L-Mandelate dehydrogenase from *Rhodotorula graminis*: cloning, sequencing and kinetic characterization of the recombinant enzyme and its independently expressed flavin domain

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The L-mandelate dehydrogenase (L-MDH) from the yeast *Rhodotorula graminis* is a mitochondrial flavocytochrome b_2 which catalyses the oxidation of mandelate to phenylglyoxylate coupled with the reduction of cytochrome *c*. We have used the N-terminal sequence of the enzyme to isolate the gene encoding this enzyme using the PCR. Comparison of the genomic sequence with the sequence of cDNA prepared by reverse transcription PCR revealed the presence of 11 introns in the coding region. The predicted amino acid sequence indicates a close relationship with the flavocytochromes b_2 from *Saccharomyces cerevisiae* and *Hansenula anomala*, with about 40% identity to each. The sequence shows that a key residue for substrate specificity in *S. cerevisiae* flavocytochrome b_2, Leu-230, is replaced by Gly in L-MDH. This substitution is likely to play an important part in determining the different substrate specificities of the two enzymes. We have developed an expression system and purification protocol for recombinant L-MDH. In addition, we have expressed and purified the flavin-containing domain of L-MDH independently of its cytochrome domain. Detailed steady-state and pre-steady-state kinetic investigations of both L-MDH and its independently expressed flavin domain have been carried out. These indicate that L-MDH is efficient with both physiological (cytochrome *c*, $k_{cat} = 225\ s^{-1}$ at 25 °C) and artificial (ferricyanide, $k_{cat} = 550\ s^{-1}$ at 25 °C) electron acceptors. Kinetic isotope effects with [2-^{2}H]mandelate indicate that H–C-2 bond cleavage contributes somewhat to rate-limitation. However, the value of the isotope effect erodes significantly as the catalytic cycle proceeds. Reduction potentials at 25 °C were measured as −120 mV for the 2-electron reduction of the flavin and −10 mV for the 1-electron reduction of the haem. The general trends seen in the kinetic studies show marked similarities to those observed previously with the flavocytochrome b_2 (L-lactate dehydrogenase) from *S. cerevisiae*.

INTRODUCTION

Flavocytochrome b_2 from the yeasts *Saccharomyces cerevisiae* and *Hansenula anomala* is a mitochondrial L-lactate dehydrogenase (L-LDH) that transfers electrons directly to cytochrome *c* [1,2] and enables these organisms to utilize lactate as the sole source of carbon and energy [3]. The red yeast, *Rhodotorula graminis*, is one of few microorganisms capable of utilizing L-mandelate (L-2-hydroxy-2-phenylacetate) as a substrate for growth [4,5]. The first enzyme of the catabolic pathway, L-mandelate dehydrogenase (L-MDH), has been isolated and shown to share several features with the L-LDHs (flavocytochromes b_2) from *S. cerevisiae* and *H. anomala* [6,7]. These enzymes all contain FMN and protoporphyrin IX, their sizes are very similar and the N-terminal sequences indicate that they may be homologues [6].

Flavocytochrome b_2 from *S. cerevisiae* is a tetramer of identical subunits, each consisting of two distinct domains [8]. The N-terminal cytochrome domain is related to cytochrome b_5, whereas the FMN-containing C-terminal domain has a separate evolutionary history, with relatives found in plants, animals and bacteria. These relatives all oxidize 2-hydroxyacids, but their substrates differ in size, chemical nature and metabolic role. Members of this family include the plant peroxisomal glycolate oxidase [9], *Pseudomonas putida* L-MDH [10], *Mycobacterium smegmatis* lactate mono-oxygenase [11] and a long-chain 2-hydroxyacid dehydrogenase from rat kidney [12].

We have reported previously that Leu-230 of *S. cerevisiae* flavocytochrome b_2 is a major determinant of substrate specificity [13]. The crystal structure [8] shows that this side-chain is in contact with the methyl group of pyruvate, the product of lactate oxidation. L-Mandelate is not a substrate for this enzyme but acts as a competitive inhibitor. It is obvious from modelling studies that mandelate cannot be accommodated in an equivalent position because of steric interference between the Leu-230 side-chain and the phenyl ring of mandelate. Sequence and structure comparisons show that Leu-230 is replaced by Trp in glycolate oxidase [9,14] and Ala in the L-MDH from *P. putida* [10]. Apparently, therefore, a large amino acid side-chain in this position correlates with a smaller preferred substrate, whereas a smaller side-chain permits access to larger substrates.

Mechanistic studies with *R. graminis* L-MDH have shown some interesting differences between this enzyme and the L-LDH activity of *S. cerevisiae* flavocytochrome [7,15]. However, studies to date with L-MDH have been limited to steady-state experiments, since the enzyme can only be isolated in small quantities from *R. graminis*. We have developed an efficient heterologous expression system to enable more detailed investigation of the mechanism of this enzyme. Studies with the intact enzyme are limited by the fact that spectral changes at the flavin cofactor are masked by the intensely absorbing haem group. To resolve this problem we have also expressed the flavin domain independently. In this paper we describe a detailed kinetic characterization of recombinant forms of both wild-type L-MDH and its independently expressed flavin domain.

Abbreviations used: L-MDH, L-mandelate dehydrogenase; L-LDH, L-lactate dehydrogenase; KIE, kinetic isotope effect; PAL, phenylalanine ammonia lyase; RT-PCR, reverse transcription PCR.

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MATERIALS AND METHODS

Strains and plasmids

R. graminis GX6000 (American Type Culture Collection 20804) was used as a source of RNA and DNA and was maintained, grown, harvested and stored as described previously [16]. *Escherichia coli* strains TG1, JM109 and NF1 were used as hosts for recombinant plasmids. The plasmids pTZ19r and pTZ18r were used for cloning [17] and the expression vector pRC23 [18] was used to direct expression of L-MDH. *E. coli* was routinely grown in Luria broth supplemented where appropriate with 100 μg ml^{-1} ampicillin. For expression of recombinant enzyme, growth was in Terrific Broth supplemented with 150 μg ml^{-1} ampicillin.

DNA isolation

Chromosomal DNA was isolated from a 10 ml of stationary-phase culture grown in YPD medium [1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose]. The cells were harvested by centrifugation and resuspended in 1 ml of breakage buffer (0.9 M sorbitol/14 mM 2-mercaptoethanol/50 mM sodium phosphate buffer, pH 7.5). The cells were then disrupted by vortexing with acid-washed glass beads. To this suspension 50 μl of 0.5 M EDTA, pH 8.0, was added, vortexed briefly and then 50 μl of 10 % SDS and 100 μl proteinase K solution (5 mg ml^{-1}) were added to help lysis. The mixture was mixed well and incubated at 65 °C for 30 min, then extracted with 1:1 phenol/chloroform and the DNA was precipitated by addition of 0.5 ml of absolute ethanol.

Plasmid DNA and single-stranded plasmid DNA were isolated from *E. coli* transformants as described previously [19,20]. M13KO7 was used as helper phage for single-stranded DNA production.

Isolation of RNA

RNA was isolated from a 100 ml culture of *R. graminis* grown until mid-exponential phase in medium containing D,L-mandelate. The cells were harvested and resuspended in 1 ml of TNE (50 mM Tris/HCl, pH 7.5/100 mM NaCl/5 mM EDTA). Acid-washed glass beads were used to disrupt the cells with vigorous vortexing for 2 min. Then 4 ml of TNE, 0.2 ml of 20 % SDS and 4 ml of phenol were rapidly added and the suspension was vortexed for another 2 min. The mixture was spun for 15 min to separate the phases. The aqueous phase containing RNA was removed and extracted with 1:1 phenol:chloroform until a clear interphase was achieved. The upper phase containing the RNA was removed and to this 0.1 vol. of 3 M Na acetate, pH 5.5, and 2 vol. of 100 % ethanol were added to precipitate the RNA.

Before use in reverse transcription (RT)-PCR, contaminating DNA was removed from the RNA by treatment with DNaseI. A 100 μl mixture containing 100 μg of total RNA and 10 units of DNaseI in 20 mM Tris/HCl, pH 8.4/12 mM $MgCl_2$/50 mM KCl was incubated at 37 °C for 1 h. The reaction was stopped by heating to 65 °C for 10 min and extraction with phenol/chloroform (1:1). The RNA was precipitated with ethanol, pelleted, dried and dissolved in 50 μl of H_2O.

First strand cDNA synthesis

A mixture of 1 μl of oligo(dT)$_{12-18}$ (500 μg/ml) and 15–20 μg of total *R. graminis* RNA (treated with DNase) in 10 μl of sterile distilled water was heated to 70 °C for 10 min and then quickly chilled on ice. The contents of the tube were collected by brief centrifugation, mixed with 4 μl of 5 × first strand buffer (Gibco-BRL), 2 μl of 0.1 M dithiothreitol, 2 μl of 5 mM dNTPs and 1 μl (200 units) of Superscript™ II RNase H$^-$ reverse transcriptase (Gibco-BRL) and incubated at 37 °C for 1 h. The products were used immediately for PCR or stored at −20 °C

RT-PCR

Forward primer H1549 (GGAATTCGAYGCNCARCTNCCNGTNAARCA) and reverse primer N7501 (TCGAAGCTTRTGYTTNGCNACYTTCNGC) were designed for amplification of a short segment of the L-MDH coding sequence using the N-terminal sequence of the isolated enzyme [6]. Unfractionated *R. graminis* RNA (15 μg) was reverse transcribed in a 20 μl reaction mixture as described above. PCR was performed in a 50 μl reaction mixture with 15 mM $MgCl_2$, 5 pmol of each primer, 200 μM dNTPs and 1 μl of the reverse transcriptase reaction products. After denaturing at 94 °C for 2 min, 40 cycles were carried out as follows: 94 °C for 40 s, 52 °C for 1 min, 72 °C for 1 min. The reaction was completed by a further 7 min incubation at 72 °C.

For amplification of the complete L-MDH coding sequence, the forward primer RI1 (CAACCCGGGATGGATGCTCAGCTGCCGGT) and the reverse primer RI2 (CGAAGCTTCTACTCGGGCACCCACCG) were used. Reverse transcription and PCR were carried out as above, except that the first three cycles comprised 40 s at 94 °C, 30 s at 50 °C and 90 s at 72 °C. The annealing temperature was then increased to 56 °C for a further 35 cycles.

Construction of *R. graminis* DNA libraries

Approx. 10 μg of *R. graminis* genomic DNA was digested with *Xba*I, *Sph*I, *Sac*I, *Bam*HI, *Hin*dIII, *Pst*I and *Eco*RI and separated on a 0.8 % agarose gel and transferred to a Hybond-N (Amersham) nylon membrane. The membrane was pre-hybridized for 1 h at 65 °C, then hybridization was carried out overnight at 65 °C. The probe was prepared from recombinant pTZ19r containing the 85 bp product of RT-PCR that had been labelled by the random priming method [21]. After washing with increasing stringency, the membrane was allowed to dry and was autoradiographed at −70 °C. A genomic DNA library was constructed by complete digestion of genomic DNA with *Hin*dIII. The resulting fragments were ligated with plasmid pTZ19r that had also been cut with *Hin*dIII. About 6000 recombinants were screened by hybridization under the same conditions as for Southern blotting.

DNA sequence determination

The sequences of cDNA and genomic DNA were determined on both strands using the dideoxy chain-termination method [22] with the Sequenase (U.S. Biochemical Corp.) T7 polymerase. Because of the relatively high GC content of *R. graminis* DNA we found it necessary to use the dITP mixture in place of dGTP, but this gave good quality, unambiguous sequence information. The complete sequence of each DNA strand was determined using a combination of subclones and specifically designed internal primers. DNA sequence information was analysed using the Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI, U.S.A.

Western blotting

Proteins were separated by SDS/PAGE and electrophoretically transferred to a nylon membrane (Hybond-N) as described previously [23]. L-MDH was detected using antiserum raised in rabbit, followed by horse-radish peroxidase-conjugated goat

anti-rabbit IgG (Bio-Rad) as secondary antibody. After washing, enzyme activity was visualized using *o*-dianisidine as substrate.

Flavin domain expression

The L-MDH coding sequence was modified by site-directed mutagenesis using the oligonucleotide GATTGGCGGCGAATTCATGGGCAAGAATGC. This introduced an *Eco*RI cleavage site between the sequences encoding the haem- and flavin-containing domains and places an ATG initiation codon immediately preceding the codon for Gly-99, the first residue of the flavin domain. Resulting clones were screened for the introduction of the *Eco*RI site and the entire flavin domain region was sequenced to ensure that no unwanted mutations had been introduced. The DNA encoding the flavin domain was transferred to the expression vector pRC23 as an *Eco*RI–*Hin*dIII fragment. The recombinant plasmid was transformed into *E. coli* JM109 cells. Growth and expression were as for the holoenzyme.

Purification of recombinant L-MDH

The following buffers were used during the purification procedure: buffer A, 0.1 M phosphate, pH 7.0; buffer B, 30 mM phosphate, pH 7.0, with 1 mM L-lactate; buffer C, 10 mM Tris/HCl, pH 7.5. Where possible, all manipulations were carried out at 4 °C.

L-MDH was isolated from *E. coli* using the following protocol. Approx. 25 g of pelleted wet cells (from 5 litres of culture) were snap-frozen in liquid nitrogen. The frozen cells were then resuspended in 250 ml of buffer A. Lysozyme was added to a concentration of approx. 0.2 mg·ml^{-1} and EDTA to a final concentration of 1 mM. The suspension was left stirring for 60 min, after which it was centrifuged at 39000 ***g*** for 10 min to remove cell debris. The red supernatant was then applied to a DE52 (Whatman) column (10 cm × 4.5 cm) equilibrated in buffer A. The red coloured L-MDH passed straight through the column and was collected. At this stage the solution containing the enzyme had an A_{269}/A_{423} ratio of around 8. This solution was then dialysed for ~ 2 h against a 10-fold greater volume of buffer B. After dialysis, the solution was loaded onto a DEAE-Sephacel column (20 cm × 3 cm) previously equilibrated with buffer B. The L-MDH bound as a tight red band at the top of the column. The column was then washed with several volumes of buffer B. The enzyme was eluted using a gradient from 0 to 0.3 M NaCl in buffer B. Fractions with an A_{269}/A_{423} ratio of ~ 2 (30 % pure) were collected and pooled. The resulting solution was dialysed overnight against a 10-fold greater volume of buffer B. After dialysis, the protein was loaded on to a CQ Sepharose (Sigma) column (20 cm × 3 cm) equilibrated in buffer B. The column was washed with several columns of buffer B and then eluted using an identical gradient to that used for the DEAE-Sephacel column. Fractions with an A_{269}/A_{423} ratio of ⩽ 1.0 (50 % pure) were pooled and concentrated to 1 ml using a Centriprep 50 (Amicon). The concentrated solution was loaded on to an S300-HR Sephacryl gel-filtration column (1 m × 2.5 cm) which had been previously equilibrated in buffer C. The protein was eluted from the column in buffer C and fractions with an A_{269}/A_{423} ratio of ⩽ 0.5 (⩾ 95 % pure) were pooled, concentrated, snap-frozen and stored in liquid nitrogen.

Purification of L-MDH-flavin domain

Purification of the flavin domain was identical with that for the holoenzyme, with the exception that the final S300-HR Sephacryl gel-filtration column was not necessary.

Kinetic analysis

All experiments were carried out at 25 ± 0.1 °C in 10 mM Tris/HCl at pH 7.5, I 0.10. The buffer was 10 mM HCl adjusted to pH 7.5 with Tris and corrected to I 0.10 by addition of NaCl. Steady-state measurements involving enzymic oxidation of L-mandelate were performed using a Shimadzu UV2101PC spectrophotometer. Cytochrome *c* (horse type VI; Sigma) or ferricyanide (potassium salt; BDH) were used as electron acceptors as previously described [24]. Pre-steady-state kinetics were carried out using an Applied Photophysics SF.17MV stopped-flow spectrophotometer. Measurements of the rates of reduction of the cofactors of L-MDH by L-mandelate were monitored at 438.3 nm (a haem isosbestic) for the flavin and at either 557 or 423 nm for the haem group. Pre-steady-state reduction of the L-MDH flavin domain was monitored at 450 nm. Collection and analysis of data were as previously described for flavocytochrome b_2 [24,25]. Measurements of kinetic isotope effect (KIE) values were also performed as previously described [24].

Synthesis of D,L-[2-^{2}H]mandelate

D,L-[2-^{2}H] mandelic acid was prepared as the racemic mixture by a method completely different to that reported previously by Smékal et al. [7]. Although no special precautions are necessary, standard safety procedures (such as the wearing of safety spectacles) should be adopted and all operations should be carried out in a fume cupboard. Sodium metal (1.6 g, 0.07 mol) was carefully dissolved in 10 ml of ^2H_2O (99.9 % ^{2}H, 9.03 g, 0.45 mol; Fisher Acros) under a nitrogen atmosphere in a three-necked flask fitted with condenser. Because of the enormous heat development, the flask was cooled with ice. After preparation of this solution, 1.15 g (7.56 mmol) of D,L-mandelic acid was added and this mixture was boiled for 4 h. The nitrogen atmosphere was retained while the solution cooled down overnight. The following day, 120 ml of 1 M hydrochloric acid was added to the reaction mixture to obtain acidic conditions. The crude product was extracted four times with 30 ml portions of ether and the organic layer was dried with 10 g of magnesium sulphate for 2 h. This solution was filtered and the ether was removed, leaving a slightly yellow crude product. Recrystallization was carried out with $CHCl_3$ and cyclohexane and yielded white crystals of DL-[2-^{2}H]mandelic acid (0.768 g, 5.01 mmol, 66.3 % yield) with a melting point of 119 °C, in agreement with the literature value [26]. The isotopic content of this DL-[2-^{2}H]mandelic acid was determined to be ⩾ 95 % by MS using a KRATOS M. S. 50 TC spectrometer. The purity of the compound was further confirmed by ^{1}H-NMR and ^{13}C-NMR spectra recorded on a Bruker AC Spectrometer (250 MHz).

Reduction potentials

The mid-point potentials for the haem and flavin groups of L-MDH were determined using the intact enzyme and the flavin domain respectively. Reduction potentials were measured spectrophotometrically using a previously published redox potentiometry method [27]. All experiments were performed at 25 °C within a Belle Technology glove box under a nitrogen atmosphere with oxygen maintained at < 5 p.p.m. Conditions for potentiometric titrations, including mediators, electrodes and protein concentrations, were as described previously [28]. Nernst plots for both reductive and oxidative titration sequences showed no evidence of hysteresis, indicating that the systems were at equilibrium during measurements. In the case of the flavin

domain, only the potential for the two-electron couple from oxidized to reduced could be determined.

RESULTS AND DISCUSSION

Isolation of the L-MDH gene

Fully degenerate oligonucleotide primers based on the N-terminal sequence of L-MDH were used to amplify *R. graminis* cDNA by the RT-PCR. The principal product was a fragment of the expected size (85 bp). This was cloned in pTZ19r and sequenced. Of eight clones that were sequenced, two had the expected coding potential. One of these (pLM1) was then used as a probe to isolate the entire L-MDH gene from *R. graminis* DNA.

Aliquots of *R. graminis* genomic DNA were cut with various restriction enzymes and subjected to Southern blotting. Hybridization with labelled pLM1 revealed that the amplified fragment

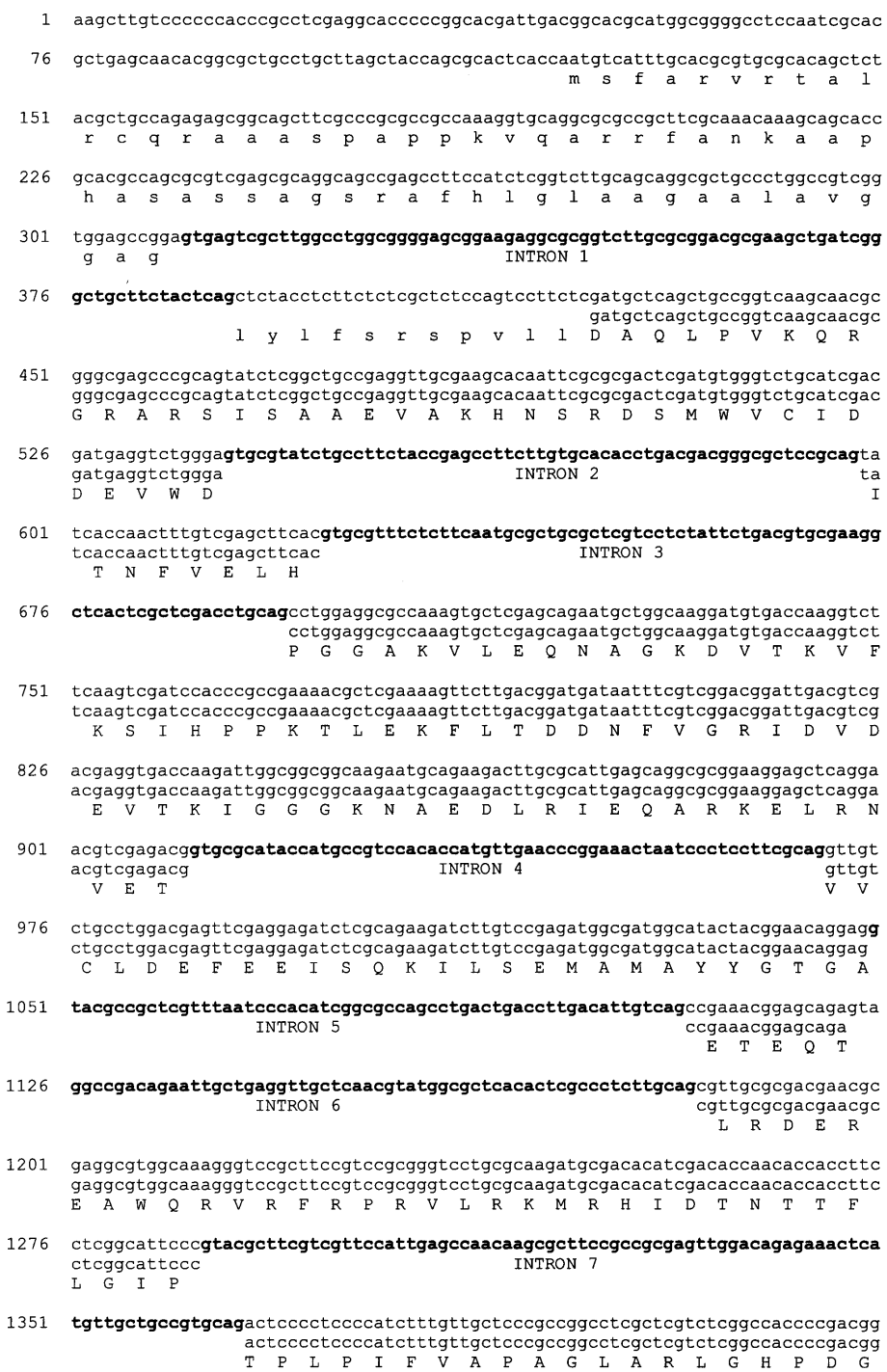

Figure 1 For legend see opposite page

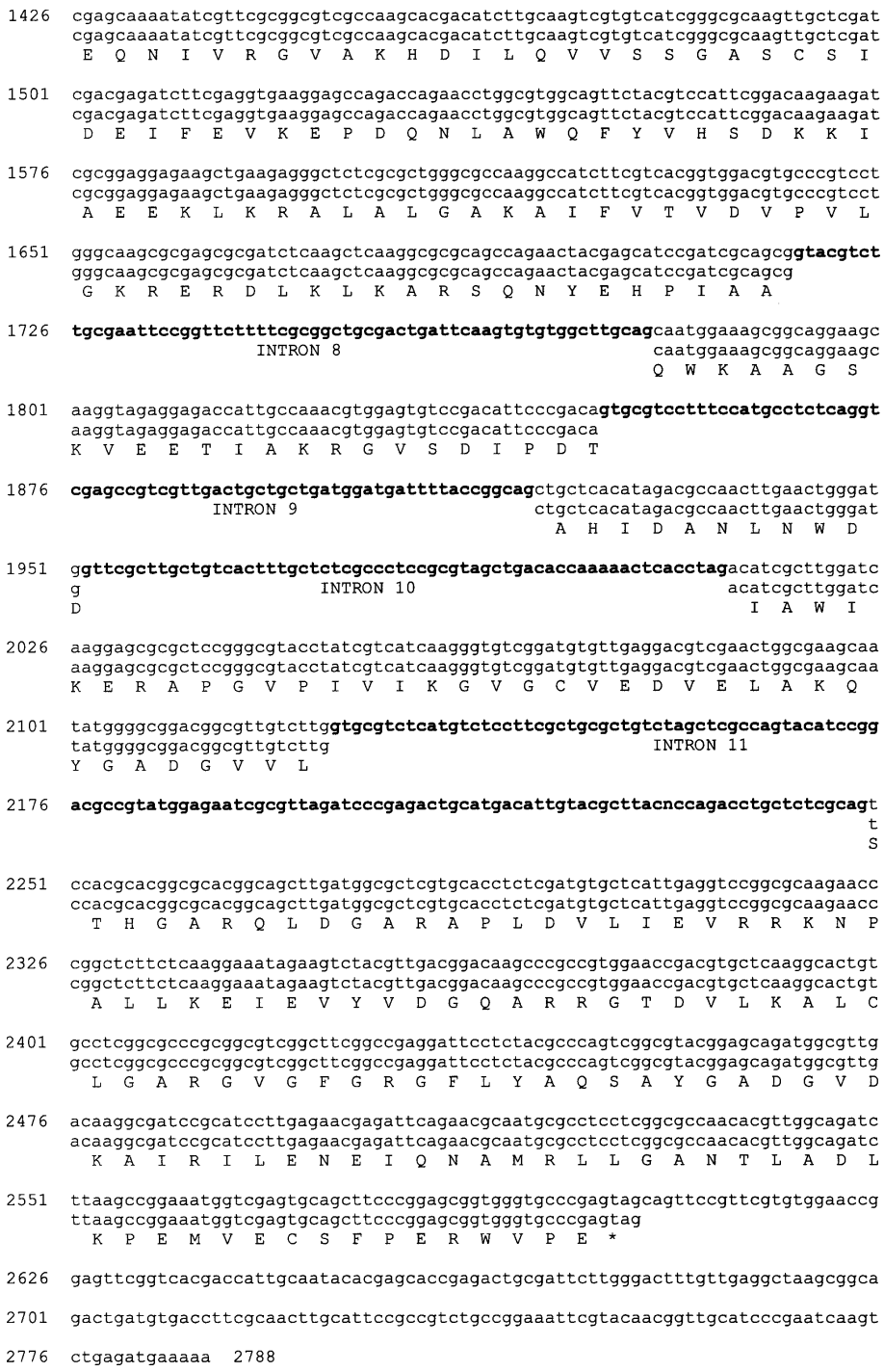

Figure 1 Complete sequence of the L-MDH coding region

The sequence of the genomic DNA (top line) is aligned with the cDNA sequence (second line), clearly showing the positions of the ten introns in the region encoding the mature enzyme. The sequences of these introns (2–11) are shown in bold face, as is the predicted intron 1, which lies upstream of the region amplified by RT-PCR. The amino acid sequence is shown below the cDNA sequence, with the predicted mitochondrial targeting sequence in lower case. These sequences have been submitted to the EMBL database with the accession numbers AJ001430 and AJ001431.

was contained on a *Hin*dIII fragment of 5.5 kb. A library of *Hin*dIII fragments of genomic DNA was constructed in pTZ19r and probed under conditions identical with those used for Southern blot hybridization. A positive clone (pLM3) was used for further DNA sequence analysis (Figure 1).

Isolation and sequencing of L-MDH cDNA

The presence of several introns in the L-MDH coding sequence was obvious from the presence of in-frame stop codons in the genomic DNA. We therefore isolated cDNA to determine the sequence of the protein encoded by this gene. This was achieved

Introns in the LMDH gene from *R. graminis*

Intron	Position	5' branchpoint 3'
1	311–391	GT.....CTGAT.....CAG
2	540–598	GT.....CTGAC.....CAG
3	624–695	GT.....CTGAC.....CAG
4	912–970	GT.....CTAAT.....CAG
5	1050–1107	GT.....CTGAC.....CAG
6	1123–1183	GT.....CTCAC.....CAG
7	1288–1366	GT.....CTCAT.....CAG
8	1717–1779	GT.....CTGAT.....CAG
9	1850–1918	GT.....CTGAT.....CAG
10	1952–2011	GT.....CTGAC.....TAG
11	2125–2249	GT.....CTTAC.....CAG

Introns in PAL genes from *R. toruloides* and *R. rubra*

GT.....CTGAC.....CAG

Introns in *S. cerevisiae*

GT.....TACTAAC.....CAG

Figure 2 Sequence features of introns

The sequences of the 5′ and 3′ ends and the putative branch points of the 11 introns in the L-MDH gene are indicated, along with their positions in the sequence. Sequence numbering is as shown in Figure 1. The corresponding sequences for other genes from *Rhodotorula* [32] and *Saccharomyces cerevisiae* [41] are shown for comparison.

using primers (RI1 and RI2) that were designed according to the known N-terminal sequence of the isolated mature protein and the C-terminal sequence predicted from the genomic DNA. The latter primer contains a *Hin*dIII cleavage site and the former was designed to incorporate both an *Xma*I cleavage site and a methionine codon immediately preceding the first codon of the mature L-MDH coding sequence to allow later expression of the cDNA. RT-PCR using these primers yielded a fragment, as expected, of approx. 1.5 kb. This was cloned in pTZ18r and pTZ19r using the *Xma*I and *Hin*dIII sites to yield the recombinant plasmids pLM5 and pLM6, which were then used to determine the complete sequence of the cDNA (Figure 1). The absence of PCR-induced mutations was apparent from the exact correspondence between the cDNA and genomic DNA sequences, except for the presence and absence of introns.

The sequence of the gene from *R. graminis* encoding L-MDH has been determined and compared with the sequence of a cDNA encoding the mature polypeptide. The G+C content of the cloned DNA is 60 % and does not differ significantly between coding and non-coding regions. The genomic sequence apparently includes 11 short introns (Figure 2). The locations of ten of these are clearly identified by alignment with the cDNA sequence. Since the cDNA extended at the 5′ end only as far as the start of mature L-MDH, there is no experimental evidence for the presence of intron 1 (Figure 1), which lies within the presequence coding region. However, the existence of this intron and its location as shown are supported by several observations. First, intron 1 shares sequence features, discussed below, with other introns in this and other *Rhodotorula* genes. Secondly, no in-frame ATG initiation codon is found in the region upstream of the sequence encoding the experimentally determined N-terminal amino acid sequence of mature L-MDH. With the intron located as shown, the N-terminal presequence is similar to the presequences of flavocytochromes b_2 from *S. cerevisiae* and *H. anomala* [29,30].

The genes encoding phenylalanine ammonia lyase (PAL) from *Rhodosporidium toruloides* [31] and *Rhodotorula rubra* [32], both of which are close relatives of *R. graminis*, revealed the presence of six and five introns respectively. All introns in both PAL genes contain the nucleotides GT at their 5′ end and CAG at the 3′ end. These introns also share the same internal consensus sequence of CTGAC, which presumably determines the RNA splicing branch point. Introns in the L-MDH gene are similar to introns in the PAL genes, with sizes ranging from 59 to 125 bp. All 11 introns have the invariant sequence of GT at their 5′ ends. Intron 10 has the sequence TAG at its 3′ end; the other ten introns have the consensus sequence CAG. The putative branch-point sequences in the introns within the L-MDH gene from *R. graminis* appear to be more variable than those in the PAL genes (Figure 2). Takahashi et al. [33] found that introns in the small nuclear RNA genes in *R. hasegawae* are similar to pre-mRNA introns and have a consensus internal sequence of CTRAC, where R is a purine (A or G). The sequences of introns in the MDH genes indicate even greater flexibility in the internal (branch-point) sequence. Only four of the eleven introns contain the sequence CTGAC, but all contain the sequence CT*n*A*y*. We have also noted flexibility in the intron sequences of the D-MDH gene from *R. graminis* (R. M. Illias, J. S. Miles, C. A. Fewson, S. K. Chapman and G. A. Reid, unpublished work; EMBL database entry AJ001428).

Mitochondrial targeting sequence

L-MDH, like flavocytochromes b_2 from *S. cerevisiae* [34], is located in the mitochondrial intermembrane space (A. Warburton and G. A. Reid, unpublished work). Targeting of flavocytochromes b_2 in *S. cerevisiae* is directed by the N-terminal presequence, which is unusually long, consisting of 80 amino acid residues [29]. This peptide is removed by two distinct proteolytic enzymes during transport to the intermembrane space [35,36]. Functionally important features of the presequence include a basic N-terminal region and a long uncharged region towards the C-terminal end that is thought to span the mitochondrial inner membrane. These features are conserved in several proteins that are targeted to the intermembrane space and are also found in the predicted L-MDH presequence.

Sequence of mature L-MDH

The sequence of L-MDH is clearly very similar to the amino acid sequences of the L-LDHs from *S. cerevisiae* and *H. anomala*. These enzymes are all flavocytochromes b_2, which are composed of distinct cytochrome and flavoprotein domains, connected by a 'hinge' peptide [8,37]. The hinge is relatively poorly conserved, as is a surface loop within the flavin-binding domain that is apparently rather flexible [30]. The crystal structure of *S. cerevisiae* flavocytochrome b_2 shows that the C-terminus, from residue 487 to 511, forms an extended structure that contacts each of the three other subunits within the homotetramer [8]. In L-MDH this C-terminal tail is absent, but this is unlikely to indicate a difference in quaternary structure. Removal of residues 489–511 from *S. cerevisiae* flavocytochrome b_2 by site-directed mutagenesis did not have a significant effect on tetramer formation [38]. Furthermore, glycolate oxidase lacks a C-terminal tail, but still has a similar 4-fold axis to that found with flavocytochrome b_2 [15]. Overall, the L-MDH sequence is 42–43 % identical with each of the other flavocytochromes b_2, whereas the two L-LDHs are 60 % identical. The flavoprotein domain of L-MDH belongs to a family of 2-hydroxyacid dehydrogenases, members of which have been found in bacteria, plants and animals, as well as in fungi. The sequences of several of these are aligned in Figure 3. Residues that have been identified, both by examination of crystal structures and by biochemical characterization of mutated enzymes, as being important for catalytic activity are all conserved in L-MDH. Whereas all enzymes in this family are L-2-hydroxyacid dehydrogenases and their catalytic

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          198                                 230
           ↓                                   ↓
Scb2 PFYVSATALC KLGNPLEGEK DVARGCGQGV TKVPQMISTL ASCSPEEIIE
Hab2 PFYISATALA KLGHP.EGEV AIAKGAGRE. .DVVQMISTL ASCSFDEIAD
 Gox PIMIAPTAMQ KMAHP.EGEY ATARAASAA. .GTIMTLSSW ATSSVEEVAS
 Hao PICISPTAFH SIAWP.DGEK STARAAQEA. .NICYVISSY ASYSLEDIVA
 Mdh PLLIGPTGLN GALWP.KGDL ALARAATKA. .GIPFVLSTA SNMSIEDLAR
 Lox PMFFAPIGVI ALC.AQDGHG DAASAQASAR TGVPYITSTL AVSSLEDIRK
LctD PVALAPVGLC GMYAR.RGEV QAAKAADAH. .GIPFTLSTV SVCPIEEVA.
LMDH PIFVAPAGLA RLGHP.DGEQ NIVRGVAKH. .DILQVVSSG ASCSIDEIFE
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Figure 3 Sequence comparison of L(+)-MDH from *R. graminis* (L-MDH) and other 2-hydroxy acid dehydrogenases

The residue numbering relates to flavocytochrome b_2 from *S. cerevisiae* (Scb2). The other sequences are: *H. anomala* flavocytochrome b_2 (Hab2), spinach glycolate oxidase (Gox), rat hydroxy acid oxidase (Hao), *P. putida* L-MDH (Mdh), *M. smegmatis* lactate oxidase (Lox) and L-LDH from *E. coli* (LctD). The amino acid sequences are shown in the vicinity of Ala-198 and Leu-230, the positions of which are indicated by the vertical arrows.

mechanisms appear to be very similar, at least for substrate oxidation, they vary considerably in their substrate selectivities. L-MDH has a strong preference for an aromatic substituent at the 2-position and is inhibited for example by L-lactate [7]. Similarly, L-mandelate inhibits the L-LDH activity of *S. cerevisiae* flavocytochrome b_2. Leu-230 has been identified as a particularly critical residue in determining substrate specificity in flavocytochromes b_2. The residue found at this position varies considerably within the L-2-hydroxyacid dehydrogenase family and there is an inverse correlation between the sizes of the substrate side-chain and the amino acid side-chain. This correlation is extended in L-MDH, where the equivalent residue is a glycine (Gly-225). Substitution of Leu-230 in *S. cerevisiae* flavocytochrome b_2 by alanine results in an enzyme with a greater selectivity towards substrates with a longer aliphatic side-chain, such as 2-hydroxyoctanoate [14], clearly demonstrating the importance of this residue. Ala-198 in *S. cerevisiae* flavocytochrome b_2 has also been identified as important in determining substrate specificity [14], and the equivalent residue in L-MDH is a glycine (Gly-195). The function of these residues is demonstrated more clearly in the accompanying paper [39], with the conversion of *S. cerevisiae* flavocytochrome b_2 into an efficient L-MDH.

Expression of L-MDH in *E. coli*

The L-MDH cDNA was isolated from pLM5 as an *Xma*I–*Hin*dIII fragment. This was ligated with the expression plasmid pKK223-3 that had been cut with the same pair of enzymes to generate pLM7. We could not detect L-MDH expression in *E. coli* JM105 transformed with this plasmid. The L-MDH cDNA was then transferred to the *E. coli* expression vector pRC23, which contains the bacteriophage λ P_L promoter as an *Eco*RI–*Hin*dIII fragment isolated from pLM7. The resulting plasmid, pLM8, was used to transform *E. coli* JM109. Total cell protein from these transformants was subjected to SDS/PAGE and L-MDH was detected by Western blotting (results not shown). JM109 does not express a cI repressor so expression under these conditions was constitutive. We found that levels of L-MDH achieved in this system were considerably higher than when pLM8 was used to drive inducible expression in *E. coli* NF1, which also expresses a temperature-sensitive cI repressor (results not shown).

The purification protocols developed for recombinant L-MDH and the independently expressed L-MDH-flavin domain have been described in detail in the Materials and methods section. Purification data for the preparation of recombinant L-MDH are summarized in Table 1. These data demonstrate that one can purify L-MDH to homogeneity with a yield of 20 mg from one litre of culture.

Table 1 Purification data for L-MDH

Details of the purification procedure are described in Materials and methods section. Amounts are expressed per litre of culture.

Step	Total protein (mg)	L-MDH (mg)	Yield (%)	Purification (fold)
Cell lysate	~ 1000	~ 140	100	1
DE52	~ 750	~ 110	79	1.1
Sephacel	~ 250	~ 80	57	1.6
Q Sepharose	~ 100	~ 50	36	3.6
S300 Sephacryl	~ 20	~ 20	14	7.1

Kinetic properties

The steady-state kinetic parameters for the oxidation of L-mandelate by both L-MDH and the L-MDH flavin domain are listed in Table 2. It is clear that the recombinant form of L-MDH can effectively catalyse the oxidation of L-mandelate with both physiological (cytochrome *c*) and artificial (ferricyanide) electron acceptors. In fact, with ferricyanide as electron acceptor, the k_{cat} value found with the recombinant L-MDH holoenzyme ($550 \pm 25\ s^{-1}$) is considerably higher than the value previously reported for the native enzyme from *R. graminis* ($109 \pm 3\ s^{-1}$) [7]. The lower activity reported for the native enzyme is explained by significant activity losses during the much longer purification protocol originally used [7]. This activity loss was almost certainly due to flavin dissociation from the enzyme, which was at very low concentration throughout the preparation. With the recombinant enzyme, the more rapid purification procedure and much higher concentrations of enzyme minimize flavin dissociation, and thus there is little activity loss.

The values of k_{cat} and K_m listed in Table 2 are of a similar magnitude to those previously reported for the oxidation of L-lactate by *S. cerevisiae* flavocytochrome b_2 (L-LDH) and its independently expressed flavin domain [28,40]. This would be consistent with the idea that both these enzymes bind and activate their respective hydroxyacid substrates in a similar manner. Another similarity between the two enzymes is the fact

Table 2 Steady-state kinetic parameters for the oxidation of L-mandelate by the L-MDH holoenzyme and its independently expressed flavin domain

All experiments were at 25 °C in Tris/HCl buffer, pH 7.5 (/ 0.10 M NaCl). Electron acceptors were used at saturating concentrations: 1 mM ferricyanide and 35 μM cytochrome *c* for the holoenzyme and 6 mM ferricyanide for the flavin domain. Values of k_{cat} are expressed as electrons transferred per second per molecule of enzyme. Values of K_m are expressed in millimolar L-mandelate. The L-MDH flavin domain has negligible activity with cytochrome *c*. ND, not determined.

	L-MDH holoenzyme		L-MDH flavin domain	
Electron acceptor	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)
Ferricyanide	550 ± 25	0.35 ± 0.02	500 ± 50	0.33 ± 0.02
Cytochrome *c*	225 ± 15	0.35 ± 0.05	ND	ND

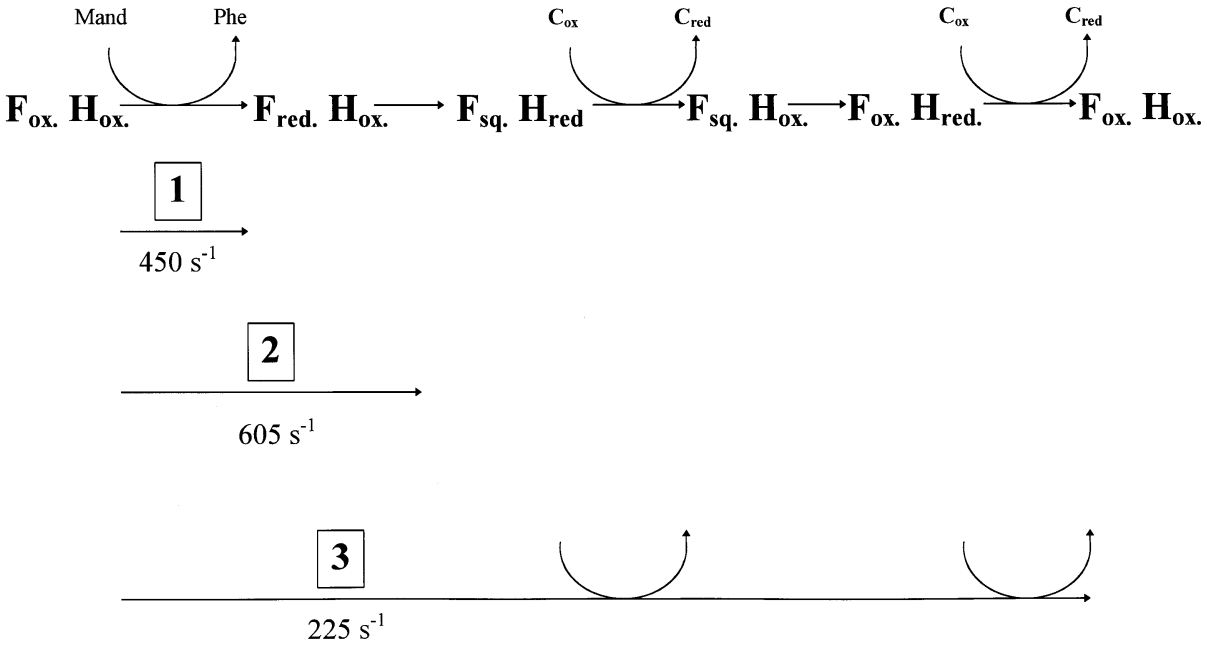
Table 3 Pre-steady-state kinetic parameters and KIE values for the reduction of the prosthetic groups in L-MDH by mandelate

All experiments were at 25 °C in Tris/HCl buffer, pH 7.5 (/ 0.10 M NaCl). Values for flavin reduction were obtained using the L-MDH-flavin domain (to avoid interference from the haem absorbances) and for haem using the L-MDH holoenzyme. Rate constants (k_F, flavin reduction; k_H, haem reduction) are expressed as the number of cofactors reduced per second. Therefore since reduction of flavin requires two electrons, values of k_F should be doubled to express them in electrons per second.

	Flavin reduction		Haem reduction	
	k_F (s^{-1})	K_d (mM)	k_H (s^{-1})	K_d (mM)
L-[2-^{1}H]Mandelate	450 (±30)	0.39 (±0.10)	605 (±50)	0.39 (±0.10)
D, L-[2-^{1}H]Mandelate	340 (±10)	0.45 (±0.07)	505 (±20)	0.49 (±0.10)
D, L-[2-^{2}H]Mandelate	81 (±3)	0.45 (±0.07)	115 (±10)	0.56 (±0.05)
KIE	4.2 (±0.3)		4.4 (±0.6)	

that the flavin domain of L-MDH, like its L-LDH counterpart [40], has negligible cytochrome *c* reductase activity. This indicates that electron flow from flavin to haem and then onto cytochrome *c* is alike in L-MDH and L-LDH.

Under pre-steady-state conditions it is possible to determine rate constants for the reduction of both the flavin and haem groups in L-MDH and these are listed in Table 3. Values for haem reduction were determined using the L-MDH holoenzyme. Data for flavin reduction, on the other hand, were acquired using the L-MDH flavin domain, in order to avoid spectral interference from the much more intense haem absorbance. The values from Table 3, in combination with steady-state data, allow us to assign rate constants to the various steps in the L-MDH catalytic cycle as shown in Scheme 1. The rate constants for flavin and haem reduction (k_F and k_H, Table 3) are of a similar order of magnitude to those reported for *S. cerevisiae* flavocytochrome b_2 [24] and its independently expressed flavin domain [40]. This indicates that



Scheme 1 Linear representation of the catalytic cycle of mandelate oxidation and cytochrome *c* reduction by L-MDH

Rate constants (25 °C) are reported for the following steps: 1, L-mandelate → flavin electron transfer (from stopped-flow); 2, L-mandelate → L-MDH-haem electron transfer (from stopped-flow); 3, L-mandelate → cytochrome *c* electron transfer (from steady-state). Abbreviations: $F_{ox.}$, oxidized flavin; $F_{red.}$, reduced flavin; $F_{sq.}$, flavin semiquinone; $H_{ox.}$, oxidized haem; $H_{red.}$, reduced haem; Mand, mandelate; Phe, phenylglyoxylate; C_{ox}, oxidized cytochrome *c*; C_{red}, reduced cytochrome *c*.

Table 4 Steady-state kinetic parameters and ^{2}H kinetic isotope effects for the oxidation of D,L-mandelate by the L-MDH and L-MDH flavin domain

Conditions were as described in Table 2, except that the normal and deuterated substrates were the racemic mixtures. The kinetic isotope effect, KIE, is defined as: $k_{cat}[^1H]/k_{cat}[^2H]$.

	L-MDH holoenzyme			L-MDH flavin domain		
Substrate (electron acceptor)	k_{cat} (s^{-1})	K_m (mM)	KIE	k_{cat} (s^{-1})	K_m (mM)	KIE
D,L-[2-^{1}H]Mandelate (ferricyanide)	300 (±10)	0.47 (±0.03)		316 (±19)	0.74 (±0.11)	
D,L-[2-^{2}H]Mandelate (ferricyanide)	88 (±4)	0.53 (±0.05)	3.4 (±0.3)	99 (±4)	0.79 (±0.08)	3.2 (±0.3)
D,L-[2-^{1}H]Mandelate (cytochrome *c*)	155 (±5)	0.78 (±0.02)		ND	ND	
D,L-[2-^{2}H]Mandelate (cytochrome *c*)	92 (±10)	1.33 (±0.10)	1.7 (±0.2)	ND	ND	ND

the microscopic steps for electron transfer are essentially the same in both enzymes.

Further kinetic characterization of L-MDH involved the determination of deuterium KIEs in both steady-state and pre-steady-state conditions (Tables 3 and 4). To measure these isotope effects it was necessary to determine rate constants using the racemic mixture, i.e. D,L-mandelate, in order to make the comparison with the isotopically labelled racemic substrate D,L-[2-^{2}H]mandelate (see the Materials and methods section). The KIE values from the steady-state experiments indicate that removal of the C-2 hydrogen contributes significantly to rate limitation with ferricyanide as electron acceptor, but much less so when cytochrome *c* is used as the acceptor. Such a trend in KIE values is similar to that reported previously for lactate oxidation by *S. cerevisiae* flavocytochrome b_2 [24]. The observation of a significant KIE value for L-MDH with ferricyanide as acceptor contradicts a previous study [7] in which no isotope effect was observed. We believe that various limitations in the previous work, e.g. very small amounts of much less active enzyme, made the determination of any KIE value very difficult, and that these results are now superseded by the present more rigorous study.

Kinetic isotope effects seen in the pre-steady-state lend support to the steady-state experiments and indicate a steady erosion of the KIE value at each step in the electron-transfer chain, e.g. approx. 4 for flavin and haem reduction, 3 for ferricyanide reduction and 2 for cytochrome *c* reduction. This decrease in KIE values parallels observations from studies with *S. cerevisiae* flavocytochrome b_2 [24], lending support to the idea that the transition states are comparable in both enzymes.

Reduction potentials

Reduction potentials for the haem and flavin groups of L-MDH were determined using the holoenzyme and the flavin domain respectively. Values at 25 °C and pH 7.5 were measured as -120 ± 10 mV for the two-electron reduction of the flavin group and -10 ± 5 mV for the haem. It was not possible to separate the two one-electron couples (i.e. oxidized/semiquinone and semiquinone/reduced) for the flavin. These values can be compared with the equivalent ones determined for *S. cerevisiae* flavocytochrome b_2 (L-LDH) which are -17 ± 3 mV for the haem group and -78 ± 5 mV for the two-electron reduction of the flavin. Thus, although the haem reduction potentials are the same within experimental error, there is a significant difference between the values for the flavin groups of the two enzymes. This difference of some 40 mV indicates that the driving force for the electron transfer from flavin to haem is substantially greater in L-MDH. This is in line with the fact that the rate constant for haem reduction in L-MDH (at 600 s^{-1}) is faster than that for L-LDH (450 s^{-1}) [24].

Conclusions

The sequence of L-MDH shows a close relationship with other flavocytochromes b_2, but the differences in substrate specificity may depend on key amino acid residues, particularly Gly-225 and Gly-195. Using the cloned cDNA, we have developed efficient expression systems and purification procedures for both the L-MDH holoenzyme and the independent L-MDH flavin domain. Both steady-state and pre-steady-state kinetic measurements show that the recombinant L-MDH is an efficient L-MDH and cytochrome *c* reductase. The kinetic and potentiometric data for L-MDH are in most cases very similar to the analogous L-LDH from *S. cerevisiae*, i.e. flavocytochrome b_2.

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