L-Mandelate dehydrogenase from *Rhodotorula graminis*: comparisons with the L-lactate dehydrogenase (flavocytochrome b_2) from *Saccharomyces cerevisiae*

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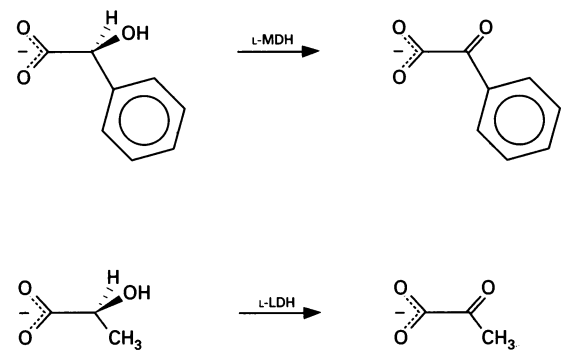
L-Lactate dehydrogenase (L-LDH) from *Saccharomyces cerevisiae* and L-mandelate dehydrogenase (L-MDH) from *Rhodotorula graminis* are both flavocytochromes b_2. The kinetic properties of these enzymes have been compared using steady-state kinetic methods. The most striking difference between the two enzymes is found by comparing their substrate specificities. L-LDH and L-MDH have mutually exclusive primary substrates, i.e. the substrate for one enzyme is a potent competitive inhibitor for the other. Molecular-modelling studies on the known three-dimensional structure of *S. cerevisiae* L-LDH suggest that this enzyme is unable to catalyse the oxidation of L-mandelate because *productive* binding is impeded by steric interference, particularly between the side chain of Leu-230 and the phenyl ring of mandelate. Another major difference between L-LDH and L-MDH lies in the rate-determining step. For *S. cerevisiae* L-LDH, the major rate-determining step is proton abstraction at C-2 of lactate, as previously shown by the ^2H kinetic-isotope effect. However, in *R. graminis* L-MDH the kinetic-isotope effect seen with DL-[2-^{2}H]mandelate is only 1.1 ± 0.1, clearly showing that proton abstraction at C-2 of mandelate is not rate-limiting. The fact that the rate-determining step is different indicates that the transition states in each of these enzymes must also be different.

INTRODUCTION

L-(+)-Mandelate dehydrogenase (L-MDH) from the yeast *Rhodotorula graminis* catalyses the oxidation of L-(+)-mandelate to phenylglyoxylate [1,2]. Recently the isolation of this enzyme, along with a number of its physical properties, have been reported [3; M. Yasin and C. A. Fewson, unpublished work]. It has become evident that there are many similarities between *R. graminis* L-MDH and the flavocytochrome b_2 from *Saccharomyces cerevisiae*. Both enzymes are tetramers of identical subunits with similar M_r values, both contain flavin mononucleotide and protohaem IX prosthetic groups and have identical electronic absorption spectra. The sequence of the first 32 amino acid residues of *R. graminis* MDH shows 50% identity with the same region of the *S. cerevisiae* flavocytochrome b_2 sequence [3; M. Yasin and C. A. Fewson, unpublished work]. Although both enzymes are α-hydroxyacid dehydrogenases, they have somewhat different substrate specificities (Scheme 1). The *S. cerevisiae* flavocytochrome b_2 is a L-(+)-lactate dehydrogenase (L-LDH) [5] unable to oxidize mandelate, whereas *R. graminis* L-MDH is unable to oxidize lactate. Thus we believe that *S. cerevisiae* L-LDH and *R. graminis* L-MDH are both flavocytochromes b_2 with mutually exclusive primary substrates. To test this idea and to probe the similarities and differences of these enzymes in more detail we have carried out extensive kinetic and mechanistic studies on both enzymes. Since the X-ray crystal structure of *S. cerevisiae* flavocytochrome b_2 has been solved to 0.24 nm resolution [6], we are able to interpret the results of our solution studies in terms of this three-dimensional information. In addition molecular-graphics investigations have allowed us to identify important residues at the active site of the enzyme and to suggest structural reasons why *S. cerevisiae* flavocytochrome b_2 is unable to catalyse the oxidation of mandelate.



Scheme 1 The reactions catalysed by L-MDH and L-LDH

The upper reaction shows the conversion of L-mandelate into phenylglyoxalate by L-MDH, and the lower reaction shows the conversion of L-lactate into pyruvate by L-LDH

MATERIALS AND METHODS

Isolation of enzymes

L-MDH from *R. graminis* (strain KGX39) was prepared as previously described [3; M. Yasin and C. A. Fewson, unpublished work (details are available from C. A. F. on request)]. Flavocytochrome b_2 (L-LDH) from *S. cerevisiae* was expressed in

Abbreviations used: L-LDH, L-lactate dehydrogenase; L-MDH, L-mandelate dehydrogenase; DCIP, dichlorophenol-indophenol; PMS, phenazine methosulphate.

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Escherichia coli [7] and isolated from frozen cells as reported elsewhere [7]. Enzyme concentrations were measured using previously published molar absorption coefficients [8].

Preparation of DL-[2-^{2}H]mandelate

DL-Mandelate ^{2}H-labelled at the C-2 position was prepared by the following procedure. Methylbenzoylformate (580 μl) was dissolved in 5 ml of ethanol. NaB^2H_4 (0.4 g in 1.5 ml of cold water) was added slowly to this solution with stirring at room temperature. After complete addition, the solution was stirred for a further 40 min, after which it was slowly added to 25 ml of 1 M NaOH. This mixture was left on ice for 2 h and then added to 5 ml of conc. HCl and left overnight at 0 °C. The product which precipitated out was collected by vacuum filtration and washed twice with 5 ml volumes of water. Dry crude product was recrystallized from ethanolic solution by the addition of light petroleum (b.p. 60–80 °C). The purity of the labelled mandelate (66% yield) was checked by t.l.c. The isotopic purity, as ascertained by ^{1}H-n.m.r. spectroscopy and mass spectrometry, was 98.6%.

Kinetic measurements

All kinetic experiments were carried out at 25±0.1 °C in Tris/HCl buffer at pH 7.5 and *I* 0.10. The buffer concentration was 10 mM in HCl with *I* adjusted to 0.10 by addition of NaCl.

Steady-state kinetic measurements involving the enzymic oxidation of 2-hydroxyacid substrates were carried out using Beckman DU62 or Pye–Unicam SP.8-400 spectrophotometers. Ferricyanide, cytochrome *c* or 2,6-dichlorophenol-indophenol (DCIP) were used as electron acceptors and reactions monitored at the following wavelengths (using previously published molar absorption coefficients): ferricyanide, 420 nm [9]; cytochrome *c*, 550 nm [10]; DCIP, 600 nm [11].

When DCIP was used as the electron acceptor, the assay mix included BSA (200 μg/ml) and phenazine methosulphate (PMS) (4.5 μM), reagents which have been previously shown to maximize rates and improve linearity of steady-state measurements [3,4]. K_m, K_i and $k_{cat.}$ values were determined from the appropriate curves using non-linear regression analysis. Such parameters were obtained from at least triplicate runs with reproducibility better than ±10%, and mean values are quoted.

Kinetic-isotope effects (KIEs) for L-MDH were determined using DL-[2-^{1}H]mandelate and DL-[2-^{2}H]mandelate as substrates.

Molecular-graphics analysis

The co-ordinates for the three-dimensional structure of *S. cerevisiae* flavocytochrome b_2 were kindly provided by Professor F. S. Mathews and were examined using the programs FRODO and SYBYL on an Evans and Sutherland ESV10 graphics system.

RESULTS

Steady-state kinetic parameters

Kinetic results for the oxidation of L-mandelate and L-lactate by *R. graminis* L-MDH or *S. cerevisiae* L-LDH with ferricyanide as electron acceptor are presented in Table 1. The most striking result is that L-MDH is unable to oxidize L-lactate and L-LDH is unable to oxidize L-mandelate. Moreover, L-lactate is a competitive inhibitor of L-MDH and L-mandelate is a competitive inhibitor of L-LDH. Values of K_i for L-lactate and L-mandelate with L-MDH and L-LDH are very similar to the K_m values seen when these compounds act as substrates with L-LDH and L-MDH (Table 1). Values of $k_{cat.}$ for the enzymes with their primary substrates are of similar magnitude, with $k_{cat.}$ for L-LDH being about 4-fold larger than that for L-MDH. The two enzymes also have similar overall efficiencies, as judged by a comparison of their $k_{cat.}/K_m$ values. Table 1 also includes results for a number of other substrates and inhibitors with the two enzymes. Of particular interest are the values for L-phenyllactate, which is a substrate for L-LDH, but an inhibitor for L-MDH. It appears that the insertion of a CH_2 group between the α-carbon and the aromatic ring of mandelate to give phenyllactate prevents L-MDH from utilizing the compound as a substrate, but allows L-LDH to do so. Steady-state kinetic parameters determined with the alternative electron acceptors cytochrome *c* and DCIP are shown in Table 2. Values of $k_{cat.}$ seen for both enzymes with these acceptors are lower (by about 2–3-fold) than the corresponding values with ferricyanide. L-LDH has slightly higher $k_{cat.}$ values with cytochrome *c* and DCIP than does L-MDH (as also observed with ferricyanide) and the K_m values for the two enzymes are similar (Table 2). These

Table 1 Steady-state kinetic parameters for L-MDH from *R. graminis* and L-LDH from *S. cerevisiae* with ferricyanide as electron acceptor

All experiments were carried out at 25 °C in Tris/HCl buffer, pH 7.5 (/0.10). The ferricyanide concentration was 1 mM. Values of $k_{cat.}$ are expressed in electrons transferred/s per mol of enzyme (since L-mandelate and L-lactate are two-electron donors, the $k_{cat.}$ values can be halved to express them in terms of mol of substrate reduced). Competitive inhibition was observed with all inhibitors, except oxalate, which exhibited mixed inhibition behaviour.

Substrate or inhibitor	Enzyme	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	K_i (mM)
L-Mandelate	L-MDH	109±3	0.27±0.03	4.04×10^5	–
	L-LDH	–	–	–	0.26±0.08
L-Lactate	L-MDH	–	–	–	0.40±0.04
	L-LDH	400±10	0.49±0.05	8.16×10^5	–
L-Phenyllactate	L-MDH	–	–	–	1.90±0.40
	L-LDH	16±1	0.18±0.04	8.88×10^4	–
Oxalate	L-MDH	–	–	–	0.078±0.001
	L-LDH	–	–	–	1.50±0.20
L-Hexanoate	L-MDH	–	–	–	0.37±0.01
	L-LDH	–	–	–	0.20±0.03

Table 2 Steady-state kinetic parameters for *R. graminis* L-MDH and *S. cerevisiae* L-LDH with alternative electron acceptors

All experiments were carried out at 25 °C in Tris/HCl buffer, pH 7.5 (/0.10). Electron acceptors were used at the following concentrations: [Cytochrome *c*], 2.2 μM; [DCIP], 68 μM. Values of $k_{cat.}$ are expressed as in Table 1. Abbreviations: L-MDH, L-mandelate dehydrogenase; L-LDH, L-lactate dehydrogenase; Cyt *c*, cytochrome *c*.

Enzyme	Substrate	Electron acceptor	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)
L-MDH	L-Mandelate	Cyt *c*	49±3	0.17±0.06	2.88×10^5
	L-Mandelate	DCIP*	36±1	0.14±0.03	2.57×10^5
L-LDH	L-Lactate	Cyt *c*	207±10	0.24±0.04	8.6×10^5
	L-Lactate	DCIP*	59±4	0.25±0.07	2.4×10^5

* Experiments with DCIP as electron acceptor had BSA (200 μg/ml) and PMS (4.5 μM) added to the buffer as previously described [3; M. Yasin and C. A. Fewson, unpublished work].

results demonstrate that both *R. graminis* L-MDH and *S. cerevisiae* L-LDH are able to utilize a number of electron acceptors and do so with similar characteristics. Both L-MDH and L-LDH show a dependence of reaction rate on the concentration of cytochrome *c* (which is the known physiological acceptor for L-LDH). The K_m values for cytochrome *c* seen for the two enzymes are quite similar, being 10 ± 1 μM with L-LDH and 22 ± 4 μM with L-MDH. This would be consistent with both enzymes interacting with cytochrome *c* in a similar fashion.

Kinetic isotope effects

It has been previously demonstrated that proton abstraction at C-2 of lactate is the major rate-limiting step in lactate oxidation by *S. cerevisiae* L-LDH [12–14]. With ferricyanide as electron acceptor and using DL-lactate ^{2}H-labelled at the C-2 position (DL-[2-^{2}H]lactate) a KIE of 5 was obtained [12]. In order to determine whether or not the same step is rate-limiting in *R. graminis* L-MDH, steady-state experiments were carried out using DL-[2-^{1}H]mandelate and DL-[2-^{2}H]mandelate as substrates and with ferricyanide as electron acceptor. Results from these experiments are presented in Table 3. The KIE value from Table 3 of 1.1 ± 0.1 clearly shows that there is no significant ^{2}H isotope effect and this indicates that the rate-limiting step in L-MDH is different from that in L-LDH and must be later in the reaction pathway. This also means that the transition states in the two enzymes must be different.

Table 3 ^{2}H KIE for *R. graminis* L-MDH

All experiments were carried out at 25 °C in Tris/HCl buffer, pH 7.5 (/ 0.10). Ferricyanide was used as the electron acceptor at a concentration of 1 mM. Values of $k_{cat.}$ are expressed as in Table 1. KIE = $k_{cat.}(^1H)/k_{cat.}(^2H)$.

DL-[2-^{1}H]Mandelate		DL-[2-^{2}H]Mandelate		
$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}$ (s^{-1})	K_m (mM)	KIE
84 ± 5	0.34 ± 0.06	79 ± 4	0.75 ± 0.07	1.1 ± 0.1

Molecular graphics

To provide a structural basis for why *S. cerevisiae* L-LDH is

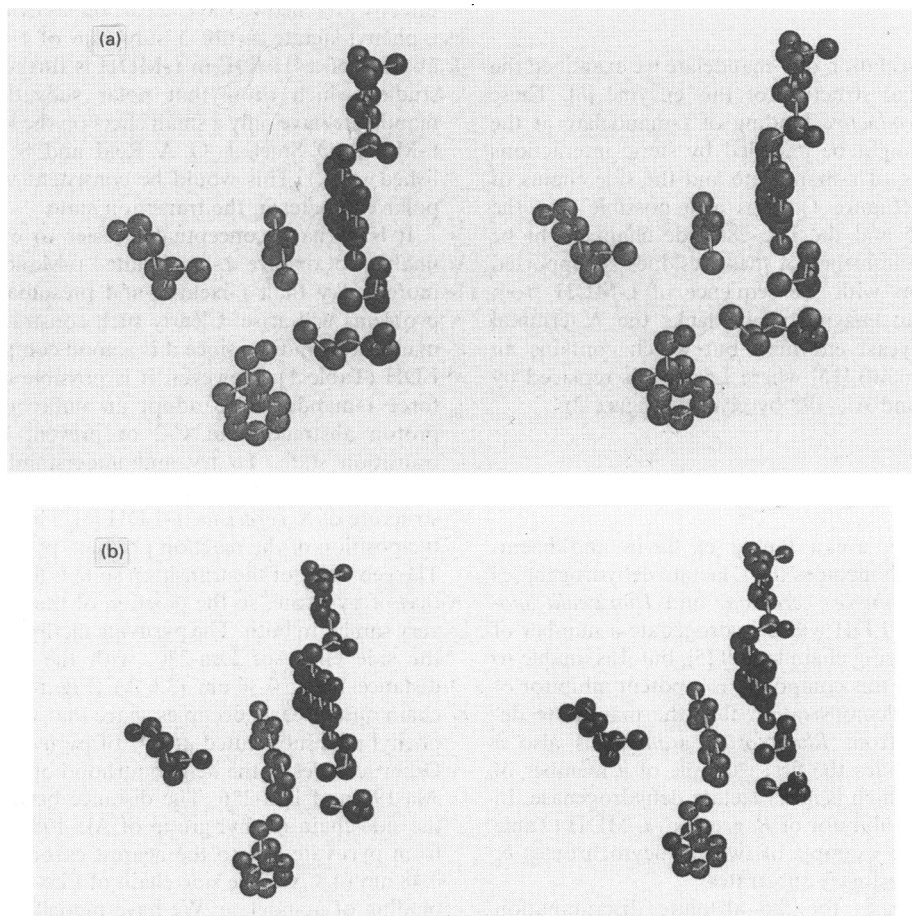

Figure 1 (a) A parallel-vision stereoscopic view of the active site of *S. cerevisiae* L-LDH and (b) modelling phenylglyoxalate in the L-LDH active site

(**a**) Pyruvate is shown (top centre) with the methyl group pointing downwards. Residues with side chains close to the methyl group are Leu-286 (left), Phe-325 (bottom), Leu-230 (bottom right) and Ala-198 (right). FMN is also shown (top right). (**b**) The view is essentially as in (**a**), except that Leu-230 has been replaced by alanine and the methyl group of pyruvate has been replaced by a phenyl ring.

```
L-LDH  QLKSLLPPLD NIINLYDFEY LASQTLTKQA WAYYSSGAND EVTHRENHNA  160
L-MDH  .......MSQ NLFNVEDYRK LRQKRLPKMV YDYLEGGAED EYGVKHNRDV   43
Con    ---------- N--N--D--- L----L-K-- --Y---GA-D E-----N---

                                        *
L-LDH  YHRIFFKPKI LVDVRKVDIS TDMLGSHVDV PFYVSATALC KLGNPLEGEK  210
L-MDH  FQQWRFKPKR LVDVSRRSLQ AEVLGKRQSM PLLIGPTG.. .LNGALWPKG   90
Con    -----FKPK- LVDV------ ---LG----- P-----T--- -L---L----

                 *
L-LDH  DVARGCGQGV TKVPQMISTL ASCSPEEIIE AAPSDKQIQW YQLYVNSDRK  260
L-MDH  DLALARAATK AGIPFVLSTA SNMSIEDLAR QCDGD...LW FQLYV.IHRE  136
Con    D-A------- ---P---ST- ---S-E---- ----D----W -QLYV---R-

                          *
L-LDH  ITDDLVKNVE KLGVKALFVT VDAPSLGQRE KDM..KLKFS NTKAGPKAMK  308
L-MDH  IAQGMVLKAL HTGYTTLVLT TDVAVNGYRE RDLHNRFKIP MSYSAKVVLD  186
Con    I----V---- --G---L--T -D----G-RE -D-----K-- ----------

                                        *
L-LDH  K......... .........T NVEESQGASR A.....LSKF IDPSLTWKDI  335
L-MDH  GCLHPRWSLD FVRHGMPQLA NFVSSQTSSL EMQAALMSRQ MDASFNWEAL  236
Con    ---------- ---------- N---SQ--S- -------S-- -D-S--W---

L-LDH  EELKKKTKLP IVIKGVQRTE DVIKAAEIGV SGVVLSNHGG RQLDFSRAPI  385
L-MDH  RWLRDLWPHK ILVKGLLSAE DADRCIAEGA DGVILSNHGG RQLDCAISPM  286
Con    --L------- I--KG----E D-------G- -GV-LSNHGG RQLD----P-

L-LDH  EVLAETMPIL EQRNLKDKLE VFVDGGVRRG TDVLKALCLG AKGVGLGRPF  435
L-MDH  EVLAQSVA.. .....KTGKP VLIDSGFRRG SDIVKALALG AEAVLLGRAT  329
Con    EVLA------ -----K---- V--D-G-RRG -D--KAL-LG A--V-LGR--

L-LDH  LYANSCYGRN GVEKAIEILR DEIEMSMRLL GVTSIAELKP DLLDLSTLKA  485
L-MDH  LYGLAARGET GVDEVLTLLK ADIDRTLAQI GCPDITSLSP DYLQ......  373
Con    LY-----G-- GV------L- --I------- G---I--L-P D-L-------

L-LDH  RTVGVPNDVL YNEVYEGPTL TEFEDA                           511
L-MDH  .NEGVTNTAP VDHLIGKGTH A......                           393
Con    ---GV-N--- --------T- -------
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Figure 2 Alignment of the amino acid sequences of the flavodehydrogenase domain of *Saccharomyces* flavocytochrome b_2 (L-LDH) and the L-MDH from *Pseudomonas putida*

The consensus (Con), where both sequences are identical, is shown below. The positions of Ala-198, Leu-230, Leu-286 and Phe-325 of L-LDH are indicated by asterisks above the aligned sequences.

unable to catalyse the oxidation of L-mandelate we examined the known three-dimensional structure of this enzyme [6]. These studies indicate that *productive* binding of L-mandelate at the active site of L-LDH might be impeded by steric interactions between the phenyl ring of L-mandelate and the side chains of Leu-230 and Ala-198 (Figure 1). It is also possible that the phenyl ring of Phe-325 and the Leu-286 side chain might be involved. The probable influence of these residues is supported by making comparisons with the sequence of L-MDH from *Pseudomonas putida* (an enzyme which lacks the *N*-terminal haem domain of the yeast enzymes, but which contains an homologous FMN domain) [15] where Leu-230 is replaced by the less bulky alanine and Ala-198 by glycine (Figure 2).

DISCUSSION

The flavocytochromes b_2 are a family of flavin and haem-containing enzymes which includes the L-lactate dehydrogenases (L-LDHs) from *Saccharomyces cerevisiae* and *Hansenula anomala*. The *S. cerevisiae* L-LDH will dehydrogenate a number of L-α-hydroxyacids of differing chain length [5], but it is unable to utilize mandelate. In fact this compound is a potent inhibitor of L-LDH (Table 1). The demonstration that the mandelate dehydrogenase (L-MDH) from *Rhodotorula graminis* is also a flavocytochrome b_2 provides the first example of a member of this family of enzymes which is *not* a lactate dehydrogenase. In fact L-lactate is a potent inhibitor of *R. graminis* L-MDH (Table 1). We therefore have an example of two flavocytochromes b_2 with mutually exclusive primary substrates.

To understand the basis for the absolute discrimination between these two substrates we have carried out a kinetic comparison of the two enzymes. The first point to note is that K_m and K_i values for lactate or mandelate with both enzymes are very similar. Since the inhibition observed is competitive in nature, it would appear that the discrimination between substrates does *not* arise from a lack of binding. Other possible explanations are that there are differences in the orientation of the compounds at the active sites and/or differences in the nature of the transition state. If the transition states in both enzymes are essentially the same, it follows that both enzymes should have the same rate-determining step. In *S. cerevisiae* L-LDH the major rate-determining step is known to be proton abstraction at C-2 of lactate [10–12]. This was demonstrated by measuring a ^2H kinetic isotope effect, KIE, value of 5.0 for L-LDH with DL-[2-^2H]lactate as substrate and ferricyanide as electron acceptor. However, the corresponding experiment with *R. graminis* L-MDH using DL-[2-^2H]mandelate shows an isotope effect of only 1.1 (Table 3). This indicates that the rate-determining step in L-MDH does not involve proton abstraction at C-2 of mandelate. It follows therefore that, in L-LDH, the C–H bond is being broken in the transition state, but in L-MDH it is not. The transition state in L-MDH would appear to occur after proton abstraction and may involve the resulting carbanion at the C-2 position, which might be stabilized by the aromatic ring of mandelate. This would not, of course, be possible for lactate, which has a methyl group in the equivalent position. This proposal is supported somewhat by the finding that, although α-hydroxyacids with hydrophobic side chains can bind at the active site of L-MDH, they lack the aromatic ring and therefore do not undergo catalysis (O. Smékal, M. Yasin, C. A. Fewson, G. A. Reid and S. K. Chapman, unpublished work). It is also clear that, in L-MDH, the substrate must have the aromatic ring directly attached to the C-2 carbon, as evidenced by the fact that L-phenyl-lactate is not a substrate of L-MDH (Table 1). The absence of a ^2H KIE in L-MDH is further supported by recent studies which show that polar substituents on the ring of mandelate have only a small effect on the kinetic parameters with L-MDH (O. Smékal, G. A. Reid and S. K. Chapman, unpublished work). This would be consistent with a somewhat non-polar character in the transition state.

It is perhaps conceptually easier to explain why L-LDH is unable to oxidize L-mandelate. L-Mandelate is considerably more bulky than L-lactate, and presumably, in L-LDH, steric problems will arise. Clearly such constraints do not prevent L-mandelate binding, since it is a good competitive inhibitor for L-LDH (Table 1). However, it is possible that steric interactions force L-mandelate to adopt an unfavourable orientation for proton abstraction at C-2 or prevent the formation of the transition state. To try and understand this, we carried out molecular-modelling studies on the known three-dimensional structure of *S. cerevisiae* L-LDH [6]. The crystal structure shows the position of the reaction product, pyruvate, at the active site. The geometry of the transition state is likely to be very similar to that of pyruvate, so the position of the methyl group should be very similar in both. The pyruvate methyl group projects towards the side chain of Leu-230, with the shortest carbon–carbon distance being 0.36 nm (3.6 Å) (Figure 1a). The Leu-230 side chain quite clearly occupies space that would be demanded by a phenyl ring substituted at C-2 of pyruvate (Figures 1a and 1b). Other residues in the neighbourhood of C-3 of pyruvate include Ala-198 and Leu-286. The distance between pyruvate C-3 and the side-chain methyl group of Ala-198 is 0.43 nm (4.3 Å), and from pyruvate C-3 to the nearest carbon atom of Leu-286 it is 0.48 nm (4.8 Å). The side chain of Phe-325 may also restrict the binding of mandelate. We have modelled phenylglyoxalate, the product of mandelate dehydrogenation, at the active site of *S. cerevisiae* L-LDH after substitution of Leu-230 by Ala (Figure 1b). In this model we have retained the positions of all three pyruvate carbon atoms. In this position the phenyl ring is not sterically hindered by the side chains mentioned above. However,

the phenyl ring of Tyr-254, a catalytically important residue [9,16], restricts the orientation of the mandelate ring. Nevertheless, this modelling indicates that, with only a minor alteration to the L-LDH active site, it should be possible to allow productive binding of mandelate. This will be tested in future work by introducing the appropriate site-directed mutations. In this context it is interesting that the L-MDH from *Pseudomonas putida* contains less bulky amino acids at the positions occupied by Leu-230, Ala-198 and Leu-286 in L-LDH (Figure 2).

CONCLUSIONS

From our comparative study of the flavocytochromes b_2 from *R. graminis* and *S. cerevisiae* we draw the following conclusions.

(i) Although both enzymes are α-hydroxyacid dehydrogenases, they have mutually exclusive primary substrates.

(ii) The *R. graminis* enzyme requires an aromatic ring *directly* attached to the α-carbon of the hydroxyacid for it to act as a substrate.

(iii) The rate-determining step is different in the two enzymes, and therefore the transition states in each enzyme must be different. In the *S. cerevisiae* enzyme the C_α–H bond is being broken in the transition state and in the *R. graminis* enzyme it is not.

(iv) Productive binding of L-mandelate in *S. cerevisiae* flavocytochrome b_2 is sterically impeded by Leu-230. This may be the crucial residue in the discrimination by the *S. cerevisiae* enzyme against L-mandelate.

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