, and then 700 ml of buffer A containing 0.075 M NaCl, all at a rate of 90 ml/h. L(+)-Mandelate dehydrogenase was eluted with a linear 0.075–0.3 M NaCl gradient in buffer A (total volume was 1 litre) at a flow rate of 51 ml/h. Fractions containing more than 25% of the activity of the peak fraction were pooled and dialysed overnight against buffer A.

Step 3: chromatography on a DEAE-Sepharose CL-6B column

The dialysed DEAE-Sephacel pool was applied at 30 ml/h to a column (2.6 cm × 45 cm) of DEAE-Sepharose CL-6B which had been equilibrated with buffer A. After loading, the column was washed, at a flow rate of 90 ml/h, with 100 ml of buffer A and then 900 ml of buffer A containing 0.08 M NaCl. L(+)-Mandelate dehydrogenase was eluted with a linear 0.08–0.2 M NaCl gradient in buffer A (total volume was 1 litre) at a flow rate of 22 ml/h. Fractions containing more than 25% of the activity of the peak fraction were pooled and brought to 20% satn. with $(NH_4)_2SO_4$ and stirred on ice for at least 30 min.

Step 4: chromatography on a phenyl-Sepharose CL-4B column

The DEAE-Sepharose pool containing $(NH_4)_2SO_4$ was applied at 30 ml/h to a column (2.6 cm × 10.8 cm) of phenyl-Sepharose CL-4B which had been equilibrated with 20 mM glycylglycine buffer, pH 7.5, containing $(NH_4)_2SO_4$ to 25% satn. After loading, the column was washed at 40 ml/h with 20 mM Tris/HCl buffer, pH 7.5, containing $(NH_4)_2SO_4$ (25% satn.) until the A_{280} of the effluent returned to the baseline level. The column was then washed with 300 ml of 20 mM glycylglycine buffer, pH 7.5, containing $(NH_4)_2SO_4$ (20% satn.) and then with 90 ml of 20 mM glycylglycine buffer, pH 7.5, containing $(NH_4)_2SO_4$ (12% satn.). L(+)-Mandelate dehydrogenase was eluted with 20 mM glycylglycine buffer, pH 7.5, containing $(NH_4)_2SO_4$ (7.5% satn.) at a flow rate of 28 ml/h into tubes containing sufficient of a solution of Triton X-100 at 5 mg/ml to give a final concentration of 0.5 mg of Triton X-100/ml. Fractions with peak activity were pooled.

Step 5: storage

The pooled fractions were stored at −20 °C or brought to 90% satn. with $(NH_4)_2SO_4$ and then the precipitate was collected by centrifugation and stored at 4 °C.

Enzyme assays

All enzyme assays were carried out at 27 °C using plastic cuvettes with 1 cm light-paths. One unit of enzyme activity is defined as the conversion of 1 μmol of substrate per minute.

L(+)-Mandelate dehydrogenase activity was routinely measured in 3 ml assay mixtures containing: 66.7 mM potassium phosphate buffer (pH 7.9), 53 μM 2,6-dichloroindophenol, 200 μg of BSA, 10 μM *N*-methylphenazonium methosulphate, 8.33 mM L(+)-mandelate (pH 7.9), and enzyme to initiate the reaction. The molar absorption coefficients of 2,6-dichloroindophenol were determined under all the experimental conditions used and a decrease in A_{600} of 7.22 units was taken to correspond to the oxidation of 1 μmol of substrate at pH 7.9. In some experiments alternative electron acceptors were used in the assays and 2,6-dichloroindophenol and *N*-methylphenazonium methosulphate were replaced by 8.33 μM cytochrome *c* or 1.66 mM potassium ferricyanide. Changes in absorbance of 6.6 units at 550 nm in the case of cytochrome *c* or of 0.34 units in the case of ferricyanide were taken to correspond to the oxidation of 1 μmol of substrate. L(+)-Lactate dehydrogenase activity was measured in 3 ml reaction mixtures containing 66.7 mM potassium phosphate buffer (pH 7.9), 0.33 mM EDTA, 53 μM 2,6-dichloroindophenol, 200 μg of BSA, 6.6 mM L(+)-lactate (pH 7.9) and enzyme to initiate the reaction (Morton et al., 1961). Published methods were used to measure the activities of D(−)-mandelate dehydrogenase (Baker and Fewson, 1989), NADPH-cytochrome *c* reductase (Mahler, 1955), α-mannosidase (Boehringer-Mannheim, 1973a) and fumarase (Boehringer-Mannheim, 1973b).

Amino-acid composition

Approx. 200 μg of purified L(+)-mandelate dehydrogenase was dialysed at 4 °C for 3 days against five 2 litre batches of 1 mM potassium phosphate buffer, pH 7.5, and then for 1 day against 5 litres of 0.1% SDS in distilled water. Samples of dialysed enzyme (approx. 6 μg) were lyophilized and then dissolved in 1 ml of 5.65 M HCl. Tubes were flushed with N_2, frozen and then sealed under vacuum. Triplicate samples were each hydrolysed at 110 °C for 24 h, 48 h and 72 h. The samples were then lyophilized at −20 °C, dissolved in 1 ml of water and lyophilized again; this procedure was repeated twice. A further triplicate set of dialysed samples were oxidized with performic acid. Performic acid was prepared by mixing 1 ml of 30% (v/v) H_2O_2 and 9 ml of 98–100% (v/v) formic acid. The mixture was left for 1 h at room temperature and then at 0 °C for 15 min. Lyophilized enzyme samples (approx. 6 μg) were dissolved in 1 ml of performic acid and oxidized for 4 h at room temperature. Samples were then lyophilized and redissolved three times in 1 ml of distilled water. The oxidized samples were then hydrolysed for 24 h as described above. Amino-acid analysis was carried out by Dr. D. G. Campbell, Department of Biochemistry, University of Dundee, Dundee, U.K., using a Waters PICO-TAG amino-acid analyser in which amino acids were quantified as the phenylthiohydantoin derivatives.

N-terminal sequence

Purified L(+)-mandelate dehydrogenase was dialysed at 4 °C for five 24 h periods, each against 2 litres of 0.1% SDS in distilled water, and then lyophilized at −20 °C. The N-terminal sequence was determined on three occasions by Dr. J. N. Keen, Protein Sequencing Unit, Department of Biochemistry and Molecular Biology, University of Leeds, Leeds, U.K., using the general methods described by Findlay et al. (1989). The enzyme was sequenced by Edman degradation; the phenylthiohydantoin–amino-acid derivatives were identified by a reverse-phase (C_{18})

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

Full details of the purification procedure are given in the Experimental section.

	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
Extract	250	701	3300	0.21	100	1
Dialysed $(NH_4)_2SO_4$ fraction	70	667	879	0.76	96	3.6
DEAE-Sephacel, dialysed	175	623	144	4.33	90	20
DEAE-Sepharose CL6B	90	590	4.8	123	85	586
Phenyl-Sepharose CL4B	90	252	1.4	180	37	857

h.p.l.c. system, quantified at 269 nm with serine and confirmed with threonine at 313 nm.

Isoelectric focusing, native and denaturing PAGE, extraction and quantification of flavin, and determination of protein concentrations

The PhastGel isoelectric-focusing system (Pharmacia, Uppsala, Sweden) covering the range pH 3–9 and calibrated with proteins of known pI values was used according to the manufacturer's instructions.

Native and SDS/PAGE and protein staining were carried out as described by Baker and Fewson (1989), and flavin was extracted and quantified essentially as described by Allison et al. (1985). Protein concentrations were determined by the method of Lowry et al. (1951), with BSA as a standard.

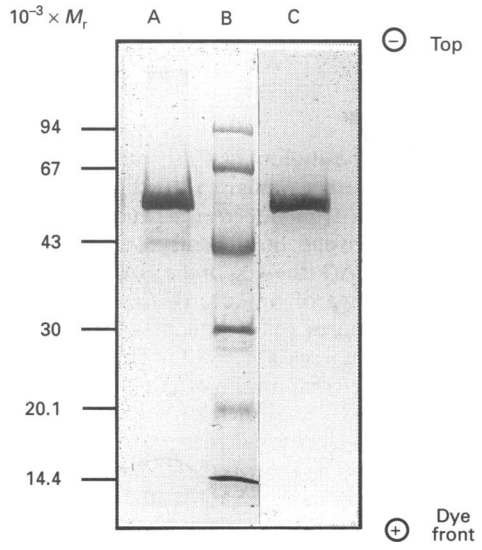

Figure 1 Electrophoresis of L(+)-mandelate dehydrogenase from *R. graminis* KGX 39

L(+)-Mandelate dehydrogenase was purified as described in the Experimental section and then concentrated by vacuum dialysis. The freshly purified and concentrated enzyme (track C) was run on a 10% (w/v) polyacrylamide slab gel containing 0.1% SDS and then stained for protein with Coomassie Brilliant Blue G250. After storage at −20 °C for approx. 2 months, a sample of the same preparation was electrophoresed under the same conditions (track A). Track B contained M_r markers.

RESULTS AND DISCUSSION

Purification of L(+)-mandelate dehydrogenase

As first indicated by Durham (1984), there was some evidence that L(+)-mandelate dehydrogenase is associated with membranes or cellular organelles. While almost 100% of the NAD^+-dependent D(−)-mandelate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and fumarase activities were found in the supernatant fraction after centrifuging extracts (prepared in the absence of Triton X-100) at 113000 $g_{av.}$ for 2 h, only 70–80% of the L(+)-mandelate dehydrogenase activity was in the supernatant and the remainder of the activity was found in the pellet. About 15–30% of the activities of NADPH-cytochrome *c* reductase (usually found in the endoplasmic reticulum) and α-mannosidase (presumably from the vacuolar membranes) were also precipitated under these conditions. Inclusion of Triton X-100 in the extraction buffer ensured that almost all of the L(+)-mandelate dehydrogenase was found in the 'soluble' fraction and Triton X-100 was also included in buffers used during the purification in order to obtain maximum recovery of activity.

Table 1 summarizes the results of a typical purification of L(+)-mandelate dehydrogenase. The DEAE-Sephacel column was a useful step and the degree of purification achieved depended on washing the column at pH 6.5, which is above the isoelectric point of the enzyme. Chromatography on a DEAE-Sepharose CL-6B column was particularly effective, presumably because both the gel-filtration and ion-exchange properties of the column were exploited. Hydrophobic chromatography on a phenyl-Sepharose column was vital in order to remove a few minor contaminants, but there was a significant loss of activity at this stage because Triton X-100 had to be omitted as it seemed to be precipitated on to the column material by the $(NH_4)_2SO_4$. This purification scheme yielded approx. 1 mg of protein from 70 to 90 g of cells. In nine independent preparations, the range of specific activity of the purified enzyme was 160–210 units/mg of protein with 32–37% recovery. The enzyme gave a single band on denaturing PAGE (Figure 1) and on non-denaturing PAGE. After prolonged storage of the enzyme at −20 °C, an extra minor protein band of lower M_r (approx. 45000) was sometimes seen (Figure 1).

Relative molecular mass and subunit structure

The apparent native M_r of L(+)-mandelate dehydrogenase was determined by gel filtration of both purified enzyme and extracts of *R. graminis* through calibrated Sephacryl S-300 columns. The apparent native M_r was 239900 (S.D. = 8400, $n = 3$) for the purified enzyme and 235000 (S.D. = 5200, $n = 3$) for the enzyme

Table 2 Amino-acid composition of L(+)-mandelate dehydrogenase from *R. graminis* and its comparison with the amino-acid compositions of L(+)-lactate dehydrogenases from the yeasts *S. cerevisiae* and *H. anomala*

The amino-acid composition of L(+)-mandelate dehydrogenase from *R. graminis* was determined as described in the Experimental section. The means of three independent determinations are given and each value in parentheses represents the mean of four, ten and four replicates respectively except for valine, leucine, isoleucine, serine and threonine. Valine, leucine and isoleucine values are the means of results for six 72 h acid hydrolyses, whereas values for serine and threonine were obtained by extrapolation to zero time of hydrolysis. Approximate numbers of amino acids per subunit were calculated from a subunit M_r value of 59100 and an average residue M_r of 108. Tryptophan could not be determined in this way but there is one tryptophan residue in the N-terminal sequence (Figure 2) and there may be others. The amino-acid sequences of the L(+)-lactate dehydrogenases from *S. cerevisiae* (Guiard, 1985; Lederer et al., 1985) and *H. anomala* (Black et al., 1989) were used to calculate their amino-acid compositions.

	L(+)-Mandelate dehydrogenase from *R. graminis*		L(+)-Lactate dehydrogenase from *S. cerevisiae*	L(+)-Lactate dehydrogenase from *H. anomala*
Amino acid	Composition (mol %)	Approximate no. of amino acids per subunit	No. of amino acids per subunit	No. of amino acids per subunit
Asx	8.83 (7.37, 9.3, 9.83)	48	56	54
Glx	11.34 (10.43, 11.36, 12.22)	61	53	62
Ser	4.6 (4.0, 4.7, 5.1)	25	27	25
Gly	8.82 (9.23, 8.24, 8.99)	47	35	35
His	1.67 (1.85, 1.61, 1.54)	9	8	13
Arg	7.52 (7.93, 7.46, 7.18)	40	20	28
Thr	4.47 (4.8, 4.3, 4.3)	24	24	19
Ala	10.91 (11.08, 10.83, 10.83)	59	36	37
Pro	5.34 (5.5, 5.2, 5.3)	29	32	28
Tyr	1.64 (1.85, 1.63, 1.44)	9	16	14
Val	9.69 (9.67, 9.65, 9.76)	52	43	33
Met	0.39 (0.71, 0.33, 0.12)	2*	9	11
Cys	0.44 (0.31, 0.52, 0.48)	2*	7	3
Ile	6.47 (6.51, 6.48, 6.43)	35	29	39
Leu	9.61 (9.89, 9.54, 9.4)	52	51	44
Phe	2.9 (2.94, 2.87, 2.89)	16	14	18
Lys	6.81 (6.55, 6.94, 6.94)	37	47	33

* Probably underestimates.

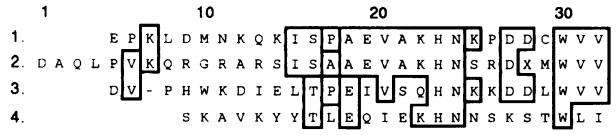

Figure 2 Comparison of the N-terminal amino-acid sequences of L(+)-mandelate dehydrogenase from *R. graminis*, L(+)-lactate dehydrogenase from *S. cerevisiae*, L(+)-lactate dehydrogenase from *H. anomala* and microsomal cytochrome b_5 of beef

The N-terminal sequence of L(+)-mandelate dehydrogenase from *R. graminis* (2) was determined three times, and comprehensive details of the determinations have been deposited as Supplementary Publication SUP 50172 at the British Library Document Supply Centre. The sequences for L(+)-lactate dehydrogenase from *S. cerevisiae* (1) (Guiard, 1985; Lederer et al., 1985), L(+)-lactate dehydrogenase from *H. anomala* (3) (Black et al., 1989) and microsomal cytochrome b_5 (4) (Guiard et al., 1974; Guiard and Lederer, 1976; Haumont et al., 1987) were taken from the literature.

in extracts. The subunit M_r value of the purified enzyme determined by SDS/PAGE was 59100 (S.D. = 840, $n = 26$) using nine different batches of enzyme. Comparison of the native and subunit M_r values and the fact that a single N-terminal sequence was found (see below) suggests that the enzyme is homotetrameric. In this respect it is very similar to the L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala* which are homotetramers with subunit M_r values of 57500 and 58000 respectively (Chapman et al., 1991), whereas the bacterial NAD(P)-independent membrane-bound L(+)-lactate and L(+)-mandelate dehydrogenases have M_r values of 40000–44000 (Hoey et al., 1987).

Amino-acid composition

The amino-acid composition of L(+)-mandelate dehydrogenase from *R. graminis* is rather similar to the compositions of L(+)-lactate dehydrogenase from *S. cerevisiae* and *H. anomala* (Table 2). Pairwise comparisons of the enzymes were made using calculated values of $S\Delta Q$ (the sum of the squares of the difference between the percentage of residues of the two proteins being compared) as measures of relatedness (Marchalonis and Weltman, 1971; Vanni et al., 1990). This showed that the similarity between the composition of L(+)-mandelate dehydrogenase and that of L(+)-lactate dehydrogenase from *S. cerevisiae* ($S\Delta Q = 53$) or L(+)-lactate dehydrogenase from *H. anomala* ($S\Delta Q = 43$) was almost as great as the similarity between the two L(+)-lactate dehydrogenases ($S\Delta Q = 30$), which share about 60% overall sequence identity (Black et al., 1989).

N-terminal sequences

The N-terminal sequence of L(+)-mandelate dehydrogenase from *R. graminis* showed strong similarities to L(+)-lactate dehydrogenases from *S. cerevisiae* and *H. anomala* and similarity to the microsomal cytochrome b_5 of beef, which is known to be homologous with the haem domains of the two yeast lactate dehydrogenases (Chapman et al., 1991) (Figure 2).

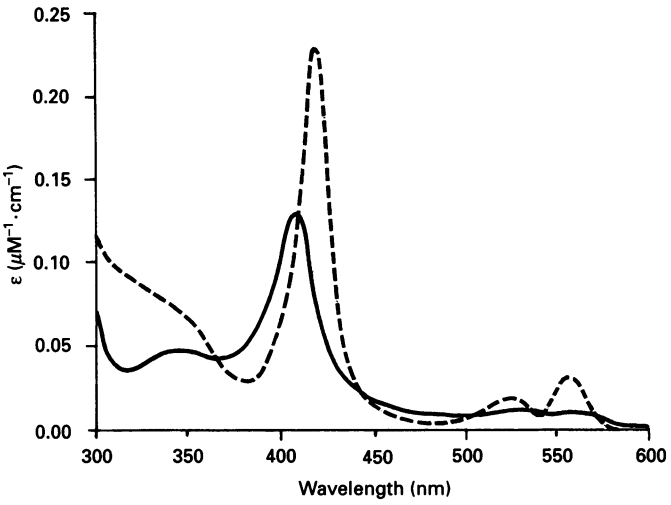

Figure 3 Absorption spectra of L(+)-mandelate dehydrogenase from *R. graminis*

L(+)-Mandelate dehydrogenase was purified as described in the Experimental section, concentrated to 100 μg/ml (1.67 μM) by vacuum dialysis and then scanned before (——) and after (---) incubation with 10 mM L(+)-mandelate.

Haem and flavin cofactors

L(+)-Mandelate dehydrogenase had broad absorption peaks at 560 nm and 530 nm and a sharp peak at 413 nm, and after reduction with substrate there were absorption peaks at 557, 528 and 423 nm (Figure 3). This absorption pattern is characteristic of a *b*-type cytochrome. The amount of haem present was calculated to be approx. 1 mol/59600 g of protein using an $\epsilon_{ox.}$ at 413 nm of 130 $mM^{-1} \cdot cm^{-1}$ (Labeyrie et al., 1978). This value is extremely close to the subunit M_r value of 59100 and so it is likely that each enzyme subunit contains one haem group.

The absorption spectra of trichloroacetic acid extracts of the enzyme were very similar to those of standard FAD and FMN solutions, with absorption peaks of 445 and 372 nm. Excitation at 440 nm gave identical emission fluorescence spectra for the trichloroacetic acid extract and for standard FAD and FMN solutions and the fluorescence was abolished by the addition of a few crystals of sodium dithionite. The addition of *Naja naja* snake venom resulted in a 9-fold increase in the fluorescence of a standard solution of FAD at 523 nm, but had no effect on the fluorescence spectra of either FMN or the extract. These results suggest the presence of FMN as a non-covalently bound prosthetic group. The amount of FMN present in the extract was calculated to be 0.55 (S.D. = 0.086, $n = 4$) mol/mol of subunit. The remaining pellet contained a further 0.2–0.25 mol of FMN/mol of subunit, perhaps because the Triton X-100 prevented complete extraction. Therefore in total the purified enzyme contains at least 0.8 mol of FMN/mol of subunit and so it seems likely that the native enzyme contains one molecule of non-covalently bound FMN/subunit.

Effects of pH on the enzyme

The isoelectric point of the enzyme could not be determined by chromatofocusing because it was inactivated at pH 5 or below; however, isoelectric focusing showed that the pI value was 4.4.

Purified L(+)-mandelate dehydrogenase had a fairly broad pH profile with half-maximal activity at pH values of about 6.2 and 9.0 and a pH optimum of 7.9 in phosphate buffer. An almost identical response to pH was found for the enzyme in crude extracts.

Electron acceptors

L(+)-Mandelate dehydrogenase, like the yeast L(+)-lactate dehydrogenases (Chapman et al., 1991), could use 2,6-dichloroindophenol, mammalian cytochrome *c* and potassium ferricyanide as electron acceptors and the relative activities for L(+)-mandelate dehydrogenase determined under the conditions described in the Experimental section (1:1.18:3.03 respectively) were very similar to those reported for L(+)-lactate dehydrogenase from *S. cerevisiae* (Morton et al., 1961), as were the apparent K_m values for 2,6-dichloroindophenol (27 and 34 μM respectively for the *R. graminis* and *S. cerevisiae* enzymes) and cytochrome *c* (40 and 44 μM respectively).

$k_{cat.}$ and apparent K_m and $V_{max.}$ values

The $k_{cat.}$ value with L(+)-mandelate as substrate was 50.8 s^{-1} (S.D. = 4.6, $n = 7$). The apparent K_m value of the purified enzyme for L(+)-mandelate was 266 μM (S.D. = 17; $n = 6$), which was identical with the value obtained using crude extract (266 ± 19 μM, $n = 3$). This value was obtained at pH 7.9 in phosphate buffer, and was similar to that obtained using Tris buffer at pH 7.5 (270 ± 30 μM; Smékal et al., 1993). The apparent K_m values for L(+)-mandelate in phosphate buffer increased considerably at higher and lower pH values (e.g. to 650 and 1100 μM at pH values of 8.8 and 6.5 respectively).

Substrate specificity and the inhibitory effects of certain substrate analogues

L(+)-Mandelate dehydrogenase is stereospecific for its substrate and D(−)-mandelate was not oxidized at a detectable rate, even at a concentration of 20 mM. However, D(−)-mandelate competitively inhibited L(+)-mandelate dehydrogenase activity with an apparent K_i value of 1.98 mM (S.D. = 0.18, $n = 3$). L(+)-Hexahydromandelate was also a competitive inhibitor (apparent $K_i = 0.33$ mM).

Effects of salts, thiol-blocking agents and other possible inhibitors

The enzyme was neither activated nor inhibited by any of the following salts at a concentration of 1 mM in the reaction mixture: NaCl, KCl, $CaCl_2$, $MgCl_2$, $MgSO_4$, $(NH_4)_2SO_4$; nor was the activity significantly affected by 10 mM concentrations of EDTA, 2,2′-bipyridyl, pyrazole, 8-hydroxyquinoline or sodium azide. Iodoacetate and iodoacetamide gave little or no inhibition at 10 mM and *N*-ethylmaleimide was only slightly inhibitory (35% at 10 mM). However, $HgCl_2$ and 4-chloromercuribenzoate strongly inhibited L(+)-mandelate dehydrogenase; thus, 10 min pre-incubation of the enzyme on ice with either of these compounds at 1 μM resulted in about 50% inhibition and there was no evidence for substrate protection. These results are broadly similar to those found for L(+)-lactate dehydrogenase from yeast (references in Chapman et al., 1991).

None of the following possible metabolic effectors, or their analogues, had any significant effect on enzyme activity when tested at 1 mM: acetyl-CoA, ADP/Mg^{2+}, ATP/Mg^{2+}, benzene sulphonate, citrate, glucose, glyceraldehyde, glycolate, isocitrate, DL-malate, oxaloacetate, 2-oxoglutarate, phenylacetate, phenylglyoxylate, phenylpyruvate, phenylsuccinate, pyruvate, succinate or tartrate. This observation is in line with the general finding that peripheral metabolic pathways in micro-organisms seem not to be subject to feedback control (Fewson, 1988, 1992).

Conclusions

The similarity in properties of the purified enzyme and of the enzyme in extracts (pH optimum, native M_r and apparent K_m value) suggest that L(+)-mandelate dehydrogenase was purified from *R. graminis* with little or no effect on its properties.

L(+)-Mandelate dehydrogenase from *R. graminis* is very similar to the L(+)-lactate dehydrogenases from the other two yeasts, *S. cerevisiae* and *H. anomala*. All three of these enzymes are tetramers of similar subunit M_r, contain both FMN and haem as cofactors, can use 2,6-dichloroindophenol, ferricyanide and cytochrome *c* as electron donors, have similar amino-acid compositions and N-termini, and possess broadly similar kinetic and related properties. Our results are compatible with the notion that L(+)-mandelate dehydrogenase, like L(+)-lactate dehydrogenase, is located in the intermembrane space of mitochondria. Furthermore, the appearance on prolonged storage of a lower-M_r fragment (Figure 1) would be consistent with either proteolysis between a haem domain and a flavodehydrogenase domain or with the existence of a proteinase-sensitive loop, as in L(+)-lactate dehydrogenase (Chapman et al., 1991). The only substantial respect in which the enzymes differ is that they show exactly reciprocal behaviour with respect to L(+)-lactate and L(+)-mandelate: L(+)-lactate is a substrate for the L(+)-lactate dehydrogenase and a competitive inhibitor of the L(+)-mandelate dehydrogenase, whereas L(+)-mandelate is a substrate for the L(+)-mandelate dehydrogenase and a competitive inhibitor of L(+)-lactate dehydrogenase (Smékal et al., 1993). This mutual substrate exclusivity raises substantial questions about substrate-binding and catalytic mechanisms that should be amenable to experimental tests (Smékal et al., 1993). There is strong evidence for homology among the N-terminus of L(+)-mandelate dehydrogenase, cytochrome b_5 and the haem domains of the two yeast L(+)-lactate dehydrogenases (Figure 2). Evidence for homology among the flavin domains of the three dehydrogenases is only circumstantial so far, being based on gross properties of the enzymes (e.g. size, amino-acid composition, lactate binding, etc., as listed above). However, it also has to be recalled that L(+)-mandelate dehydrogenase of *Pseudomonas putida* is almost exactly the same size as the flavodehydrogenase domain of the yeast L(+)-lactate dehydrogenases and it is without doubt homologous with them (Tsou et al., 1990). It seems very likely that both bacterial and yeast mandelate dehydrogenases have arisen as a result of recruitment of 2-hydroxy-acid dehydrogenases from other metabolic pathways, presumably by gene duplication and/or mutation (Tsou et al., 1990; Fewson, 1992). Of the NAD(P)-independent dehydrogenases discovered so far, bacterial L(+)-lactate and L(+)-mandelate dehydrogenases seem to be single-domain flavodehydrogenases, whereas the yeast L(+)-lactate and L(+)-mandelate dehydrogenases consist of a haem domain and a flavodehydrogenase domain. The yeast mandelate dehydrogenase and lactate dehydrogenases could have evolved directly one from the other, or from a common ancestor in which the haem domain and the flavodehydrogenase domain were already fused; alternatively, there may have been independent fusion of the domains, in which case the flavin domains of mandelate and lactate dehydrogenases may show a different degree of identity than do the haem domains, or may even, although this seems unlikely, turn out to be unrelated.

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