L(+)-Mandelate dehydrogenase of the yeast *Rhodotorula graminis*

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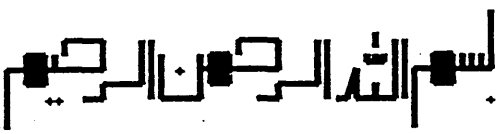

Begin with the name of 'Allah' the most Gracious and the most Merciful

To my parents

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ABBREVIATIONS

The abbreviations used are those recommended by the Biochemical Journal, London, in the instuctions to authors, [Biochem. J. (1992) 281, 1-19] with the following additions.

AU	Absorbance units
DCIP	2, 6-Dichloroindophenol
DTT	Dithiothreitol
fplc	Fast protein liquid chromatography (Pharmacia system)
K_m	Apparent Michaelis constant
NBT	Nitro Blue Tetrazolium
NCIB	National Collection of Industrial Bacteria,Aberdeen, U. K.
NCYC	National Collection of Yeast Cultures, Norwich, U. K.
OD	Optical density
PAGE	Polyacrylamide-gel electrophoresis
PMS	N -Methylphenazonium methosulphate
PQQ	Pyrroloquinoline quinone
PSI	Pounds per square inch
RPM	Revolutions per minute
SDS	Sodium dodecyl sulphate
TEMED	*N,N,N',N'*-Tetramethylenediamine
Vmax	Apparent maximum velocity

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Summary

1. *Rhodotorula graminis* KGX-39 is an imperfect yeast and can grow on mandelate as sole source of carbon and energy. D(–)-Mandelate dehydrogenase and L(+)-mandelate dehydrogenase oxidise the respective enantiomers of mandelate to phenylglyoxylate which in turn is metabolized by peripheral and secondary metabolic pathways to succinate and acetyl-CoA and these enter into the central metabolic pathways. The objective of the work described in this thesis was to purify and characterise the L(+)-mandelate dehydrogenase and to compare it with other similar prokaryotic and eukaryotic enzymes.

2. An assay system was developed for measuring L(+)-mandelate dehydrogenase activity reproducibly and accurately. DCIP reduction was measured in the presence of PMS in a buffered reaction mixture that also contained bovine serum albumin or Triton X-100 in order to ensure maximum and linear rates.

3. A reproducible procedure was developed for the purification of L(+)-mandelate dehydrogenase to homogeneity using a combination of hydrophobic interaction and ion-exchange chromatography and gel filtration. The method yields approximately 1 mg of L(+)-mandelate dehydrogenase from 70-90 g (wet weight) of cells. The enzyme was homogeneous as judged by denaturing and non-denaturing polyacrylamide gel electrophoresis and is stable on storage at -20^{0}C.

4. Preliminary experiments indicate that L(+)-mandelate dehydrogenase appears to be membrane associated.

5. L(+)-Mandelate dehydrogenase is thermolabile but is stable in the presence of Triton X-100 or other non-ionic detergents or bovine albumin serum and L(+)-mandelate.

6. The apparent subunit molecular weight is 59,100, as determined by SDS-polyacrylamide gel electrophoresis. The apparent native molecular weight of the purified enzyme is 240,000 and the enzyme in the crude extract has an apparent native molecular weight of 235,000. L(+)-Mandelate dehydrogenase therefore appears to be a tetrameric protein.

7. L(+)-Mandelate dehydrogenase catalyses an essentially irreversible reaction.

8. L(+)-Mandelate dehydrogenase is stereospecific for its substrate and can oxidise a wide range of substituted mandelates. D(–)-Mandelate and D(–)- or L(+)-hexahydro-mandelate are not substrates but are competitive inhibitors of the enzyme. In addition a wide range of aliphatic 2-hydroxy acids which are not substrates, can inhibit enzyme activity and very similar behaviour was observed for the L(+)-mandelate dehydrogenase of *Acinetobacter calcoaceticus*.

9. L(+)-Mandelate dehydrogenase is neither activated nor inhibited by a wide range of salts, metal ions and metal chelating agents, nor is its activity affected by a range of possible metabolic inhibitors such as ADP or ATP or Mg^{+2} or acetyl-CoA or glucose or succinate or benzaldehyde.

10. The enzyme has an optimum pH value of 7.9 and the pI value of 4.4.

11. Flavin was extracted from L(+)-mandelate dehydrogenase with trichloroacetic acid and was identified and quantified by absorption and fluorescence spectroscopy. There is approximately 0.8 moles of FMN per subunit of L(+)-mandelate dehydrogenase. The oxidised and reduced absorption spectra of the native L(+)-mandelate dehydrogenase suggested the presence of haem and there is almost exactly 1 mole of haem per subunit.

12. In addition to DCIP, the enzyme can use cytochrome *c* and ferricyanide as electron acceptors and in this respect resembles L(+)-lactate dehydrogenase of *Saccharomyces cerevisiae* but not L(+)-mandelate dehydrogenase or L(+)-lactate dehydrogenase of *A. calcoaceticus* or L(+)-mandelate dehydrogenase of *Pseudomonas putida* which cannot use cytochrome *c* as an electron acceptor.

13. The apparent K_m value for L(+)-mandelate is 266 μM at pH 7.9.

14. L(+)-Mandelate dehydrogenase activity is not affected by iodoacetate or iodoacetamide but is strongly inhibited by *p*-chloromercuribenzoate and mercuric chloride.

15. The amino acid composition was determined.

16. The sequence of the first 32 *N*-terminal amino acids was determined.

17. Antisera were prepared against L(+)-mandelate dehydrogenase of *R. graminis* and gave strong immunological reactions with L(+)-mandelate dehydrogenases of *R. graminis* KGX 39, *R. graminis* NCYC 980 and yeasts DB2, DB3 and L(+)-lactate dehydrogenase of *S. cerevisiae* but not with other mandelate and lactate dehydrogenases tested.

18. Anti-L(+)-mandelate dehydrogenase of *A. calcoaceticus* was found to cross-react with L(+)-mandelate dehydrogenase of *P. putida* but not with the enzyme from *R. graminis.*

19. Significant amino acid compositional similarities were found among L(+)-mandelate dehydrogenase of *R. graminis* and L(+)-lactate dehydrogenases of *S. cerevisiae* and *Hansenula anomala*. Such similarities were also found among L(+)-lactate dehydrogenase of *Escherichia coli*, L(+)-mandelate dehydrogenase of *P. putida* and glycolate oxidase of spinach.

20. Significant homologies were found when the *N*-terminal sequence of L(+)-mandelate dehydrogenase of *R. graminis* was compared with the *N*-terminal sequence of the haem-binding domain of L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala* and microsomal cytochrome b_5 from beef. Other work has shown that there are homologies amongst L(+)-lactate dehydrogenase of *A. calcoaceticus* and L(+)-mandelate dehydrogenases of *A. calcoaceticus* and *P. putida* and glycolate oxidase of spinach (all contain FMN but not haem), and between these enzymes and the FMN-binding domains of the L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala*.

21. Considering the overall similarities among L(+)-mandelate dehydrogenases and lactate dehydrogenases from both bacteria and yeasts, it is suggested that mandelate dehydrogenases of *A. calcoaceticus*, *P. putida* and yeasts *R. graminis*, DB2 and DB3 have almost certainly evolved by recruitment of 2-hydroxy acid dehydrogenases of other metabolic pathways.

CHAPTER 1

INTRODUCTION

1.1 Nature of metabolism and evolution of peripheral metabolic pathways

The central metabolic pathways represent the heart of metabolism . In addition to the central metabolic pathways such as the Krebs cycle and glycolysis, some microorganisms have acquired more specialised, less common, secondary pathways (Fig. 1.1). Examples include pathways for the degradation of catechol and protocatechuate, the *meta* -cleavage pathways and amino acid degrading pathways. Lastly, there are so called 'peripheral' pathways in which organic molecules are converted into relatively few key intermediates such as catechol and protocatechuate (Fewson, 1981; Dagley, 1988). These, in turn, are degraded via the *ortho* and *meta* -ring cleavage pathways (secondary pathways) to yield compounds that enter the central amphibolic pathways (Fig. 1.1). The central metabolic pathways have revealed some fascinating evolutionary relationships but their study perhaps does not reveal more recent evolutionary events. Peripheral pathways may have evolved more recently than the central metabolic pathways and knowledge of the peripheral pathways may in some respects provide better understanding of enzyme evolution than does knowledge of the central metabolic pathways. The mandelate pathway is one of the important peripheral pathways that has been studied (Fewson, 1988b). Unlike the central metabolic pathways, peripheral pathways would appear to be subject to less evolutionary constraint partly because they are generally not subjected to feedback or similar types of controls and are not tightly integrated into closely-related areas of metabolism. Microbial peripheral pathways may evolve more rapidly as the chances of genetic changes facilitated by transformation and/or plasmid mediated transfer are greater. Indeed, many of the enzymes of peripheral pathways are plasmid-encoded (e.g. Williams, 1981) and even in organisms in which they are chromosomally-encoded, mechanisms of transformation and of non-conjugal DNA transfer have been demonstrated (e.g. Ahlquist *et al.*, 1980; Vakeria *et al.*, 1985). Microorganisms that possess peripheral pathways are often nutritionally versatile and so if mutation leads to the loss of a particular enzymic activity, the organism may still be able to grow on other carbon sources. As many of the substrates of peripheral pathways are uncommon and may be only occasionally present in the environment, the genes encoding peripheral enzymic pathways may lie dormant for substantial lengths of time and therefore

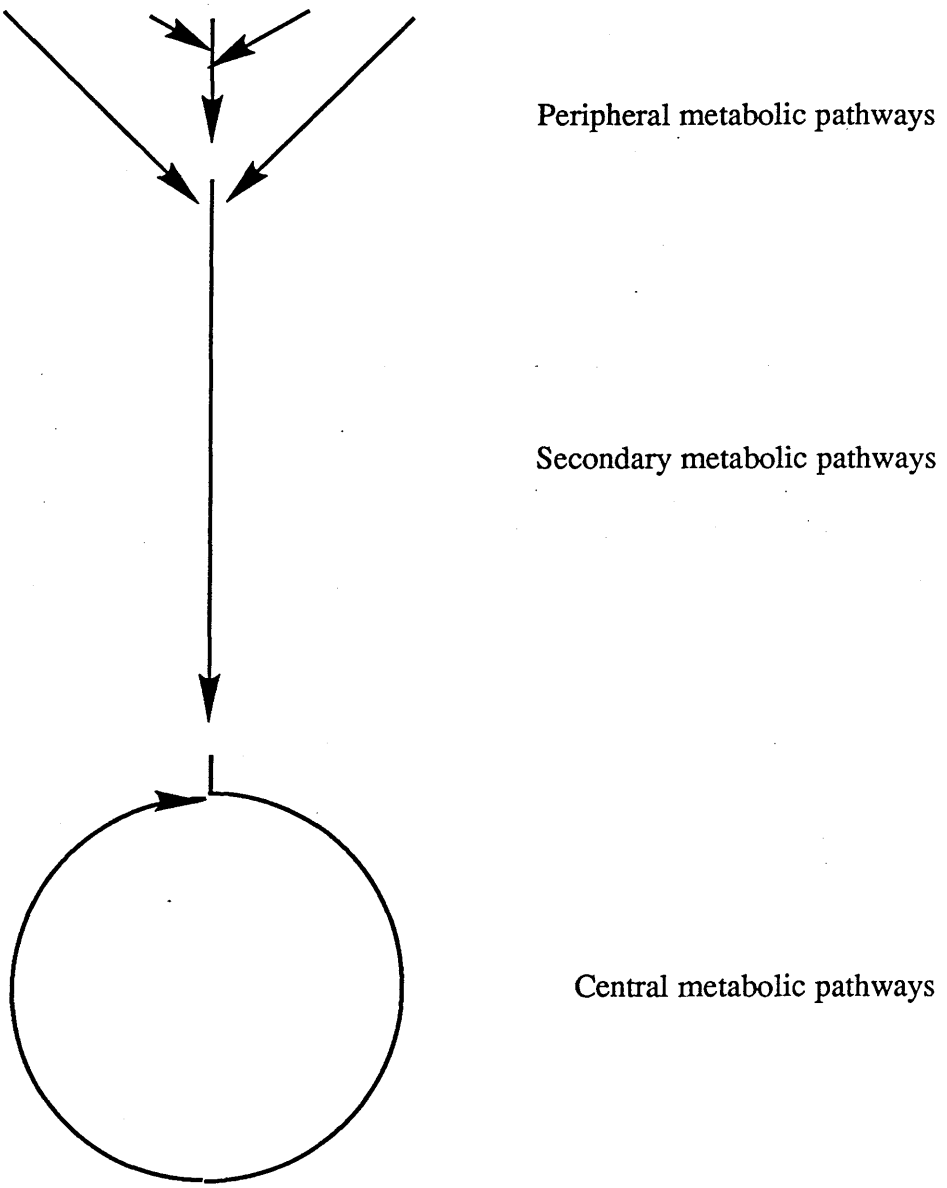


Figure 1.1 Schematic representation of peripheral, secondary and central metabolic patways

mutations could occur without apparent deleterious effects. Despite all this, very little is known about the evolution of peripheral metabolic pathways.

The study of mandelate pathway enzymes in prokaryotic and eukaryotic microorganisms has been shown to serve as a good model system for the study of evolution of a peripheral pathway (Fewson, 1988b & 1992). Sequence analysis and crystallographic studies of some of the mandelate pathway enzymes in *Pseudomonas* have suggested a possible mechanism by which the pathway may have evolved in bacteria (Neidhart *et al.*, 1988; Ranson *et al.*, 1988; Tsou *et al.*, 1990). In contrast, little is known about mandelate pathway enzymes in fungi. Only an NAD^+-dependent D(–)-mandelate dehydrogenase from the yeast *Rhodotorula graminis* KGX 39 has been purified and characterised (Baker & Fewson, 1989; Baker, 1990; Baker *et al.*, 1992).

This thesis is concerned with purification and characterisation of L(+)-mandelate dehydrogenase from the yeast *Rhodotorula graminis* KGX 39 in order to study the evolution of the mandelate pathway in microorganisms in continuation of related work done on different microorganisms in Glasgow, Japan, Germany and the USA (Kennedy & Fewson, 1968 a,b; Yeh *et al.*, 1982; Durham,1984; Durham *et al.*, 1984; Allison *et al.*, 1985a; Allison & Fewson 1986; Yamazaki & Maeda, 1986a,b; Hoey *et al.*, 1987; Fewson, 1988b; Hummel *et al.*, 1988; Baker & Fewson; Tsou *et al.*, 1990; 1989; Fewson, 1992).

1.2 The yeast *Rhodotorula graminis*

1.2.1 *Rhodotorula* as a genus

The main groups of yeasts are: (a) ascosporogenous yeasts, (b) basidiosporogenous yeasts, and (c) imperfect yeasts. Imperfect yeasts do not form ascospores or basidiospores. Sporulation may be absent because imperfect yeasts are haploid and no opposite mating types are known. Imperfect yeasts may resemble the other two groups, but differ from them in not forming sexual spores (Kregor-van Rij, 1984). Members of the genus *Rhodotorula* belong to the group of imperfect yeasts, class Hyphomycetes. The number of species in the genus *Rhodotorula* varies as the criteria of speciation varies. Fell *et al.* (1984) recognised the following eight species: *Rhodotorula acheniorum, R. araucariae,*

R. aurantiaca, R. glutinis, R. graminis, R. lactosa, R. minuta and *R. rubra* and *R. glutinis* is the type species. However Barnett *et al.* (1983) listed 18 species: *R. acheniorum, R. araucariae, R. aurantiaca, R. bogoriensis, R. diffluens, R. fujisanense, R. glutinis, R. graminis, R. ingeniosa, R. javanica, R. lactosa, R. marina, R. minuta, R. mucilaginosa, R. muscorum, R. pallida, R. pilatii* and *R. pilimane.* Some other species have since been isolated, e.g. *R. grinbergsil* (Ramirez & Gonzalez, 1984). Classification of the genus has been confused by the use of a large number of generic synonyms such as *Chromotorula, Cryptococcus, Flavotorula, Mycotorula, Sporobolomyces, Torula* and *Torulopsis* and an even larger number of species synonyms (Fell *et al.,* 1984). Cells are usually spherical, oval or elongated and are present singly or in pairs or sometimes in short chains or clusters. They reproduce asexually by multilateral budding, and do not form ascospores or ballistospores, although some strains of *R. glutinis* represent opposite mating types of the perfect basidiomycetous yeast *Rhodosporidium toruloides* (Banno, 1967). Some strains may produce pseudomycelium or true mycelium. Visible carotenoid pigments are synthesised giving red, pink, orange or yellow coloured colonies. Many members of the genus give mucous colonies due to capsule formation, whilst others give pasty or dry and wrinkled colonies (Fell *et al.,* 1984). Metabolism in all members of the genus *Rhodotorula* is strictly aerobic and they are unable to ferment (Fell *et al.,* 1984). Inositol is not assimilated but nitrate is assimilated by all species except *R. minuta* and *R. rubra* (Fell *et al.,* 1984).

Members of the genus *Rhodotorula* are the most widely distributed yeasts in both natural and man-made environments, and have been isolated from a wide range of sources including air, animal tissues, beer, birds, brine, butter, cheese, dung, dust, fish, fresh and marine water, fruits, grass, insects, jam, leather, live and decaying plant debris, malt syrup, man, oil wells, pickled cucumbers, shrimps, and soil (Cook *et al.,* 1960; Spencer *et al.,* 1970; Fell *et al.,* 1984; Baker, 1990). Moreover, *R. glutinis, R. graminis, R. minuta, R. mucilaginosa, R. rubra* and *R. texensis* have been isolated from polluted water and sewage during examination of natural yeast populations (Cook *et al.,* 1960). In a similar study, Spencer *et al.* (1970) isolated 129 yeasts from the South Saskatchewan River, of which 30 were *Rhodotorula* species.

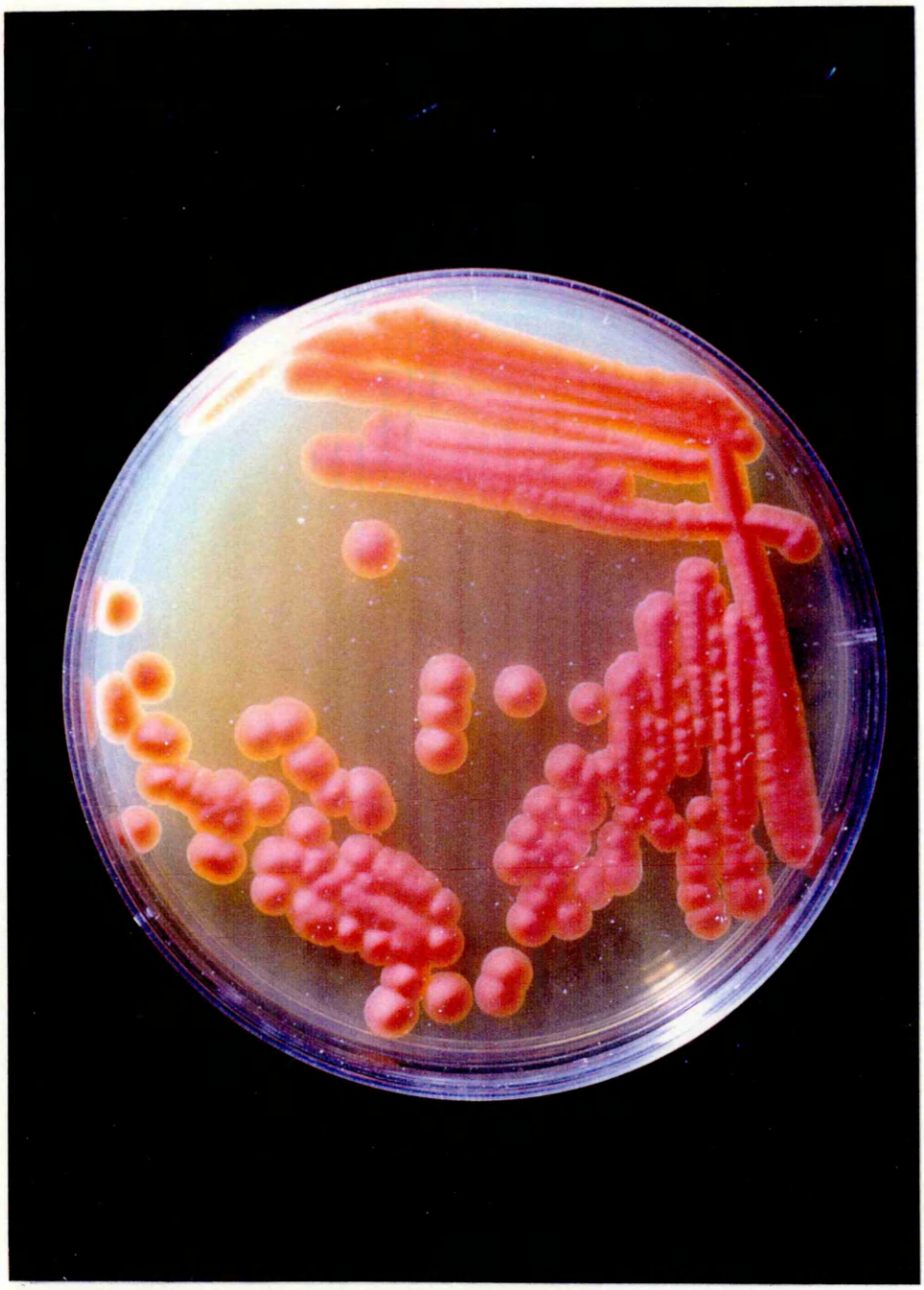


Figure 1.2 *Rhodotorula graminis* KGX 39 growing on Sabouraud-dextrose agar

Mills *et al.* (1971) isolated *R. minuta, R. glutinis* and *R. rubra* from sewage and found that several strains of these species could grow on a variety of aromatic compounds including resorcinol, phloroglucinol, salicylate and gentisate. This is consistent with the observation that several species of *Rhodotorula*, especially *R. rubra* and *R. glutinis*, can use phenol or phenolic compounds as a sole source of carbon and energy (Mills *et al.*, 1971; Walker, 1973; Katayama-Hirayama *et al.*, 1991). A strain of *R. glutinins* can grow on sinapic acid, a monomer of hard-wood lignin (Gupta *et al.*, 1986). It degrades sinapic acid into syringate in the absence of glucose but further metabolism of syringate to 3-*O* -methylgallic acid, gallic acid and 2,6-dimethoxy-1,4-benzoquinone occurs only in the presence of glucose as a co-metabolite (Gupta *et al.*, 1986). Co-metabolism of aromatic compounds is also a feature of some other species of *Rhodotorula* such as *R. glutinis*, *R. mucilaginosa* and *R. minuta* which can co-metabolise the plant phenylpropanoid, rutin (Westlake & Spencer, 1966).

R. macerans and *R. mucilaginosa* have been shown to grow on vanillic and ferulic acids which represent breakdown products of beech, pine or spruce soft wood lignins (Cain *et al.*, 1968; Cook & Cain, 1974). Similarly the isolation of *R. grinbergsii* from decaying wood (Ramirez & Gonzalez, 1984) indicates that wood decaying processes might involve these organisms. These examples illustrate the nutritional diversity of the genus *Rhodotorula* and their significant contribution to overall microbial breakdown and recycling of aromatic compounds in the environment and this becomes even more significant since many natural and man-made aromatic compounds are recalcitrant to breakdown and/or toxic (Fewson, 1981, 1988a).

1.2.2 *Rhodotorula graminins* as a species, including strain KGX 39

Rhodotorula graminis (synonym: *Rhodotorula rosa*) has the following characteristics:

(a) Cells are spherical or ovoidal or elongated, (2.5-7) x (4-15) μm and are present in pairs or in short chains or clusters.

(b) Forms pink or red colonies due to the presence of carotenoid pigments. Colonies have a shining soft texture and are sometimes mucous (Durham *et al.*, 1984; Fell *et al.*, 1984).

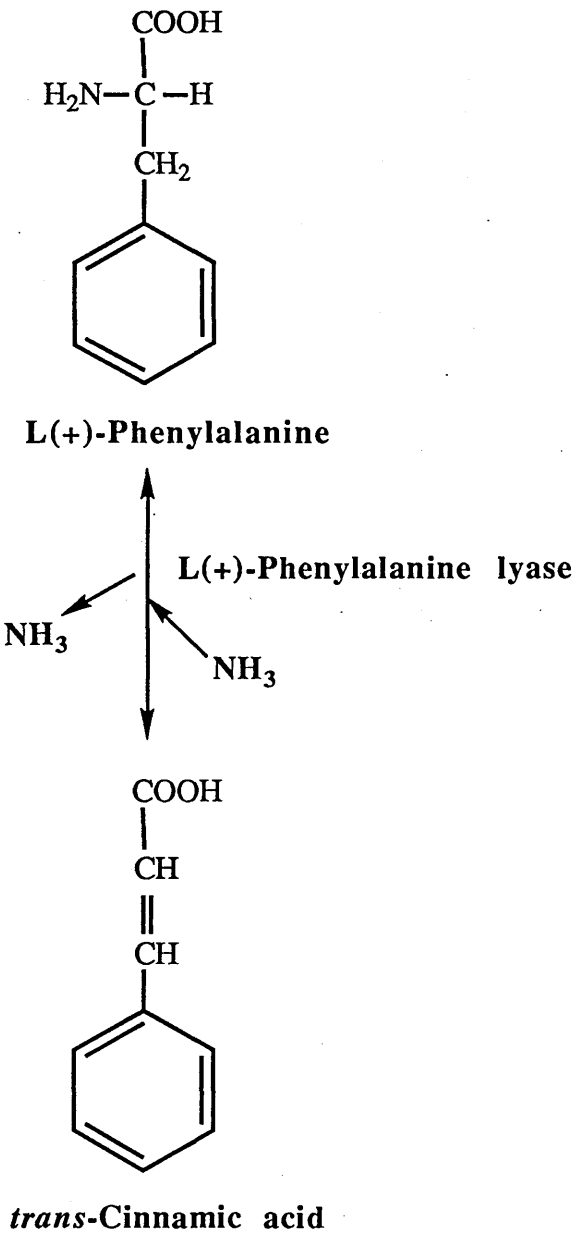


Figure 1.3 Reversible deamination of L(+)-phenylalanine to *trans*-cinnamic acid in *R. graminis, R. glutinis* and *R. rubra.*

(c) Has the highest guanine plus cytosine content of DNA (70 mol %) among members of the genus *Rhodotorula* (Fell *et al.*, 1984).

(d) Strains have been isolated from plants, marine water and soil (Durham *et al.*, 1984; Fell *et al.*, 1984).

R. graminis strain KGX 39 (Fig. 1.2) originally isolated from soil, can grow on vitamin-free medium. This strain can assimilate glucose, galactose, mannitol, ribitol, sucrose, raffinose, citrate and succinate but cannot assimilate maltose, lactose, starch, inositol, erithritol or melezitose (Durham *et al.*, 1984; Fell *et al.*, 1984). It can also utilise aromatic compounds such as benzoate, benzyl alcohol, D,L-mandelate, L(+)-phenylalanine, protocatechuate and salicylate as a sole sources of carbon and energy and nitrate or nitrite as source of nitrogen (Durham *et al.*, 1984; Fell *et al.*, 1984; McNamee *et al.*, 1984; Middleton, 1988).

1.2.3 Applications of *Rhodotorula* species

Enzymes from species of *Rhodotorula* have received special attention because of their potential practical clinical and industrial importance. For example, the enzyme phenylalanine ammonia lyase from *R. glutinis, R. graminis* and *R. rubra* catalyses the deamination of L(+)-phenylalanine to *trans* -cinnamate and ammonia (Fig. 1.3) allowing these organisms to grow on L(+)-phenylalanine as a sole source of carbon, nitrogen and energy (Yamada *et al.*, 1981; Orndorff *et al.*, 1988). However the reaction is reversed when the enzyme is incubated in the presence of high concentrations of *trans*-cinnamate and ammonia at elevated pH and the enzyme from *Rhodotorula graminis* and *R. rubra* has been used in the commercial production of L(+)-phenylalanine (Hamilton *et al.*, 1985; Orndorff *et al.*, 1988). The inducible phenylalanine ammonia lyase from a mutant strain *R. graminis* GX 6000 has been found to be more commercially useful because of its physiological traits than the enzyme from *R. rubra* (Orndorff *et al.*, 1988). Four to five times more enzyme is produced in mutant strain GX 6000 and the enzyme has six to seven times higher specific activity. This enzyme has the half life which is three to five fold longer than the enzyme from *R. rubra* (Orndorff *et al.*, 1988).

D(-)-mandelic acid L(+)-mandelic acid

Figure 1.4 Enantiomers of mandelic acid

L(+)-Phenylalanine ammonia-lyase also has potential for the medical treatment of phenylketonuria. The enzyme from *R. rubra* has been used to produce diet containing a low-phenylalanine content suitable for phenylketonuric patients (Watanabe *et al.*, 1988). Phenylalanine ammonia lyase from *R. glutinis* has been studied as a possible treatment for phenylketonuria by administering it directly in the alimentary tract where it deaminates phenylalanine to reduce blood levels of phenylalanine after ingestion of a normal meal (Hoskins *et al.*, 1980).

Species of *Rhodotorula* have also been used for other biotransformation processes such as the synthesis of chiral cyclopentenones and carbonyl reduction (Allenmark & Andersson, 1988; Okano *et al.*, 1988).

The lipid-soluble, carotenoid pigments from an undisclosed pink yeast, presumably a species of *Rhodotorula*, have been used to enhance the colour of farmed salmon which lacks the characteristic pinkness of wild salmon because of a deficiency of the pigment astaxanthin (Highfield, 1987).

1.3 Mandelate and related compounds in the environment

Mandelate (2-hydroxy-2-phenylacetic acid, α-hydroxyphenylacetic acid, phenylglycollic acid) is a naturally occurring aromatic 2-hydroxy acid, which exists in two enantiomeric forms, D(-)-mandelic acid (R-form) and and L(+)-mandelic acid (S-form) (Fig. 1.4). The pK_a of its carboxyl group is 3.37, and so under most natural conditions it exists as the carboxylate mandelate anion (Fewson, 1988b). Although free mandelate is not abundant in natural soil and water environments, mandelate and a variety of related compounds are continually released into the environment from several sources. For example mandelate and various mandelate analogues are produced by algae, fungi, and higher plants and animals (Fewson, 1988b). Mandelate is found in tissues of wheat leaves and grapes (Fewson, 1988b) and many plants such as almond, peach and apricot accumulate mandelate-related compounds such as glycosides of D(–)-and L(+)-mandelonitrile [e.g. (R)-prunasin, (S)- sambunigrin] and the β-gentiobioside of D(–)-mandelonitrile (Conn, 1981). Other mandelate-related compounds include glycosides of either mandelonitrile or hydroxy-mandelonitrile such as (R)-holocalin, (S)-zierin, amygdalin, vicianin and lucumin

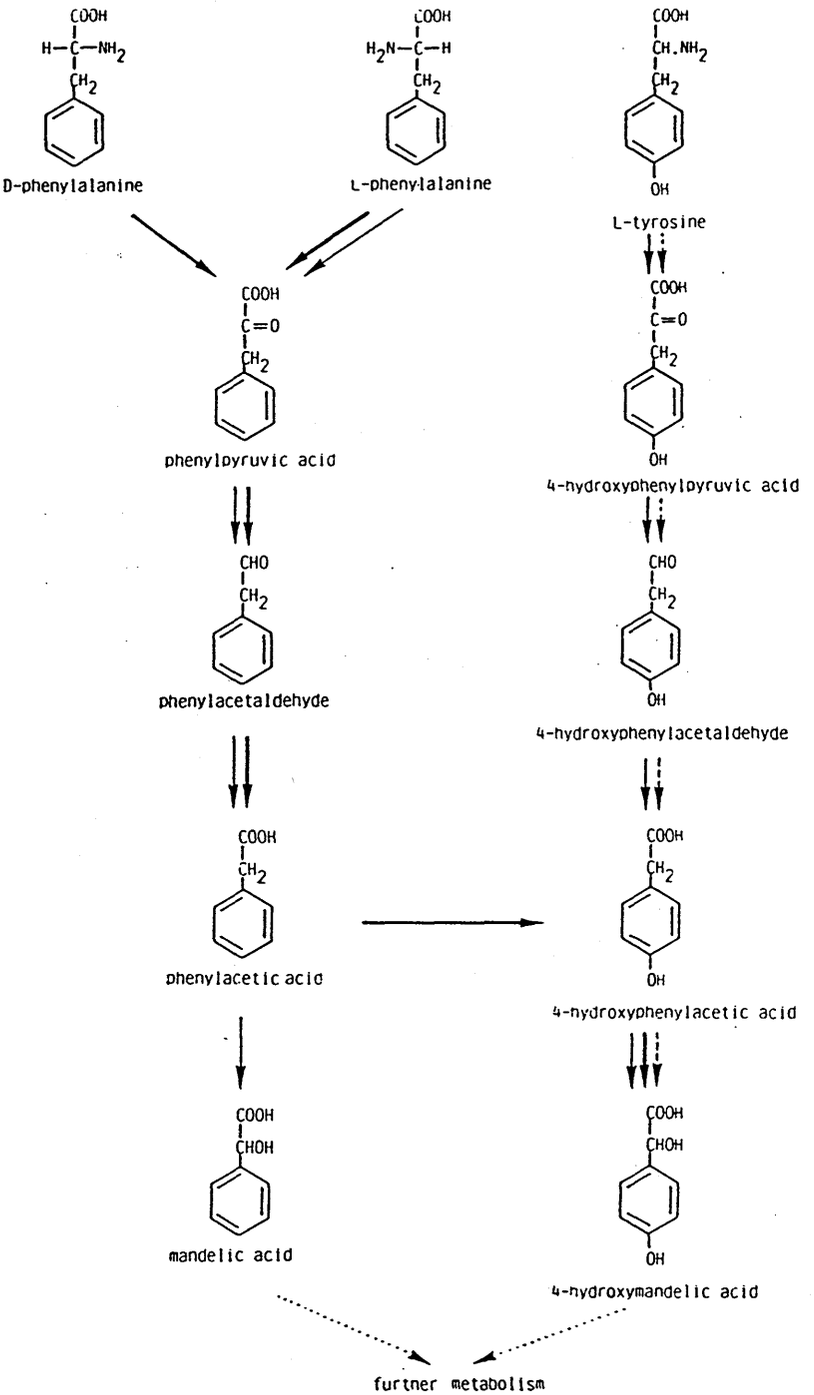


Figure 1.5 Tentative pathway for the formation of mandelate and 4-hydroxy-mandelate by *Aspergillus niger* (⟶), the phytoplankton *Isochrysis galbana* and *Navicula incerta* (⟶) and the red alga *Odonthalia floccosa* (- - ➔). From Fewson (1988b).

(Conn, 1981). These glycosides of mandelonitrile are synthesised from L(+)-phenylalanine via mandelonitrile rather than from mandelate itself. Breakdown of glycosides by glucosidase releases mandelonitrile which in turn is hydrolysed by mandelonitrile lyase to give HCN and benzaldehyde (Borner, 1960; Conn, 1981; Xu *et al.*, 1986). In addition to plants, a variety of arthropods such as the Lepidoptera, Coleoptera, Chilopoda and Diplopoda synthesize and store substantial amounts of D-mandelonitrile or its glycosides which they convert to benzaldehyde and HCN as a repellent (Duffey & Towers, 1978). It is possible that mandelonitrile released into the environment may give rise to free mandelate by hydrolytic reactions (Fewson, 1988b). In addition, some plants synthesise ring-substituted mandelates such as 3-carboxymandelate and 3-carboxy-4-hydroxymandelate, which are precursors in the biosynthesis of (3-carboxyphenyl) glycine and (3-carboxy-4-hydroxyphenyl) glycine respectively (Larsen & Wieczorkowska, 1975). L(+)-Phenylalanine, phenylacetate and L(+)-tyrosine are converted into mandelate and 4-hydroxymandelate by a wide range of organisms (Fig. 1.5). For example the fungus *Aspergillus niger* produces 4-hydroxymandelate when grown on L(+)-phenylalanine or phenylacetate (Fig. 1.5; Kishore *et al.*, 1974 & 1976). Another fungus *Polyporus hispidus* produces 4-hydroxymandelate from L(+)-phenylalanine and L(+)-tyrosine (Perrin & Towers, 1973) and the related fungus *P. tumulus* seems to produce 4-hydroxymandelate, 2,5-dihydroxymandelate and 3,4-dihyroxymandelate from shikimate, according to results of isotopic incorporation studies (Crowden, 1967). The phytoplankton *Isochrysis galbana* and *Navicula incerta* produce mandelate and 4-hydroxymandelate when grown on L(+)-phenylalanine and L(+)-tyrosine (Fig. 1.5; Landymore *et al.*, 1978) and the red alga *Odonthalia* produces 4-hydroxymandelate when grown on L(+)-tyrosine (Fig. 1.5; Manley & Chapman, 1979).

Mandelate and phenylglyoxylate are also released in the natural environment from animal urine. The normal mandelate concentration in human urine is 1-15 μM and in the pathological condition of phenylketonuria it is as much as 600 μM (Luthe *et al.*, 1983). Large amounts of vanillylmandelate (4-hydroxy-3-methoxymandelate) are found in urine of patients suffering from neuroblastoma i.e. neural crest tumours such as pheochromocytomas and ganglioneuromas (Voorhees & Gardner, 1961; Gitlow *et al.*, 1973; Rampini *et al.*, 1989; Mellor & Gallacher, 1990; Nishi *et al.*, 1990). 3,4-Dihydroxymandelate,

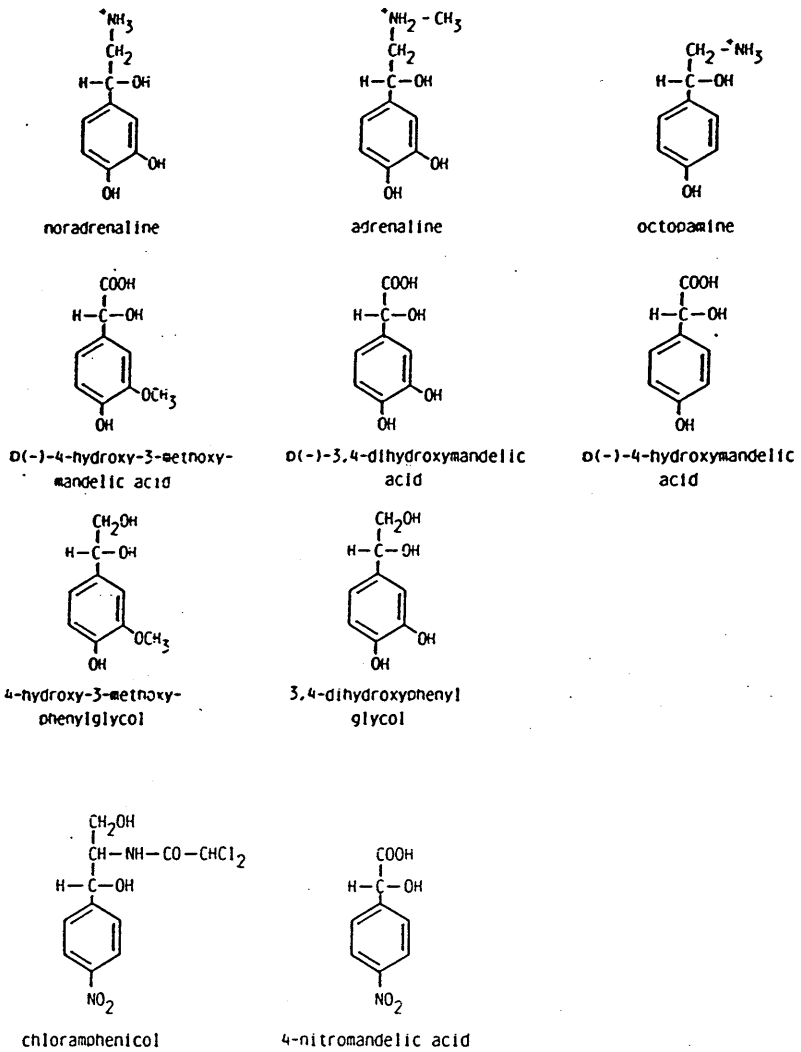


Figure 1.6 Adrenaline, octopamine, chloramphenicol and other strutural analogues of mandelic acid. From Fewson (1988b).

4-hydroxy-3-methoxymandelate, 4-hydroxymandelate, 3,4-dihydroxyphenylglyoxylate and 4-hydroxy-3-methoxy phenylglyoxylate are breakdown products of adrenaline (epinephrine) and noradrenaline (norepinephrine) (Fig. 1.6) and are excreted in substantial amounts in normal animal urine and faeces (Armstrong *et al.*, 1957; Axelrod & Savedra, 1977; Sze & Dagley, 1987; Fewson, 1988b). Mandelate is also excreted in the urine by mammals exposed to styrene (Chakrabarti, 1979; Wilson *et al.*, 1983; Korn *et al.*, 1984; Truchon *et al.*, 1990), ethylbenzene or acetophenone (Sullivan *et al.*, 1976), or after administration of the stimulant pemoline (Cummins & Perry, 1969; Luthe *et al.*, 1983). Mandelate is also a component of certain drugs such as cyclandelate (3,3,5-trimethyl-cyclohexanyl mandelate), a vasoactive substance used to improve red blood cells deformation (Middleton *et al.*, 1983) and methenamine mandelate is a urinary antiseptic (Greenwood & Slack, 1981; Kevorkian *et al.*, 1984). D(-)-Mandelate is a component of various semisynthetic penicillins, cephalosporins and antiobesity agents (Yamazaki & Maeda, 1986a,b; Vasic-Racki *et al.*, 1989; Hosono *et al.*, 1990). A diluted mandelic acid solution is occasionally used to prevent urinary tract infection associated with urethral catheterization (Van Putten, 1979). Use of these mandelate-containing drugs may result in the excretion of mandelate and related compounds into the environment.

Although free mandelate does not seem to be abundant in most natural environments, a large number of organic compounds can be channelled into mandelate or mandelate pathway intermediates. Examples include the conversion of phenylacetate, phenylglyoxal, mandelamide, and mandelate esters into mandelate; D(–)- or L(+)-phenylglycine into phenylglyoxylate; 4-hydroxyphenylglycine into 4-hydroxyphenylglyoxylate; and of benzoin, mandelonitrile and toluene into benzaldehyde (Fig. 1.7; Hegeman, 1966a; Worsey & Williams, 1975; Van den Tweel *et al.*, 1986; Yamazaki & Maeda, 1986a; Fewson, 1988b; Gonzalez & Vicuna, 1989). Mandelate itself may therefore be absent from a particular environment but several mandelate analogues, related organic compounds and/or precursors of mandelate as well as breakdown products of adrenaline, noradrenaline and octamine mentioned above, may be present in abundance in that ecosystem and that may account for the commonness of the mandelate pathway in microorganisms (Sze & Dagley, 1987; Fewson, 1988b).

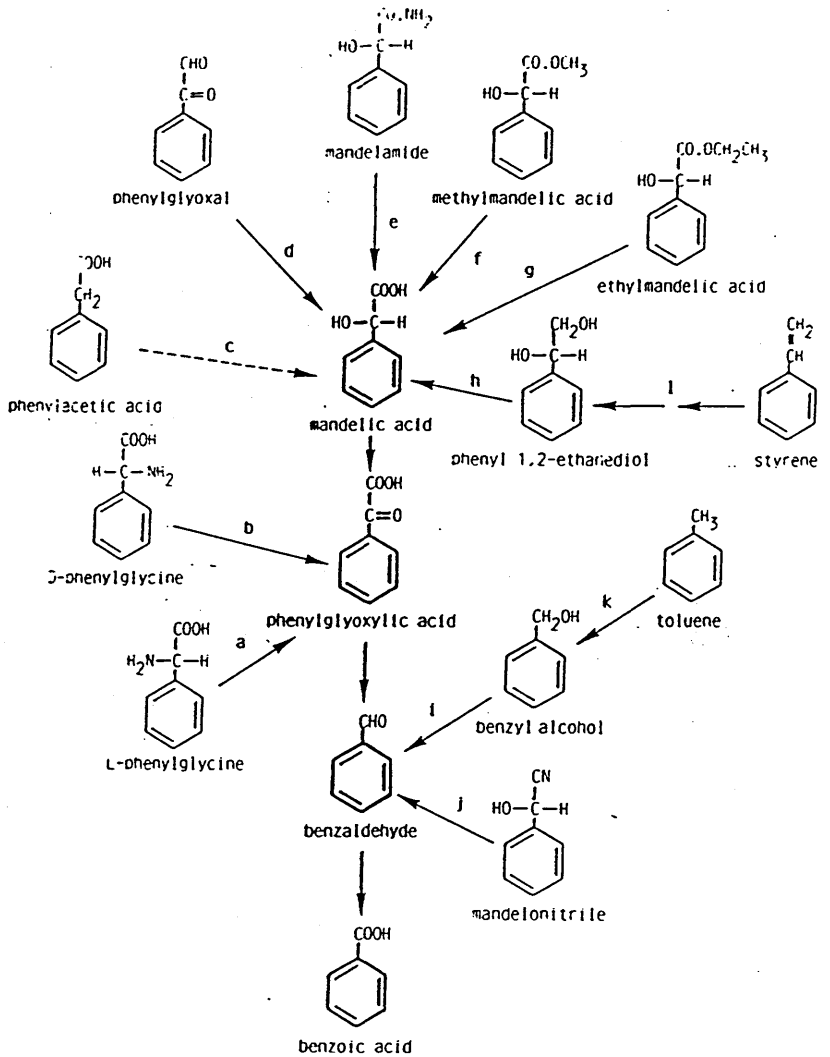


Figure 1.7 Degradation of several precursors to mandelate or mandelate pathway intermediates. Examples of various convertions are:
a and b, *Pseudomonas putida* and *Flavobacterium* species; c, *Penicillium chrysogenum* , mechanism uncertain; d, *Pseudomonas putida, Lactobacillus* species, *Bacillus* species; e, *Pseudomonas putida*; f and g, *Pseudomonas putida*; h, *Pseudomonas putida* ; i, *Acinetobacter calcoaceticus* , *Pseudomonas putida*; j, *Pseudomonas putida* containing TOL plasmid; l, mammalian tissues. From Fewson (1988b).

1.4 Microbial metabolism of mandelate

A variety of prokaryotes and eukaryotes can use either L(+)-mandelate or D(-)-mandelate or both as sole source of carbon and energy to support growth (Table 1.1). Amongst prokaryotes, numerous bacteria including *Acinetobacter*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Nocardia*, *Pseudomonas* and *Rhizobium* species have been reported to grow on either L(+)-mandelate or D(-)-mandelate or both (Table 1.1; Stanier *et al.*, 1966; Stevenson, 1967; Baumann *et al.*, 1968; Hegeman *et al.*, 1970; Rosenberg, 1971; Strzelczyk *et al.*, 1972; Bhat & Vaidyanathan, 1976a; Palleroni, 1984; Fewson, 1988b; Chen *et al.*, 1989). Amongst eukaryotes, several fungi including *Byssochlamys fulva* and *Rhodotorula graminis* and other unidentified yeasts can grow on either L(+)-mandelate or D(–)-mandelate or both (Hegeman *et al.*, 1970; Jamaluddin *et al.*, 1970; Ramakrishna Rao & Vaidyanathan, 1977; Iyayi & Dart, 1980; Dart & Iyayi, 1981; Durham, 1984; Durham *et al.*, 1984, Baker, 1990 and present project). *Aspergillus niger* and *Neurospora crassa* can co-oxidise mandelate in the presence of other carbon sources but do not seem able to use it as sole carbon source to support growth (Jamaluddin *et al.*, 1970; Ramakrishna Rado & Vaidyanathan, 1977). Although mandelate metabolism has been found in many bacteria and fungi, it is less well understood in fungi than in bacteria.

1.4.1 Bacterial metabolism of mandelate

In aerobic organisms, the first step of the pathway attacking mandelate can be racemization, ring hydroxylation or stereospecific dehydrogenation of mandelate to phenylglyoxylate (Fig. 1.8a, b', b"). The subsequent pathway involves decarboxylation and oxidation to benzoate or 4-hydroxybenzoate. These compounds are further oxygenatively converted into catechol or protocatechuate which feed into the secondary pathways and are converted into succinate and acetyl-CoA which enter the central metabolic pathways (Fewson, 1992).

In *A. calcoaceticus* the stereospecific dehydrogenation of mandelate to phenylglyoxylate is achieved by a pair of flavin-linked enzymes, L(+)-mandelate dehydrogenase and D(–)-mandelate dehydrogenase (Fig. 1.8b',b"). *A. calcoaceticus* strain NCIB 8250 has only L(+)-mandelate dehydrogenase and so can grow on only

Table 1.1 Microorganisms that can use mandelate

Bacteria

D,L-Mandelate	L(+)-Mandelate	D(–)-Mandelate
Acinetobacter calcoaceticus	*Acinetobacter calcoaceticus*	*Acinetobacter calcoaceticus*
Azotobacter beijerinckii	*Bacillus sphaericus*	*Lactobacillus curvatus*
Pseudomonas convexa	*Pseudomonas aeruginosa*	*Pseudomonas putida*
Pseudomonas putida	*Pseudomonas caryophylli*	
	Pseudomonas fluorescens	
	Pseudomonas multivorans	
	Pseudomonas putida	

In addition, certain *Arthrobacter* species, *Arthrobacter -Corynebacterium* species; *Bacillus* species and *Nocardia* species can grow on mandelate but the enantiomers used are not known.

Fungi

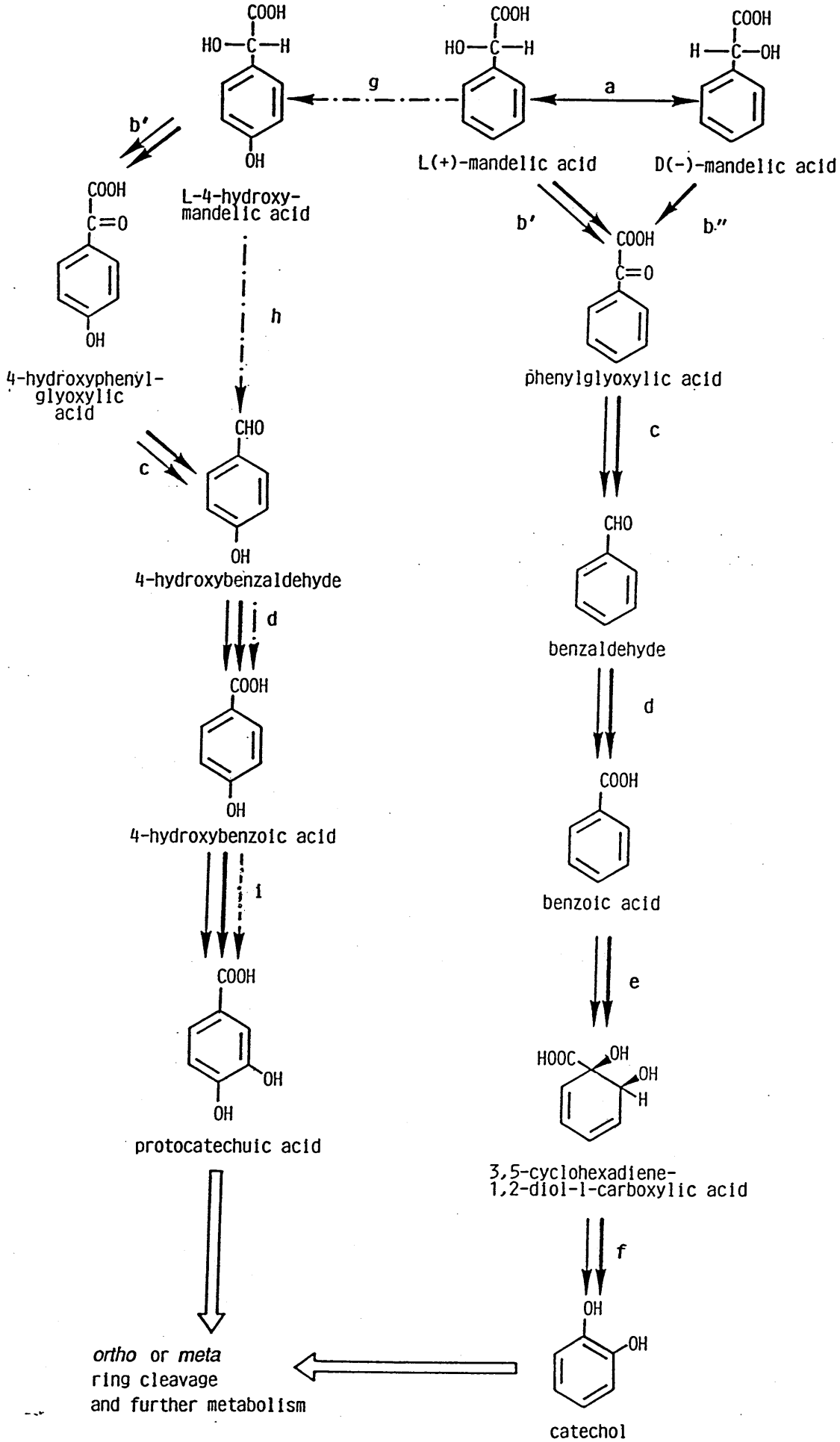
D, L-Mandelate	L(+)-Mandelate	D(–)-Mandelate
Aspergillus flavus	*Aspergillus flavus*	*Aspergillus flavus*
Byssochlamys fulva	*Byssochlamys fulva*	*Byssochlamys fulva*
Aspergillus niger	*Aspergillus niger*	*Aspergillus niger*
Neurospora crassa	*Neurospora crassa*	*Neurospora crassa*
Polyporus tumulosus	*Polyporus tumulosus*	*Polyporus tumulosus*
Rhodotorula graminis	*Rhodotorula graminis*	*Rhodotorula graminis*
Unidentified yeasts	Unidentified yeasts	Unidentified yeasts

Adapted from Fewson (1988b).

L(+)-mandelate (Kennedy & Fewson 1988b), whereas strain EBF 65/65 has only D(–)-mandelate dehydrogenase and so can grow only on D(+)-mandelate (Hills & Fewson, 1983a). However, mutant strain EBF 65/174 has both L(+)-mandelate dehydrogenase and D(–)-mandelate dehydrogenase activities and can grow on both enantiomers (Hills & Fewson 1983a, b). Phenylglyoxylate is decarboxylated and oxidised in two successive steps to give benzaldehyde and benzoate by phenylglyoxylate decarboxylase and benzaldehyde dehydrogenase respectively (Fig. 1.8c,d; Barrowman & Fewson, 1985; MacKintosh & Fewson, 1988a; Chalmers & Fewson, 1989a). Phenylglyoxylate decarboxylase (Fig. 1.8c) catalyses a TPP-dependent decarboxylation of phenylglyoxylate to benzaldehyde. Benzaldehyde is oxidised to benzoate by benzaldehyde dehydrogenase I, one of a pair of NAD^+-dependent benzaldehyde dehydrogenases found in *A. calcoaceticus* (Fig. 1.8d; MacKintosh & Fewson, 1988a; Chalmers & Fewson , 1989a). The two isofunctional enzymes benzaldehyde dehydrogenase I and benzaldehyde dehydrogenase II occur at the point where the mandelate and benzyl alcohol pathways converge (see Fig. 1.7). However, different induction mechanisms have evolved to induce the two enzymes of these two pathways. Mandelate dehydrogenase, phenylglyoxylate decarboxylase and benzaldehyde dehydrogenase I are induced by phenylglyoxylate (Livingstone & Fewson, 1972; Beggs & Fewson, 1977), whereas benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II are induced by benzaldehyde or benzyl alcohol (Livingstone *et al.,* 1972). Benzoate is then oxygenatively converted to 3,5-cyclohexadiene-1, 2-diol-1-carboxylate by benzoate 1, 2-oxygenase (Fig. 1.8e) and then decarboxylated to catechol (Fig. 1.8f). Catechol is then metabolised by second stage pathway enzymes and is converted into the amphibolic intermediates succinic acid and acetyl-CoA which enter the central metabolic pathways (Fig.1.8).

P. putida strain NCIB 9494 grows on both enantiomers of mandelate, however unlike *A. calcoaceticus* it does not possess two stereospecific mandelate dehydrogenase but it converts D(–)-mandelate into L(+)-mandelate by means of a soluble, metal-dependent (Mg^{2+} or Mn^{2+}) mandelate racemase (Fig. 1.8a; Hegeman *et al.,* 1970; Fee *et al.*, 1974a,b; Maggio *et al.*, 1975; Kenyon & Hegeman, 1979; Whitman *et al.*, 1985). L(+)-Mandelate is then oxidised to phenylglyoxylate by an FMN-linked, membrane-bound L(+)-mandelate

Figure 1.8 Metabolism of mandelate in *A. calcoaceticus* (⟶), *P. putida* (⟶) and *P. convexa* (- - ➔). Enzymes: a, mandelate racemase; b′, L(+)-mandelate dehydrogenase; b″, D(–)-mandelate dehydrogenase; c, phenylglyoxylate decarboxylase; d, benzaldehyde dehydrogenase; e, benzoate 1,2-oxygenase; f, 3,5-cyclohexadiene-1,2-diol-1-carboxylate dehydrogenase; g, L(+)-mandelate 4-hydroxylase; h, L(+)-4-hydroxymandelate oxidase; i, 4-hydroxybenzoate 3-hydroxylase. Adapted from Fewson (1992).


L(+)-mandelic acid
D(-)-mandelic acid
L-4-hydroxy-mandelic acid
4-hydroxyphenyl-glyoxylic acid
phenylglyoxylic acid
4-hydroxybenzaldehyde
benzaldehyde
4-hydroxybenzoic acid
benzoic acid
protocatechuic acid
3,5-cyclohexadiene-1,2-diol-1-carboxylic acid
catechol
ortho or meta ring cleavage and further metabolism
a
b'
b''
c
d
e
f
g
h
i

dehydrogenase (Tsou *et al.*, 1990, Fewson, 1992). Phenylglyoxylate is decarboxylated to benzaldehyde by a TPP-dependent phenylglyoxylate decarboxylase and then benzaldehyde is oxidised to benzoate by an NAD^+ and/or an $NADP^+$-dependent benzaldehyde dehydrogenase (Fig. 1.8d; Hegeman 1966a, b, c; Hegeman 1970; Tsou *et al.*, 1990). The benzoate formed is then converted to catechol by the action of benzoate oxygenase and 3,5-cyclohexadiene-1,2-diol-1-carboxylate dehydrogenase (Fig. 1.8 e, f; Gunsalus *et al.*, 1953; Hegeman, 1966a, b, c; Hegeman, 1970; Tsou *et al.*, 1990) as in *A. calcoaceticus*. Although most of the strains of *P. putida* use the pathway described above, one strain converts L(+)-mandelate to D(–)-mandelate by mandelate racemase and this is then oxidised to phenylglyoxylate by D(–)-mandelate dehydrogenase (Kenyon & Hegeman, 1979). Some other strains of *P. putida* do not possess a racemase and therefore can grow only on D(–)-mandelate (Hegeman *et al.*, 1970).

P. aeruginosa grows on only L(+)-mandelate as it has an L(+)-mandelate dehydrogenase but no D(–)-mandelate dehydrogenase or mandelate racemase but apart from that it uses the same pathway as *P. putida* (Rosenberg, 1971).

P. convexa , which is probably a strain of *P. putida* (Hegeman *et al.*, 1970), has a mandelate racemase and therefore can grow on both enantiomers of mandelate. However it differs from other bacteria in hydroxylating the L(+)-mandelate by L(+)-mandelate 4-hydroxylase (Fig. 1.8g; Bhatt & Vaidyanathan, 1976b). The 4-hydroxymandelate is decarboxylated to 4-hydroxybenzaldehyde, without forming 4-hydroxyphenylglyoxylate, by a membrane-bound, FAD, Mn^{2+} and O_2-dependent 4-hydroxymandelate oxidase (Fig. 1.8h; Bhat & Vaidyanathan, 1976c). The 4-hydroxybenzaldehyde is oxidised to 4-hydroxy-benzoate which is then hydroxylated at position 3 of the ring to form protocatechuate instead of catechol as in *A. colcoaceticus* and *P. putida* (Bhat *et al.*, 1973; Bhat & Vaidyanathan, 1976a, b, c).

Some enzymes whose exact function is not known but which can act as mandelate dehydrogenases are found in a wide range of bacteria such as *Lactobacillus curvatus, L. delbrueckii, L. bulgaricus, L. casei, L. brevis, L. fructivorans, L. leichamannii, L. parvus, L. lactis, Leuconostoc dextranicum, Leuconostoc mesenteroides* and *Streptococcus faecalis* as well as *Bacillus thuringiensis, Xanthomonas oryzae* and *Pichia*

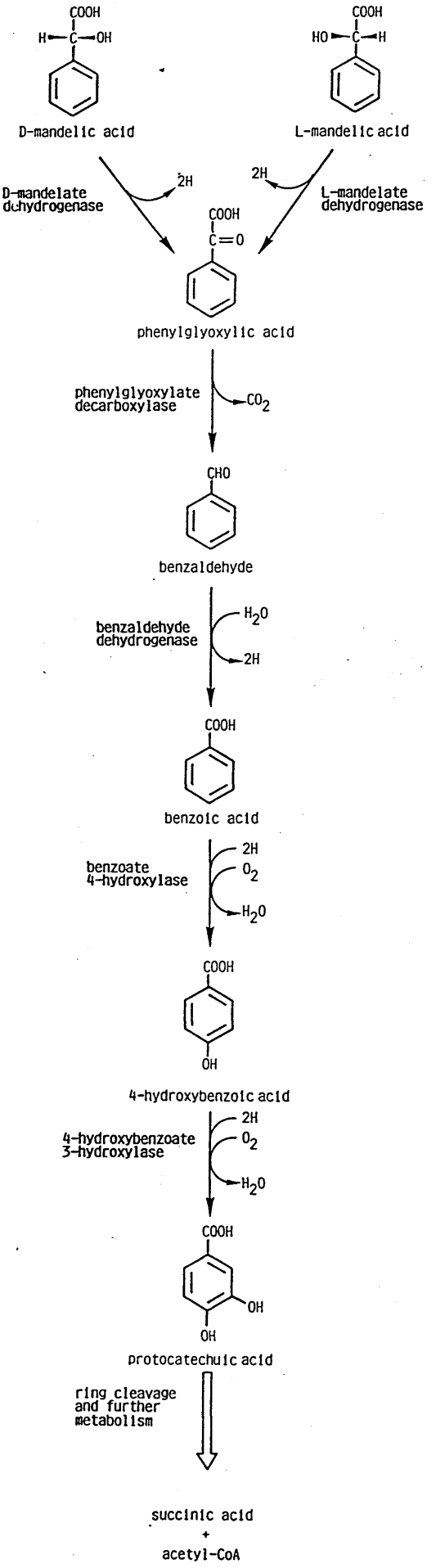


Figure 1.9 Metabolism of mandelate in *A. niger*, *N. crassa* and *R. graminis*.

quercum (Yamazaki & Maeda, 1986a, Hummel *et al.*, 1988; Hummel & Kula, 1989). However, in general these organisms do not seem to use mandelate as source of carbon and energy. Mandelate dehydrogenase activities were discovered during the screening of extracts of bacteria.

In many organisms the enzymes involved in converting mandelate into benzoate can tolerate substitutions on the benzene ring of the substrate so these organisms can completely or partially metabolise ring-substituted mandelates such as 4-hydroxymandelate (Fig. 1.8), 3, 4-dihydroxymandelate, vanillylmandelate and other related compounds (Fig. 1.6; Fewson, 1988b).

1.4.2 Fungal metabolism of mandelate

Metabolism of mandelate is less well characterized in fungi than in bacteria. The first step is oxidation either by D(–)-mandelate dehydrogenase or L(+)-mandelate dehydrogenase, as in most bacteria. In *R. graminis* there is a pair of stereospecific mandelate dehydrogenases (Fig. 1.9). *Aspergillus niger* strain UBC 814 also contains two stereospecific mandelate dehydrogenases, one that is a soluble, dye-linked L(+)-mandelate dehydrogenase which is stimulated by FAD and FMN and the other membrane bound D(–)-mandelate dehydrogenase cannot use 2,6-dichloroindophenol as an electron acceptor (Jamaluddin *et al.*, 1970). In another strain of *A. niger* both D(–)-mandelate dehydrogenase and L(+)-mandelate dehydrogenase are present in the soluble as well as the particulate fraction and both enzymes can use $NADP^+$ and to lesser extent NAD^+ as cofactor (Kishore *et al.*, 1976). It is not known whether D(–)-mandelate dehydrogenase in *A. niger* strain UBC 814 can use NAD^+ as cofactor or not. *Bassochlamys fulva* also has an NAD^+-dependent mandelate dehydrogenase but its specificity for the substrate is not known (Iyayi & Dart, 1986).

The phenylglyoxylate is decarboxylated to benzaldehyde by a TPP-dependent phenylglyxoylate decarboxylase (Fig. 1.9) in *A. niger, N. crassa* and *R. graminis* although in no case has the enzyme been fully characterized (Jamaluddin *et al.*, 1970; Kishore *et al.*,1976; Ramakrishna Rao & Vaidyanathan, 1977; Durham, 1984). Benzaldehyde is oxidised to benzoate by an $NADP^+$- or NAD^+-dependent benzaldehyde dehydrogenase in

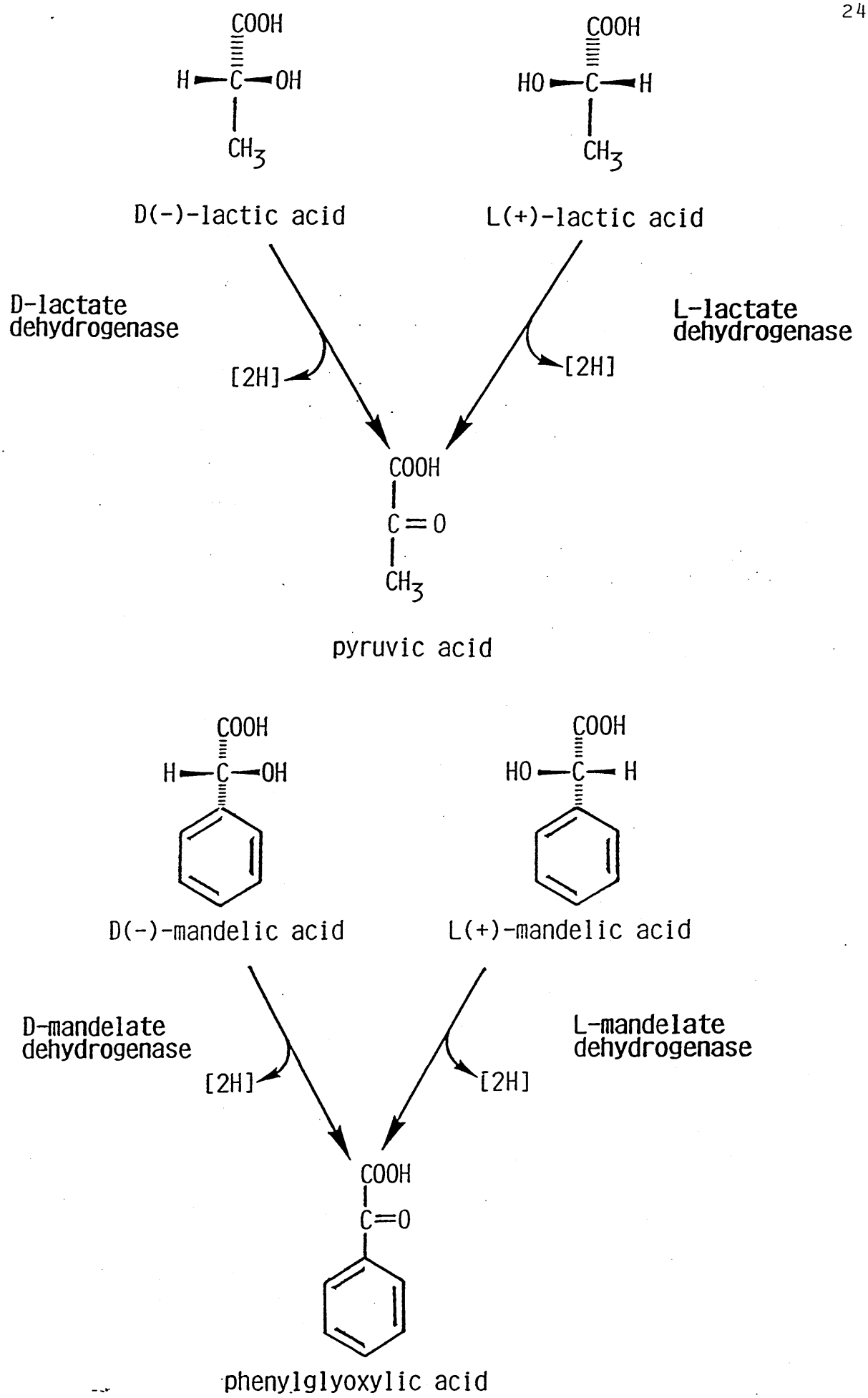


Figure 1.10 Metabolism of lactate and mandelate in microorganisms.

A. niger (Jamaluddin *et al.*, 1970) whereas in *R. graminis* there is only one NAD^+-dependent benzaldehydrogenase (Durham 1984). In *R. graminis* benzoate is also formed from benzyl alcohol by an NAD^+-dependent benzyl alcohol dehydrogenase (Middleton, 1988). None of these latter enzymes has been purified and adequately characterized.

Benzoate is hydroxylated to 4-hydroxybenzoate, instead of forming 3,5-cyclohexadiene-1,2-diol-1-carboxylate as in bacteria, (Fig. 1.9) by benzoate 4-hydroxylase which is a tetrahydropteridine, NADPH and O_2-dependent soluble enzyme in *A. niger* , whereas, in *R. graminis* it is membrane-associated flavoenzyme (Reddy & Vaidyanathan, 1975; McNamee & Durham, 1985). The 4-hydroxybenzoate is then further hydroxylated by a soluble NADPH-dependent 4-hydroxybenzoate-3-hydroxylase to form protocatechuate in *R. graminis* (McNamee & Durham, 1985). The protocatechuate is ring-cleaved and fed into the secondary metabolic pathway and converted into amphibolic intermediates such as succinate and acetyl-CoA which enter the central metabolic pathways.

Protocatechuate is produced during fungal metabolism of many aromatic compounds (Cain *et al.*, 1968; Cook & Cain, 1974; McNamee & Durham, 1985). However, when *R. graminis* was grown on salicylate or phenol it followed the catechol pathway for the degradation of these compounds (Durham *et al.*, 1984; Katayama-Hiryama *et al.*, 1991). *R. graminis* possesses ring cleavage enzymes for both catechol and protocatechuate. Therefore it possesses both branches of the β-ketoadipate pathway for the degradation of aromatic compounds e.g. mandelate and phenylalanine are degraded via protocatechuate and salicylate and phenol are degraded via catechol (Durham *et al.*, 1984; Katayama-Hiryama *et al.*, 1991).

1.5 2-Hydroxy acid dehydrogenases

This thesis is primarily concerned with mandelate dehydrogenases. These enzymes are directly comparable with lactate dehydrogenases (Fig. 1.10), being members of the general class of 2-hydroxy acid dehydrogenases. Mandelate can thus be regarded as an analogue of lactate with an aromatic ring in place of a methyl group.

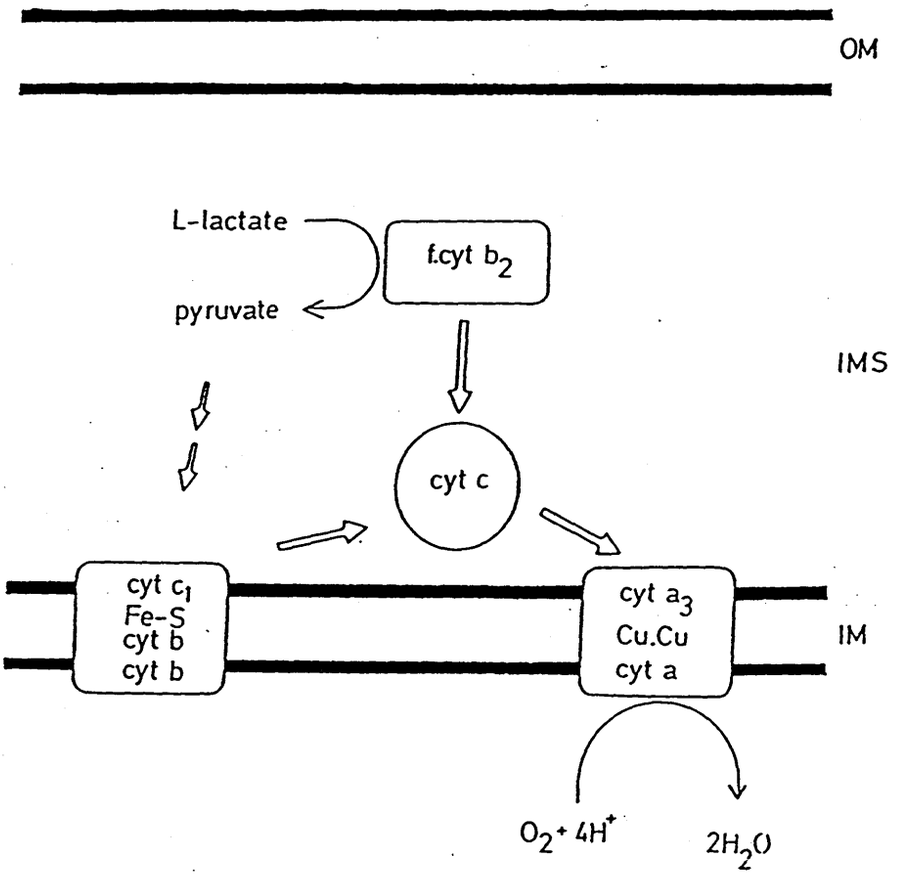


Figure 1.11 The topological arrangement of electron transfer involving L(+)-lactate dehydrogenase of *S. cerevisiae* (f.cyt b_2) in mitochondria; OM, outer membrane; IMS, intermembrane space; IM, inner membrane; cyt, cytochrome. From Chapman *et al.* (1991).

1.5.1 Properties of aliphatic 2-hydroxy acid dehydrogenases

There are various quite different types of 2-hydroxy acid dehydrogenases using different cofactors. Amongst lactate dehydrogenases, for instance, there are NAD^+- and $NADP^+$-linked soluble L(+)-lactate and D(–)-lactate dehydrogenases, FMN- and FAD-containing membrane-bound dehydrogenases, and flavin and haem containing dehydrogenases (Garvie, 1980; Chapman *et al.*, 1991). The L(+)-mandelate dehydrogenase of *R. graminis* is an $NAD(P)^+$-independent enzyme but $NAD(P)^+$-dependent aliphatic 2-hydroxy acid dehydrogenases are not discussed in this section because they are largely irrelevant to the experimental work described in this thesis.

Flavocytochrome b_2 L(+)-lactate dehydrogenase (cytochrome *c* oxidoreductase) of *Sacchromyces cerevisiae* is possibly the best studied NAD(P)-independent 2-hydroxy acid dehydrogenase. It is a soluble enzyme and is present in the intermembrane space of the mitochondria (Daum *et al.*, 1982). L(+)-Lactate dehydrogenase catalyses the oxidation of L(+)-lactate to pyruvate (Fig. 1.10) with electrons being transferred to cytochrome *c* and then to cytochrome *c* oxidase (Fig. 1.11; Appleby & Morton, 1954; Chapman *et al.*, 1991). This enzyme has a pH optimum of between 7 and 8 (Hatefi & Stiggal, 1976; Chapman *et al.*, 1991). The enzyme can oxidise a broad range of L(+)-2-hydroxy acids and the best activity is found with L(+)-lactate (Dickens & Williamson, 1956; Morton *et al.*, 1961). The enzyme is stereospecific for its substrate and it is inhibited by D(–)-lactate (Somlo & Slonimski, 1966; Baudras, 1971). It cannot use aromatic substrate analogues but it is strongly competitively inhibited by mandelate (Morton *et al.*, 1961); other inhibitors include pyruvate (Lederer, 1978; Somlo & Slonimski, 1966; Tegoni *et al.*, 1986; Genet & Lederer, 1990), propionate (Genet & Lederer, 1990), sulphite (Lederer, 1978) and oxalate (Somlo & Slonimski, 1966; Blazy *et al.*, 1976b; Lederer, 1978). The native enzyme has a M_r of 230,000 (Labeyrie & Baudras, 1972; Mevel-Nino, 1972) and a subunit M_r of 57,500 (Pjot & Groudinskey, 1970). The homotetrameric nature of the enzyme was confirmed by X-ray crystallography (Fig. 1.12; Mathews & Xia, 1987; Xia *et al.*, 1987; Xia & Mathews, 1990). Each subunit contains one molecule each of protohaem and FMN (Fig. 1.13; Chapman *et al.*, 1991). The complete amino acid sequence has been determined and contains 511 residues (Guiard, 1985; Lederer *et al.*, 1985). *In vitro* the enzyme can use methylene blue, cytochrome *c* , DCIP or

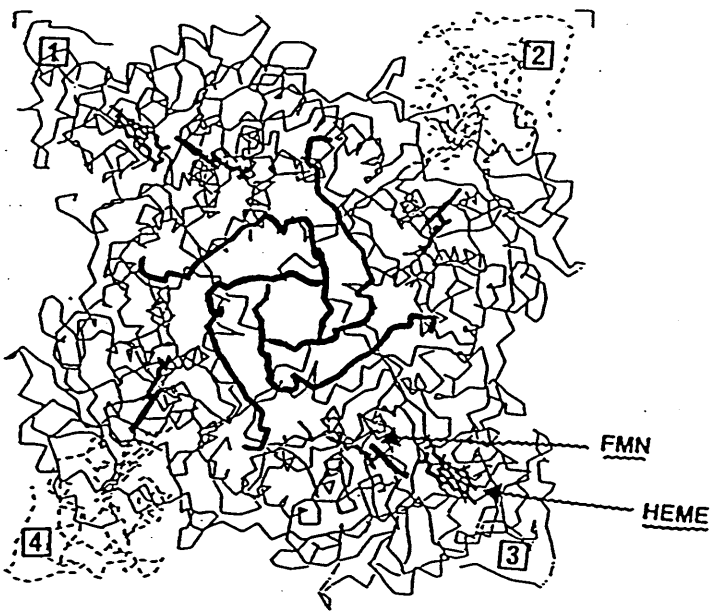


Figure 1.12 The three-dimensional structure of L(+)-lactate dehydrogenase of *S. cerevisiae*. The FMN and heme groups are highlighted looking down the fourfold axis of symmetry. The four subunits are numbered 1 to 4. The shaded portions in subunits 2 and 4 represent the two haem domains, disordered in the structure. From Xia and Mathews (1990).

ferricyanide as electron acceptors (Appleby & Morton, 1959; Chapman *et al.*, 1991). Chapman *et al.* (1991) have postulated a domain structure of flavocytochrome b_2, which consists of a smaller haem domain and a bigger flavo-dehydrogenase domain. The two domains are linked by a "hinge" region. A "tail" of C-terminus consisting of residues 489-511 that wraps around the centre of the enzyme and makes close contacts with the rest of the three-subunit structures (White *et al.*, 1989; Xia & Mathews, 1990). Amino acid residues at the active site appear to make significant interactions with the substrate and/or FMN (Mathew & Xia, 1987, Xia *et al.*,1987; Xia & Mathews 1990).

The other flavocytochrome b_2 L(+)-lactate dehydrogenase characterized most fully to date is from *Hansenula anomala*. It exhibits catalytic and kinetic properties which are generally similar to those of L(+)-lactate dehydrogenase of *S. cerevisiae* with which it is strongly homologous (Lederer, 1974; Capeillere-Blandin *et al.*, 1975 & 1986; Blazy *et al.*, 1976a; Iwatsub *et al.*, 1977; Chapman *et al.*, 1991). It has a native M_r of about 230,000 and a subunit M_r of about 58,000 (Lebeyrie & Baudras, 1972) and is homotetrameric (Chapman *et al.*, 1991). The native structure is important for activity because dissociation of the subunits at low ionic strength produces inactive monomers. However, these monomers can be reassociated with recovery of activity (Prats, 1978). This enzyme has a similar domain structure to that described for L(+)-lactate dehydrogenase of *S. cerevisiae* and also contains one haem and one FMN per subunit (Gervais *et al.*, 1977; Chapman *et al.*, 1991). The amino acid sequence deduced from the cloned gene for the L(+)-lactate dehydrogenase of *H. anomala* (Black *et al.*, 1989; Risler *et al.*, 1989) shows 60% overall identity with L(+)-lactate dehydrogenase from *S. cerevisiae* (see section 4.7).

Many other microorganisms contain membrane bound lactate dehydrogenases (Garvie, 1980; Fewson, 1992). Two stereospecific lactate dehydrogenases can be induced separately in *A. calcoaceticus* by either D(-)-lactate or L(+)-lactate (Fewson & O'Donnell, 1981; Allison *et al.*, 1985a) and both enzymes are membrane-bound and NAD-independent (Allison *et al.*, 1985a; Allison & Fewson 1986). L(+)-Lactate dehydrogenase is FMN-dependent whereas D(–)-lactate dehydrogenase contains FAD but neither enzyme contains haem. L(+)-Lactate dehydrogenase has a subunit M_r of 40,000 and D(–)-lactate dehydrogenase has a subunit M_r of 63,000 (Allison *et al.*, 1985a; Allison & Fewson 1986).

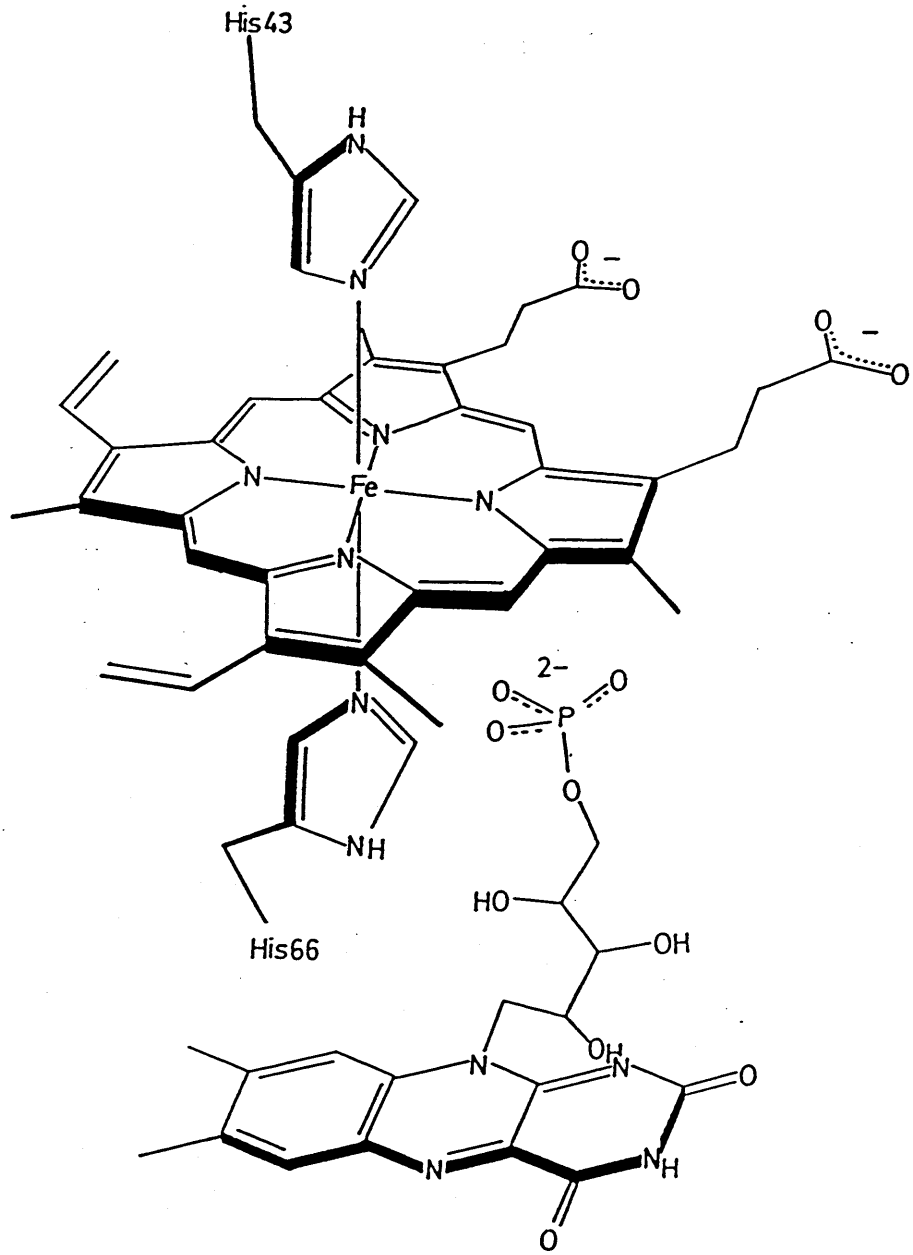


Figure 1.13 Structure of prosthetic groups of L(+)-lactate dehydrogenase of *S. cerevisiae* ; protohaem IX and flavin mononucleotide (FMN). From Chapman *et al.* (1991).

The enzymes can be solubilized in active form by detergents, especially by Triton X-100 and Lubrol PX. Neither enzyme is affected by a range of chelating agents but both are susceptible to several thiol-blocking reagents especially *p* -chloromercuribenzoate and $HgCl_2$ (Allison *et al.*, 1985a; Allison & Fewson, 1986; Hoey *et al.*, 1987). Both lactate dehydrogenases have been recently purified to homogeneity and limited amino terminal sequences have been determined (C.A. Fewson, personal communication). L(+)-Lactate dehydrogenase from *E. coli* is also membrane-bound and can be solubilised by detergent treatment (Futai & Kimura, 1977). It has a M_r of 43,000 and contains a mole of FMN per mole of subunit (Futai & Kimura, 1977). It is heat-labile and can be stabilised by the presence of detergents (Kimura & Futai, 1978). It has been purified and its amino acid composition has been determined (Kimura & Futai, 1978) but unfortunately it has not yet been cloned. The D(–)-lactate dehydrogenase from *E. coli* has an M_r of 64,613 (determined from its gene sequence) and contains FAD as prosthetic group (Futai 1973; Campbell *et al.*, 1984; Rule *et al.*, 1985). *A. calcoaceticus* has an L(+)-mandelate dehydrogenase and a D(–)-mandelate dehydrogenase which are extremely similar in properties to the equivalent lactate dehydrogenases and these enzymes are discussed in detail in section 1.5.2 below.

Glycolate oxidase of spinach is another flavoprotein which catalyses the oxidation of L(+)-2-hydroxyacids. In higher animals it is perhaps involved in the production of oxalate by oxidizing glycolate via glyoxylate (Schuman & Massey, 1971). The enzyme is present in leaves of higher plants where it oxidises glycolate to glyoxylate and water in the process of photorespiration (Tolbert, 1981). Both mammalian and plant glycolate oxidases usually exist as tetrameric or octameric enzymes with subunit Mr of 43,000 (Frigerio & Harbury, 1958, Schwam *et al.*, 1979, Behrends *et al.*, 1982). Glycolate oxidase from spinach contains FMN as prosthetic group and it has been purified to homogeneity (Lindqvist & Branden, 1979; Volokita & Somerville, 1987). The amino acid sequence has been determined both by peptide sequencing (Cederlund *et al.*, 1988) and by sequencing DNA (Volokita & Somerville, 1987). The catalytic mechanism for the transfer of electrons is similar to that of the lactate dehydrogenase of *S. cerevisiae* (Branden *et al.*, 1987; Xia *et al.*, 1987). The three-dimensional structure has recently been determined by X-ray

crystallography studies (Lindqvist & Branden, 1985 & 1989) and the enzyme has a high degree of homology with the FMN-binding domain of L(+)-lactate dehydrogenase of *S. cervisiae* (Branden *et al.*, 1987), a reflection of the 37% sequence identity between the two enzymes (Lindqvist & Branden, 1989).

L(+)-2-Hydroxy acid oxidase from rat kidney is another FMN-containing protein (Blanchard *et al.*, 1945 & 1946; Urban *et al.*, 1988). Originally it was recognised as an L(+)-amino acid oxidase (Blanchard *et al.*, 1945) and later it was shown to be capable of oxidising 2-hydroxy acids of long chain homologues of glycolate as well as aromatic 2-hydroxy acids such as phenyllactate and mandelate (Blanchard *et al.*, 1946; Nakano & Danowski, 1966). The carbanion mechanism for the oxidation of L(+)-lactate to pyruvate suggested for L(+)-lactate dehydrogenase from *S. cerevisiae* (Lederer, 1974; Chapman *et al.*, 1991) is compatible with mechanistic studies made with L(+)-2-hydroxy acid oxidase from rat kidney (Cromartie & Walsh, 1975, Lederer, 1984; Urban *et al.*, 1984 & 1988). The rat kidney hydroxy acid dehydrogenase has been purified (Blanchard *et al.*, 1945; Nakano & Danowski, 1966; Cromartie & Walsh, 1975; Duley & Holmes, 1976) and its amino-terminal sequence is homologous with that of glycolate oxidase of spinach (Volvokita & Somerville, 1987) and L(+)-lactate dehydrogenase of *S. cerevisiae* (Guiard, 1985; Lederer *et al.*, 1985).

Several microorganisms contain 2-hydroxy acid dehydrogenases that can oxidise both aliphatic and aromatic substrates such as phenyllactate or 4-hydroxyphenyllactate or even mandelate itself; examples include the L(+)- and D(–)-2-hydroxyisocaproate dehydrogenases of *Neisseria gonorrhoea* and *L. casi* respectively and D(–)-lactate dehydrogenase of *L. confusus* and *Candida maltosa* (Bode *et al.*, 1986; Yamazaki & Maeda 1986a; Batnager *et al.*, 1989; Hummel & Kula, 1989; Hendry *et al.*, 1990).

1.5.2 Properties of mandelate dehydrogenases

L(+)-Mandelate dehydrogenase of *R. graminis* was purified and characterized during the present project and is described in later chapters. However, D(–)-mandelate dehydrogenase of *R. graminis* had already been purified and fairly fully characterized. It is an NAD^+-dependent soluble dimeric enzyme with subunit M_r of 30,000 and can interconvert

mandelate and phenylglyoxylate at different pH values (Baker & Fewson, 1989; Baker, 1990; Baker *et al.*, 1992). D(–)-Mandelate dehydrogenase of *R. graminis* seems similar to NAD^+-dependent D(–)-mandelate dehydrogenase from *L. curvatus* and 2-hydroxy-isocaproate dehydrogenase of *S. faecalis*. All three enzymes are dimeric, have similar subunit M_r values of about 30,000 to 38,000, pH optimum of 8.5 to 9.5, similar K_m value for NADH of about 36 μM and none is inhibited by metal chelating agents but all are affected by some thiol-blocking reagents (Yamazaki & Maeda, 1986a; Hummel *et al.*, 1988; Baker & Fewson, 1989). In contrast, L(+)-mandelate dehydrogenases from the bacteria *A. calcoaceticus* and *P. putida* are FMN-dependent and catalyse an essentially irreversible reaction (Hoey *et al.*, 1987; Tsou *et al.*, 1990; Fewson, 1992). Neither the L(+)-mandelate dehydrogenases nor the D(–)-mandelate dehydrogenase from *A. calcoaceticus* appear to contain haem. D(–)-Mandelate dehydrogenase from *A. calcoaceticus* contains FAD and it also catalyses an essentially irreversible reaction (Allison *et al.*, 1985a,b). Both D(–)- and L(+)-mandelate dehydrogenase from *A. calcoaceticus* are inhibited by the opposite enantiomer of the substrate, have similar dependence on pH, temperature, and several metal chelating agents have no effect on their activity (Allison *et al.*, 1985a,b; Hoey *et al.*, 1987). L(+)-Mandelate dehydrogenase has a subunit M_r of 44,000 and D(–)-mandelate dehydrogenase has a subunit M_r of 60,000 (Allison *et al.*, 1985a; Allison & Fewson, 1986; Hoey *et al.*, 1987). Both enzymes are inhibited by oxalate and are susceptible to inhibition by $HgCl_2$ or *p*-chloromercuribenzoate (Hills & Fewson 1983a; Allison *et al.*, 1985a,b; Hoey *et al.*, 1987). L(+)-Mandelate dehydrogenase from *P. putida* is an FMN-linked membrane-bound enzyme which has a M_r of 44,000 as estimated by SDS-PAGE which is in agreement with the M_r of 43,352 deduced from the nucleotide sequence (Tsou *et al.*, 1990; Fewson, 1992). L(+)-Mandelate dehydrogenase from *P. putida* has been solubilized in an active form using the same detergent treatment as that developed for L(+)-mandelate dehydrogenase of *A. calcoaceticus* and it has also been purified almost to homogeneity using the same procedure as developed for L(+)-mandelate dehydrogenase of *A. calcoaceticus*. (C.A. Fewson & A.J. Scott, unpublished results). None of the other mandelate dehydrogenases from either bacteria or fungi (see section 1.4.1 & 1.4.2) has been characterised to any significant extent.

1.6 Methods for studying molecular evolution

Understanding the basis of molecular evolution is a highly important challenge to modern science. The fundamental method for protein comparison is comparison of DNA or protein sequences. Nucleotide sequences are compared in the same general way as amino acid sequences. Several examples have illustrated the fact that generally the rate of nucleotide substitution for DNA is higher than that for amino acids replacements in a protein. Part of the explanation for the difference in rate is redundancy of the genetic code allowing the degenerate nucleotide in a codon to change without effecting the amino acid sequence (nucleotide changes are known as synonymous nucleotide substitutions). For example, comparison of the nucleotide sequence coding region for mouse lactate dehydrogenase B shows 86% identity with that of human isoenzyme but only eight of the 139 nucleotide differences resulted in corresponding amino acid substitutions, at positions 10, 13, 14, 17, 52, 132, 236 and 317 giving 97.6% of identity in the amino acid sequences (Hiraoka *et al.*, 1990). Nucleotide substitutions that result in amino acid change are known as non-synonymous substitutions (Hiraoka *et al.*, 1990). Comparison of different proteins has shown that the rate of non-synonymous substitutions varies greatly, whereas the rate of synonymous substitutions is uniform among different proteins with a maximum of 3-4 times difference (Hartl & Clark, 1989).

Amino acid sequence comparisons have been used extensively to reveal possible evolutionary relationships among proteins. This method is now commonly used as new sequence data become available. Advantages include the fact that one can compare thousands of proteins using computerised searches of data bank sequences. The popular comparison methods include FASTA, BLASTA and GCG BESTFIT programs and all these programs' versions are continually renewed with new data (Argos *et al.*, 1991). All these methods allow the introduction of gaps to accommodate insertions or deletions that may have occurred during the evolution of genetic material. Gap penalties and other parameters set for different programs affect alignment results and sometimes result in wrong alignments. The FASTA program uses some of the scoring schemes developed by Needleman and Wunsh (1970), Sellers (1974) and Dayhoff *et al.*(1983) for rapid recognition of best alignment (Pearson & Lipman, 1988). The FASTA algorithm allows a homology search of one

sequence with databases containing more than 20,000 sequences (Argos *et al.,* 1991) and it also allows a rapid comparison, but it may not be sensitive for revealing distant relationships. However, for sensitivity and accuracy, the RAM 250 scoring matrix provides the best alignment between two sequences and it is based on the observed frequency of substitutions in 71 groups of closely related proteins (Dayhoff *et al.,* 1978) but it usually takes many hours to run on a VAX computer.

Until methods were developed to clone and sequence genes coding for proteins and to sequence proteins, indirect comparisons were used to evaluate homologies amongst proteins. The success of indirect methods depends on how closely related are the gene sequences of the proteins concerned. A number of indirect methods have been used to search for homologies, such as comparison of amino acid composition, enzyme kinetics, copurification, proteolytic behaviour, stoichiometry, stereochemistry of reactions, regulation of pathways and three-dimensional structures.

1.6.1 Amino terminal and internal peptide amino acid sequences

Complete protein sequences are difficult to obtain and it is very common to obtain short amino-terminal sequences. Such sequences can be aligned by eye or by computer analysis and sometimes during such comparisons good homology is found. However, in cases where no homology is found, care should be taken about drawing conclusions because the proteins compared may have short spans of conserved residues which do not happen to include those studied. At the same time homology searched of short sequences may give rise to false mismatches.

In certain cases blocked amino-termini may not allow sequencing and the general way to obtain sequence information for such proteins is to prepare peptide fragments by proteolytic cleavage. The purified internal peptide then can be sequenced for comparison with other related proteins. Such internal peptides also can be used for multiple probes for cloning of genes.

1.6.2 Amino acid composition

Amino acid compositions of proteins are easier to obtain than amino acid sequences. Comparison of amino acid composition can be used to quantify the degree of evolutionary relatedness among proteins which are already known to be related on the basis of other criteria. Yeh *et al.* (1982) suggested that it is quite unusual to obtain composition homology for proteins which are not homologous as judged by sequence information. Mostly such comparisons have been focused on isofunctional gene products in different organisms (Yeh *et al.,* 1982; Kagawa & Bruno, 1988; Vanni *et al.*, 1990). However, compositional relatedness has also been found among enzymes of β-ketoadipate pathways in *Acinetobacter* which suggests their common evolutionary origin (Yeh *et al.,* 1982).

1.6.3 Enzyme kinetics and physical characteristics

In the literature different kinetic and physical characteristics have been cited as criteria that can be used to indicate evolutionary relationships. However, none of these methods is considered to be a good guide to evolutionary relationships, mainly because they sometimes fail to show relationships between highly homologous proteins and partly because amino acid substitutions can have large effects on enzyme kinetic constants (Hartley, 1984).

1.6.4 Three-dimensional structure

Although sequence comparisons give valuable information, some proteins have evolved so rapidly or so far that the divergent but related proteins have so little homology that it is beyond recognition (Schultz, 1981). In such cases comparison of three-dimensional structures may be very useful in assessing homology among proteins because in the process of protein evolution, conformation of backbone is conserved much longer than the amino acid sequence and gives clues about structural evolution which may reveal even distant relationships (Schultz, 1981). For example Lebioda & Stec (1988) found that enolase has only 15% amino acid sequence homology with pyruvate kinase and triosephosphate isomerase but share similar folding patterns for their structural domains and subunits. Their analysis of structural homology among enolase, pyruvate kinase and triose phosphate isomerase suggested the existence of a common ancestral multifunctional enzyme.

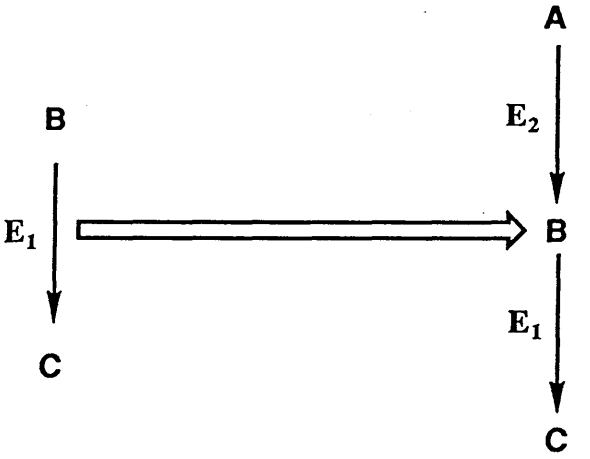
They also concluded that structural and sequence evidence suggest that muconate lactonizing enzyme and enolase may have evolved from a common ancestor.

1.6.5 Immunological cross-reactions

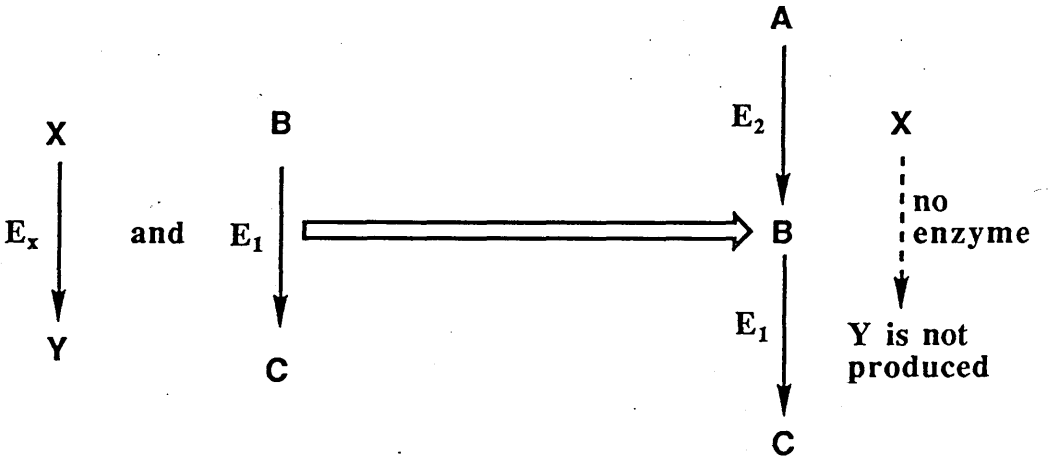
Immunological studies have been used to establish evolutionary affinities within families of isoenzymes (e.g. Arnon & Neurath, 1969; Arnheim *et al.*, 1971; Prager & Wilson, 1971 a,b). A polyclonal antiserum which contains antibodies directed against many different antigenic determinant sites is obtained when a purified protein is injected into the rabbit or sheep or goat (Johnstone & Thorpe, 1987; Catty, 1988). Polyclonal antibodies are widely used in preference to monoclonal antibodies because related proteins share different numbers of antigenic determinant sites depending upon the degree of dissimilarity (Campbell, 1984; Van Regenmortel, 1987). Polyclonal antisera are of a multispecific nature and therefore cannot detect antigenic differences at the individual epitope level of related proteins (Catty, 1988) but because of their broad specificity can recognise some of the structural features. Polyclonal antisera can thus be used to detect structural similarities (Kimura & Futai, 1978; Schwartz *et al.*, 1980; Hirabayashi *et al.*, 1987; Hue *et al.*, 1989) or sequence homologies (Prager & Wilson, 1971a,b; Benyamin, 1986). There appears to be a direct relationship between the degree of cross-reaction and the extent of sequence divergence. This relationship predicts that immunological cross-reaction will not take place for proteins with less than 60% sequence homology in the native form. However, a sequence homology of only 40% is required for cross reaction with antibodies raised against proteins denatured by reduction or carboxymethylation (Arnheim *et al.*, 1971; Arnon & Maron, 1971; Zakin *et al.*, 1978; Pekkala-Flagen & Ruoslahti, 1982).

Immunological cross-reactions depend on the immunogenicity of the epitopes. Epitopes which are highly immunogenic will promote detection of cross-reactions. The amino acids which are usually found in antigenic determining sites, such as histidine, lysine, alanine, leucine, aspartate and arginine, are particularly important in this regard (Scheidtmann, 1989).

a Simple retrograde evolution : E_2 evolved by gene duplication and subsequent mutation of E_1



b Mutation and recruitment : E_x is mutated and recruited to a new function as E_2, but in the absence of gene duplication X cannot be converted into Y.



c Duplication, mutation and recruitment : E_x is duplicated, the product of one copy can continue to convert X into Y while the second copy can mutatio to give E_2 thus extending the pathway

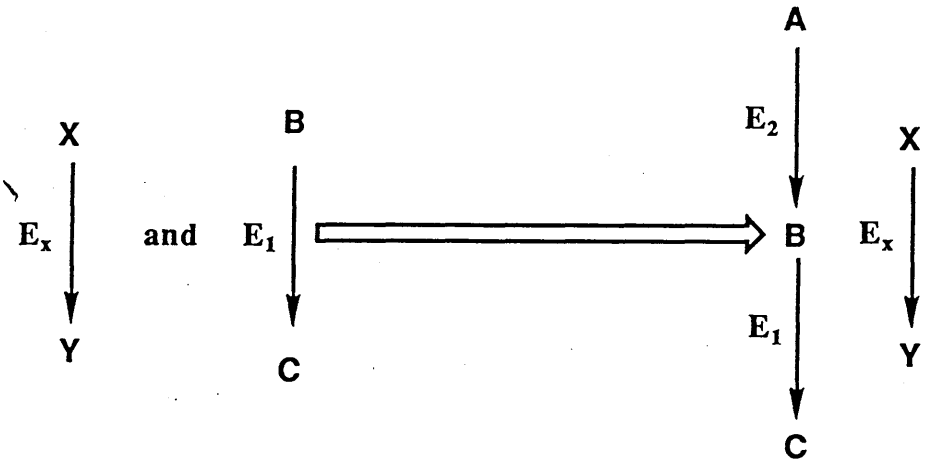


Figure 1.14 Gene duplication and mutation with retrograde evolution or recruitment : extension of pathway from B ⟶ C to become A ⟶ B ⟶ C

1.7 The evolution of metabolic pathways

The idea of retrograde evolution (Fig. 1.14; Horowitz, 1945 & 1965) together with concepts of gene duplication (Lewis, 1951) and gene recruitment (Fig. 1.14; Jensen, 1976; Parsot *et al.*,1987) are generally considered to account for evolution of most metabolic pathways. If an organism which could grow on substrate 'B' as a sole source of carbon and energy, encountered an ecosystem which contained very little or no 'B' but did contain 'A', then there might be a duplication of the gene that codes for an enzyme E_1 that converts B to C. One copy of the gene might then undergo mutation to enable it to encode enzyme E_2 that converts A to B and so there would be retrograde evolution to utilise a new substrate (Fig. 1.14a). Subsequent duplication and mutation might give rise to genes which could evolve enzymes able to transform precursor molecules into A; however, this is most likely to happen if reaction mechanisms of consecutive steps are similar. Alternatively, the new enzyme might be recruited from an entirely different pathway in which 'X' is converted into 'Y' (Fig. 1.14b, c). Applying these ideas to the metabolism of aromatic compounds, for example (Fig. 1.7), C might be benzoate, B benzaldehyde and A benzyl alcohol. In this case benzyl alcohol dehydrogenase might have evolved by duplication of benzaldehyde dehydrogenase or by the recruitment of an alcohol dehydrogenase from another pathway. Neither the *N* -terminal amino acid sequences nor immunological experiments provide evidence in favour of an evolutionary relationship between benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase of *A. calcoaceticus* (Chalmers *et al.*, 1991). It is more likely that the two enzymes have been recruited to the present function from some other pathway rather than evolved by retrograde evolution (Chalmers *et al.*, 1991).

Glycolysis and other central metabolic pathways are the best conserved pathways but one can find only little evidence to support the Horowitz hypothesis of retrograde evolution by successive gene duplication. The one possible example is enolase and pyruvate kinase, two consecutive enzymes, which are homologous and share similar three-dimensional structures (Lebioda & Stec, 1988). Both enzymes bind to the same phosphoenolpyruvate ligand and require two Mg^{2+} cations per active site (Lebioda & Stec, 1988). However, Forthergill-Gilmore (1986a, b) could find no evidence for homologies among other

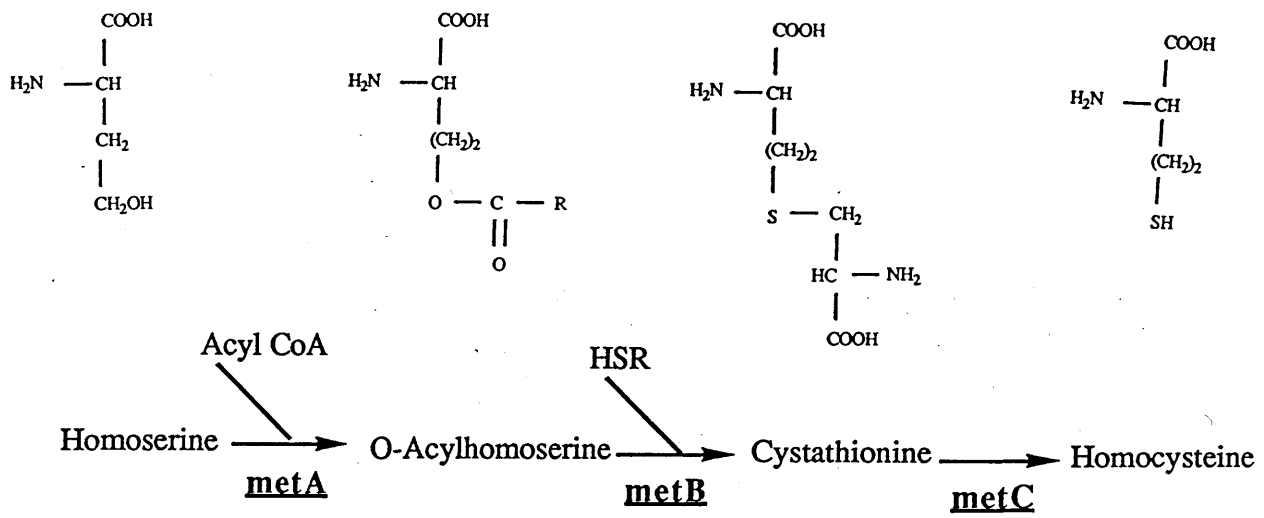


Figure 1.15 Some of the reactions involved in the methionine biosynthetic pathway. The names of the *E. coli* genes encoding the relevant enzymes are given: metA, homoserine transsuccinylase; metB, cystathionine-γ-synthase; metC, cystathionine-β-lyase. The radical R represent cysteine and the acyl group can be either succinyl or acetyl. Adapted from Parsot *et al.* (1986)

glycolytic enzymes, so although retrograde evolution may have contributed to the evolution of some metabolic pathways, it probably did not account for the appearance of glycolysis.

Cystathionine-γ-synthase and cytathionine-β-lyase of *E. coli* catalyse two consecutive steps in methionine biosynthesis (Fig. 1.15). The genes coding for the two enzymes have been sequenced and the amino acid sequences show a total of 36% homology (Fig. 1.16; Belfaiza *et al.*, 1986). So in this case retrograde evolution probably did occur, unless each enzyme was individually recruited from another pathway.

However, many other examples contradict the hypothesis of retrograde evolution by simple gene duplication, but point instead to recruitment of genes from one pathway to another. Many peripheral pathways and pathways for the biosynthesis of amino acids are thought to have originated as a result of recruitment of genes from other related pathways. Jensen (1976) proposed that many pathways existed at a low levels of expression due to fortuitous reactions and that these pathways recruited suitable enzymes from other pathways to amplify their reactions. Parsot *et al.* (1987) have shown that recruitment of genes probably played an important role in establishing the biosynthetic pathways for threonine, isoleucine and tryptophan in *E. coli* and *Bacillus subtilis*. The metabolic diversity of modern organisms and the substrate ambiguity of many other enzymes makes gene recruitment a very plausible mechanism to give rise to new metabolic pathways. The substrate ambiguity provides the start of fortuitous reactions which is then amplified by gene recruitment. For instance in the isoleucine biosynthetic pathway threonine dehydratase is homologous with threonine synthase, the preceding enzyme in the threonine biosynthetic pathway, and both probably evolved from a common ancestor; the remaining four enzymes are non-specific enzymes of the valine biosynthetic pathway (Fig. 1.17; Parsot *et al.*, 1987).

The mandelate pathway enzymes in *A. calcoaceticus* , *P. putida* and *R. graminis* seem to have originated by recruitment. The fact that mandelate can be attacked by three types of enzymes, catalysing racemization, stereospecific dehydrogenation or ring hydroxylation (Fig. 1.8a, b', b", d), is a clear indication of the different evolutionary origins of these enzymes. Similarly, the metabolism of mandelate is carried out by successive enzymes having very different properties. Mandelate racemase, which is formed only in

Figure 1.16 Comparison of the amino acid sequences of cystathionine-γ-synthase (B) and cystathionine-β-lyase (C). Stars indicate identical residues and conservative replacements (I/L/V, D/E, R/K, T/S) are indicated by + signs. From Belfaiza *et al.* (1986).

P. putida biotype A, is soluble, tetrameric, contains four identical subunits of M_r of 69,500 and has an absolute requirement for a divalent metal ion for activity (Hegeman *et al.*, 1970; Kenyon & Hegeman, 1970; Fee *et al.*, 1974a, Maggio *et al.*, 1975). Mandelate racemase, therefore has marked differences from the next enzyme, L(+)-mandelate dehydrogenase, which in *P. putida* is an FMN-dependent monomer of Mr 44,000 (Tsou *et al.*, 1990; Fewson, 1992; section 1.5.2). The L(+)-mandelate dehydrogenase in *A. calcoaceticus* is very similar to the *P. putida* enzyme (Fewson, 1992) but D(–)-mandelate dehydrogenase from *R. graminis* is a soluble, NAD-dependent enzyme (Baker & Fewson, 1989). The next enzyme is phenylglyoxylate decarboxylase which is a soluble, TPP-dependent, allosteric, tetrameric enzyme with an apparent subunit M_r of 58,000 (Hegeman, 1970; Barrowman & Fewson, 1985; Reynolds *et al.*, 1988; Weiss *et al.*, 1988). Phenylglyoxylate decarboxylase is thus very different from any of the preceding enzymes of the pathway and from the NAD-dependent, K^+ activated benzaldehyde dehydrogenases from *P. putida* and *A. calcoaceticus* which catalyse the following step (Stachow *et al.*, 1967; Livingstone *et al.*, 1972; Chalmers *et al.*, 1989a, b). Furthermore, mandelate racemase, L(+)-mandelate dehydrogenase and phenylglyxoylate decarboxylase from *P. putida* , which catalyse successive steps in mandelate metabolism have different chemical mechanisms and show no evidence of sequence homology (Tsou *et al.*, 1990). Therefore, it is difficult to imagine that retrograde evolution by simple gene duplication could explain the origin of the mandelate pathway in *P. putida.* Recent evidence has suggested that the mandelate pathways enzymes might have evolved by recruitment of genes from other pathways. L(+)-Mandelate dehydrogenase from *P. putida* has been cloned and sequenced and is homologous with other 2-hydroxy acid dehydrogenases (Tsou *et al.*, 1990; Fewson, 1992 and see section 4.7) Similarly the phenylglyoxylate decarboxylase sequence is homologous with that of other TPP-dependent enzymes e.g. pyruvate oxidase from *E. coli* , pyruvate decarboxylase from *S. cerevisiae* and *Zymomonas mobilis* and acetolactate synthase from *E. coli* and *S. cerevisiae* (Squires *et al.*, 1983; Grabau & Cronan, 1986; Kellerman *et al.*, 1986; Conway *et al.*, 1987; Tsou *et al.*, 1990). Similarities between L(+)-mandelate dehydrogenase and other 2-hydroxyacid dehydrogenases and between phenylglyoxylate decarboxylase and other TPP-dependent decarboxylases strongly suggest that these two mandelate pathway enzymes

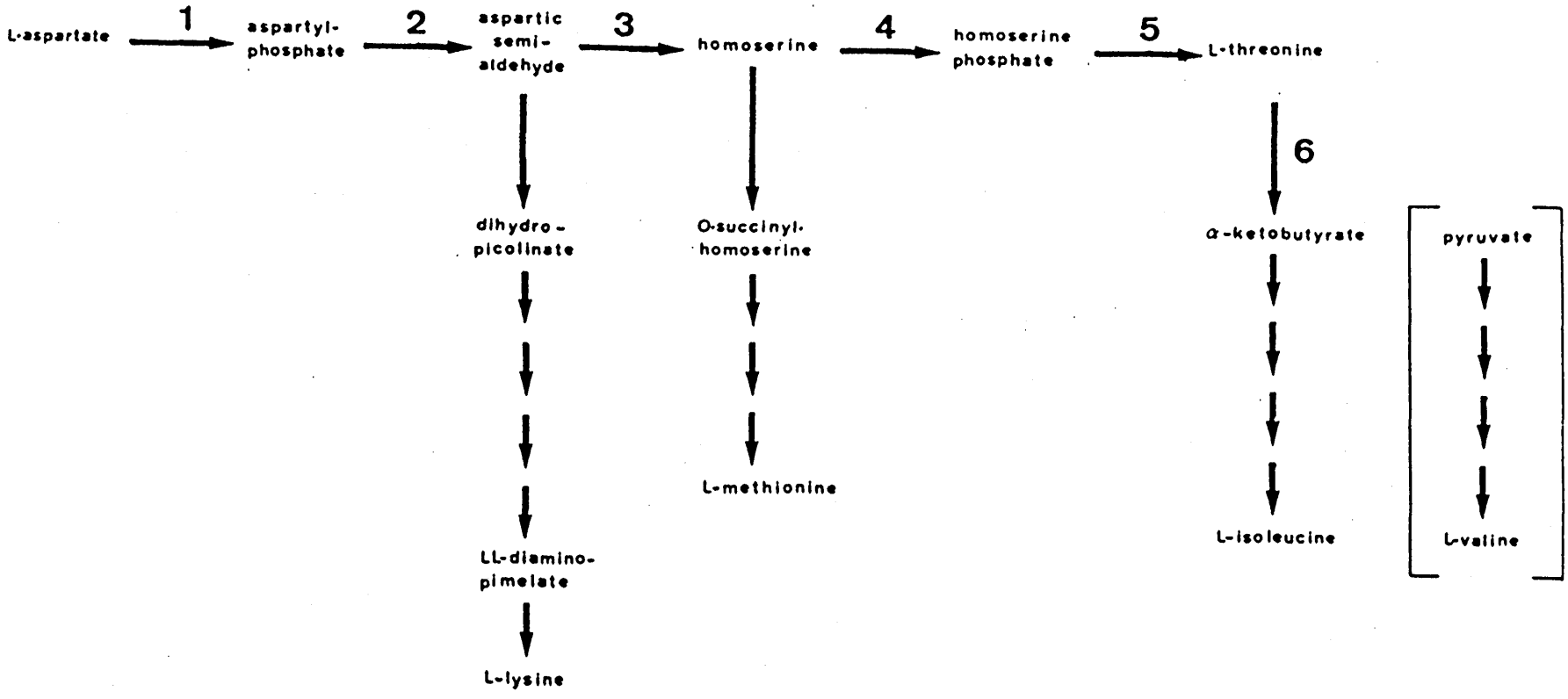


Figure 1.17 The schematic representation of lysine, methionine and isoleucine biosynthetic pathways

The branch-point metabolites of the threonine pathway are presented on the top line. The four steps of the valine pathway which are catalysed by the same four enzymes (acetohydroxy acid synthaseI, II, III; acetohydroxy acid isomeroreductase; dihydroxy acid dehydratase and branched-chain amino acid aminotransferase) involved in the isoleucine pathway are indicated in brackets. Numbers above the arrows refer to the enzymes indicated: 1, aspartokinase; 2, aspartic semialdehyde dehydrogenase; 3, homoserine dehydrogenase; 4, homoserine kinase; 5, threonine synthase; 6, threonine dehydratase. From Parsot (1986).

might have evolved by recruitment of enzymes from other metabolic pathways already established (Tsou *et al.*, 1990). Another much more surprising example to strengthen the idea of the recruitment theory comes from the evidence that mandelate racemase and *cis* , *cis* -muconate lactonizing enzyme have α/β barrel structures which are superimposeable on one another (Tsou *et al.*, 1990). Although the primary sequences show only about 26% identity, the secondary, tertiary and quaternary structures of the two enzymes are superimposable (Tsou *et al.*, 1990). It seems likely that these two enzymes were recruited to their present functions from the same ancestor.

1.8 Aims and scope of this thesis

Most previous work to study the mandelate pathway had been concentrated on prokaryotic enzymes. The only exception was D(–)-mandelate dehydrogenase from the yeast *Rhodotorula graminis* which was purified and characterised as the present work was being started (Baker & Fewson, 1989; Baker, 1990; Baker *et al.*, 1992). Therefore L(+)-mandelate dehydrogenase of *R. graminis* was selected for a similar study so that at least one pair of enzymes could be compared in both prokaryotes and eukaryotes. This enzyme had not previously been purified and all that was known about it from preliminary work on crude extracts was that (a) it was inducible, (b) it could be assayed with DCIP and PMS as electron acceptors but there was no activity with NAD, (c) it was stereospecific for its substrate, (d) it had a pH optimum of 7.0 and a half-life of 45 min at 52^0C, and (e) it might be membrane-associated because activity was found in both the membrane and supernatant fractions (Durham, 1984).

The overall aim of the present work was to characterise L(+)-mandelate dehydrogenase of *R. graminis* and to compare it with similar bacterial and yeast enzymes. In the preliminary stages it was found that L(+)-mandelate dehydrogenase of *R. graminis* is a rather labile enzyme and therefore efforts were made to increase its stability. The first aim was then to purify the enzyme to homogeneity so that its physical, chemical and kinetic properties could be determined. The other aim was to determine its amino-terminal sequence and amino acid composition so that these could be used to evaluate possible homologies with flavin-linked or NAD^+-dependent mandelate dehydrogenases and other 2-hydroxy acid dehydrogenases. In addition it was planned to raise polyclonal antisera against L(+)-mandelate dehydrogenase of *R. graminis* in order to study immunological interactions amongst different mandelate and lactate dehydrogenases. The ultimate aim was that this study would help to find answers to some questions related to the evolution of mandelate pathways enzymes and specifically about possible recruitment gene for mandelate dehydrogenase from other genes for 2-hydroxy acid dehydrogenases. The data obtained would also help to to add the knowledge of the range and classification of mandelate and lactate dehydrogenases.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., except for those listed below.

Acetic acid, acrylamide, ethanediol, formic acid, *N,N'*-methylene-bis-acrylamide, NaCl, orthophosphoric acid, SDS and urea were from FSA Laboratory Supplies, Loughborough, U.K.

Ammonium sulphate, glucose and sucrose were from Formachem (Research International)Ltd., Strathaven, Midlothian, U.K.

Brilliant Blue G-250 was from Serva Feinbiochemica, Heidelberg, FRG.

2,6-Dichlorobenzoic acid, 2,6-dihydroxybenzoic acid, 3, 5-dihydroxybenzoic acid, D, L-4-fluoromandelic acid, glycolic acid, glyoxylic acid, D, L-phenyllactic acid, phenylpyruvic acid and trichloroacetic acid were from Koch Light Laboratories Ltd., Colnbrook, Bucks., U.K..

D, L-3,4-Dihydroxymandelic acid was from Calbiochem, Los Angeles, USA.

Dithiothreitol (DTT), NAD^+ (free acid), oxaloacetic acid, 2-oxoglutaric acid, and Tris (base) were from Boehringer Corporation, Lewes, Sussex, U.K.

Ethanol was from James Burrough (F.A.D.) Ltd., Witham, Essex, U.K.

Ethyl acetate, formaldehyde, formic acid and nitric acid were from May and Baker Ltd., Dagenham, Essex, U.K.

D, L-3-Hydroxymandelic acid, D, L-4-hydroxymandelic acid, D(–)-mandelic acid, L(+)-mandelic acid and phenylglyoxylic acid were from Fluka Chemicals Ltd., Glossop, Derbyshire, U.K.

D, L-4-Chloromandelic acid, D(–)-hexahydromandelic acid, L(+)-hexahydro-mandelic acid, D, L-mandelamide and thiopheneglyoxylic acid were from Aldrich Chemical Company Ltd., Gillingham, Dorset, U.K.

Acetyl-CoA (lithium salt), ADP (disodium salt), ATP (disodium salt), bicine, 4-chloro 1-naphthol, 2, 2'-dipyridyl, 5, 5'-dithiobis(2-nitrobenzoic) acid, *N* -ethylmaleimide, guanidine hydrochloride, FAD (disodium salt), FMN (sodium salt),

D, L-2-hydroxycaproic acid, D, L-2-hydroxyisocaproic acid, D, L-2-hydroxymandelic acid, D, L-4-hydroxy-3-methoxymandelic acid, D, L-3-hydroxy-4-methoxymandelic acid, D, L-4-hydroxyphenyl-lactic acid, 4-hydroxyphenyl L(+)-lactic acid, D, L-2-hydroxyvaleric acid, D, L-2-hydroxy-isovaleric acid, 8-hydroxyquinoline, iodoacetamide, isocitric acid, D(–)-lactic, Lubrol PX, Lubrol WX, D(+)-malic acid, D, L-mandelic acid isoamyl ester, D, L-2-methoxymandelic acid, D, L-3-methoxymandelic acid, Mes, Mops, Nitro Blue Tetrazolium , oxaloacetic acid, β-phenyl L(+)-lactic acid, pyruvic acid, sodium azide and Tween 20 and were from Sigma Chemical Company, Poole, Dorset, U.K.

Silver nitrate was from Johnson Matthey, Royston, Herts, U.K.

All chemicals were of the highest grade commercially available.

2.1.2 Chromatography media

All chromatography media and prepacked columns were from Pharmacia LKB Biotechnology, Upsala, Sweden.

2.1.3 Proteins and enzymes

All proteins were obtained from Sigma Chemical Company, Poole, Dorset, U.K. except for those listed below.

Bovine serum albumin (fraction V) was from Wilfred Smith Ltd., Edgeware, London, U.K.

Combithek Calibration Kit for Gel Filtration Chromatography, alcohol dehydrogenase (horse liver), pig heart lactate dehydrogenase, rabbit muscle pyruvate kinase, horse spleen ferritin and horse heart cytochrome *c* were from Boehringer Corporation, Lewes, Sussex, U.K.

Low M_r standards for SDS-PAGE, which included rabbit muscle phosphorylase b, bovine serum albumin, ovalbumin, bovine erythrocyte carbonic anhydrase, bovine milk α-lactalbumin and soya bean trypsin inhibitor were from Pharmacia Ltd., Milton Keynes, Bucks., U.K.

Purified or semipurified D(–)-mandelate dehydrogenases from *R. graminis* or

L(+)-mandelate dehydrogenases from various organisms other than *R. graminis* were gifts from Mr A. J. Scot, Department of Biochemistry, University of Glasgow.

2.1.4 Miscellaneous materials

Antisera raised against D(–)-mandelate dehydrogenase of *Acinetobacter calcoaceticus* and *Rhodotorula graminis* and L(+)-mandelate dehydrogenase of *Acinetobacter calcoaceticus* were gifts from Dr. D.P. Baker, Dr. M.E. Hoey, and Dr. I.D. Hamilton, Department of Biochemistry, University of Glasgow.

Chromatography and filter papers were from Whatman International Ltd., Maidstone, Kent, U.K.

Freund's complete and incomplete adjuvants were from Difco Laboratories, Detroit, MI, USA.

Heat-inactivated horse serum was from Gibco, Paisley, Strathclyde, U.K.

N_2 and O_2 were from British Oxygen Corporation, Guildford, Surrey, U.K.

Nescofilm was from Nippon Shoji Kaisha Ltd., Osaka, Japan.

Nitrocellulose was from Scheicher and Schuell, Dassel, FRG.

Normal donkey serum, normal rabbit serum and donkey anti-rabbit IgG peroxidase conjugate were from Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K.

Polybuffer PB 74 was from Pharmacia Ltd., Milton Keynes, Bucks, U.K.

Visking tubing was from Scientific Instrument Centre, Eastleigh, Hants, UK .

2.2. General methods

2.2.1 pH measurements

The pH values of most solutions were determined using a pH meter (Model 7010; E.l.L. Ltd., Cumbernauld, Glasgow, U.K.) connected to a combined glass electrode (Probion Ltd., Glenrothes, Fife, UK). The pH values of small volumes were measured with a pH meter (Radiometer type M26, Copenhagen, Denmark) fitted with a GK 2302 micro pH electrode. Calibration of the electrodes was carried out immediately before use with solutions of known pH (BDH buffer tablet, one tablet dissolved in 100 ml distilled water).

2.2.2 Conductivity measurements

The conductivity values of solutions were determined with a Radiometer (type CDM2e). Specific conductivity values were determined by using the equation:

$$\text{Specific conductivity} = \frac{\text{reading (mS)}}{\text{cell constant (cm)}} \quad \text{mS/cm (milli-Siemens/cm)}$$

2.2.3 Protein estimation

The methods of Lowry *et al.*, (1951) and Bradford (1976) were used to estimate the protein concentration of samples, using BSA solution as standard. During enzyme purification, the A_{280} values of column effluents were measured . The presence of protein in the DEAE-Sephacel or Phenyl Sepharose effluents was monitored with an LKB (Milton Keynes, Bucks., UK) Uvicord 2138 monitor at A_{280} and the presence of protein in effluents of FPLC (Pharmacia) columns was measured with a Pharmacia UV -1 monitor.

2.2.4 Glassware

Glassware was washed by immersing in a solution of 1% (w/v) Haemo-sol [Alfred Cox (Surgical) Ltd., Coulsdon, Surrey, U.K.] and then thoroughly rinsed with tap water and finally with distilled water. Glassware for amino acid composition and amino acid sequencing was immersed in boiling 10% (v/v) nitric acid for 30 min and then rinsed thoroughly with distilled water.

2.2.5 Dialysis

Dialysis tubing was prepared by boiling for 15 min in 1% (w/v) EDTA twice and then rinsed and boiled 3 times in distilled water for 15 min and stored in 20% (v/v) ethanol at 4^0C. Before use, visking tubing was again boiled once in distilled water for 15 min and then rinsed extensively with distilled water. Samples were dialysed overnight against three 2 l changes of buffer or as otherwise stated.

2.2.6 Preparation of chromatography media

DEAE-Sephacel, DEAE-Sepharose, Phenyl Sepharose, Sephacryl S300 HR and Ultrogel ACA-34 were supplied pre-swollen and were poured directly into columns. All columns were prequilibrated with 10 column volumes of appropriate buffer before use. DEAE-Sephacel and DEAE-Sepharose columns were regenerated after use by washing with 1M NaCl, and Phenyl Sepharose columns was regenerated with 6 M urea.

All columns were stored in 0.02% sodium azide.

2.2.7 Lyophilization

Purified samples were frozen in appropriate vessels by placing them in methanol/dry ice. The tops were covered with Nescofilm and the film punctured with a needle. These frozen samples were placed into a desiccator attached to an Edward's freeze dryer (Edwards High Vacuum type E2M2, Crawley, Sussex, England, U.K.) and lyophilised.

Yeast cultures were lyophilised in glass ampoules using an Edwards Speedivac centrifugal freeze dryer (Edwards High Vacuum Ltd, Crawley, Sussex, England, U.K.) according to the manufacturer's instructions.

2.2.8 Microfuge

Small samples, as generally used during immunological studies, were centrifuged in Eppendorf tubes at 15,000 g for 10 min in an Eppendorf 3200 centrifuge.

2.3 Microorganisms: maintenance, growth, harvesting and disruption

2.3.1 Source of microorganisms

(a) Yeasts

All yeasts and bacteria were obtained from Professor C.A. Fewson, Department of Biochemistry, University of Glasgow, but, originally they were obtained from the sources indicated below.

Rhodotorula graminis strain KGX 39, originally isolated from soil, was obtained from Dr. D.R. Durham, Genex Corporation, Gaithersburg, MD 29877, USA.

Rhodotorula graminis strain NCYC 980 was from the National Collection of Yeast Cultures, Norwich, Norfolk, U.K.

Mandelate-utilising yeasts, DB2 and DB3, tentatively recognised as *Rhodotorula*, were isolated from rabbit faeces and garden compost respectvely by Dr. D.P. Baker (Baker, 1990)

(b) Bacteria

Acinetobacter calcoaceticus mutant strain C1219 [NC1B 11457 (constitutive for D(–)-mandelate dehydrogenase, L(+)-mandelate dehydrogenase, benzaldehyde dehydrogenase 1 and phenylglyoxylate decarboxylase (Hills & Fewson, 1983a) were obtained from Professor C.A. Fewson, Department of Biochemistry, University of Glasgow.

Pseudomonas putida strain NCIMB 9494 and *Lactobacillus curvatur*s (NCIMB 9710) were obtained from The National Collection of Industrial & Marine Bacteria Ltd , Aberdeen, Scotland, U.K.

Streptococcus faecalis (IFO 12964) was obtained from the Institute of Fermentation, Osaka, Japan.

2.3.2 Maintenance of cultures

All yeasts were maintained as lyophilized cultures in a filter-sterilized mixture of 3 parts of heat-inactivated horse serum to 1 part 30% (w/v) glucose and they were also maintained in Sabouraud-dextrose agar at 4^0C and in 50% (v/v) glycerol at -80^0C.

A. calcoaceticus mutant strain C1219 was maintained in complex medium containing D, L-mandelate (MMB broth, Allison *et al.*, 1985a) and *L. curvatur*s and *S. faecalis* were maintained in Oxoid MRS broth (Man *et al.*, 1960). *P. putida* was maintained in nutrient broth. All these bacteria were stored at 4^0C.

2.3.3 Growth media

Minimal media

(a) Mandelate salts medium

The mandelate salts medium was prepared by mixing first 2 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$ and 1.5 g of D, L-mandelic acid in 900 ml of distilled water. Then pH was adjusted to 7.0 with NaOH and the volume made up to 1 l. The medium was then autoclaved and then 20 ml of sterile 2% (w/v) $MgSO_4$ $7H_20$ was added (Kennedy & Fewson, 1968a, b; Livingstone & Fewson, 1972).

Complex medium

(b) MMB broth

The medium contained 26 g of Oxoid nutrient broth, 1.5 g of D, L-mandelic acid, 0.9 g of L(+)-glutamic acid.HC1, 2 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$ and 0.4 g of $MgSO_4.7H_2O$ in distilled water. The pH was adjusted to 7.0 with NaOH and the volume made up to 1 l. In large scale cultures 0.005% poly (propylene glycol) 2025 was added as an antifoam (Allison *et al.*, 1985b).

(c) MMC broth

The MMC medium was prepared as MMB medium except that 1.5 g of lactic acid was also added per l of medium.

(d) Oxoid MRS broth

Oxoid MRS broth was prepared according to the manufacturer's instructions.

(e) Oxoid nutrient broth

Oxoid nutrient broth (Oxoid CMI) was prepared according to the manufacturer's instructions.

(f) Oxoid Sabouraud-dextrose broth and agar

Both Oxoid Sabouraud-dextrose broth and Oxoid Sabouraud-dextrose agar were prepared according to the manufacturer's instructions.

2.3.4 Sterilization

(a) Moist heat

Growth media were sterilized by autoclaving at 109^{0}C (5 p.s.i.) for 50 min (up to 500 ml) or 60 min (500 ml to 2.1) and more than 10 1 of medium was sterilized at 120^{0}C (15 p.s.i.) for 75 min. The success of sterilization was judged using Browne's tube (Type three, Albert Browne Ltd., Leicester, Leicestershire, UK). Disposable apparatus were also sterilized by autoclaving at 109^{0}C (5 p.s.i.) for 50 min.

(b) Dry heat

Glass pipettes were sterilized by heating at 160^{0}C for 105 min. The success of sterilization was again judged using Browne's tubes (Type one)

(c) Filtration

Growth media containing heat-labile compounds were sterilized by filtration through Millipore (Watford, Herts., UK) 0.22 µm pore size filters. Small volumes were sterilized using Nalgene disposable sterile filter units with 0.22 µm pore size (Sybron Corporation, Rochester, N.Y. USA.)

(d) Ethylene oxide

Plastic pipettes were sterilized with ethylene oxide (Anprolene) for 12 h in a sterilising unit (AN74, H.W. Anderson Products Ltd., Clacton-on-Sea, Essex, UK) and sterilization was judged by Anprolene exposure indicators (AN80) or by steritest units (AN80).

2.3.5 Growth of microorganisms

Growth of yeasts

(a) Growth of yeasts in minimal medium

Lyophilized cultures of yeasts (*R. graminis* strain KGX 39, *R. graminis* strain NCYC 980, DB2 and DB3) were rehydrated with sterile distilled water and streaked onto Sabouraud-dextrose agar and grown at 30^{0}C for a few days. Five loopfuls of culture were aseptically transferred to 400 ml of minimal medium in a 2 l conical flask, fitted with a polystyrene foam bung (A. & J. Beveridge Ltd., Edinburgh, U.K.) and grown on a rotary shaker (200 r.p.m, M.K.V. Orbital Shaker, L.H. Engineering Corporation Ltd., Stoke Poges) at 30^{0}C for 24 h.

(b) Growth of *R. graminis* KGX 39 in MMB broth

Large amounts of *R. graminis* KGX 39 were grown in MMB broth in a 10 l fermenter (Braun Biostat V; F.T. Scientific Instruments, Tewkesbury, Glos., UK) fitted with a 20 l reservoir (Allison *et al*., 1985b). Lyophilized cultures of *R. graminis* strain KGX 39 was rehydrated with sterile distilled water and streaked onto Sabouraud-dextrose agar and grown at 30^{0}C for 3 days. A loopful of culture was aseptically transferred to 400 ml of complex medium in a 2 l conical flask and grown on a rotary shaker at 30^{0}C for 24 h. The entire culture was then used to inoculate the fermenter, which contained 10 l of the complex medium. The fermenter was operated at 30^{0}C with an aeration rate of 4 l of sterile air min^{-1} and stirring at setting 2.5 (approx. 250 r.p.m.). After growth for 24 h, 9.5 l of culture were removed for harvesting and 9.5 l of fresh medium was transferred from the reservoir. After a further 24 h of growth, this process was repeated.

Growth of bacteria

(a) Growth of *A. calcoaceticus* strain C1219 in MMC broth

A. calcoaceticus was grown in MMC broth by Mr. A.J. Scott. Stock culture (0.1 ml) was aseptically transferred to 50 ml of MMC broth (in a 250 ml conical flask) and grown on a rotary shaker (200 r.p.m.) at 30^{0}C overnight. Then the entire culture was aseptically transferred to 400 ml of fresh MMC broth (in a 2 l conical flask) and grown on a rotary shaker (200 r.p.m.) at 30^{0}C overnight. The 400 ml of culture was aseptically transferred to inoculate the 10 l fermenter fitted with a 20 l reservoir. The fermenter was operated at 23^{0}C with an aeration rate of 4 l of sterile air min^{-1} and stirring at setting 2.5 (approx. 250 r.p.m.). After growth for 15 h, 9.5 l of culture were removed for harvesting and 9.5 l of fresh medium was transferred from the reservoir. After a further 15 h of growth, this process was repeated.

(b) Growth of *S. faecalis* and *L. curvatus* on MRS broth

Both *L. curvatus* and *S. faecalis* were grown in MRS broth. Stock culture (1 ml) was aseptically transferred to 50 ml of MRS broth (in a 250 ml conical flask) and grown on a rotary shaker (200 r.p.m.) at 30^{0}C for 8 h. Then the entire culture was aseptically transferred to 400 ml of fresh MRS broth (in a 2 l conical flask) and grown on a rotary shaker (200 r.p.m.) at 30^{0}C for 72 h and harvested.

(c) Growth of *P. putida* on minimal mandelate medium

Stock nutrient broth (0.1 ml) of *P. putida* culture was aseptically transferred to 50 ml of MMB broth minus L(+)-glutamic acid.HC1 (in a 250 ml conical flask) and grown on a rotary shaker (200 r.p.m.) at 30°C overnight. Then the entire culture was aseptically transferred to 400 ml of fresh MMB broth minus L(+)-glutamic acid.HC1 (in a 2 1 conical flask) and grown on a rotary shaker (200 r.p.m.) at 30°C overnight. Then this culture was aseptically transferred to 4 l of minimal medium with 10 mM D, L-mandelate (in a 10 1 round flask with magnetic stirrer and sterile air supplied at a rate of 500 ml min^{-1}) and grown for about 15 h at 30°C overnight and then harvested the cells.

2.3.6 Optical density measurements

Growth of microorganisms was monitored by measuring the optical density at 595 nm, relative to the centrifuged medium, with an LKB Ultrospec spectrophotometer (Model 4050). Samples with an optical density of more than 0.5 were diluted with distilled water so that optical density did not exceed this value.

2.3.7 Harvesting and storage of cells

Cultures of of small volumes (50-200 ml) were harvested by centrifugation at 12,000 g for 15 min in an M.S.E. High speed 18 centrifuge (Model No. 69650, MSE Ltd., London, UK) at 4°C. Larger volumes were harvested in 750 ml polypropylene bottles (maximum volume 500 ml) at 5,000 r.p.m. for 15 min in an M.S.E. Mistral 6L centrifuge. Each pellet was resuspended twice in ice cold water equal to the original culture volume and recentrifuged. The pellets from the 10 l fermenter were combined, made to 6 fractions of 50 ml each with ice cold 20 mM Tris/HCl, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} and centrifuged at 12,000 g for 30 min in M.S.E. High speed 18 centrifuge. The supernatants were decanted and the pellets stored at -20°C.

2.3.8 Cell disruption

Cell disruption was carried out at 0-4^{0}C.

Cell pellets were suspended in 2 volumes of the ice cold 20 mM Tris/HCl, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} and broken by 4 passages through an ice cold French Pressure cell (FA-073 or FA-003 models, American Instrument Company, Silver Spring, MD, USUA) at a pressure of 98 MPa (14,300 lb in^{-2}) and at 0-4^{0}C. The homogenate was centrifuged at 12,000 g for 30 min to remove intact cell and debris and the supernatant was known as cell-free extract.

2.3.9 Preparation of membrane fractions and high speed supernatants

Cell-free extracts (Methods 2.3.8) were centrifuged at 113,000 g_{av} for 2 h. The high speed supernatant of *R. graminis* known as crude extract, was used to purify L(+)-mandelate dehydrogenase. The pellets were washed with appropriate buffer twice by resuspension and centrifugation at 113,000 g_{av} for 2 h each time. The washed pellet membrane fractions were resuspended in appropriate buffers and stored at -20^{0}C.

2.3.10 Preparation of Triton extract of bacteria

Membrane fractions (prepared as during in Methods 2.3.9) were diluted to 20 mg protein ml^{-1} and Triton X-100 was added to final concentration of 0.5 mg of detergent (mg protein)$^{-1}$. The suspensions were stirred for 30 min and then centrifuged at 113,000 g_{av} for 2 h.

2.4 Enzyme assays

An LKB Ultrospec spectrophotometer was routinely used for assays during enzyme purification and a Philip's spectrophotometer (PU 8700, UV/visible, Cambridge, U.K.) was used for enzyme characterization and kinetic experiments. Assays were carried out in 3 ml plastic cuvettes with 1 cm light-path at 27^{0}C which was maintained using an attached heating unit. Gilson (Gilson Medical Electronics, Villiers-le-Bell, France), Pipetmen pipettes and Finn variable pipettes [Jencons (Scientific) Ltd., Leighton Buzzard, Beds., U.K.] were used to dispense solutions. Assays were initiated by the addition of enzyme (usually 50 µl; more

active enzyme was diluted in appropriate buffer so that 50 µl of the enzyme sample gave a change in absorbance of 0.05-0.12 min^{-1}) and the components of the reaction mixture were mixed using 'plumpers' (Calbiochem, San Diego, California, USA.). The change in absorbance was followed for 2 min. All assays were carried out at least in duplicate and control assays were run containing all constituents except substrate and in some cases a non-enzyme control was also run. One unit of enzyme activity is defined as 1 µmol of substrate converted min^{-1} and specific activities are given as units $(mg\ of\ protein)^{-1}$.

2.4.1 Assay of NAD^+-dependent D(–)-mandelate dehydrogenase

The enzyme was assayed in 1ml reaction mixtures (Baker & Fewson, 1989) containing:

800 µl of 250 mM Bicine/NaOH buffer (pH 9.5) (200 mM assay concentration)

50 µl of 20 mM NAD^+ (1 mM assay concentration)

50 µl of 100 mM D(–)-mandelate (pH 7.0) (5 mM assay concentration)

Enzyme

Distilled water to 1 ml

The rate of NAD^+ reduction was followed at 340 nm. The molar absorption coefficient of NAD^+ at 340 nm used was $6.3 \times 10^3\ M^{-1}\ cm^{-1}$ (Bergmeyer, 1985)

2.4.2 Assay of DCIP-linked L(+)-mandelate dehydrogenases in yeasts

L(+)-Mandelate dehydrogenase activities of yeasts were assayed by following the phenazine methosulphate (PMS)-dependent reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm. The assay developed during these studies was based on method described by Hoey *et al.* (1987) and the amount of BSA was lowered in the assay in order to obtain maximal activity. The enzymes were assayed in 3 ml reaction mixture containing:

2.0 ml of 0.1 M potassium phosphate buffer, pH 7.9, (66.6 mM assay concentration)

100 µl of 1.6 mM DCIP (53 µM assay concentration)

100 µl of 0.2% (w/v) bovine serum albumin (200 µg in assay mixture)

100 µl of 250 mM L(+)-mandelate, pH 7.9 (8.33 mM assay concentration)

100 µl of 0.3 mM PMS (10 µM assay concentration)

Enzyme

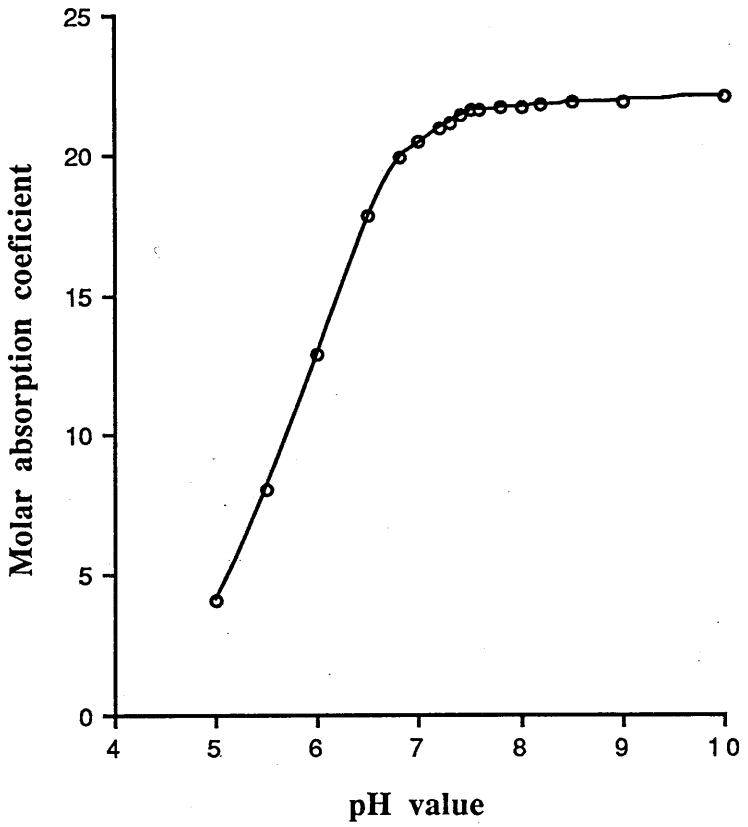


Figure 2.1 The pH dependence of the molar absorption coefficient of 2,6-dichlorophenol indophenol

A sample of the sodium salt of DCIP was dried to a constant weight over P_2O_5 and under vacuum for 24 h at 37°C. Various 1.6 mM DCIP solutions were then prepared using 100 mM potassium phosphate buffers at pH values from 5 to 10. The absorptions of the DCIP solutions at various pH values were measured at room temperature in cuvettes of 1 cm light-path using a Pye-Unicam SP8-100 and a LKB Ultrospec spectrophotometer, which gave identical values. Molar absorpticn coefficients at the different pH values were caculated using the formula:

$$\varepsilon_{600} = A_{600} / C.D$$

where ε is the molar absorption coefficient

A is the absorption at 600 nm

C is the concentration of DCIP

D is the thickness of the absorbing medium

Distilled water to 3 ml.

A decrease in A_{600} of 7.22 units (Fig. 2.1) was taken to correspond to the oxidation of 1 µmol of substrate at pH 7.9.

2.4.3 Assay of ferricyanide or cytochrome *c*-linked L(+)-mandelate dehydrogenases of *R. graminis*

The enzymes were assayed in 3 ml reaction mixture containing:

2.0 ml of 0.1 M potassium phosphate buffer, pH 7.9, (66.6 mM assay concentration)

100 µl of 2.5 mM cytochrome *c* (83.3 µM assay concentration) or 100 µl of 50 mM potassium ferricyanide (1.66 mM assay concentration)

100 µl of 0.2% (w/v) bovine serum albumin (200 µg in assay mixture)

100 µl of 250 mM L(+)-mandelate, pH 7.9, (8.33 mM assay concentration)

Enzyme

Distilled water to 3 ml.

Changes in absorbance of 6.6 units at 550 nm (in the presence of cytochrome *c*) and 0.34 units at 420 nm (in the presence of potassium ferricyanide) were taken to correspond to the oxidation of 1 µmol of substrate at pH 7.9 (Appleby & Morton, 1959; Lebeyrie *et al.*, 1978; Dawson *et al.*, 1986)

2.4.4 Assay of DCIP-linked L(+)-lactate dehydrogenase in yeasts

L(+)-Lactate dehydrogenase activity of yeast was assayed by following the phenazine methosulphate (PMS)-dependent reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm. The assay developed during these studies was based on methods described by Morton *et al.* (1961) and Lebeyrie *et al.* (1978). The enzymes were assayed in 3 ml reaction mixture containing:

2.0 ml of 0.1 M potassium phosphate buffer with 0.5 mM EDTA, pH 7.9 (66.6 mM of potassium phosphate buffer with 0.33 mM of EDTA assay concentration)

100 µl of 1.6 mM DC1P (53 µM assay concentration)

100 µl of 0.2% (w/v) bovine serum albumin (200 µg in assay mixture)

100 µl of 200 mM L(+)-lactate, pH 7.9 (6.6 mM assay concentration)

Enzyme

Distilled water to 3 ml.

A decrease in A_{600} of 7.22 units (Fig. 2.1) was taken to correspond to the oxidation of 1 µmol of substrate at pH 7.9.

2.4.5 Assay of ferricyanide or cytochrome *c*-linked L(+)-lactate dehydrogenase in *S. cerevisiae*

The enzymes were assayed in 3 ml reaction mixture containing:

2.0 ml of 0.1 M potassium phosphate buffer with 0.5 mM EDTA, pH 7.9,

(66.6 mM of potassium phosphate buffer with 0.33 mM of EDTA assay concentration)

100 µl of 2.5 mM cytochrome *c* (83.33 µM assay concentration) or 100 µl of

50 mM potassium ferricyanide (1.66 mM assay concentration)

100 µl of 0.2% (w/v) bovine serum albumin (200 µg in assay mixture)

100 µl of 200 mM L(+)-lactate, pH 7.9 (6.66 mM assay concentration)

Enzyme

Distilled water to 3 ml.

A change in absorbance of 6.6 units at 550 nm (in the presence of cytochrome *c*) and 0.34 units at 420 nm (in the presence of potassium ferricyanide) were taken to correspond to the oxidation of 1 µmol of substrate at pH 7.9 (Appleby & Morton, 1959; Lebeyrie *et al.*, 1978; Dawson *et al.*, 1986).

2.4.6 Assay of DCIP-linked bacterial mandelate and lactate dehydrogenases

L(+)-Mandelate dehydrogenase activities of *A. calcoaceticus* and *P. putida* , L(+)-lactate dehydrogenase activity of *A. calcoaceticus*, D(–)-mandelate dehydrogenase and D(–)-lactate dehydrogenase activities of *A. calcoaceticus* were assayed exactly as L(+)-mandelate dehydrogenase of *R. graminis* (Methods 2.4.3) except that the assay contained 5 mM L(+)-mandelate or L(+)-lactate or 2 mM D(–)-mandelate or D(–)-lactate as substrates.

2.4.7 Assay of NADPH cytochrome *c* oxidoreductase

NADPH cytochrome *c* oxidoreductase assay was based on methods described by Haas (1955) and Mahler (1955). The enzyme was assayed in 3 ml reaction mixture containing:

2.56 ml of 25 mM potassium phosphate buffer, pH 7.3 (21.3 mM assay concentration)

100 µl of 0.1M magnesium chloride (3.33 mM assay concentration)

100 µl of 6 mg NADPH ml^{-1} (0.6 mg/assay mixture)

20 µl of 0.5% (w/v) glucose 6-phosphate dehydrogenase (100 µg/assay mixture)

100 µl of 2.4% (w/v) of cytochrome (2.4 mg/assay mixture)

The above mixture was incubated for 5 min at 27°C and then the enzyme was added to measure reduction of cytochrome *c* at 550 nm. A change in absorbance of 6.6 unit at 550 nm was taken to correspond to the reduction of 1 µmol of substrate at pH 7.3 (Haas, 1955; Appleby & Morton, 1959).

2.4.8 Assay of α-mannosidase

α-Mannosidase was assayed based on methods described by Van der Wilden *et al.* and in Boehringer Mannheim (1973b). The enzyme was assayed in a 3 ml reaction mixture containing:

2.68 ml of 0.1 M Tris/HCl buffer, pH 7.2 (89 mM assay concentration)

200 µl of 24 mM *p*-nitrophenyl-α-mannoside (1.6 mM assay concentration)

20 µl of 20% (w/v)Triton X-100 (4 mg/assay mixture)

Enzyme sample (usually 10-20 µl)

Distilled water to 3 ml.

A change in absorbance of 6.16 units at 400 nm was taken to correspond to the hydrolysis of 1 µmol of substrate at pH 7.2 (Boehringer Mannheim, 1973b).

2.4.9 Assay of fumarase

Fumarase was assayed based on methods described by Hill and Teippel (1971) and Boehringer Mannheim (1973c). The enzyme was assayed in a 3 ml reaction mixture containing:

2.05 ml of 0.1 M potassium phosphate buffer, pH 7.6 (68 mM assay concentration)

20 µl of 750 mM L(+)-malate (5 mM assay concentration)

25-50 µl of enzyme sample

Distilled water to 3 ml.

A change in absorbance of 0.81 units at 240 nm was taken to correspond to the conversion of 1 µmol of substrate at pH 7.6 (Boehringer Mannheim, 1973c).

2.4.10 Assay of glyceraldehyde-3-phosphate dehydrogenase

The glyceraldehyde-3-phosphate dehydrogenase (Krebs, 1955 & Velick and Furfine,1963) assay was based on method described by Boehringer Mannheim (1973a). The enzyme was assayed in 3 ml reaction mixture containing:

2.6 ml of phosphate-cysteine buffer i.e. 1 ml of 0.3 M cysteine-HCl, pH 8.5 and 50 ml of 0.03 M potassium phosphate buffer, pH 8.5 (26 mM assay concentration)

100 µl of 7.6 mM NAD (0.25 mM assay concentration)

Enzyme

The mixture was incubated at 27°C for 5 min to activate the enzyme in the presence of cysteine and then added:

20 µl of 7.6 mM glyceraldehyde 3-phosphate (50.66 µM assay concentration)

20 µl of 0.17 M sodium arsenate (1.13 mM assay concentration)

Distilled water to 3 ml.

An change in absorbance of 2.1 units at 340 nm was taken to correspond to the oxidation of 1 µmol of substrate at pH 8.5 (Boehringer Mannheim, 1973a).

The substrate was prepared as follows: Glyceraldehyde 3-phosphate diethylacetal (100 mg) was incubated with 1.5 g of Dowex-50 or Dowex-100 in 6 ml distilled water in boiling water bath for three min and centrifuged at 12,000 g_{av}. The supernatant containing glyceraldehyde 3-phosphate was collected. Glyceraldehyde 3-phosphate was extracted twice in a similar way.

2.5 Analysis of initial enzyme velocities and determination of kinetic coefficients

The kinetic data were analysed using the Direct Linear method (Eisenthal & Cornish-Bowden, 1974) using an Enzpack computer program (Williams, 1985). Although the values given in this thesis were from the Direct Linear method, the data were also analysed using Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plotting methods, again using the Enzpack computer programme and they all gave very similar results.

2.6 Standard deviations

Means and standard deviations were calculated using a Casio Scientific calculator (fx-82B). The number of experiments used to obtain the mean and standard deviation are given in parentheses.

2.7 Enzyme purification

All steps were carried out at a temperature of 0-4^{0}C.

2.7.1 Preparation of the crude extract

Crude extract was prepared from approximately 70-90 g of *R. graminis* cells suspended in 2 volumes of 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} and then French Pressing and centrifuging (Methods 2.3.8 & 2.3.9). The high speed supernatant was used as starting material for enzyme purification.

2.7.2 Ammonium sulphate fractionation

Solid ammonium sulphate (low in heavy metals for enzyme work) was added to crude extract in 15 min to give 40% saturation. When all the ammonium sulphate had been added, the solution was stirred for another 60 min and the precipitate was removed by centrifuging at 52,000 g_{av}. More ammonium sulphate was added in the resulting supernatant to give 60 % saturation and again centrifuged the mixture at 52,000 g_{av} for 20 min. The precipitated protein was collected and resuspended in 20 mM Tris/HCl buffer,

pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. This solution was dialysed overnight against 5 l of 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}.

2.7.3 Chromatrography on DEAE-Sephacel

Dialysed ammonium sulphate sample was applied to a DEAE-Sephacel column (2.6 cm x 24 cm) which had been equilibrated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. Once loaded, the column was washed with: (a) 200 ml of 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}; (b) 700 ml of 20 mM Tris/HC1 buffer, pH 6.5, containing 0.5 mg Triton X-100 ml^{-1}; and (c) 700 ml 0.075 M NaCl in 20 mM Tris/HCl buffer, pH 7.5 containing 0.5 mg Triton X-100 ml^{-1} at a rate of 90 ml h^{-1}. L(+)-Mandelate dehydrogenase was eluted with a linear 0.075-03M NaC1 gradient in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} (total vol. = 1000 ml) at a flow rate of 51 ml h^{-1}. Fractions containing more than 25 % of the activity of the peak fraction from DEAE-Sephacel were pooled together. The pooled fractions of this column were then dialysed overnight against 5 l of 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}.

2.7.4 Chromatography on DEAE-Sepharose CL-6B

The dialysed DEAE-Sephacel pool was applied at 30 ml h^{-1} to a DEAE-Sepharose CL-6B column (45 cm x 2.6 cm) pre-equilibrated with 10 volumes of 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. Once loaded, the column was washed with buffers (a) 100 ml of 20 mM Tris/HCl buffer, pH 7.5 containing 0.5 mg Triton X-100 ml^{-1} and (b) 900 ml of 0.08 M NaCl in 20 mM Tris/HC1 buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} at a rate of 90 ml h^{-1}. L(+)-Mandelate dehydrogenase was eluted with a linear 0.08-0.2M NaCl gradient in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} (total vol. = 1000 ml) at a flow rate of 22 ml h^{-1}. Fractions containing more than 25 % of the activity of the peak fraction from DEAE-Sepharose CL-6B were pooled together. The pooled fractions of this column were then brought to 20% saturation with ammonium sulphate (low in heavy metals for enzyme work) by adding solid ammonium sulphate over a period of 15 min and then the solution was stirred for at least 30 min on ice.

2.7.5 Chromatography on Phenyl Sepharose CL-4B

The DEAE-Sephacel pool saturated with 20% ammonium sulphate was applied at 30 ml h^{-1} to a Phenyl Sepharose column (10.8 cm x 2.6 cm) pre-equilibrated in 20 mM glycylglycine buffer, pH 7.5, containing 25% saturation ammonium sulphate. Once loaded, the column was washed at 40 ml h^{-1} with 20 mM Tris/HCl buffer, pH 7.5, until the A_{280} of the effluent returned to the baseline. The column was then washed with: (a) 300 ml of 20 mM glycylglycine buffer, pH 7.5, containing 20% saturation ammonium sulphate and (b) 90 ml of 20 mM glycylglycine buffer, pH 7.5, containing 12% saturation ammonium sulphate. L(+)-Mandelate dehydrogenase was eluted with 20 mM glycylglycine buffer, pH 7.5, containing 7.5% saturation ammonium sulphate at a flow rate of 28 ml h^{-1} into test tubes containing enough Triton X-100 ml^{-1} to give a final cocentration of 0.5 mg ml^{-1}. Fractions containing more than 25 % of the activity of the peak fraction from Phenyl Sepharose CL-4B were pooled together.

2.7.6 Storage of purified L(+)-mandelate dehydrogenase

The pooled fractions from step 2.7.5 were stored at -20^{0}C or precipitated with 90% ammonium sulphate saturation and the precipitate was kept at 4^{0}C.

2.8 Polyacrylamide-gel electrophoresis (PAGE)

2.8.1 Stock solutions

PAGE was carried out according to the method of Laemmli (1970) and gels were prepared from the following stock solutions:

Solution A: 3.0 M Tris/HC1 (pH 8.8) containing 0.23% (v/v) *N,N,N',N'* - tetramethylenediamine (TEMED) (stored at room temperature)

Solution B: 28% (w/v) acrylamide containing 0.735% (w/v) *N,N'*-methylene-bis-acrylamide (deionised with amberlite) (stored at room temperature and used within one month)

Solution C: 100 mM Tris/HC1 (pH 6.8) containing 0.8% (w/v) SDS and 0.25% (v/v) TEMED (for native gels omit SDS) (stored at room temperature)

Solution D: (reservoir buffer): 25 mM Tris/192 mM glycine containing 0.5% (w/v) SDS (for native gels, omit SDS) and 0.01% (v/v) β-mercaptoethanol was added before use. (stored at 4°C)

Solution E: 20% (w/v) SDS (stored at 30°C)

2.8.2 Gel plates

Glass plates (dimensions 9.5 x 20 cm and 10 x 20 cm) were washed in Decon 75, to ensure they were free of silicon grease, then washed with scouring powder and thoroughly rinsed with tap water and distilled water. The plates were assembled using 1.5 mM teflon spacers and silicon grease and placed into a home-made gel-casting box suitable for making up to 4 gels at a time.

2.8.3 SDS-PAGE

(a) Resolving gel [10% (w/v) acrylamide]

An SDS-resolving gel solution was prepared containing 25 ml solution A, 71.5 ml solution B, 1 ml solution E and 100 ml of distilled water. The solution was mixed thoroughly and degassed. Then 150 mg of ammonium persulphate was added, the mixture swirled gently and thoroughly degassed again . The gel mixture was poured between glass plates in a gel casting box and overlaid with a thin layer of n-propanol. The gels were left to polymerise. After the gel had set, the n-propanol was poured off and the gel was thoroughly washed with distilled water. The tops of the gels were blotted dry with blotting paper.

(b) Stacking gel [5.6% (w/v) acrylamide]

An SDS-stacking gel solution was prepared containing 17.5 ml of solution B, 10 ml of solution C, and 55 ml of distilled water. The solution was mixed thoroughly and degassed. Then 150 mg of ammonium persulphate was added and the mixture was thoroughly degassed again. The gel mixture was poured on top of the resolving gels and 18-track Teflon combs were placed on tops and the gels left to polymerise. The gels were used within one week of preparation.

(c) Sample preparation

Protein samples for SDS-PAGE were prepared using the following stock solutions: Solution F (tracker dye mixture): 10 ml of 500 mM Tris/HC1 (pH 8.8), containing 1.5 g of SDS, 8 g of sucrose and 100 µg of Pyronin Y to give an intense pink colour. This solution was then heated and filtered through cotton wool prior to use.

Solution G: 2M-DTT

Solution H: To 1 vial low M_r standards (Pharmacia), 400 µl of distilled water and 100 µl solution F were added. The solution was heated to 100°C for 5-10 min prior to use.

The samples for electrophoresis were prepared by mixing solutions in the following way: 10-40 µl of sample, 10 µl of distilled water, 5-10 µl of solution F, 2.5 µl solution G. All samples were then heated at 100^0C for 5 min prior to loading. Volumes of up to 50 µl (including solution H as standard marker) were loaded per track using a Hamilton glass syringe (Hamilton Corporation, Reno, NV, USA).

(d) Electrophoresis conditions

The samples were electrophoresed at 80 mA per slab gel until the dye front almost reached the bottom of the gel. The gel apparatus was cooled by continuously circulating an ice-cooled water.

2.8.4 Non-denaturing PAGE

(a) Resolving gel [6% (w/v) acrylamide]

A non-denaturing-resolving gel solution was prepared exactly as described in Methods 2.8.3a except that SDS was omitted from all the solutions and the solution contained 25 ml solution A, 42.9 ml solution B and 129.6 ml of distilled water.

(b) Stacking gel [5.6% (w/v) acrylamide]

A non-denaturing stacking gel solution was prepared exactly as described in Methods 2.8.3b except that SDS was omitted from all the solutions and the solution contained 17.5 ml of solution B, 10 ml of solution C and 55 ml of distilled water.

(c) Sample preparation

Native enzyme samples were mixed with an ice cold solution containing 6 µl of glycerol, 5 µl of of 0.01% Bromophenol Blue and made up to 60 µl with reservoir buffer. A volume of 10-40 µl was loaded per track using a Hamilton glass syringe.

(d) Electrophoresis conditions

Native gels were electrophoresed at 30 mA for 30 min prior to samples being loaded. Native enzyme samples were electrophoresed at the same current until the dye front almost reached the bottom of the gel. Gels were cooled as in Methods 2.8.3d

2.8.5 Staining of gels

2.8.5.1 Protein staining

(a) Coomassie Blue staining

Routinely, proteins in the gels were stained using the method of Lumsden and Coggins (1977). Gels were immersed in 0.1% Coomassie Brilliant Blue G-250 in methanol/acetic acid/distilled water (50:10:40 by volume) for 1 h at 60^0C. Gels were destained in methanol/acetic acid/distilled water (10:10:80 by volume) at the same temperature.

(b) Silver staining

Gels loaded with little amount of proteins were silver-stained by the method of Wray *et al.* (1981)

2.8.5.2 Activity staining

Non-denaturing gels were activity stained by a procedure adapted from the method of MacKintosh and Fewson (1988a) for benzyl alcohol dehydrogenase. Non-denaturing gels run as described in 2.8.4 were soaked in 6.7 mM sodium pyrophosphate buffer, pH 7.5, containing 0.5 mg of Triton X-100 ml^{-1} for 30 min at 4^0C to remove β-mercaptoethanol. The gels were stained for L(+)-mandelate dehydrogenase activity by immersing in 66.7 mM sodium pyrophosphate buffer, pH 7.5, containing 0.5 mg of Triton X-100 ml^{-1}, 8.3 mM L(+)-mandelate, 550 µM PMS and 55 µM Nitro Blue Tetrazolium in the dark for about

10 min at 30^{0}C. L(+)-Mandelate dehydrogenase activity was detected as a purple precipitate of formazan.

2.8.6 Gel scanning

Coomassie-stained gels (Methods 2.8.5 1a) were scanned with an LKB Model No. 2202 Ultroscan laser densitometer.

2.9 Analysis of flavin in purified enzyme

The flavin content of L(+)mandelate dehydrogenase from *R. graminis* was analysed using the method described by Allison *et al.* (1985b). Purified L(+)-mandelate dehydrogenase (150 µg to 1 mg of purified enzyme in 100-500 µl of 20 mM phosphate buffer, pH 7.5, containing more than 0.5 mg Triton X-100 ml^{-1}) was incubated with (10% w/v) trichloracetic acid (TCA) at 4^{0}C for 15 min. Then the mixture was centrifuged at 15,000 g for 15 min at 4^{0}C. The protein precipitate was twice re-extracted with TCA in the same way. The supernatants were combined and TCA in the combined extract (supernatants) was extracted twice with four volumes of diethylether. Residual diethylether was removed by flushing the sample with nitrogen gas. The extract was neutralised by addition of 0.5 ml of 1M potassium phosphate buffer, pH 7.5 and made to a final volume of 3 ml with 100 mM potassium buffer, pH 7.5. The absorption and emission spectra of the extract were recorded in a Philip's spectrophotometer (PU 8700, UV/visible, Cambridge, U.K.) and a Perkin-Elmer 3000 fluorescence spectrophotometer respectively using quartz cuvettes . The absorption and emission spectra were then compared with absorption and emission spectra of standard solutions of FAD and FMN.

2.10 Immunological methods

2.10.1 Production of polyclonal antisera

Purified L(+)-mandelate dehydrogenase (300-720 µg) was emulsified in 1 ml of Freund's complete adjuvant and made to 2 ml with distilled water. The emulsions were injected subcutaneously at six shaved sites on the back and sides of two rabbits. Booster injections were prepared by mixing 1 ml of purified L(+)-mandelate dehydrogenase

Table 2.1 Summary of steps involved in the preparation of antisera

Polyclonal antisera were raised in two New Zealand rabbits by Dr. I.D. Hamilton, Department of Biochemistry, University of Glasgow according to Home Office regulations.

Rabbit No.	Amount of protein in initial injection (μg)	Amount of protein in booster injection (μg)	Days between first and booster injection	Days between booster injection and first bleeding and No. of first bleeding in parentheses	Days between booster injection and second bleeding and No. of second bleeding in parentheses
1153	300	240	62	19 (1153:1)	30 (1153:2)
1154	720	250	72	15 (1154:1)	24 (1154:2)

(240-300 µg) with 1 ml of Freund's incomplete adjuvant and injected as before. The rabbits were bled twice on two occasions as shown in Table 2.1. The amounts of purified enzyme injected and times of injections are also shown in Table 2.1. The blood samples were allowed to clot at 4°C overnight and the clotted material was removed by centrifugation. The antisera were stored at -20°C. All subsequent immunological studies were carried out at 0-4°C.

2.10.2 Immunoinhibition assay

Purified enzymes or enzyme extracts were diluted in 20 mM Tris/HCl buffer, pH 7.5. containing 0.5 mg Triton X-100 ml^{-1}. Appropriate concentrations of purified enzyme or enzyme extracts (usually 200 µl) were incubated with undiluted antisera (usually 200 µl) for 1 h on ice. Enzyme samples were also incubated with 20 mM Tris/HCl buffer, pH 7.5 containing 0.5 mg Triton X-100 ml^{-1} or normal rabbit serum as controls. After 1 h the remaining enzyme activities in the supernatants were measured.

The positive cross-reaction between purified L(+)-mandelate dehydrogenase from *R. graminis* and homologous antisera was quantified by immunotitration. Serial dilutions (1:2 to 1:8192) of antisera in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} were prepared and incubated with appropriate concentrations of purified enzyme for 1 h. The remaining enzyme activities in the supernatants were then assayed. Control experiments were carried out at all dilutions using enzyme samples incubated with 20 mM Tris/HCl buffer, pH 7.5. containing 0.5 mg Triton X-100 ml^{-1} or serial dilutions of normal rabbit serum.

2.10.3 Immunoprecipitation assay

Immunoprecipitation studies were carried exactly as described in Methods 2.10.2 except that after the incubation period of 1 h the immunocomplexes were precipitated by centrifuging for 10 min in an Eppendorf 3200 centrifuge at 4°C and enzyme activities in the supernatants were measured. The remaining enzyme activity was expressed as a percentage relative to the activity of an enzyme sample that had been incubated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}.

Positive cross-reactions between anti-L(+)-mandelate dehydrogenase of *R. graminis* and purified L(+)-mandelate dehydrogenase of *R. graminis* or enzyme extracts from other yeasts were quantified by immunotitration. Serial dilutions (1:2 to 1:8192) of antisera in 20 mM Tris/HCl buffer, pH 7.5. containing 0.5 mg Triton X-100 ml^{-1} were prepared and incubated with appropriate concentrations of enzyme for 1 h. After 1 h the immunocomplexes were precipitated by centrifuging for 10 min in an Eppendorf 3200 centrifuge at 4^0C and the remaining enzyme activities in the supernatants were assayed. Control experiments were carried out at all dilutions using enzyme samples incubated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} or serial dilutions of normal rabbit serum.

2.10.4 *Staphylococcus aureus* (protein A) immunoprecipitation assay

(a) Preparation of *S. aureus* cells

The *S. aureus* cells were a gift from Dr. I.D. Hamilton and were prepared as described by Kessler (1975). The cells were washed three times by centrifugation (Methods 2.2.8) and resuspended to their original volume in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}.

(b) Immunoprecipitation assay with protein A

Purified enzymes or enzyme extracts were diluted in 20 mM Tris/HCl buffer, pH 7.5. containing 0.5 mg Triton X-100 ml^{-1}. Appropriate concentrations of purified enzyme or enzyme extracts (usually 200 µl) were incubated with undiluted antisera (200 µl) for 1 h on ice. Then 400 µl of *S. aureus* cells was added. After a further 1 h on ice, with occasional mixing, the samples were microfuged at 4^0C (Methods 2.2.8) and the supernatants were assayed for enzyme activity.

2.10.5 Immuno-blotting (Western blotting)

Immuno-blotting was carried out essentially as described by Towbin *et al.* (1979) and Batteiger *et al.* (1982), using the following buffers

(a) Blot transfer buffer A: 190 mM glycine, 25 mM Tris, 0.02% (w/v) SDS and 20% (v/v) methanol.

(b) Buffer B: 10 mM Tris buffer, pH 7.2.

(c) Buffer C: 20 mM Tris buffer, pH 7.2, containing 150 mM NaCl.

(d) Buffer D: 20 mM Tris buffer, pH 7.2, containing 150 mM NaCl, 0.5% (v/v) Tween 20.

(e) Buffer E: 20 mM Tris buffer, pH 7.2 containing 150 mM NaCl and 5% (v/v) normal donkey serum.

(f) Buffer F: 50 ml buffer B mixed prior to use with 10 ml methanol containing 30 mg of 4-chloro 1-naphthol and 150 µl of 4 % H_2O_2 (6.5 ml of distilled water and 1 ml of H_2O_2).

(a) SDS-PAGE

SDS-polyacrylamide gels were prepared and proteins samples were run on SDS-polyacrylamide gels as described in Methods 2.8.3.

(b) Electroblotting

The proteins were transferred from SDS-polyacrylamide gels to nitrocellulose paper (Anderman and Corporation, Kingston Upon Thames, Surrey,U.K.) using a BIO-RAD Trans-Blot tank (placed in a container with ice) containing blot transfer buffer A at a constant voltage of 70 for 4 h.

(c) Development of blot

The nitrocellulose papers were then soaked in buffer E overnight. The nitrocellulose papers were then rinsed twice with buffer C and incubated with buffer D containing 5% (v/v) normal donkey serum and 0.5 % (1 : 200 dilution) antiserum for 90 min at 23^0C. After this the nitrocellulose papers were washed four 12 min periods in buffer D and then washed with buffer C. The nitrocellulose papers were placed in buffer E to which 0.1 % donkey anti-rabbit IgG conjugated to peroxidase was added and incubated at 23^0C for about 90 min. The nitrocellulose papers were washed five 12 min periods in buffer C and then rinsed twice with buffer B. Finally nitrocellulose papers were placed in freshly prepared buffer F until bands appeared (approx. 5-20 min) and then they were washed in excess distilled water. The nitrocellulose paper was then dried between sheets of filter papers and stored in dark.

2.11 PhastGel isoelectric focussing

PhastGel isoelectric focussing gel (Pharmacia Laboratory Separation Division, Uppsala, Sweden), covering the pH range 3 to 9 was used. The gel consists of homogeneous polyacrylamide containing Pharmalyte carrier ampholytes. The gel was prefocussed at a constant current of 2.5 mA for about 10 min to form the pH gradient. The proteins of known pI points and purified L(+)-mandelate dehydrogenase samples were applied onto the pre-focussed gel by means of a PhastGel sample applicator (8 x 2 µl volumes). Proteins migrated to their isoelectric points at 15^0C in 30 min at a constant current of 2.5 mA (2000 V). After the proteins reached to their isoelectric points, the gel was transferred to a silver staining tank. The gel was stained for 90 min at 20^0C as shown below (PhastGel silver staining system, Pharmacia):

1.	20% (w/v) TCA	9 min
2.	Methanol/acetic acid/distilled water (40:10:50)	14 min
3.	Milli-Q water	4 min
4.	25 % (w/v) Glutardialdehyde	14 min
5.	Milli-Q water	4 min
6.	Mille-Q water	4 min
7.	20% (v/v) Ethanol	9 min
8.	Ammonical $AgNO_3$	
	[97 ml 2% (v/v) Ethanol + 1 ml 20% (w/v) NaOH	14 min
	1 ml of 14.8 M NH_4 OH + 1ml of 20% (w/v) Ag NO_3	
	(added last)]	
9.	20% (v/v) Ethanol	2 min
10	20% (v/v) Ethanol	2 min
11.	Developer	3 x 5 min
	(0.04% (v/v) formaldehyde+ 0.6 mM citric acid)	
12.	10% (v/v) acetic acid and, 5% (v/v) glycerol	10 min

2.12 Amino acid composition

(a) Dialysis

Approximately 200 μg of purified L(+)-mandelate dehydrogenase was used to determine its amino acid composition. The enzyme was dialysed at 4^0C for three days against 2 l of 1 mM potassium phosphate buffer, pH 7.5, with five changes and then dialysed for 24 h against 5 1 of 0.1 % SDS (w/v) in distilled water.

(b) Hydrolysis

Dialysed enzyme samples (approx. 6 μg each) were lyophilized and then dissolved in 1 ml of 5.65 M HCl. Tubes were flushed with nitrogen, frozen and sealed under vacuum. Triplicate samples were hydrolysed at 110^0C for 24 h, 48 h and 72 h. The samples were then lyophilized at -20^0C and dissolved in one ml distilled water and lyophilized again. This procedure was repeated twice.

(c) Performic acid oxidation

A further set of triplicate dialysed samples were oxidised 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 (to generate performic acid) followed by 15 min at 0^0C prior to use. Lyophilized enzyme (approx. 6 μg) was dissolved in 1 ml of performic acid and oxidised for 4 h at room temperature. Samples were then lyophilized and redissolved 3 times with 1 ml of distilled water and lyophilized each time. The oxidised enzyme was hydrolysed for 24 h as in Methods 2.12b.

(d) Analysis

Amino acid analysis was carried out by Dr. D. Campbell, Department of Biochemistry, Medical Science Institute, University of Dundee. The amino acid composition was determined using a Waters PICO-TAG amino acid analyser. The amino acids were quantified by the spectrophotometric detection of the phenylthiohydantoin (PTH)-amino acid derivatives.

2.13 Protein sequencing

(a) Dialysis

Purified L(+)-mandelate dehydrogenase was dialysed at 4^0C for five 24 h periods against 2 l of 0.1% SDS in distilled water and then lyophilized at -20^0C.

(b) Sequencing

The amino terminal sequence of L(+)-mandelate dehydrogenase was determined on three occasions by Dr. J. Keen at the SERC Sequencing Unit, Department of Biochemistry and Molecular Biology, University of Leeds, using the method of Findlay *et al.* (1989). The enzyme was sequenced by automated Edman degradation (Laursen, 1971). PTH-amino acid derivatives were identified by a reverse phase (C_{18}) HPLC system. The PTH-amino acids were quantified at 269 nm, serine and threonine residues were confirmed by detection at 313 nm.

2.14 Safety

(a) Microbiological

Microbiological safety practices were applied as described by COSHH rules and the University of Glasgow Safety Handbook 'Guidelines for Safety'(1987). All cultures were killed by autoclaving before disposal and any spillage was immediately swabbed with 10% (v/v) n-propanol.

(b) Chemical

All chemicals, especially compounds with known toxic, irritant or carcinogenic properties, were handled with care and from 1990, COSHH assessments were completed for all procedures involving potential hazards.

CHAPTER 3

PURIFICATION OF L(+)-MANDELATE DEHYDROGENASE FROM *RHODOTORULA GRAMINIS* STRAIN KGX 39

3.1 Introduction

L(+)-Mandelate dehydrogenase from the bacteria *Acinetobacter calcoaceticus* and *Pseudomonas putida* and D(–)-mandelate dehydrogenase from the bacteria *A. calcoaceticus, Lactobacillus curvatus* and *Streptoloccus faecalios* and from the yeast *Rhodotorula graminis* have been purified and partially characterised (Allison *et al.*, 1985a, b; Allison & Fewson, 1986; Yamazaki & Maeda, 1986a; Hoey *et al.*, 1987; Hummel *et al* ., 1988; Baker & Fewson, 1989; Tsou *et al.*, 1990; Fewson, 1992). It is therefore possible to compare at least a few D(–)-mandelate dehydrogenases from prokaryotic and eukaryotic organisms. Such a comparison has not been available for L(+) mandelate dehydrogenase and that is why L(+)-mandelate dehydrogenase from a yeast was purified and partially characterised in the present work.

Initial studies on mandelate utilisation by *R. graminis* indicated the presence of a dye-linked L(+)-mandelate dehydrogenase and a soluble NAD-dependent D(–)-mandelate dehydrogenase (Durham, 1984). The D(–)-mandelate dehydrogenase has been purified and characterised (Baker & Fewson, 1989; Baker, 1990; Baker *et al.*, 1992), and therefore a reliable procedure for the purification of L(+)-mandelate dehydrogenase from *R. graminis* KGX 39 was required in order to characterise it and allow comparison with other mandelate and lactate dehydrogenases.

3.2 Growth of cells and preparation of cell-free extract

3.2.1 Growth of *R. graminis* KGX 39

R. graminis which had been grown in defined medium containing mandelate (Methods 2.3.5a) contained high activities of D(-) and L(+)-mandelate dehydrogenases [0.22 μmol min^{-1} (mg protein)$^{-1}$ and 0.39 μmol min^{-1} (mg protein)$^{-1}$ respectively] but the yield of cells (3.33 g wet weight l^{-1}) was rather low. A higher yield of *R. graminis* cells (9.6 g wet weight l^{-1}) could be obtained by growth in defined media with glucose as carbon source, but these cells contained no detectable D(–)- or L(+)-mandelate dehydrogenase activity. However, cells grown in complex medium containing mandelate (Methods 2.3.5b) gave quite high specific activities of the D(-) and L(+)-mandelate dehydrogenases [0.18 to 0.21 μmol min^{-1} (mg protein)$^{-1}$ and 0.33 μmol min^{-1} (mg protein)$^{-1}$ respectively] and a high

Table 3.1 Distribution of L(+)-mandelate dehydrogenase and marker enzymes in subcellular fractions prepared from *R. graminis*

R. graminis cells were resuspended in two volumes of 20 mM phosphate buffer, pH 7.5, and French pressed (Methods 2.3.8). The cell-free extract (Methods 2.3.8) was centrifuged at 113,000 g (Methods 2.3.9). The high speed supernatant and high speed pellets, after resuspension in 20 mM phosphate buffer, were assayed for D(–)-and L(+)-mandelate dehydrogenases, glyceraldehyde-3-phosphate dehydrogenase, fumarase, cytochrome *c* reductase and α-mannosidase activities (Methods 2.4.1, 2.4.2, 2.4.7-2.4.10). The percentage distribution of enzyme activity is based on the total activity present in the two fractions.

Enzyme	Location	Distribution (%) in fractions	
		High speed	
		Supernatant	Pellet
L(+)-Mandelate dehydrogenase	?	80	20
D(–)-Mandelate dehydrogenase	Soluble	100	0
Glyceraldehyde-3-phosphate dehydrogenase	Cytoplasmic	98	2
Fumarase	Mitochondrial	92	8
NAD(P)H cytochrome *c* reductase	Endoplasmic reticulum	70	30
α-Mannosidase	Vacuolar membranes	83	17

Table 3.2 Solubilization of L(+)-mandelate dehydrogenase with Triton X-100, SDS and NaCl

R. graminis cells were resuspended in two volumes of 20 mM phosphate buffer, pH 7.5 and French pressed (Methods 2.3.8). Extracts were incubated in 20 or 50 mM phosphate buffer or in 20 mM phosphate buffer containing Triton X-100 or SDS or NaCl for one hour and then centrifuged at 12,000 g for 30 min. The pellets were discarded and supernatants were again centrifuged at 113,000 g for 2 h. The high speed supernatants and pellets resuspended in 20 mM phosphate buffer were assayed for enzyme activity (Methods 2.4.2). The percentage distribution of enzyme activity is based on the total activity present in the high speed supernatant and pellet. The relative solubilisation of L(+)-mandelate dehydrogenase into the high speed supernatant under different conditions was calculated, taking the high speed supernatant prepared in 20 mM phosphate buffer, pH 7.5, as 100%.

Sample	Phosphate buffer (mM)		Triton X-100 (mg / mg of protein)			SDS (mg / mg of protein)			NaCl (M)		
	20	50	0.07	0.3	0.5	0.07	0.3	0.5	0.2	0.5	1.0
High speed supernatant (%)	77	80	83	94	97	70	93	93	80	80	70
High speed pellet (%)	23	20	17	6	3	30	7	7	20	20	30
Relative release of L(+)-mandelate dehydrogenase (%)	100	90	108	148	146	92	92	6	98	84	39

yield of *R. graminis* cells (12-15 g wet weight l^{-1}). Therefore *R. graminis* grown in complex medium containing mandelate was used for most of the preliminary work and for all the purifications.

3.2.2 Distribution and solubilisation of L(+)-mandelate dehydrogenase from *R. graminis*

3.2.2.1 Localization of L(+)-mandelate dehydrogenase

Preliminary results suggested that L(+)-mandelate dehydrogenase from *R. graminis* may be membrane-associated (Durham, 1984 & present project). Attempts were therefore made to test this. After high speed centrifugation L(+)-mandelate dehydrogenase showed a similar distribution pattern to α-mannosidase and NAD(P)H cytochrome *c* reductase with a small but significant fraction of enzymes in the high speed pellet (Table 3.1). D(–)-Mandelate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase were present in the high speed supernatant and very little or no detectable activity in the pellet. Similar experiments repeated 15 times showed that 70-80% of L(+)-mandelate dehydrogenase activity appeared in the high speed supernatant whereas 20-30% was present in the pellet.

3.2.2.2 Extraction of L(+)-mandelate dehydrogenase with Triton X-100, SDS and NaCl

Triton X-100 or SDS released L(+)-mandelate dehydrogenase activity into the high speed supernatant fraction (Table 3.2) but the enzyme was not 'solubilised' in the presence of 50mM phosphate buffer, pH 7.5, or 0.2-1M NaCl in 20mM phosphate, buffer pH7.5. High NaCl and SDS concentrations inactivated the enzyme. Therefore, solubilisation of L(+)-mandelate dehydrogenase was achieved by including 0.3-0.5 mg of Triton X-100 (mg protein)$^{-1}$ in the cell-free extract (Table 3.2) and this increased the measurable activity in the high speed supernatant by about 50%.

In some subsequent experiments 0.5 mg of Triton X-100 (mg protein)$^{-1}$ was added to cell-free extracts and this resulted in the release of 95-99% of the enzyme into the high speed supernatant with high specific activity. However, some of the other membrane proteins released by this concentration of the detergent could not be removed by different

methods employed during the purification procedure. Therefore in the final purification procedure, cells were suspended in two volumes of 20mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} and this gave a good release of L(+)-mandelate dehydrogenase in the supernatant without extensive release of other membrane proteins which may have caused problems at later stages of purification.

3.3. Enzyme activity and stability

3.3.1 Preliminary studies on optimising the assay conditions.

The assay conditions for D(–)- and L(+)- lactate and mandelate dehydrogenases from *A. calcoaceticus* had been optimised (Allison *et al.*, 1985a, b; Allison & Fewson, 1986; Hoey, 1986; Hoey, *et al.*, 1987) and these conditions were used as the starting point for the present work. Both lactate and mandelate dehydrogenases require BSA and PMS in the reaction mixture for maximum activity with linear rates. Similarly, when the L(+)-mandelate dehydrogenase in extracts of *R. graminis* was assayed in the absence of BSA, the change in absorbance was not linear but fell off with time. To obtain a maximum and linear rate of enzyme activity for more than 2 min, 67 to 100 μg of BSA ml^{-1} and 10 nmol of PMS ml^{-1} were included in the assay mixture.

3.3.2 Effects of different buffers on the stability of L(+)-mandelate dehydrogenase during purification

L(+)-Mandelate dehydrogenase in a high speed supernatant was precipitated by 40-60% ammonium sulphate saturation (Methods 2.7.2). The precipitate was dissolved in 20mM phosphate buffer, pH 7.5. One ml samples were then gel filtered through Sephacryl S-300 using different buffers. The buffers used to pre-equilibrate the column and elute the L(+)-mandelate dehydrogenase included 20mM Tris/HCl, phosphate, Mops, Hepes and glycylglycine buffers,pH 7.5. The elution pattern was similar with each buffer but less than 50% of the enzyme was recovered when Tris/HCl or phosphate buffer was used. However 70%, 81%, 91% of L(+)-mandelate dehydrogenase activity was recovered when Hepes, Mops and glycylglycine buffers were used respectively. Glycylglycine buffer (20mM,

pH 7.5) was then used for DEAE-Sephacel chromatography. The L(+)-mandelate dehydrogenase was eluted using a 0-0.5 M NaCl linear gradient. The pH values of the fractions containing L(+)-mandelate dehydrogenase activity had increased from 7.5 to 9.0. A similar rise in pH was observed when 20mM phosphate buffer, pH 7.5 was used. Preliminary results showed that L(+)-mandelate dehydrogenase is inactivated very rapidly at pH 5.9 or pH 9.0, so the increase in pH to 9.0 was not desirable. Although the use of glycylglycine buffer was continued for hydrophobic interaction chromatography where this rise in pH did not occur. Therefore 20mM Tris/HCl buffer was used for DEAE-Sephacel and DEAE-Sepharose CL-6B columns and with this buffer the pH remained constant. The low recovery of the enzyme with 20mM Tris/HCl, pH7.5, noted above in the gel filtration experiments was avoided by including Triton X-100 or substrate or both in the buffer.

3.3.3 Effect of chelators, detergents and salts on the stability of L(+)-mandelate dehydrogenase in crude extracts during dialysis

The L(+)-mandelate dehydrogenase was rather unstable when crude extracts prepared in 20mM phosphate buffer, pH 7.5, were dialysed against 20mM phosphate buffer, pH 7.5. Several potential stabilising agents were investigated in an attempt to stabilise the enzyme during dialysis. Crude extract [specific activity 0.2 units (mg protein)$^{-1}$] was diluted five-fold with 20mM phosphate buffer, pH 7.5, containing 10-20 mM $MgSO_4$ or 0.1-10 mM DTT or 2-10 mM EDTA or 0.1-1 mg sodium deoxycholate ml^{-1} or 0.1-2 mg of Lubrol PX ml^{-1} or 1-2 mg of Lubrol WX ml^{-1} or 1-2 mg of Triton X-100 ml^{-1} or 1-2 mg of Triton 114 ml^{-1} or 1-2 mg of Tween 20 ml^{-1} or 1-2 mg of BSA ml^{-1} or 5-20 % (w/v) glycerol or 5-20 % (w/v) ethanediol, and then dialysed against the same buffer for 24 h. Buffers containing 0.5 mg of Lubrol PX or Lubrol WX or Triton X-100 or Triton 114 or Tween 20 or BSA ml^{-1}, were found to give the best recovery of the enzyme and indeed a 15-20 % increase in activity was observed with Triton X-100, Lubrol, BSA or Tween 20. Ethanediol, EDTA and glycerol were also effective in protecting the enzyme activity to some degree. DTT interferes with the assay of the enzyme reaction and so no definite conclusion could be drawn about its effect on the stability of the enzyme.

Table 3.3 Effect of number of passages through the French press on the release of L(+)-mandelate dehydrogenase and D(–)-mandelate dehydrogenase from *R. graminis* KGX 39

R. graminis grown in minimal medium containing mandelate (Methods 2.3.5a) was French pressed one to six times immediately after harvesting (Methods 2.3.8) and cell-free extracts were prepared (Methods 2.3.8). A sample of cells which had been stored at -20^{0}C for one month was also disrupted. The cell-free extracts were assayed for L(+)-mandelate dehydrogenase and D(–)-mandelate dehydrogenase activity (Methods 2.4.2 &2.4.1 respectively). Mandelate dehydrogenase activities were calculated as percentages of the enzyme activities present in low speed supernatant (Methods 2.3.8) which had been French pressed four times. The values represent means of two independent experiments and the numbers in parentheses represent the individual results obtained. The percentage of enzyme activity released is based on the activity present in four times French Pressed extracts.

Type of cells used	No. of passages through French pressure cell	LMDH activity (%) released into cell-free extract	DMDH activity (%) released into cell-free extract
Frozen cells	1	32 (30, 33)	51 (65, 37)
	2	84 (83, 84)	73 (74, 71)
	3	88 (78, 97)	91 (97, 85)
	4	100	100
	5	98 (89, 106)	100 (101, 99)
	6	92 (91, 92)	102 (103, 99)
Freshly harvested cells	1	41 (43, 38)	63 (69, 57)
	2	74 (85, 63)	88 (90, 82)
	3	85 (93, 77)	99 (103, 95)
	4	100	100
	5	102 (106, 98)	107 (106, 107)
	6	94 (97, 91)	103 (106, 100)

Glycylglycine buffer (20 mM, pH 7.5) containing 0.5 mg Triton X-100 ml^{-1} and 20% ammonium sulphate saturation was used to bind the L(+)-mandelate dehydrogenase onto a Phenyl Sepharose column. Continuous washing with the same buffer destroyed the column and no enzyme could be recovered with low (7.5%) ammonium sulphate saturation. It is possible that presence of ammonium sulphate in the buffer resulted in the precipitation of Triton X-100 onto the column material and the presence of a very high Triton X-100 concentration on the column surface inactivated the enzyme. Therefore, Triton X-100 was omitted from the buffer containing 5-20% ammonium sulphate saturation used for hydrophobic interaction chromatography on Phenyl Sepharose.

There was a progressive loss in activity when no Triton X-100 was included in the 20mM Tris/HCl buffer, pH 7.5, during ammonium sulphate precipitation, gel filtration, ion-exchange chromatography and hydrophobic-interaction chromatography and only 5-10% of the original enzyme was recovered, with a specific activity of 21-26 units mg^{-1} of purified protein. However, with the inclusion of 0.5 mg Triton X-100 ml^{-1} in the 20mM Tris/HCl buffer, pH 7.5, percentage recovery increased by 3-7 fold with specific activity of 160-210 μmol (mg of purified protein)$^{-1}$.

3.4 Development of a purification procedure

3.4.1 Disruption of *R. graminis* KGX 39

A French pressure cell with a capacity of up to 45 ml of liquid was used to break open *R. graminis* (Methods 2.3.8). Four passages released maximum enzyme activity into the high speed supernatant whereas one passage released about one third of this activity (Table 3.3)

3.4.2 Precipitation of enzyme activity by ammonium sulphate

Precipitation of enzyme activity by ammonium sulphate was tested as a possible first step of purification (Methods 2.7.2). Approximately 50% of the total protein precipitated between 0-40% ammonium sulphate saturation and one to nine percent of the L(+)-mandelate dehydrogenase activity, whereas 90-100% L(+)-mandelate dehydrogenase activity precipitated between 40-60% ammonium sulphate saturation giving a three to five-fold

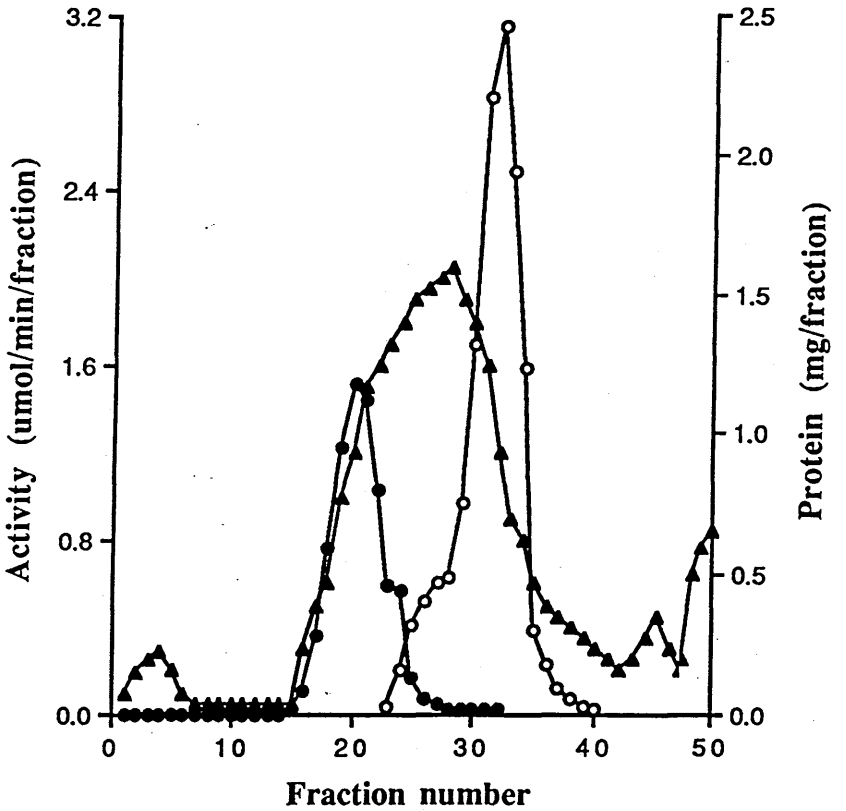


Figure 3.1 Gel filtration of L(+)-mandelate dehydrogenase and D(–)-mandelate dehydrogenase

A 40-60% saturation ammonium sulphate fraction of a crude extract prepared from 5 g of cells was applied to a column of Ultrogel ACA-34 (2.5 cm x 72 cm) which had been equilibrated with 20 mM phosphate buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. After loading the sample, the enzyme was eluted with 20 mM phosphate buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} at a flow rate of 21 ml h^{-1}. Fractions of 5.0 ml were collected and each fraction was assayed for enzyme activity as described in Methods 2.4.2. Protein in each fraction was estimated by using the method of Bradford, 1976 (Methods 2.2.3).

(•) L(+)-Mandelate dehydrogenase activity; (○) D(–)-mandelate dehydrogenase activity; (▲) protein.

purification (Table 3.4). Most of the D(–)-mandelate dehydrogenase also precipitated between 40-60% ammonium sulphate saturation.

However, diluted extracts or partially purified L(+)-mandelate dehydrogenase samples were difficult to precipitate with only 40-60% ammonium sulphate saturation and gave 40-90% recovery even with 100% ammonium sulphate saturation.

This step was found very useful and was included in the final purification procedure.

3.4.3 Gel filtration through Ultrogel ACA-34 or Sephacryl S-300

The L(+) mandelate dehydrogenase precipitated with 40-60 % ammonium sulphate saturation was dissolved in 20mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} to give a final concentration of 10 mg of protein ml^{-1} and was subjected to gel filtration chromatography using either Ultrogel ACA-34 or Sephacryl S-300. D(–)-mandelate dehydrogenase and L(+)-mandelate dehydrogenase eluted at different volumes (Figure 3.1).

The crude extract obtained from 80-90 g of *R. graminis* was then precipitated with 40-60% ammonium sulphate saturation. This sample, containing 50 mg of protein ml^{-1}, could not be applied onto Ultrogel ACA-34 or Sephacryl S-300 columns (72 x 2.6 cm) because of its viscosity. Considering the rather low amount of protein that could be handled during gel filtration and the relatively poor purification (2-3 fold), this technique was not included in the final purification scheme. An FPLC Superose 12 column was also tried, at room temperature, and much of the enzyme activity was lost, so this column was not included in the final purification scheme either.

3.4.4 Ion-exchange chromatography on DEAE-Sephacel

A 40-60% ammonium sulphate saturation fraction was dialysed against 20mM Tris/HCl buffer pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} (Methods 2.2.5). The dialysed sample was applied onto a DEAE-Sephacel column.(Methods 2.7.3) and the column was washed with 20mM Tris/HCl buffer pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. The L(+)-mandelate dehydrogenase was then eluted with a linear NaCl gradient of 0-0.5 M in 20mM Tris/HCl buffer pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. This step gave a

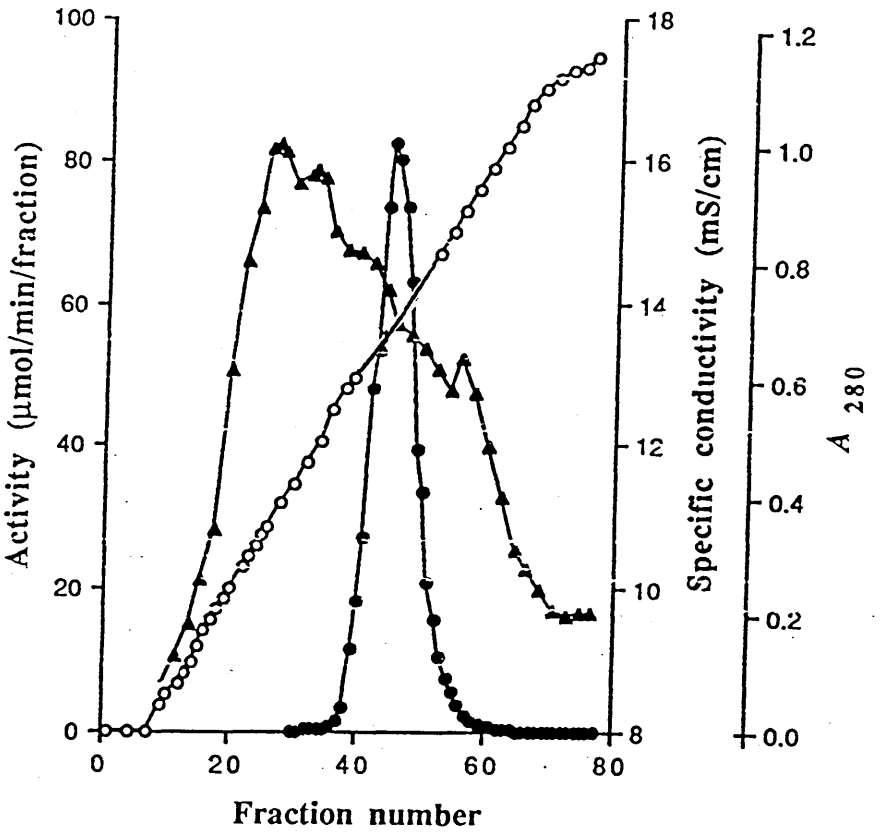


Figure 3.2 Ion-exchange chromatography of L(+)-mandelate dehydrogenase on DEAE-Sephacel

A 40-60% saturation ammonium sulphate fraction which had been dialysed against 20 mM-Tris/HC1 buffer, pH 7.5, containing 0.5 mg Trition X-100 ml^{-1}(Methods 2.2.5), was applied to a column of DEAE-Sephacel (2.6 cm x 24 cm) which had been equilibrated with the dialysis buffer (Methods 2.7.3). After loading the sample, the column was washed as described in Methods 2.7.3. Elution of L(+)- mandelate dehydrogenase was carried out with a linear gradient of 0.075-0.3M-NaCl in 20 mM-Tris buffer, pH 7.5 (Methods 2.7.3). Fractions of 12.2 ml were collected during elution of L(+)-mandelate dehydrogenase with the NaCl gradient. Each fraction was assayed for enzyme activity as described in Methods 2.4.2.

(•) L(+)-Mandelate dehydrogenase activity; (▲) A_{280}; (○) specific conductivity.

6-7 fold purification. Later, the washing of the column was improved and it involved washing with 20mM Tris/HCl buffer pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}, then 20mM Tris/HCl buffer pH 6.5, containing 0.5 mg Triton X-100 ml^{-1} and lastly 20mM Tris/HCl buffer pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} with 0.075 M NaCl (Methods 2.7.3). The enzyme was eluted with a linear NaCl gradient of 0.075-0.3M in 20mM Tris/HCl buffer pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} (Methods 2.7.3). This improved method of washing and elution resulted in a 25-30 fold purification with 80-90% recovery. A representative elution profile of L(+)-mandelate dehydrogenase activity and protein is shown in Figure 3.2. The large capacity for protein of the ion-exchange column made this step ideal for purification of ammonium sulphate precipitate of extract from 80-90 g of *R. graminis* cells. As a large amount of protein could be handled by DEAE-Sephacel column, giving a good increase in purification, this step was included in the final purification procedure.

3.4.5 Ion-exchange chromatography on DEAE-Sepharose CL-6B

A contaminant of M_r 95000 could not be removed using ammonium sulphate precipitation, gel filtration through Ultrogel ACA-34, ion-exchange chromatography on DEAE-Sephacel or hydrophobic interaction chromatography on Phenyl Sepharose but was easily removed by using a long DEAE-Sepharose CL-6B column (45 x 2.6cm). This column was used as an anionic-exchanger as well as a gel filtration column.

The pooled active fractions from the DEAE-Sephacel column were dialysed against 20mM Tris/HCl buffer pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} and were applied onto a DEAE-Sepharose CL-6B column (Methods 2.7.4). The column was then washed with 20mM Tris/HCl buffer pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} followed by 20mM Tris/HCl buffer pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} with 0.08 M NaCl. The enzyme was eluted by applying a shallow linear 0.08-0.2M NaCl gradient at a flow rate of 22 ml^{-1}. This step gave 23-28 fold purification with 75-85% recovery of enzyme activity. This step was included in the final purification procedures. A representative elution profile of the L(+)-mandelate dehydrogenase activity and protein is shown in Figure 3.3. Initially this step was used after ammonium sulphate precipitation and gel filtration but in the final

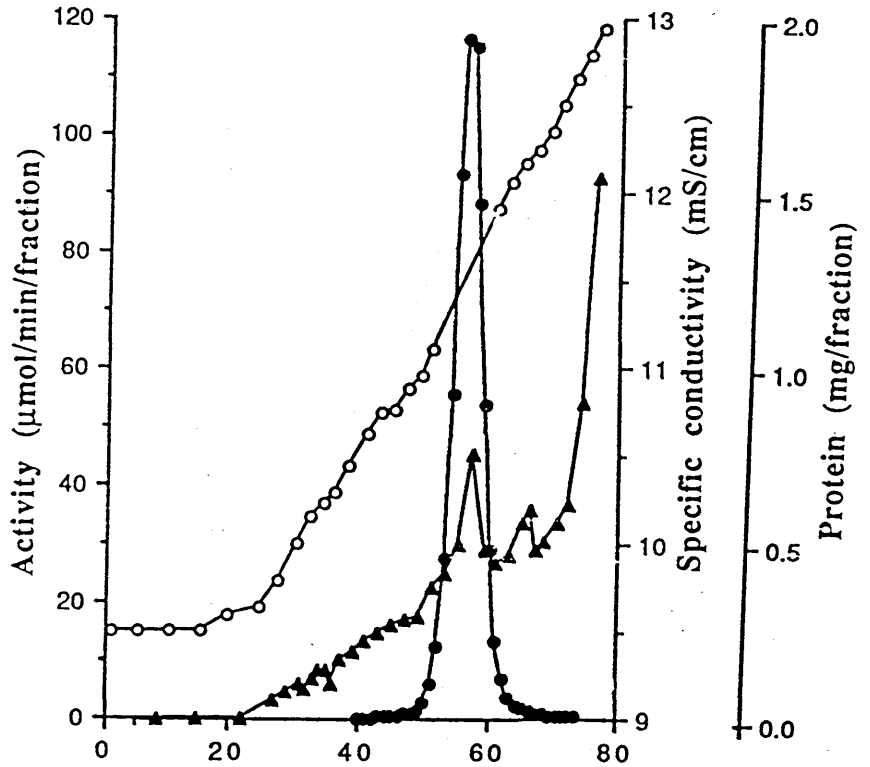


Figure 3.3 Ion-exchange chromatography of (L+)-mandelate dehydrogenase on DEAE-Sepharose CL-6B

A DEAE-Sephacel pool was dialysed against 5 l of 20 mM-Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} for 6 h. The dialysed sample was applied onto a column of DEAE-Sepharose CL-6B (2.6 x 45 cm) which had been equilibrated with 20 mM-Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} (Methods 2.7.4). After loading the sample, the column was washed with buffers (Methods 2.7.4). Elution of L(+)-mandelate dehydrogenase was carried out with a linear gradient of NaCl in 20 mM-Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} (Methods 2.7.4). Fractions of 10 ml were collected during elution of the enzyme each fraction was assayed for enzyme activity as described in Methods 2.4.2.

(●) L(+)-Mandelate dehydrogenase activity; (▲) protein; (○) conductivity.

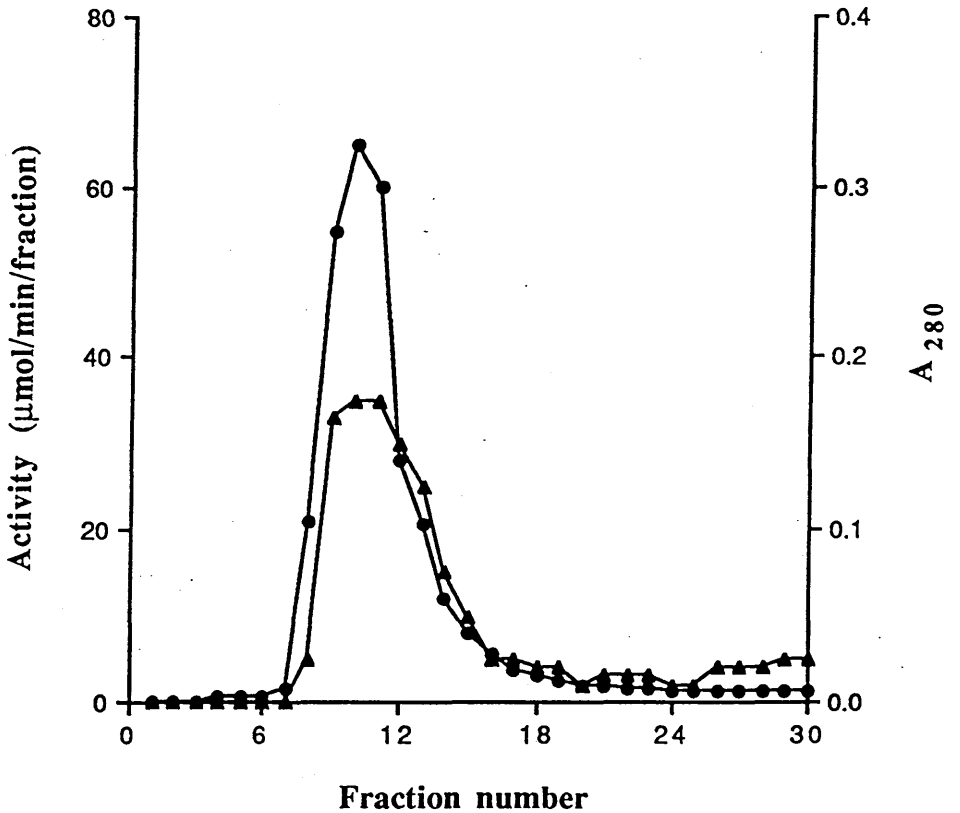


Figure 3.4 Hydrophobic interaction chromatography of L(+)-mandelate dehydrogenase on Phenyl Sepharose CL-4B

Fractions containing more than 25% of the activity of the peak fraction from DEAE-Sepharose CL-6B were pooled together and ammonium sulphate was added to give a final saturation of 20%. This sample was applied to a column of Phenyl Sepharose CL-4B (2.6 cm x 10.8 cm) that had been equilibrated with 20 mM-glycylglycine buffer, pH 7.5, containing 25% saturation ammonium sulphate (Methods 2.7.5). After loading the sample, the column was washed with buffers containing 20% and 12% saturation ammonium sulphate (Methods 2.7.5). Elution of L(+)-mandelate dehydrogenase was carried out with 20 mM-glycylglycine buffer, pH 7.5, containing 7.5% saturation ammonium sulphate (Methods 2.7.5). The enzyme was eluted into test tubes containing sufficient Triton X-100 to give a final concentration of 0.5 mg ml^{-1}. Fractions of 10.2 ml were collected during elution of L(+)-mandelate dehydrogenase. Each fraction was assayed for enzyme activity as described in Methods 2.4.2 and protein in each fraction was monitored by taking absorbance at 280 nm.

(•) L(+)-Mandelate dehydrogenase activity; (▲) A_{280}.

purification scheme it was used after the DEAE-Sephacel column because it resulted in better purification. The combination of the ammonium sulphate precipitation, DEAE-Sephacel and DEAE-Sepharose CL-6B steps gave almost pure enzyme; indeed only by overloading SDS-PAGE gels with 5 μg of purified protein from the DEAE Sepharose CL-6B pool could minor contaminants be detected.

3.4.6 Behaviour of the enzyme on hydrophobic-interaction chromatography

Preliminary experiments indicated that L(+)-mandelate dehydrogenase of *R. graminis* would bind to Phenyl Sepharose at 20% ammonium sulphate saturation and could be eluted between 5-10% ammonium sulphate saturation (Methods 2.7.5). Although the Phenyl Sepharose column did not yield a large degree of purification, it was important because all the remaining contaminants in the pool from the DEAE Sepharose CL-6B step were removed. Approximately half of the enzyme activity was lost only during this step. A representative elution profile of L(+)-mandelate dehydrogenase and protein from hydrophobic interaction chromatography is shown in Figure 3.4

3.4.7 Concentration of the purified L(+)-mandelate dehydrogenase

Concentration of purified enzyme from the Phenyl Sepharose column was attempted by three methods: (a) water absorption with carboxymethyl cellulose, (b) DEAE Sephacel ion exchanger chromatography, and (c) vacuum dialysis.

(a) Use of carboxymethyl cellulose resulted in the extraction of a yellow colour into the purified enzyme solution that interfered with certain aspects of the characterization and so carboxymethyl cellulose was not used to concentrate the enzyme for further work.

(b) The enzyme could be concentrated using a small DEAE-Sephacel column, and elution with 20mM Tris/HCl buffer pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} and 0.3 M NaCl. But the enzyme was not stable in the presence of 0.3 M NaCl and therefore this method was not used to concentrate the enzyme because a desalting step would have been required.

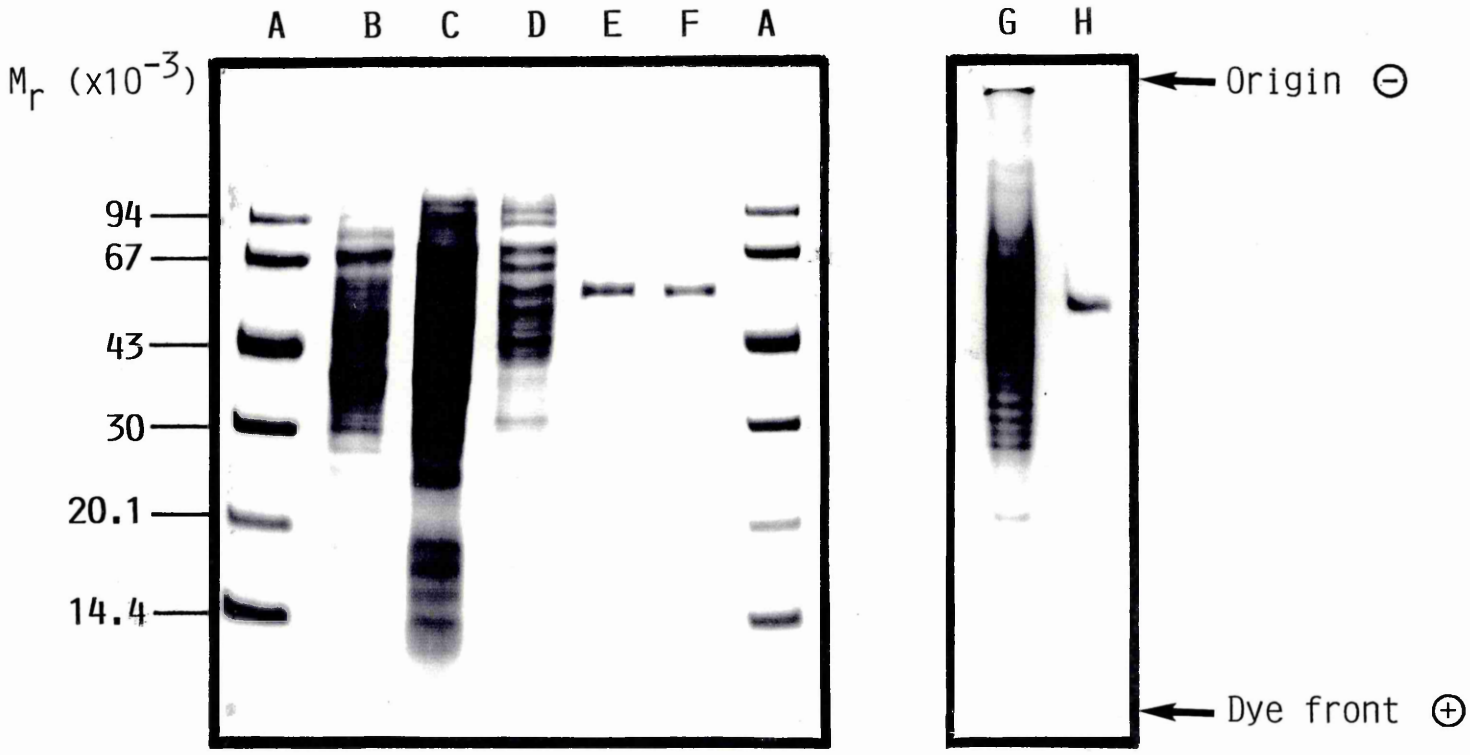
(c) Vacuum dialysis of the purified enzyme resulted in 40-60% loss of activity but this method was used for most purposes because of its convenience.

Figure 3.5 Purification of L(+)-mandelate dehydrogenase from *Rhototorula graminis* as monitored by SDS-PAGE and non-denaturing-PAGE

The 10% (w/v) SDS-polyacrylamide gel with a 5.6% stacking gel and the 6% (w/v) non-denaturing-polyacrylamide gel with a 5.6% stacking gel were run as described in Methods 2.8.3 and 2.8.4 respectively. Both gels were stained for proteins with Commassie Blue (Methods 2.8.5.1a). Samples for the SDS-polyacrylamide gel and the non-denaturing-polyacrylamide gel were prepared as described in Methods 2.8.3c and 2.8.4c respectively.

SDS-polyacrylamide gel stained for protein.
A, M_r markers; B, 60 μg of extract; C, 75 μg of the 40-60% ammonium sulphate fraction; D, 19μg of the DEAE-Sephacel pool; E, 14μg of the concentrated DEAE-Sepharose CL-6B pool; F, 12 μg of the concentrated Phenyl Sepharose pool i.e. purified L(+)-mandelate dehydrogenase

Non-denaturing-polyacrylamide gel stained for protein
G, 50 μg of the extract; H, 4 μg of the purified L(+)-mandelate dehydrogenase


A
B
C
D
E
F
A
G
H
M_r (x10^{-3})
94
67
43
30
20.1
14.4
Origin ⊖
Dye front ⊕

3.5 Final purification scheme

The final purification scheme included ammonium sulphate fractionation, DEAE-Sephacel and DEAE-Sepharose CL-6B anion exchange chromatographies and Phenyl Sepharose hydrophobic interaction chromatography (Table 3.4). This purification scheme yielded approximately one mg of protein from 70-90 g of cells. The final purification procedure was reproducible in nine independent purifications. The final purification factor varied from 650-950 depending upon the specific activity of crude extract [0.142 to 0.21 μmol (mg protein)$^{-1}$] while the percentage recovery varied between 32%-37%. The specific activity of the purified protein varied between 160-210 μmol min^{-1} (mg protein)$^{-1}$.

3.6 Storage of purified L(+)mandelate dehydrogenase

The purified L(+)-mandelate dehydrogenase eluted from the Phenyl Sepharose column using 7.5% ammonium sulphate saturation in 20mM glycylglycine buffer, pH 7.5 was unstable at O^{0}C. Storage at -20^{0}C for 2-3 months also resulted in 20-30% loss in activity. The major loss in activity usually occurred during the thawing of frozen samples and this could be avoided by adding Triton X-100 to a final concentration of 0.5 mg ml^{-1} to the pooled fractions from the Phenyl Sepharose column before storage at -20^{0}C. In addition, when the L(+)-mandelate dehydrogenase was eluted into test tubes containing Triton X-100 and substrate to give final concentrations of 0.5 mg of Triton X-100 ml^{-1} and 5mM L(+)-mandelate, no loss in activity was observed at 0^{0}C. Curiously, the pool containing Triton X-100 and L(+)-mandelate lost all its activity when stored at -20^{0}C. Therefore, samples containing 0.5 mg Triton X-100 ml^{-1} were stored at -20^{0}C but samples with substrate and Triton X-100 were precipitated with ammonium sulphate and stored on ice. Both these treatments gave better recovery of L(+)mandelate dehydrogenase activity from the Phenyl Sepharose column than collecting the fractions without any addition.

3.7 Purity of L(+)-mandelate dehydrogenase

The purified L(+)mandelate dehydrogenase gave a single band on non-denaturing and SDS-PAGE. A representative gel showing different steps of the purification is shown in Figure 3.5 and a densitometer scan of gels of purified enzyme and M_r markers shown in

Table 3.4 Purification of L(+)-mandelate dehydrogenase from *R. graminis* KGX 39

L(+)-Mandelate dehydrogenase was purified as described in (Methods 2.7). Protein concentration and enzyme activity were estimated as in Methods 2.2.3 and 2.4.3 respectively.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Purification (fold)	Yield (%)
Crude extract	250	3300	701	0.21	1	100
Ammonium sulphate 40-60% saturation	48	879	673	0.77	3.6	96
Ammonium sulphate 40-60% saturation dialysed	70	879	667	0.76	3.6	96
DEAE-Sephacel	159	148	631	4.26	20	90
DEAE-Sephacel dialysed	175	144	623	4.33	20	90
DEAE-Sepharose CL-6B	90	4.8	590	123	586	85
Phenyl Sepharose CL-4B	90	1.4	252	180	857	37

Figure 3.6 Densitometer scan of purified of L(+)-mandelate dehydrogenase from *Rhodotorula graminis* KGX 39 and M_r markers

Concentrated purified L(+)-mandelate dehydrogenase and Mr markers were resolved by 10% (w/v) SDS-PAGE with a 5.6% stacking gel (Methods 2.8.3). The SDS-polyacrylamide gel was stained for protein (Methods 2.8.5.1) and tracks with purified enzyme and M_r markers were scanned with a laser densitometer (Methods 2.8.6). a, M_r marker, from left to right; phosphorylase b, (Mr 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soya bean trypsin inhibitor (M_r 20,000) and α-lactalbumin (M_r 14,400); b, 9μg of the concentrated purified L(+)-mandelate dehydrogenase; c, control (no protein applied). The top of the gel (⟶) and the position of the tracking dye (- - ➤) are indicated.

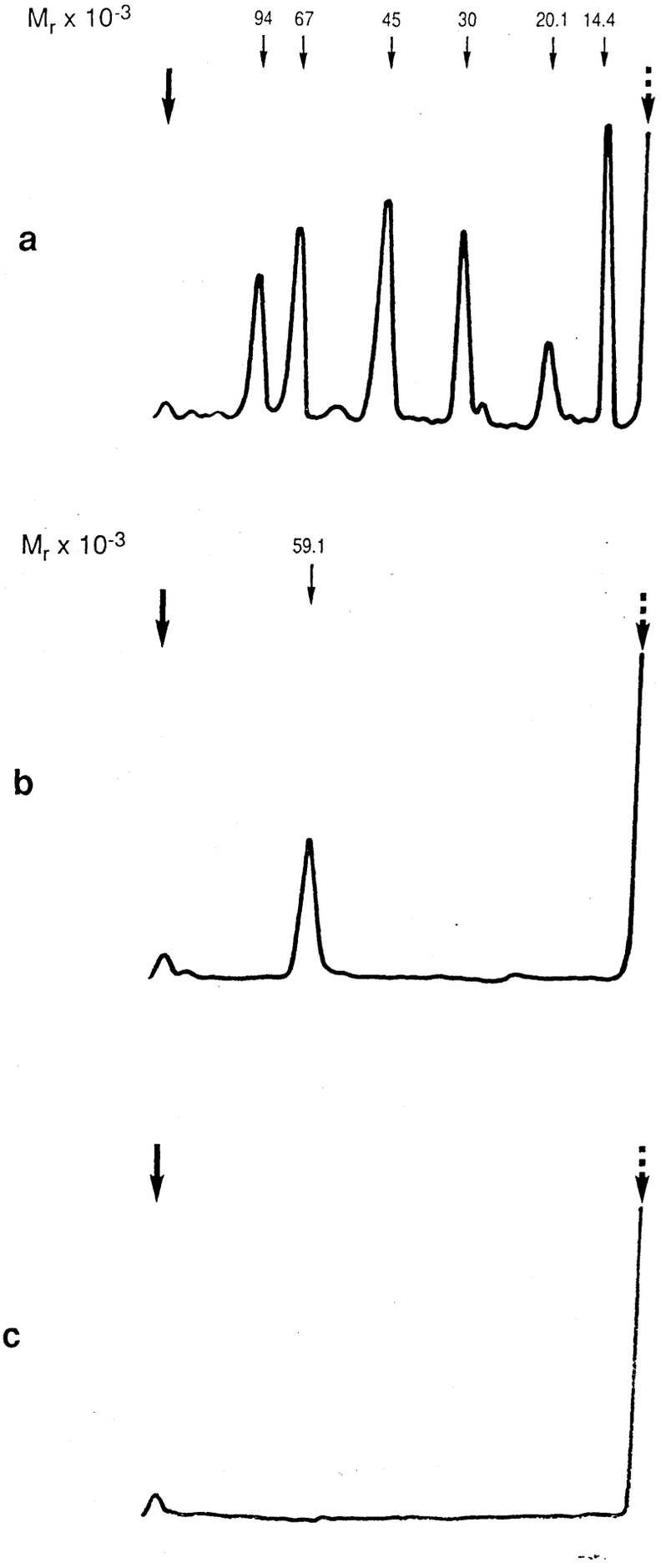

M_r x 10^{-3}
94
67
45
30
20.1
14.4
a
M_r x 10^{-3}
59.1
b
c

Figure 3.6. After long storage of enzyme at -20°C, the enzyme gave an extra minor component of lower M_r (Figure 3.7).

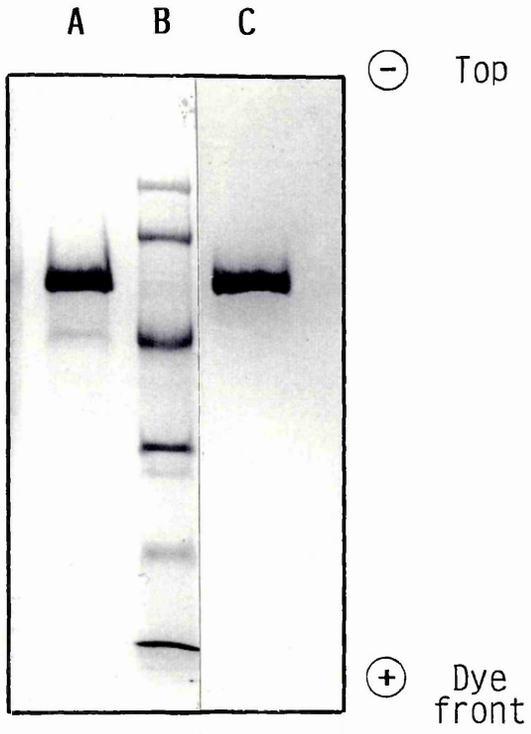
3.8 Discussion

In many bacteria, including *A. calcoaceticus*, and *P.putida* , L(+)-mandelate dehydrogenase and L(+)-lactate dehydrogenase are integral components of cytoplasmic membranes and are solubilised from the membrane fractions by Triton X-100 treatment (Allison *et al.*, 1985b; Hoey *et al.*, 1987; Fewson, 1992). L(+)-Lactate dehydrogenase from *S. cerevisiae* was found to be located between the mitochondrial membranes (Guiard, 1985; Lederer *et al.*, 1985; Chapman *et al.*, 1991). The distribution and solubilization experiments (Table 3.2, 3.3) suggest that L(+)-mandelate dehydrogenase from *R. graminis* may be loosely orgenelle- or membrane-associated and most of the enzyme (70-80%) is solubilized during cell disruption. The remaining enzyme activity can be easily 'solubilized' by Triton X-100 treatment. Gentle methods of cell disruption and sucrose-density centrifugation together with measurement of activity of the marker enzymes or immunolocalization with gold labeling (Boddingius & Dijkman, 1990; Mizoguchi *et al.*, 1990; Nickerson *et al.*, 1990; Ohishi *et al.*, 1990) are perhaps needed to identify the extent and type of association, which was not investigated during the present studies.

A reliable method for the purification of L(+)-mandelate dehydrogenase has been developed with only two types of chromatographies; ion-exchange and hydrophobic interaction, although one of the steps also involves gel filtration. The DEAE-Sephacel column was a useful purification step and the degree of purification achieved depended on washing the column with 20mM Tris/HCl buffer at pH 6.5, which is above the isoelectric point of the enzyme (see section 4.2.5). The DEAE-Sepharose CL-6B was the most significant step in the purification scheme, possibly because both the gel filtration and ion-exchange properties of the column were exploited. The Phenyl Sepharose column could not be used at the start of the purification because of its low capacity but was very useful as the final step to remove minor contaminents and the binding and elution conditions of the enzyme indicate the presence of hydrophobic residues in it. There was a significant loss (approximately 50%) in activity at this stage, possibly because Triton X-100 had to be omitted

Figure 3.7 Effect of storage on purified L(+)-mandelate dehydrogenase

L(+)-Mandelate dehydrogenase was purified as decribed in Methods 2.7 and then was concentrated by vacuum dialysis. The freshly purified and concentrated enzyme was run on 10% SDS-polyacrylamide gel with a 5.6% stacking gel (Methods 2.8.3) and stained for protein (Methods 2.8.5.1a). After storage at -20^{0}C for approximately two months, sample from the same preparation was subjected to electrophoresis on a 10% SDS-polyacrylamide gel using the same Methods and then stained for protein (Methods 2.8.5.1a). Lanes A, 12 μg of stored purified L(+)-mandelate dehydrogenase B, M_r markers C, 12 μg of freshly purified L(+)-mandelate dehydrogenase.


A
B
C
⊖ Top
⊕ Dye front

due to the reasons given in section 3.3.3. Unfortunately no way was found of avoiding this loss of activity.

After long storage an extra minor component of lower M_r (45K) was observed in purified enzyme preparation (Fig. 3.7). It is possible that this component is produced by autolytic cleavage of the enzyme. The loss of activity of the purified enzyme on long storage may also be due to autolysis of the enzyme.

CHAPTER 4

PHYSICAL, CHEMICAL AND KINETIC CHARACTERIZATION OF L(+)-MANDELATE DEHYDROGENASE

4.1 Introduction

Only a very limited preliminary investigation of the L(+)- and D(–)-mandelate dehydrogenases in crude extracts of *R. graminis* strain KGX 39 had previously been reported (Durham, 1984; see section 1.8). The two mandelate dehydrogenases differed from each other with respect to substrate specificities, cofactor specificities, electron acceptor specificities, subcellular location, pH optima, heat stabilities and electrophoretic mobilities (Durham, 1984). The NAD-linked D(+)-mandelate dehydrogenase has previously been purified and more fully characterised in this laboratory (Baker & Fewson, 1989; Baker, 1990; Baker *et al.*, 1992). This chapter describes the physical, chemical and kinetic properties of L(+)-mandelate dehydrogenase from *R. graminis* allowing a comparison with other mandelate, lactate and related dehydrogenases that have previously been characterised. Additionally a few experiments with enzymes from other organisms are described in order to supply supplementary comparative information.

4.2 Physical and chemical characterization

4.2.1 Relative molecular mass

The native M_r was determined by gel filtration of both purified enzyme and crude extract of *R. graminis* through Sephacryl S-300 calibrated with proteins of known M_r (Fig. 4.1). The experiment was done three times and a representative result is shown in Fig. 4.1. The apparent native M_r value was estimated as 239,942 (SD= 8,419, n=3) for the purified enzyme and 235,044 (SD= 5,185, n=3) for the enzyme in crude extract. The subunit M_r value under denaturing condition was determined using SDS-PAGE (Methods 2.8.3). Gels were scanned (Methods 2.8.6) and the electrophoretic mobilities of calibrating proteins were plotted against $\log_{10}M_r$ to estimate the subunit M_r of the enzyme. A representative result is shown in Fig. 4.2. The subunit M_r of the enzyme was 59,100 (SD= 840, n=26) using nine different batches of enzymes.

Comparison of the native and subunit M_r values suggests that the enzyme is homotetrameric.

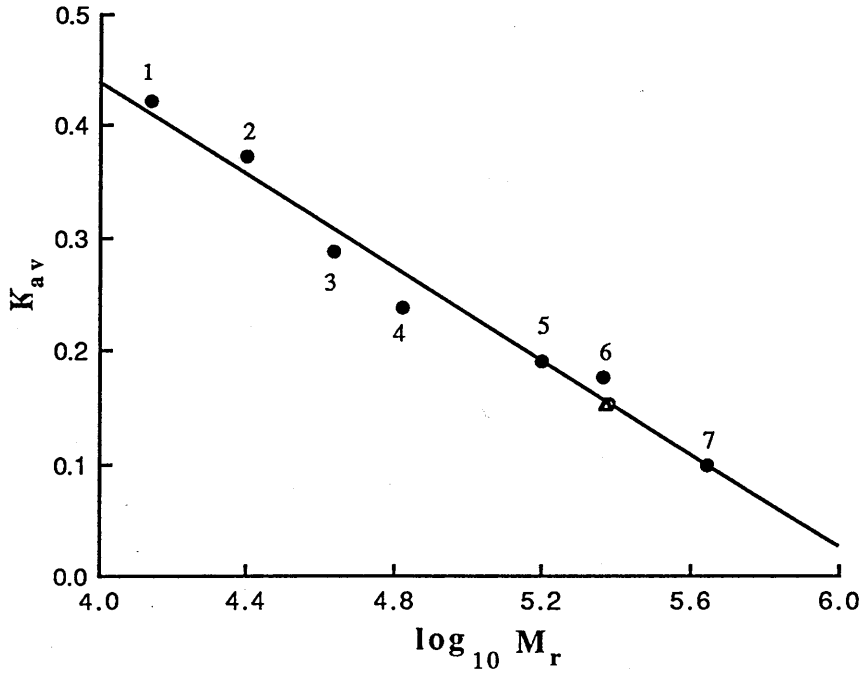


Figure 4.1 Determination of the apparent native M_r of L(+)mandelate dehydrogenase of *R. graminis* by gel filtration using Sephacryl S-300

A Sephacryl S-300 gel filtration column (90 cm long x 1.6 cm diameter) was pre-equilibrated in 20 mM phosphate buffer, pH 7.5, containing 0.15 M NaCl and 0.5 mg Triton X-100 ml^{-1}. The column was first calibrated with proteins of known M_r. Then purified L(+)-mandelate dehydrogenase and crude extract were separately filtered through the column using 20 mM phosphate buffer, pH 7.5, containing 0.15 M NaCl and 0.5 mg Triton X-100 ml^{-1} and fractions of two ml were collected. Each fraction was assayed for enzyme activity (Methods 2.4.2). Using the equation $K_{av} = (V_e - V_o)/V_t - V_o$, K_{av} values were calculated for the known proteins and plotted against their $\log_{10} M_r$. The K_{av} values for purified L(+)-mandelate dehydrogenase and for L(+)-mandelate dehydrogenase in crude extract are indicated on the graph by (○) and (▲) respectively. Samples were run in triplicate with similar results but results of a single representative experiment are shown.

(1) Ribonuclease A (M_r 13,700)
(2) Chymotrypsinogen A (M_r 25,000)
(3) Ovalbumin (M_r 43,000)
(4) Bovine serum albumin (M_r 67,000)
(5) Aldolase (M_r 158,000)
(6) Catalase (M_r 232,000)
(7) Ferritin (M_r 440,000)

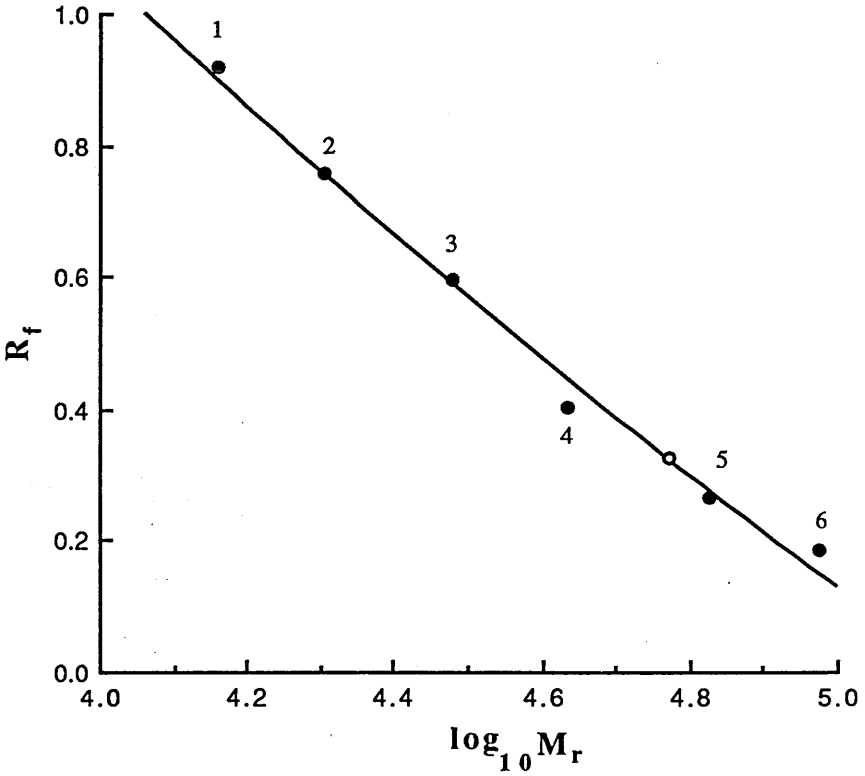


Figure 4.2 Determination of the subunit M_r of L(+)-mandelate dehydrogenase of *R. graminis* by SDS-PAGE

The marker proteins and purified L(+)-mandelate dehydrogenase were run on 10% SDS-PAGE (Methods 2.8.3) and stained for protein (Methods 2.8.5.1a). The gel was then scanned using a laser densitometer (Methods 2.8.6) and the relative mobilities (R_f) were calculated as R_f = distance travelled by protein / distance travelled by dye front. The relative mobility of L(+)-mandelate dehydrogenase is indicated by the open circle.

(1) α-Lactalbumin (M_r 14,400)
(2) Soya bean trypsin inhibitor (M_r 20,100)
(3) Carbonic anhydrase (M_r 30,000)
(4) Ovalbumin (M_r 43,000)
(5) Bovine serum albumin (M_r 67,000)
(6) Phosphorylase *b* (M_r 94,000)

Table 4.1 Amino acid composition of L(+)-mandelate dehydrogenase of *R. graminis*

The amino acid composition of L(+)-mandelate dehydrogenase of *R. graminis* was determined as described in Methods 2.12. 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 six 72 h of acid hydrolysis values whereas serine and threonine values were obtained by extrapolation to zero time of hydrolysis. Approximate numbers of amino acids per subunit were calculated from the subunit M_r of 59,100 and an average residue M_r is 108. Although tryptophan cannot be determined after acid hydrolysis, at least one residue has been determined from amino terminal sequence analysis (Table 4.2).

Amino acid	Mean (mol%)		Approximate no. of amino acids per subunit of enzyme
Asp	8.83	(7.37, 9.3, 9.83)	48
Glu	11.34	(10.43, 11.36, 12.22)	61
Ser	4.6	(4.0, 4.7, 5.1)	25
Gly	8.82	(9.23, 8.24, 8.99)	47
His	1.67	(1.85, 1.61, 1.54)	9
Arg	7.52	(7.93, 7.46, 7.18)	40
Thr	4.47	(4.8, 4.3, 4.3)	24
Ala	10.91	(11.08, 10.83, 10.83)	59
Pro	5.34	(5.5, 5.2, 5.32)	29
Tyr	1.64	(1.85, 1.63, 1.44)	9
Val	9.69	(9.67, 9.65, 9.76)	52
Met	0.39	(0.71, 0.33, 0.12)	2
Cys	0.44	(0.31, 0.52, 0.48)	2
Ile	6.47	(6.51, 6.48, 6.43)	35
Leu	9.61	(9.89, 9.54, 9.4	52
Phe	2.9	(2.94, 2.87, 2.89)	16
Lys	6.81	(6.55, 6.94, 6.94)	37
Total number of amino acids			547

Table 4.2 Amino terminal sequence of L(+)-mandelate dehydrogenase of *R. graminis*

The amino terminal sequence of L(+)-mandelate dehydrogenase was determined by Dr. J.N. Keen at the SERC Sequence Unit , Department of Biochemistry and Molecular Biology, University of Leeds (Methods 2.13). Approximately 0.2 nmol (12 μg) of enzyme was sequenced in the first two runs and approximately 0.6 nmol (36 μg) in the third run.

Cycle	Run 1		Run 2		Run 3		Deduced
	Residue	Quantity (pmol)	Residue	Quantity (pmol)	Residue	Quantity (pmol)	Sequence
1	X	-	D?	22	D?	396	D?
2	A	30	A	24	A	407	A
3	Q	29	Q	27	Q	266	Q
4	L	42	L	19	L	299	L
5	P	24	P?	3	P	64	P
6	V	27	V	8	V	148	V
7	K	3	X	-	K	133	K
8	Q	23	Q	18	Q	97	Q
9	R	27	R	21	R	64	R
10	G	19	G	8	G	58	G
11	R	16	R	10	R	21	R
12	A	19	A	9	A	75	A
13	R	13	R	6	R	3	R
14	S*	6	S*	3	S*	5	S
15	I	17	I	7	I	41	I
16	S*	6	S*	3	S*	2	S
17	A	17	A	6	A	40	A
18	A	25	A	11	A	74	A
19	X	-	E	9	E	15	E
20	V	18	V	7	V	29	V
21	A	11	A?	8	A	18	A
22	A?	10	M?	2	K	16	K?
23	K?	1	H	8	H	8	H
24	N	7	N	6	N	22	N
25	S	3	S*	2	S*	2	S
26	R	12	R	11	R	5	R
27	D?	5	D	4	D	13	D
28	S	3	X	-	D?	9	X
29	M	7	M	3	M	11	M
30	K?	1	X	-	W	2	W?
31			V?	1	V	11	V
32			X	-	V?	9	V?
33			X	-	X	-	X
34			D?	2	D	13	D?
35			D?	2			D?
36			X	-			X
37			S*?	2			S*?
38			R?	2			R?
Repetitive yield (%)		94		94		90	
Intercept (pmol)		38		19		190	

Keys * Residues confirmed at 313 nm
? Identification tentative
X Not confirmed

4.2.2 Amino acid composition

The amino acid composition of L(+)-mandelate dehydrogenase is shown in Table 4.1. Hydrophobic residues constitute approximately 50% of the total number of amino acids. The number of cysteine and methionine residues could not be determined accurately in oxidised or non-oxidised 24, 48 or 72 h acid hydrolysed samples due to their low abundance and the values in Table 4.1 represent the average of non-oxidised 24, 48 and 72 h acid hydrolysed samples. Asx (aspartic acid & asparagine), Glx (glutamic acid & glutamine) and serine values were similar in two independent determinations but were different in the third determination. Therefore the average values of these residues show greater variation as compared to other amino acids (Table 4.1).

4.2.3 Amino-terminal sequence of L(+)-mandelate dehydrogenase

Data from 3 sequencing runs is given in Table 4.2. The sequence of L(+)-mandelate dehydrogenase is well defined for 32 residues and it seems difficult to sequence to more than about 32 residues, partly because of the presence of proline (residue 5) early in the sequence which was poorly cleaved and led to lagging sequence at positions 13, 16, 19 and 21.

4.2.4 Identification of cofactors in L(+)-mandelate dehydrogenase

4.2.4.1 Identification of haem

The absorption spectra of oxidised and substrate-reduced L(+)-mandelate dehydrogenase from *R. graminis* are shown in Fig. 4.3. Oxidised enzyme gave broad absorption peaks at 560,530 and a sharp peak at 413 nm. The reduced enzyme gave absorption peaks at 557, 528 and 423nm. This absorption pattern is characteristic of a *b* -type cytochrome. The amount of haem present was calculated to be one mole per 59,565 g of protein using an ε_{oxi} at 413 nm of 130 mM^{-1} cm^{-1} (Jack & Lederer, 1974, Chapman *et al.*, 1991). This value is very close to the determined value of 59,100, the subunit M_r (Section 4.2.1) and so it is likely that each enzyme subunit contains one haem group. The absorption peak at 360 nm (oxidised form) and 336 nm (reduced form) were not used for calculations because Triton X-100 absorbs at these wavelengths.

Figure 4.3 Absorption spectra of L(+)-mandelate dehydrogenase of *R graminis*

L(+)-Mandelate dehydrogenase was purified (Methods 2.7) and concentrated by vacuum dialysis. L(+)Mandelate dehydrogenase (100 $\mu g\ ml^{-1}$ i.e. 1.67 μM) was scanned in a quartz cuvette using a Pye SP8-100 spectrometer at wavelength scan speed of 1 $nm\ s^{-1}$. The enzyme was scanned before and after incubation with 10 μmol of L(+)-mandelate for 90 minutes . The oxidised and mandelate-reduced states of the enzyme are represented by solid and dashed lines respectively.

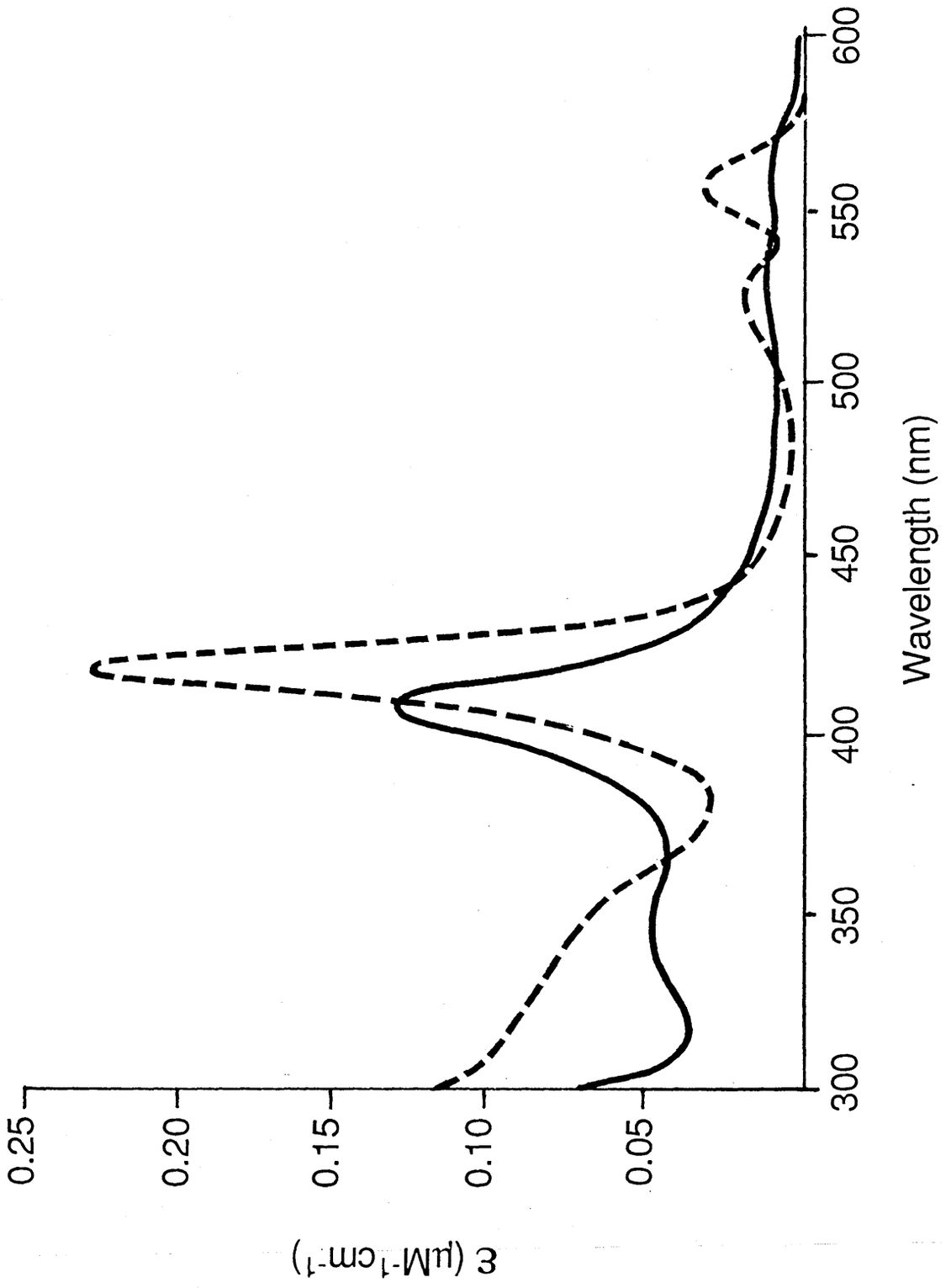

0.25
0.20
0.15
0.10
0.05
ε (μM-1cm-1)
300
350
400
450
500
550
600
Wavelength (nm)

Figure 4.4 Absorption spectra of standard solutions of FAD, FMN and a trichloroacetic acid extract of L(+)-mandelate dehydrogenase of *R. graminis*

The absorption spectra of standard solutions of (a) FAD (21.0 μM) (b) FMN (41.5 μM) and (c) a trichloroacetic acid extract of L(+)-mandelate dehydrogenase (16.67 μM) in quartz cuvette were recorded using a Pye SP8-100 spectrometer at wavelength scan speed of 1 nm s^{-1}. The standard solutions of FAD and FMN were treated with trichloroacetic acid in a similar way to the L(+)-mandelate dehydrogenase (Methods 2.9).

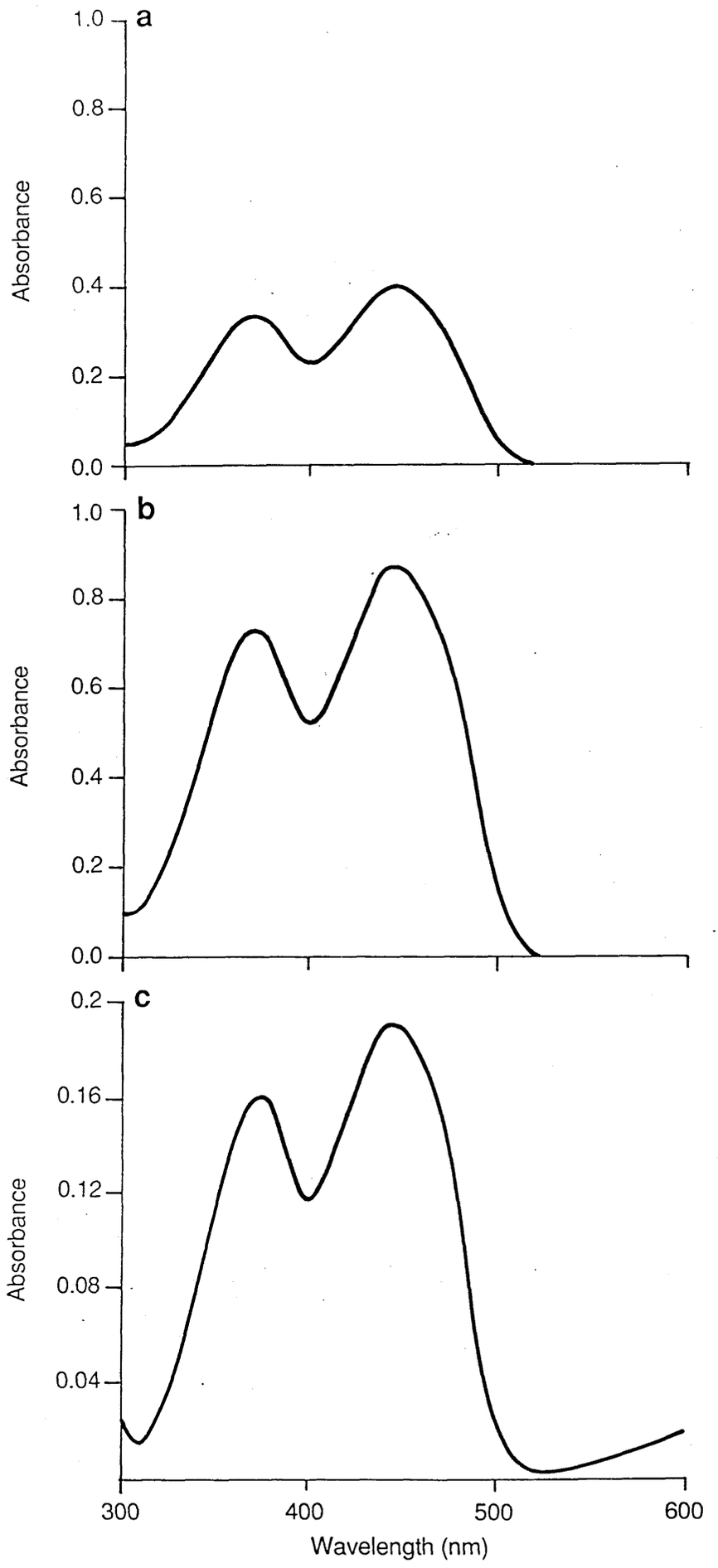
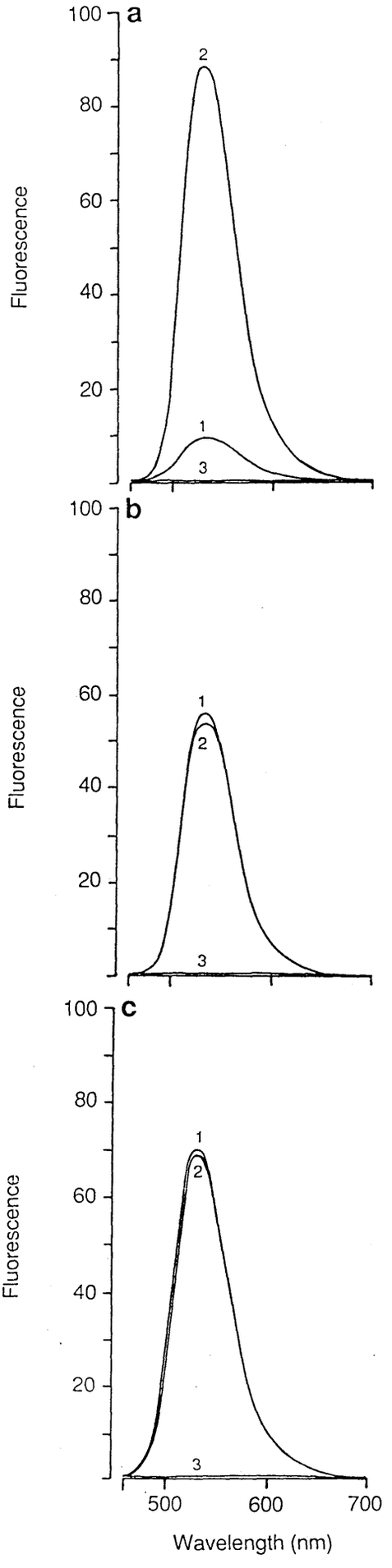

a
b
c
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0.16
0.12
0.08
0.04
300
400
500
600
Wavelength (nm)

Figure 4.5 Emission fluorescence spectra of standard solutions of FAD, FMN and a trichloroacetic acid extract of L(+)-mandelate dehydrogenase

The emission fluorescence spectra (1) of standard solutions of (a) FAD (0.15 μM), (b) FMN (1.33 μM) and (c) trichloroacetic acid extract of L(+)-mandelate dehydrogenase (190 μg) in 3 ml quartz cuvettes were recorded in a Perkin-Elmer 3000 fluorescence spectrometer (Methods 2.9). The standard solutions of FAD and FMN were processed similarily to the L(+)-mandelate dehydrogenase (Methods 2.9). The samples were excited at wavelength of 440 nm and fluorescence spectra were recorded from 450 to 700 nm. The fluorescence spectra were also recorded after mixing with few crystals of (2) *Naja naja* snake venom or (3) sodium dithionite.


a
b
c
100
80
60
40
20
Fluorescence
1
2
3
500
600
700
Wavelength (nm)

4.2.4.2 Identification of flavin

The absorption spectra of trichloroacetic acid extracts of the enzyme were very similar to those of standard FAD and FMN solutions (Fig. 4.4). The extracts gave absorption peaks at 445 and 372 nm. However on one occasion the lower wavelength absorption peak was found at 360 nm. This shift may have been due to the relatively larger amount of Triton X-100 present in the diluted extract from 0.15 mg of purified L(+)-mandelate dehydrogenase; extract from one mg of enzyme did not give this problem. Presumably this was because there was more protein relative to Triton present in the latter case.

To investigate further the nature of the flavin, the trichloroacetic acid extract of the enzyme was excited at 440 nm and gave an emission fluorescence spectrum which was identical to that of standard FAD and FMN (Fig. 4.5). The fluorescence of standard FAD, FMN and trichloracetic acid extract of the enzyme was abolished by the addition of a few crystals of sodium dithionite. The addition of a few crystals of *Naja naja* snake venom resulted in a 9-fold increase in the fluorescence of standard FAD solution at 523 nm but had no effect on the fluorescence spectra of standard FMN solution or the trichloroacetic acid extract of the enzyme (Fig. 4.5). This suggest the presence of FMN as prosthetic group of the enzyme.

The amount of FMN present was calculated to be 0.55 ± 0.086 mol/mol of subunit M_r (average of four determinations) using the absorption and fluorescence spectra values for standard FMN. The pellet obtained after treatment of the enzyme with trichloroacetic acid contained a further 0.2 to 0.25 mol of FMN per mol of subunit which may have been due to precipitation of flavin in Triton micelles. Therefore, in total, the purified enzyme contains approximately 0.8 mol of FMN per mol of subunit and so it seems likely that one mol of FMN is present per subunit of the native enzyme.

From all these results, it appears that L(+)-mandelate dehydrogenase from *R. graminis* contain equimolar concentrationsof FMN and haem. Similar results have been reported for L(+)-lactate dehydrogenase from *S. cerevisiae* and *H. anomala* (Appleby & Morton, 1954; Jacq & Lederer, 1974; Gervais *et al.*, 1983; Haumont *et al.*, 1987; Xia *et al.*,

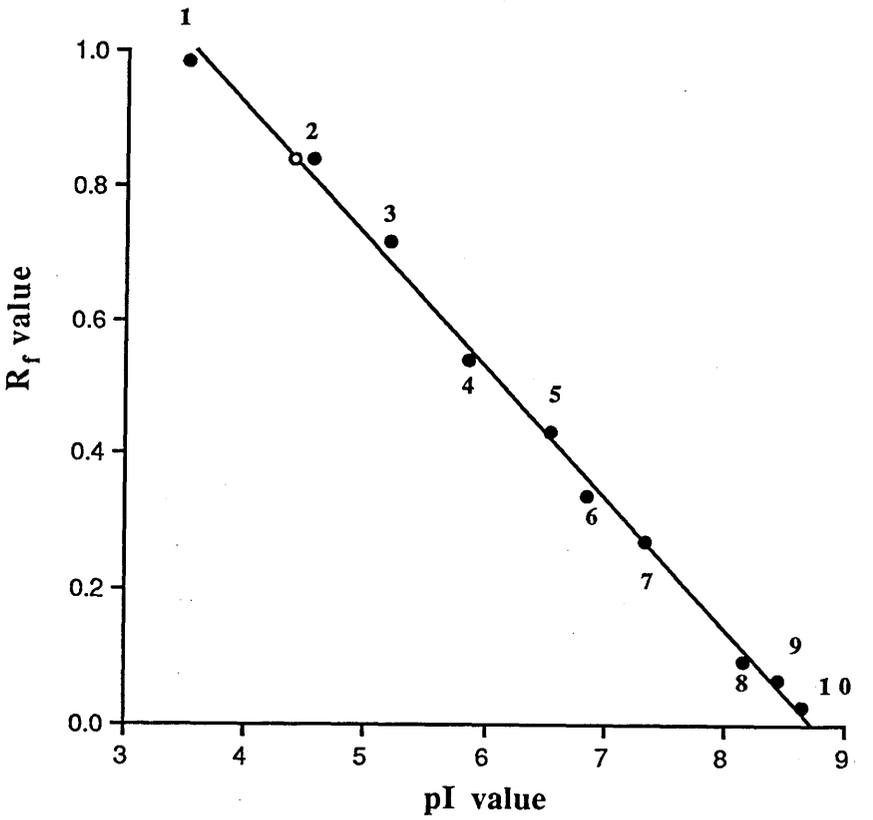


Figure 4.6 Isoelectric point of L(+)-mandelate dehydrogenase of *R. graminis*

L(+)-Mandelate dehydrogenase (0.5 µg) was run on the PhastGel system with standard proteins (Methods 2.11). Proteins were located on the the isoelectric focussing gel by silver staining (Methods 2.11). The distance travelled from the bottom of the gel was calculated for the standard proteins and for L(+)-mandelate dehydrogenase in order to calculate the pI value. The R_f value for L(+)-mandelate dehydrogenase is represented by an open circle on the graph.

(1) Amyloglucosidase (pI 3.5)
(2) Soybean trypsin inhibitor (pI 4.55)
(3) ß-Lactoglobulin A (pI 5.20)
(4) Bovine carbonic anhydrase (pI 5.85)
(5) Human carbonic anhydrase (pI 6.55)
(6) Horse myoglobin (pI 6.85)
(7) Horse myoglobin (pI 7.35)
(8) Lentil lectin (pI 8.15)
(9) Lentil lectin (pI 8.45)
(10) Lentil lectin (pI 8.65)

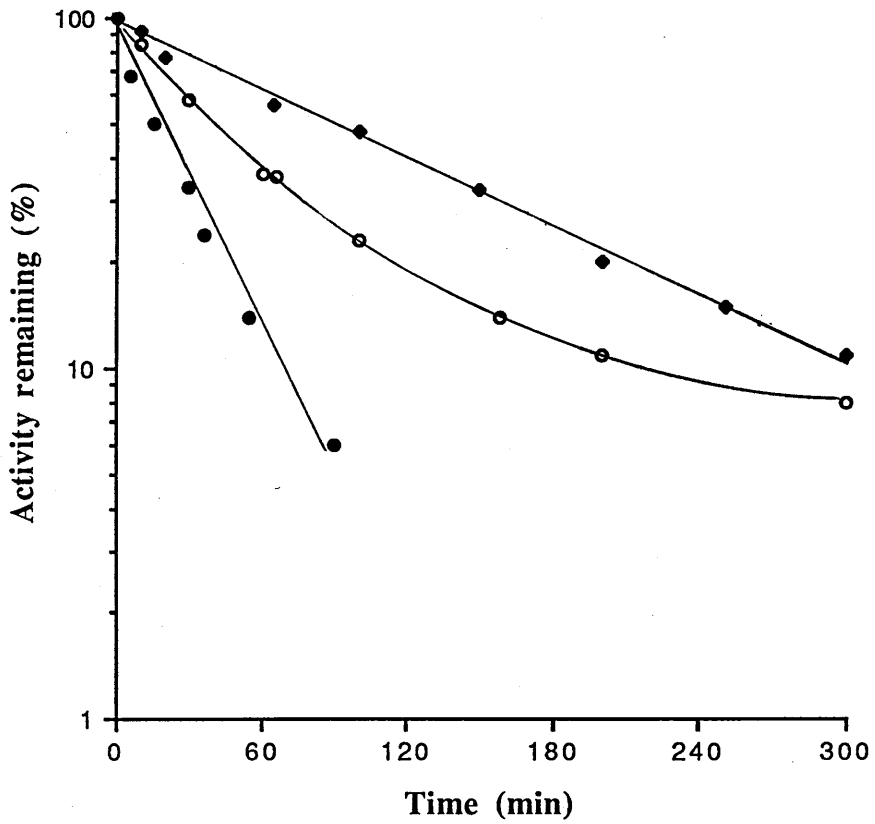


Figure 4.7 Representative plots of thermal inactivation of L(+)-mandelate dehydrogenase of *R.graminis* used to calculate time to reach 50 % inactivation at 27^{0}C

Purified L(+)-mandelate dehydrogenase of *R. graminis* was diluted 12-fold in: (•) 20 mM Tris buffer, pH 7.5; (○) 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1}; (◆) 20 mM Tris buffer, pH 7.5, containing 23 % ethanediol. All samples were incubated in duplicate at 27^{0}C. At various times after the start of incubation, samples were taken for the determination of residual L(+)-mandelate dehydrogenase activity using standard assay conditions as described in Methods 2.4.2. The concentration of L(+)-mandelate dehydrogenase in the incubation and assay mixtures were 1.25 μg ml^{-1} and 21 ng ml^{-1} respectively.

Table 4.3 Summary of thermal inactivation of L(+)-mandelate dehydrogenase of *R. graminis* when incubated under various conditions

Addition in the buffer[1]	Time (min) to reach 50% inactivation at		
	0^0C	27^0C	40^0C
Nothing	75 ± 2	19 ± 1	1.2
Glycerol (23%)	540	98	N.D.
Ethanediol (23%)	540	90	N.D.
DTT (0.92 mM)	196 ± 44	30 ± 13	N.D.
Triton (0.46mg/ml)	∞	41* ± 3	2.5
BSA (0.46mg/ml)	∞	39*	N.D.
Lubrol PX (0.46mg/ml)	∞	38*	N.D.
Triton (0.46mg/ml) with DTT (0.092 mM)	∞	42 ± 2	N.D.
D(-)-Mandelate (0.025 M)	120	38	N.D.
L(+)-Mandelate (0.025 M)	137 ± 9	69 ± 10	5.5.
Triton (0.46mg/ml) with D(-)-mandelate (0.03 M)	∞	>1000*	N.D.
Triton (0.46mg/ml) with L(+)-mandelate (0.025 M)	∞	∞	12
20 mM Tris/HCl (pH 6.8)	80	20	N.D.
20 mM Tris/HCl (pH 8.9)	61	5 ± 1	N.D.
20 mM Tris/HCl with 0.46mg Triton/ml (pH 6.8)	∞	46* ± 4	N.D.
20 mM Tris/HCl with Triton (0.46mg/ml) (pH 8.9)	∞	8 + 3	N.D.
20 mM Tris/HCl with 0.46mg Triton/ml and 0.025 M L(+)-mandelate (pH 6.8)	∞	>2000*	N.D.
20 mM Tris/HCl with 0.46mg Triton/ml and 0.025 M L(+)-mandelate (pH 8.9)	∞	630	N.D.

[1] The buffer was always 20 mM Tris/HCl, pH 7.5, values otherwise stated

* Not pseudo-first order

N.D. = Not determined

∞ = No detectable loss of activity over 600 minutes

Triton = Triton X-100

± represent SD of three determinations and other values are mean of two independependent experiments.

1987; Chapman *et al.*, 1991) rather than L(+)-lactate dehydrogenase and L(+)-mandelate dehydrogenase from *A. calcoaceticus* which possess FMN only as prosthetic group (Hoey *et al.*, 1987).

FMN added to less active L(+)-mandelate dehydrogenase preparations from *R. graminis* did not restore the original activity and paradoxically inhibited the enzyme activity even at low concentration of added FMN.

4.2.5 Isoelectric point

The isoelectric point of L(+)-mandelate dehydrogenase was determined by isoelectric focussing and found to be 4.4 (Fig. 4.6). The isoelectric point could not be determined by chromatofocussing using an FPLC Mono P chromatofocussing column because the enzyme was inactivated at pH 5.0 or below.

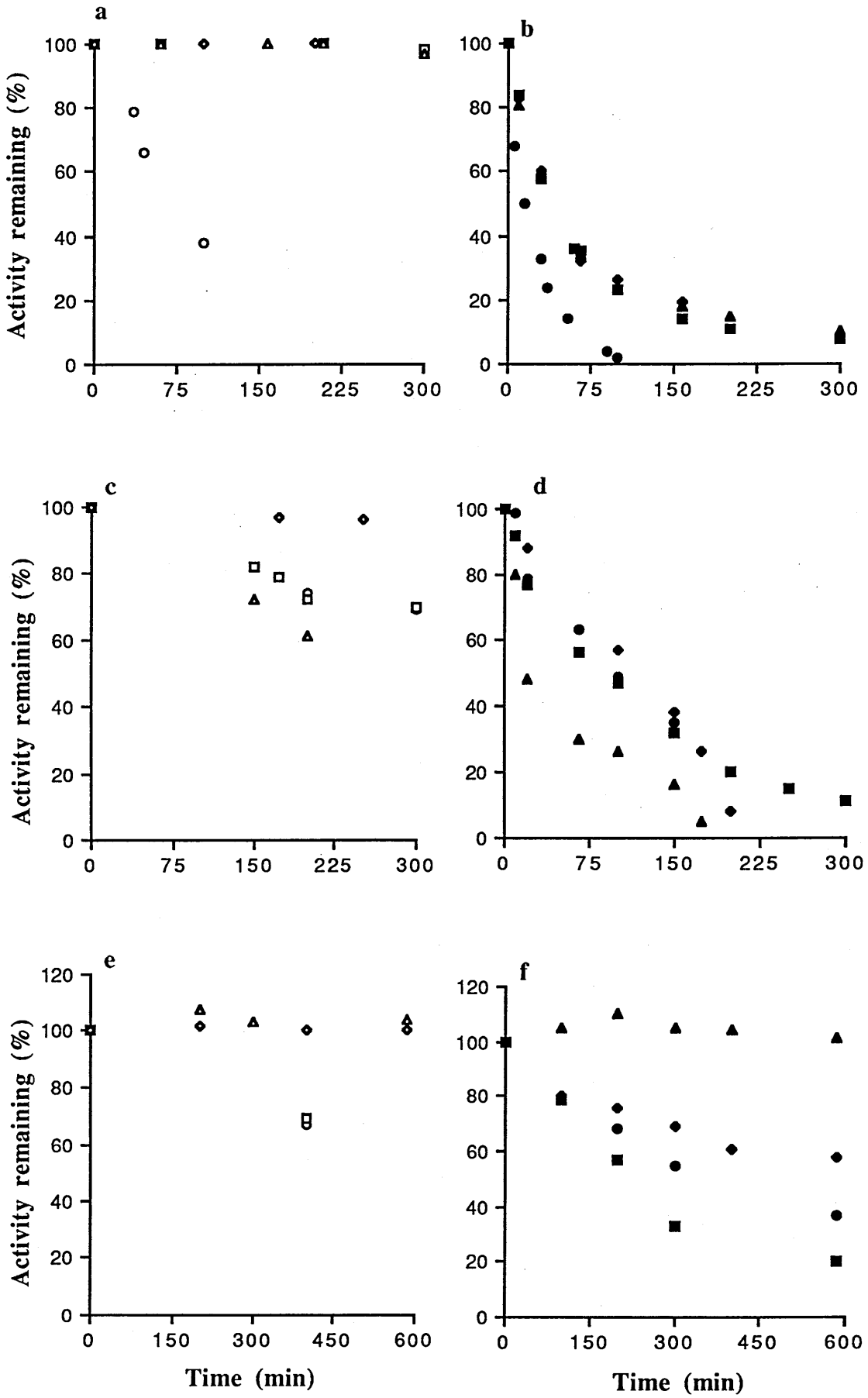
4.3 Effect of temperature on L(+)-mandelate dehydrogenase

4.3.1 Thermal stability of L(+)-mandelate dehydrogenase

Thermal stability of the enzyme was determined at various temperatures in the presence of different potential stabilizing agents. The times to achieve 50% inactivation were calculated from plots of the type illustrated in Fig. 4.7, and 50% inactivation times are summarized in Table 4.3. L(+)-Mandelate dehydrogenase was protected from loss of activity by the presence of L(+)-mandelate, D(–)-mandelate, ethanediol, glycerol, Triton X-100, Lubrol PX and BSA. In particular, substrate and Triton X-100 together stabilised the enzyme, almost completely at 27^0C and to some extent even at 40^0C (Fig. 4.8-4.11). L(+)-Mandelate dehydrogenase was less stable below pH 5.5 and especially above pH 9.5 (Fig. 4.10) even in the presence of substrate and Triton X-100 together. The effect of DTT on the stability of this enzyme was difficult to determine as it interfered with the enzyme activity. The rate of inactivation of L(+)-mandelate dehydrogenase was generally pseudo-first order but there was deviation from this pattern in the case of inactivation in the presence of detergents and BSA (Fig. 4.7).

Figure 4.8 Thermal inactivation of L(+)-mandelate dehydrogenase of *R.graminis* at 27^{0}C in the presence of various potential stabilising agents

Purified L(+)-mandelate dehydrogenase of *R. graminis* was diluted 12-fold in:
(a and b) (● , ○) 20 mM Tris buffer, pH 7.5; (□ , ■) 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1}; (▲ , ▲) 20 mM Tris buffer, pH 7.5, containing 0.46 mg BSA ml^{-1} ; (◆ , ◆) 20 mM Tris buffer, pH 7.5, containing 0.46 mg lubrol PX ml^{-1};
(c and d) (● , ○) 20 mM Tris buffer, pH 7.5, containing 23 % glycerol;
(□ , ■) 20 mM Tris buffer, pH 7.5, containing 23 % ethanediol; (▲ , ▲) 20 mM Tris buffer, pH 7.5, containing 0.092 mM DTT ml^{-1}; (◆ , ◆)) 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1} and 0.092 mM DTT ml^{-1};
(e and f) (● , ○) 20 mM Tris buffer, pH 7.5, containing 0.025 M L(+)-mandelate;
(□ , ■) 20 mM Tris buffer, pH 7.5, containing 0.025 M D(-)-mandelate;
(▲ , ▲) 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1}and 0.025 M L(+)-mandelate; (◆ , ◆)) 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1} and 0.03 M D(-)-mandelate.
All samples were incubated in duplicate at 0^{0}C (a, c, e as controls) and 27^{0}C (b, d, f). At various times after the start of incubation samples were taken for the determination of residual L(+)-mandelate dehydrogenase activity using standard assay conditions as described in Methods 2.4.2. The concentration of L(+)-mandelate dehydrogenase in the incubation and assay mixtures were 1.25 μg ml^{-1} and 21 ng ml^{-1} respectively. Results are shown for one experiment and several other replicate experiments gave very similar results.


a
b
c
d
e
f
Activity remaining (%)
Time (min)
0
20
40
60
80
100
120
0
75
150
225
300
450
600

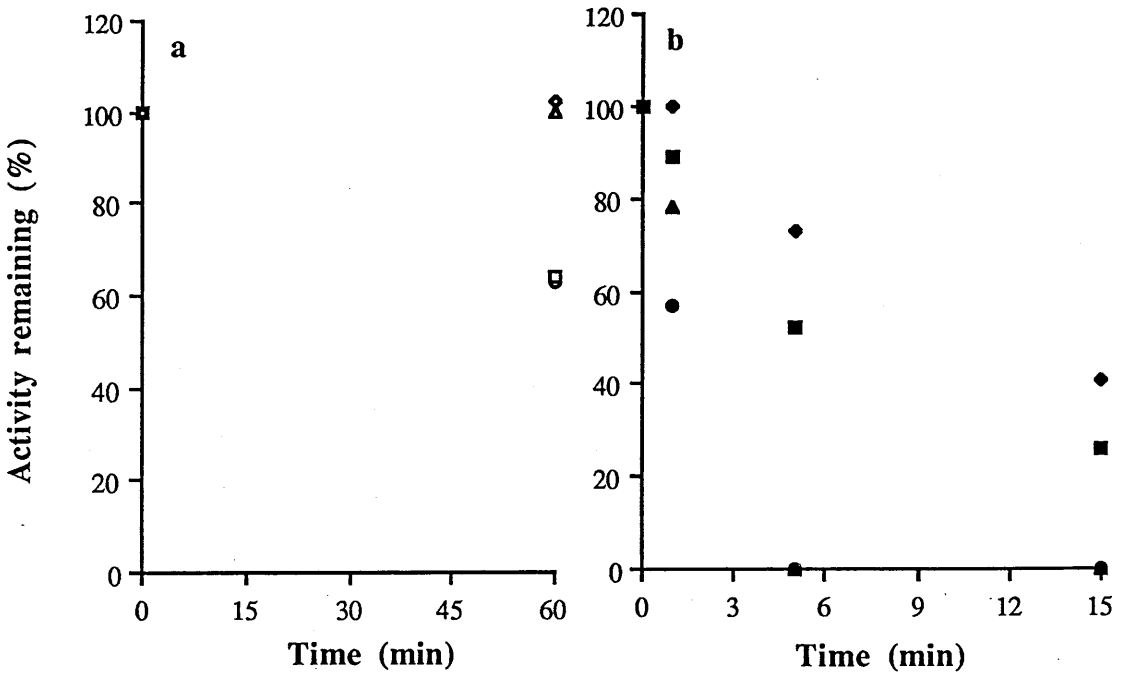


Figure 4.9 Thermal inactivation of L(+)-mandelate dehydrogenase of *R. graminis* at 40^{0}C

Purified L(+)-Mandelate dehydrgenase of *R. graminis* was diluted 12-fold in: (a) (○) 20 mM Tris buffer, pH 7.5, (▲); 20 mM Tris buffer, pH 7.5, containing 0.05 M L(+)-mandelate; (□) 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1} and (◆) 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1} and 0.05 M L(+)-mandelate. All samples were incubated in duplicate at 0^{0}C as controls for the experiment b.

(b) (●) 20 mM Tris buffer, pH 7.5, (◆); 20 mM Tris buffer, pH 7.5, containing 0.05 M L(+)-mandelate; (■) 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1} and (◆) 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1} and 0.05 M L(+)-mandelate. All samples were incubated in duplicate at 40^{0}C. At various times after the start of incubation samples were taken for the determination of residual L(+)-mandelate dehydrogenase activity using standard assay conditions as described in Methods 2.4.2. The concentration of L(+)-mandelate dehydrogenase in the incubation and assay mixtures were 1.25 μg ml^{-1} and 21 ng ml^{-1} respectively.

Figure 4.10 Thermal inactivation of L(+)-mandelate dehydrogenase of *R.graminis* at various pH values

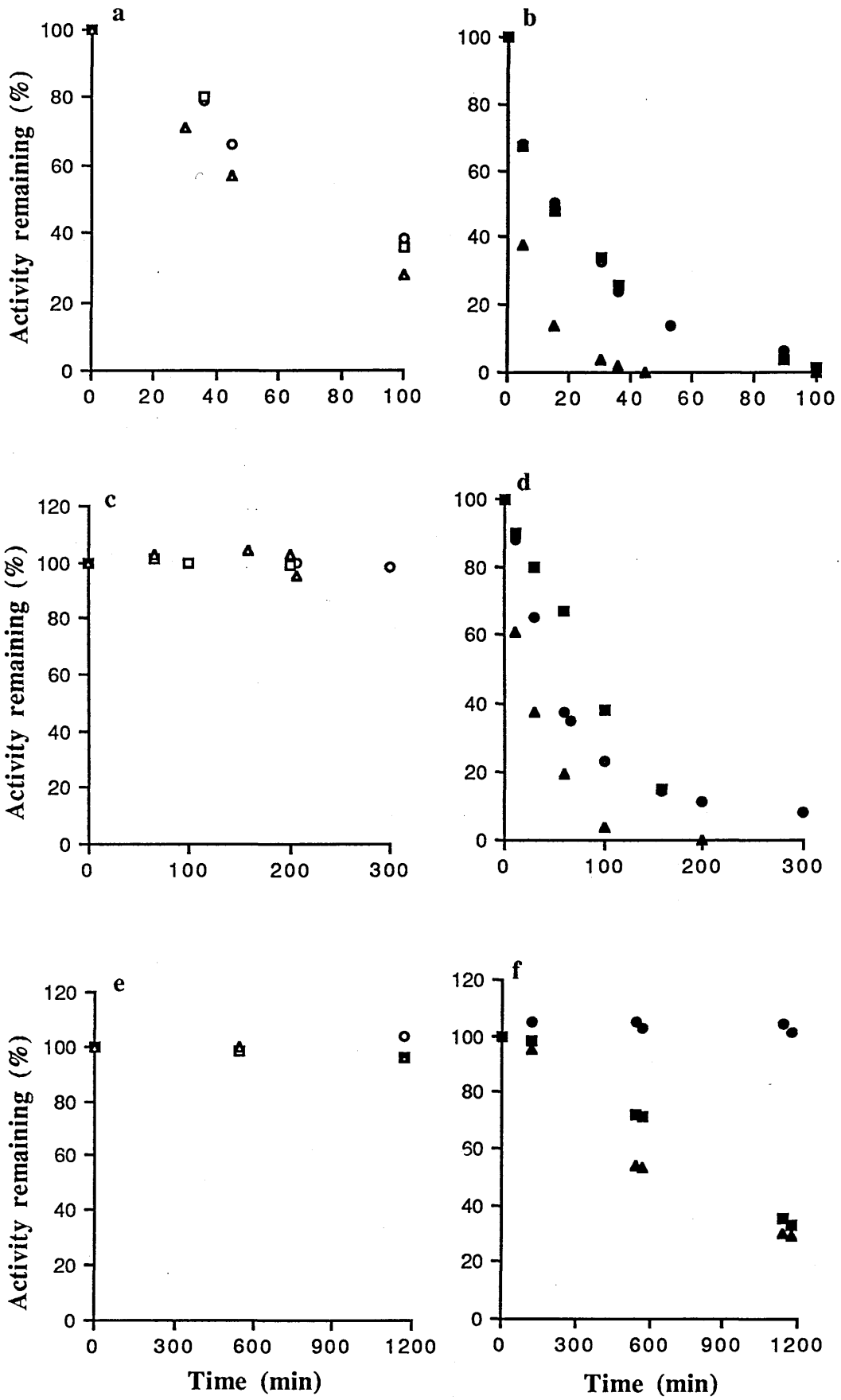
Purified L(+)-mandelate dehydrogenase of *R. graminis* was diluted 12-fold in 20 mM Tris buffer:

(a and b) (□ , ■) pH 6.8 , (○ , ●) 7.5, (△ , ▲) 8.9,

(c and d) (□ , ■) pH 6.8, (○ , ●) 7.5, (△ , ▲) 8.9, containing 0.46 mg Triton X-100 ml^{-1},

(e and f) (□ , ■) pH 6.8, (○ , ●) 7.5, (△ , ▲) 8.9, containing 0.46 mg Triton X-100 ml^{-1} and 0.025 M L(+)-mandelate.

All samples were incubated in duplicate at 0^0C (a, c, e as controls) and 27^0C (b, d, f). At various times after the start of incubation samples were taken for the determination of residual L(+)-mandelate dehydrogenase activity using standard assay conditions as described in Methods 2.4.2. The concentration of L(+)-mandelate dehydrogenase in the incubation and assay mixtures were 1.25 μg ml^{-1} and 21 ng ml^{-1} respectively. Results are shown for one experiment and other replicate experiments gave very similar results.


a
b
c
d
e
f
Activity remaining (%)
Time (min)
0
20
40
60
80
100
120
100
200
300
600
900
1200

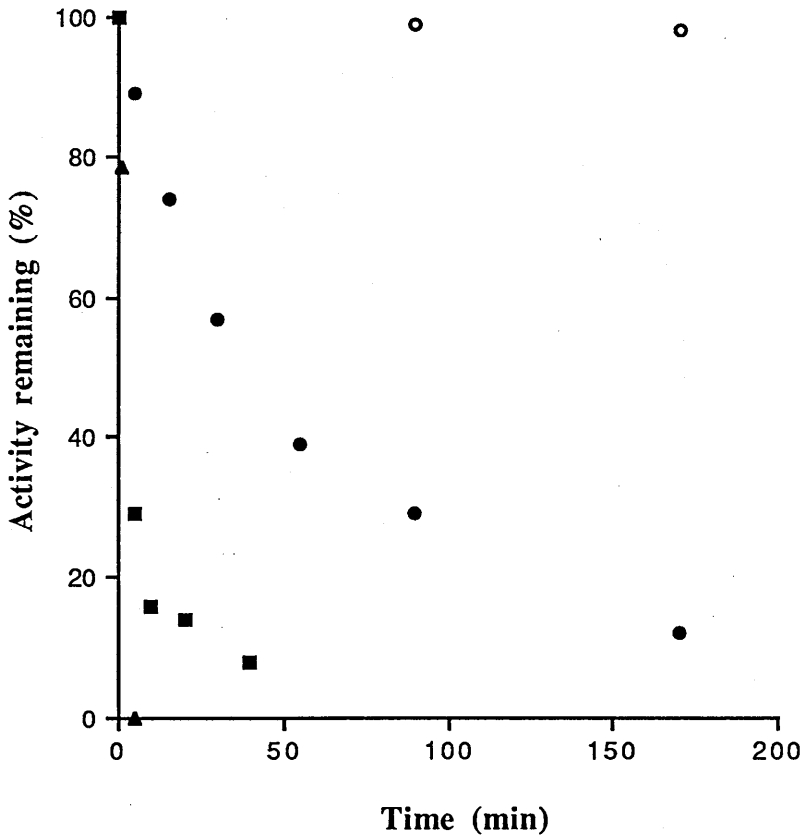


Figure 4.11 Thermal inactivation of L(+)-mandelate dehydrogenase of *R.graminis* at various temperatures

Purified L(+)-mandelate dehydrogenase of *R. graminis* diluted 12-fold in 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1} was incubated in duplicate at (○) 0^0C, (●) 27^0C, (■) 33^0C and (▲) 40^0C. At various times after the start of incubation samples were taken for the determination of residual L(+)-mandelate dehydrogenase activity using the standard assay conditions described in Methods 2.4.2. The concentration of L(+)-mandelate dehydrogenase in the incubation and assay mixtures were 1.25 μg ml^{-1} and 21 ng ml^{-1} respectively. Results are shown for one experiment and another replicate experiment gave very similar results.

Figure 4.12 Reactivation of L(+)-mandelate dehydrogenase of *R. graminis* at 0^0C and 27^0C following thermal inactivation

Purified L(+)-mandelate dehydrogenase of *R. graminis* was diluted 12-fold in: (●) 20 mM Tris buffer, pH 7.5, (■) 20 mM Tris buffer, pH 7.5, containing 0.05 M L(+)-mandelate, (▲) 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1}, (◆) 20 mM Tris buffer, pH 7.5, containing 0.46 mg Triton X-100 ml^{-1} and 0.05 M L(+)-mandelate. Three sets of replicate samples of each of this condition were inactivated at 40^0C for a total of 15 min.

(a) A set of inactivated replicate samples were incubated on ice.

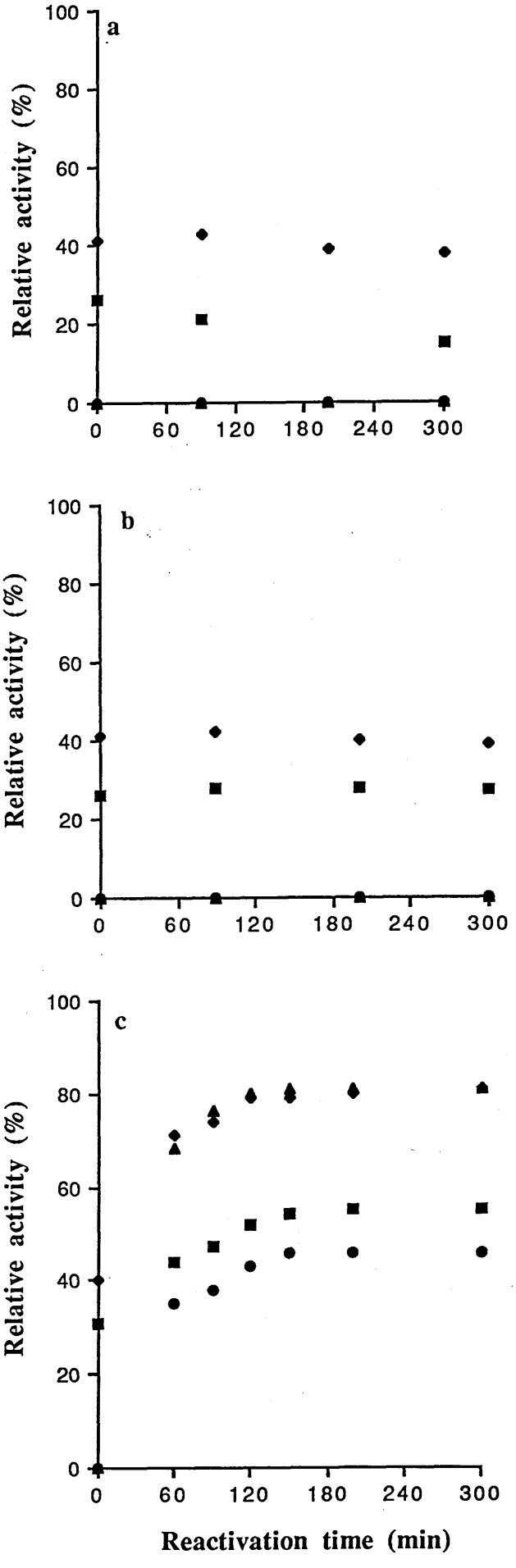
(b) To a set of inactivated samples Triton X-100 or L(+)-mandelate or both were added, whichever was missing, and the mixtures were incubated on ice.

(c) To a set of inactivated samples Triton X-100 or L(+)-mandelate or both were added, whichever was missing, and the mixtures were incubated at 27^0C.

At various times after the start of incubation, samples were taken for the determination of L(+)-mandelate dehydrogenase activity using the standard assay conditions described in Methods 2.4.2. The concentration of L(+)-mandelate dehydrogenase in the incubation and assay mixtures were 1.25 μg ml^{-1} and 21 ng ml^{-1} respectively. Results are given as mean of replicates in two independent experiments.

Pre-incubation mixture	Reaction mixture
●, LMDH + Buffer	LMDH + Buffer + LMD + Triton
▲, LMDH + Buffer + Triton	LMDH + Buffer + LMD + Triton
■, LMDH + Buffer + LMD	LMDH + Buffer + LMD + Triton
◆, LMDH + Buffer + LMD + Triton	LMDH + Buffer + LMD + Triton

LMDH, L(+)-mandelate dehydrogenase; LMD, L(+)-mandelate; Triton, Triton X-100


a
b
c
Relative activity (%)
100
80
60
40
20
0
0
60
120
180
240
300
Reactivation time (min)

4.3.2 Reactivation of thermally inactivated L(+)-mandelate dehydrogenase

L(+)-Mandelate dehydrogenase was totally inactivated in less than five minutes at 40^0C in the presence of buffer alone (Fig. 4.9, 4.11). Samples of L(+)-mandelate dehydrogenase that had been inactivated at 40^0C in the presence of buffer alone or Triton X-100 or substrate or Triton X-100 and substrate together for 15 minutes were incubated at 0^0C to measure possible reactivation. None of the samples was reactivated (Fig. 4.12). Later, in similar experiments L(+)-mandelate dehydrogenase samples were inactivated at 40^0C for 15 min in the presence of buffer alone or Triton X-100 or substrate or Triton X-100 and substrate together . Triton X-100 or L(+)-mandelate or both (whichever was missing) were added to the thermally inactivated samples. These samples were incubated at 0^0C and 27^0C. L(+)-Mandelate dehydrogenase was reactivated to 80-85% of its original activity at 27^0C in samples that had been inactivated in the presence of Triton or Triton and substrate together. The samples inactivated in the presence of substrate or buffer alone were 50-55% reactivated at 27^0C. The reactivation was appreciable at 27^0C but very little reactivation occurred at 0^0C (Fig. 4.12).

4.4 Kinetic studies

4.4.1 Development of assay conditions

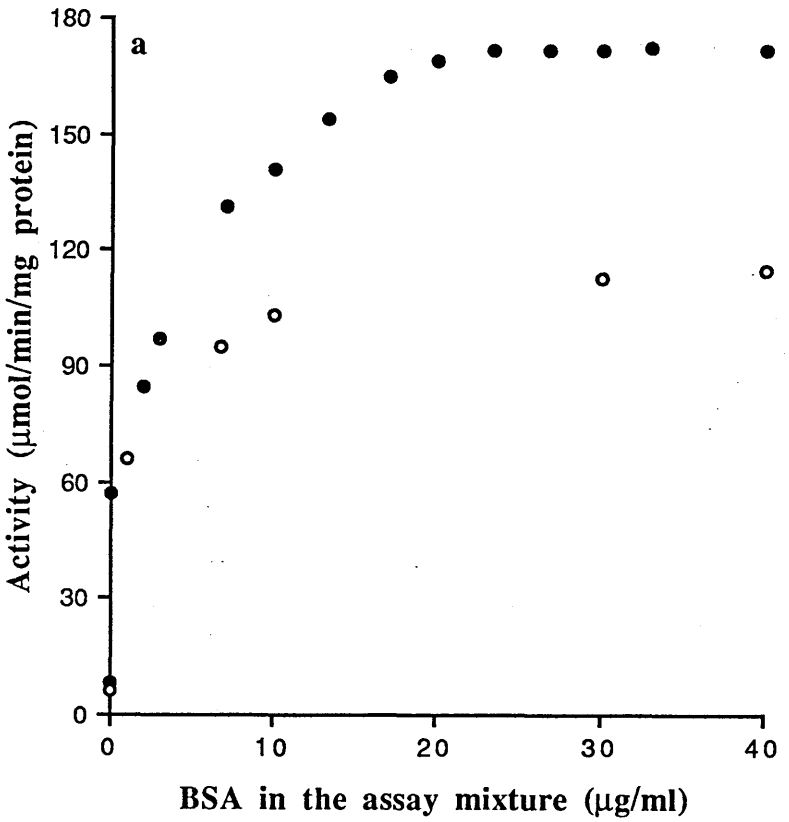
In initial experiments L(+)-mandelate dehydrogenase was assayed by following the reduction of 2,6-dichlorophenol-indophenol (DCIP) at 600 nm, basically as described by Durham (1984) with minor changes. The reaction mixture contained 200 µmol phosphate buffer, pH 7.5, 25 µmol of L(+)-mandelate, 0.16 µmol of DCIP and extract and the volume was made to three ml by adding distilled water. However, there was a constant decline of rate, producing a curved reaction plot of ΔA_{600} against time. Therefore a modified assay system was developed in order to obtained linear and maximum rates over 2-3 min. In particular, the effects of BSA, Triton X-100 and PMS were tested since these had been shown to have large effects on the activity of bacterial membrane-bound mandelate dehydrogenase (Hoey *et al.*, 1987; Allison *et al.*, 1985a; Allison & Fewson, 1986).

Figure 4.13 Effects of BSA on the activity of L(+)-mandelate dehydrogenase of *R. graminis*

Purified L(+)-mandelate dehydrogenase of *R. graminis* was diluted 12-fold in (●) 20 mM Tris buffer, pH 7.5, containing 0.5 mg Triton X-100 / ml or (○) in 20 mM Tris buffer, pH 7.5. Samples were taken for the determination of activity using the standard assay conditions described in Methods 2.4.2, except that various concentrations of BSA were included in the assay mixture. The concentration of L(+)-mandelate dehydrogenase in the assay mixture was 21 ng ml^{-1}. Results are given as means of duplicate experiment and some other experiments revealed very similar results.

(a) The increase in activity of L(+)-mandelate dehydrogenase in the presence of low concentrations of BSA in the assay mixture.

(b) The inhibitory effect of high concentrations of BSA in the assay mixture on the activity of L(+)-mandelate dehydrogenase [note the different scale from that used in (a)].


a
180
150
120
90
60
30
0
0
10
20
30
40
Activity (µmol/min/mg protein)
BSA in the assay mixture (µg/ml)

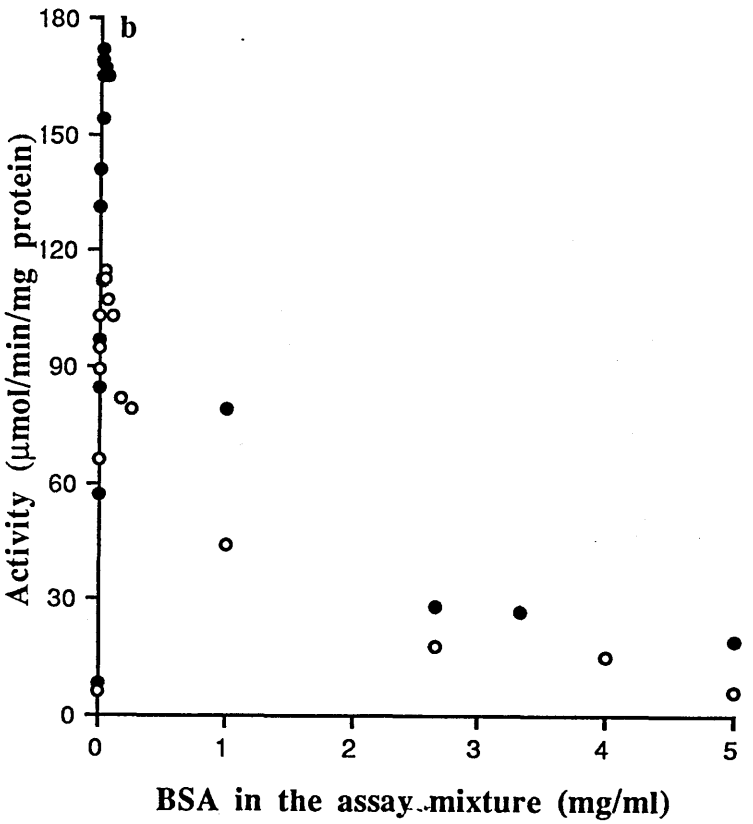
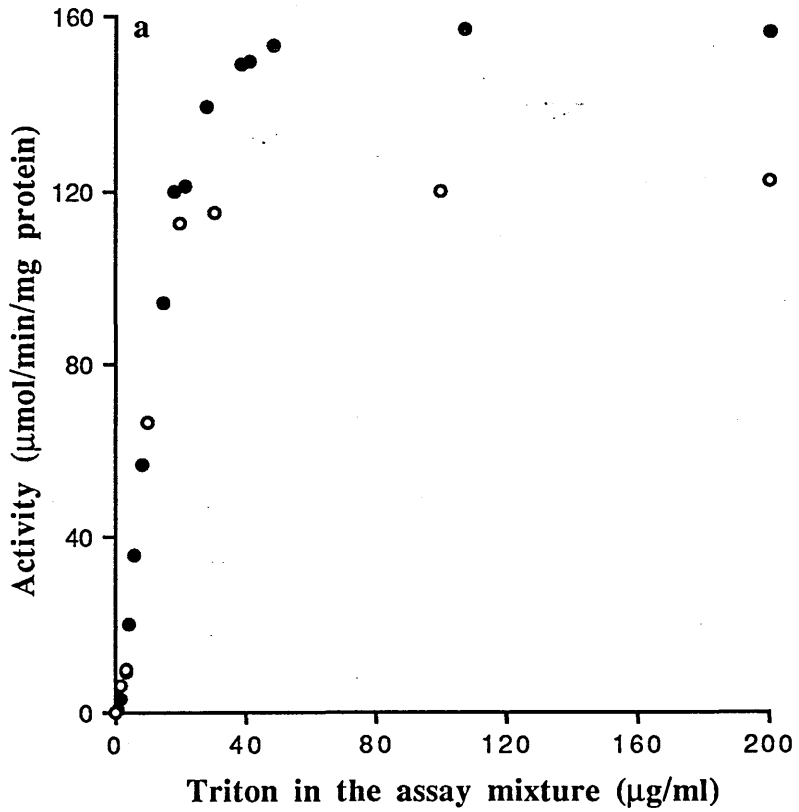

b
180
150
120
90
60
30
0
0
1
2
3
4
5
Activity (µmol/min/mg protein)
BSA in the assay mixture (mg/ml)

Figure 4.14 Effects of Triton X-100 on the activity of L(+)-mandelate dehydrogenase of *R. graminis*

Purified L(+)-mandelate dehydrogenase of *R. graminis* was diluted 12-fold in (•) 20 mM Tris buffer, pH 7.5, containing 0.5 mg Triton X-100 / ml or (○) in 20 mM Tris buffer, pH 7.5. Samples were taken for the determination of L(+)-mandelate dehydrogenase activity using the standard assay conditions described in Methods 2.4.2, except that various concentrations of Triton X-100 were included in the assay mixture. The concentration of L(+)-mandelate dehydrogenase in the assay mixture was 21 ng ml^{-1}. Results are given as means of two independent experiments in which individual results were well within 5 % of each other.

a) The increase in activity of L(+)-mandelate dehydrogenase in the presence of low concentrations of Triton X-100 in the assay mixture.

(b) The inhibitory effect of high concentrations of Triton X-100 in the assay mixture on the activity of L(+)-mandelate dehydrogenase [note the different scale from that used in (a)].


a
160
120
80
40
0
Activity (μmol/min/mg protein)
0
40
80
120
160
200
Triton in the assay mixture (μg/ml)

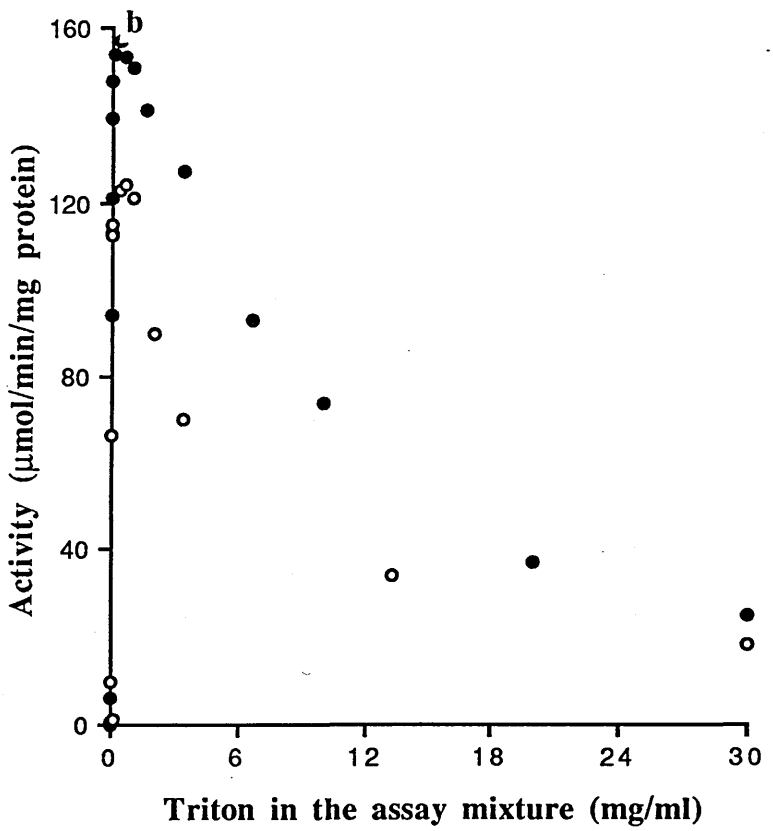
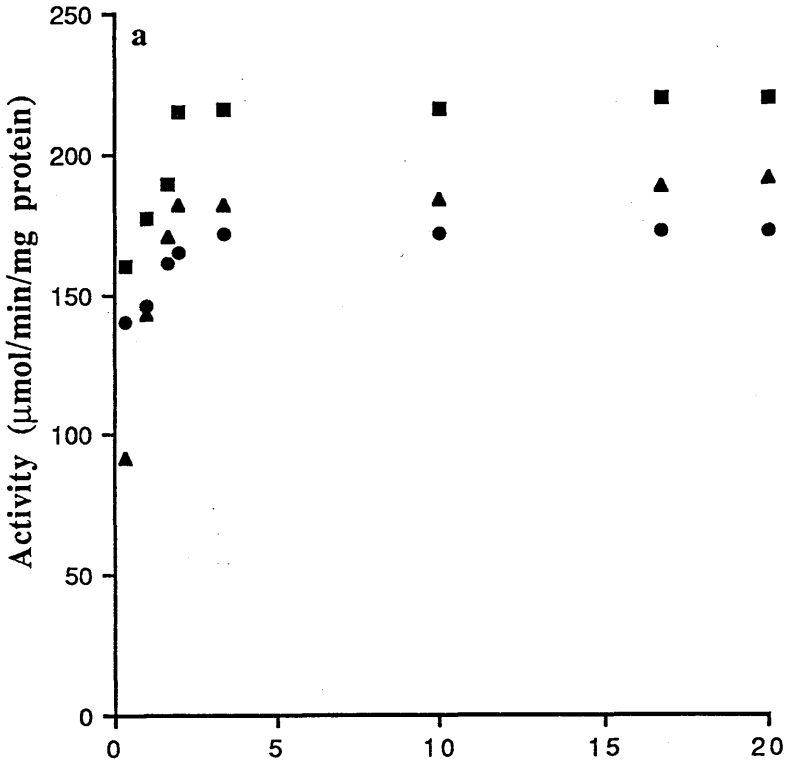

b
160
120
80
40
0
Activity (μmol/min/mg protein)
0
6
12
18
24
30
Triton in the assay mixture (mg/ml)

Figure 4.15 Effect of PMS on the activity of L(+)-mandelate dehydrogenase of *R. graminis* in the presence of various concentrations of BSA and Triton X-100

Purified L(+)-mandelate dehydrogenase of *R. graminis* was diluted 12-fold in 20 mM Tris buffer, pH 7.5 containing 0.5 mg Triton X-100. Samples were taken for the determination of activity using standard assay conditions as described in Methods 2.4.2, except that in (a) BSA was (●) 10 μg ml^{-1}, (■) 66.67 μg ml^{-1} or (▲) 1 mg ml^{-1} and in (b) Triton X-100 was (●) 10 μg ml^{-1}, (■) 333 μg ml^{-1} or (▲) 6.67 mg ml^{-1} and there were various amounts of PMS in the assay mixture. The concentration of L(+)-mandelate dehydrogenase in the assay mixture was 21 ng ml^{-1}. Results are given as means of duplicate experiments.


a
Activity (µmol/min/mg protein)
250
200
150
100
50
0
0
5
10
15
20

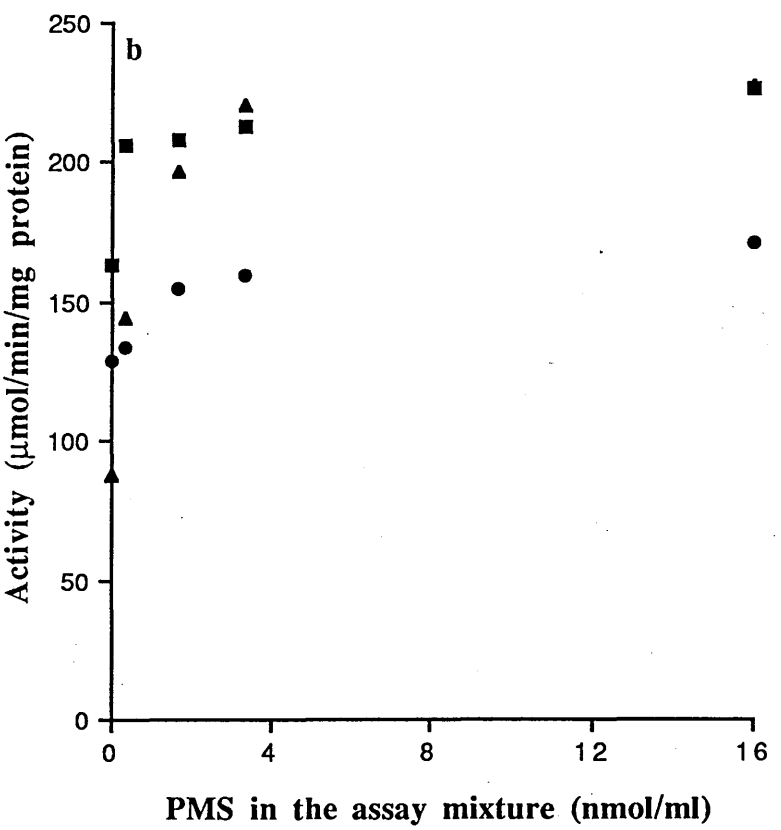

b
Activity (µmol/min/mg protein)
250
200
150
100
50
0
0
4
8
12
16
PMS in the assay mixture (nmol/ml)

Table 4.4 Summary of the effects of BSA, Triton X-100 and PMS on the activity of L(+)-mandelate dehydrogenase of *R. graminis*

Purified L(+)-mandelate dehydrogenase of *R. graminis* was diluted 12-fold in 20 mM Tris buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. Samples were taken for the determination of activity using the standard assay conditions as described in Methods 2.4.2, except that various concentrations of BSA, Triton X-100 and PMS were included in the reaction mixture. The concentration of L(+)-mandelate dehydrogenase in the reaction mixture was 21 ng ml^{-1}. Results are given as means of two independent experiments.

BSA	Triton X-100	PMS	Activity (nmol/min/mg protein)
-	-	-	**1**
-	-	++	**2**
++	-	-	**156**
-	++	-	**152**
++	++	-	**155**
-	++	++	**188**
++	++	++	**194**
+	-	-	**103**
-	+	-	**127**
+	+	-	**159**
+	-	++	**133**
-	+	++	**150**
+	+	++	**190**

++ : 66.67 μg of BSA or 300 μg of Triton X-100 or 10 nmol of PMS per ml of assay mixture or combination of the three as shown in the Table.

+ : 7 μg of BSA, 17.7 μg of Triton X-100 per ml of assay mixture or combination of the two with or without 10 nmol of PMS as shown in the Table.

Table 4.5 Comparison of the effects of BSA and PMS on purified L(+)- mandelate dehydrogenase from *R.graminis* and *A. calcoaceticus* with the effects on enzymes in extracts and washed cell membranes from *R.graminis*, *A. calcoaceticus* and *P. putida*

Purified L(+)- mandelate dehydrogenase from *R. graminis* and *A. calcoacticus* were diluted 12-fold and 30-fold in 20 mM Tris buffer, pH 7.5, respectively. *R. graminis* extract and washed cell membranes were diluted 16-fold each; *A. calcoaceticus* extract and washed cell membranes were diluted 50-fold and 100-fold respectively whereas *P. putida* extract and washed cell membranes were both diluted 50-fold each. Extracts and washed cell membranes (WCM) from *R. graminis, A. calcoaceticus* and *P. putida* were diluted in 20 mM Tris buffer, pH 7.5 . Extracts of *A. calcoaceticus* and *P. putida* already contained 1.0 mg of Triton X-100 ml^{-1} and 1% ethanediol whereas purified samples contained 0.015 mg of Triton X-100 ml^{-1} and 1% ethanediol in diluted samples. Samples were taken from these diluted samples to assay enzyme activities. The initial velocity in the presence of 3.33mg BSA ml^{-1} , 333 μM PMS and 8.33 mM L(+)-mandelate was taken as 100 % in each case. Values are given as means of three experiments in which the individual results were well within 3% of each other but in the absence of BSA the values varied by up to 8 % .

N.D. = Not determined.

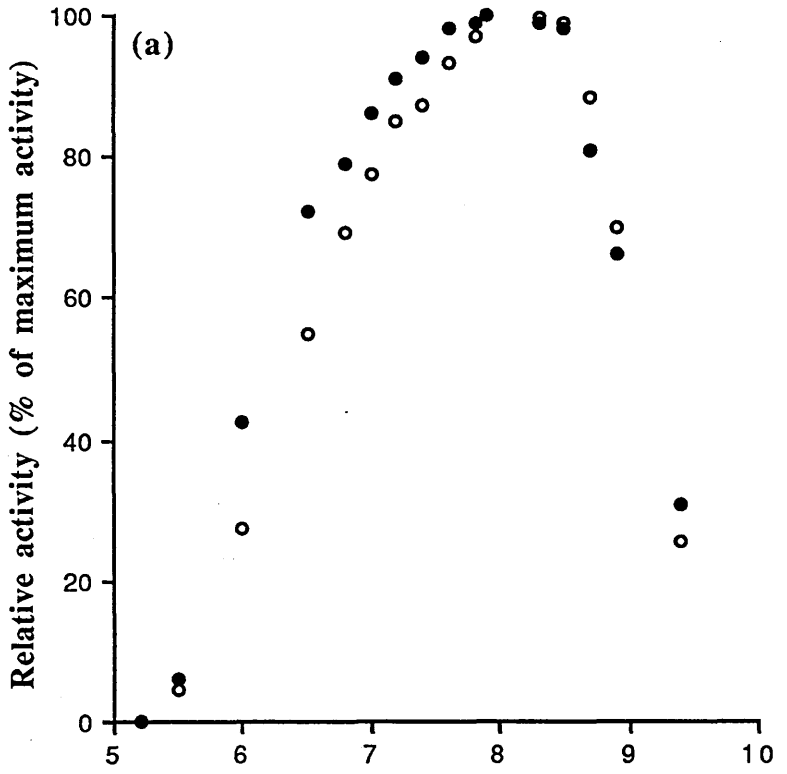
	R. graminis			*A. calcoaceticus*			*P. putida*	
	Pure enzyme	Extract	WCM	Pure enzyme	Extract	WCM	Extract	WCM
Decrease (%) in activity if PMS was omitted from the reaction mixture with								
3.333 mg BSA ml^{-1}	75	78	77	72	76	82	87	86
66.67 µg BSA ml^{-1}	12	18	17	6	7	23	43	45
Decrease (%) in activity if BSA was omitted from the reaction mixture with								
333 µM PMS	69	0	0	44	46	0	0	0
3.33 µM PMS	92-99	5	0	47	42	40	54	47
Decrease (%) in activity if 66.67 µg BSA ml^{-1} and 3.33 µM PMS were present in the reaction mixture	0	0	0	0	3	18	40	43
Decrease (%) in activity if 3.33 mg BSA ml^{-1} and 3.33 µM PMS were present in the reaction mixture	15	18	N.D.	12	N.D.	N.D.	N.D.	N.D.
Decrease (%) in activity if both BSA and PMS were omitted from the reaction mixture	92-99	22	12	53	51	44	56	54

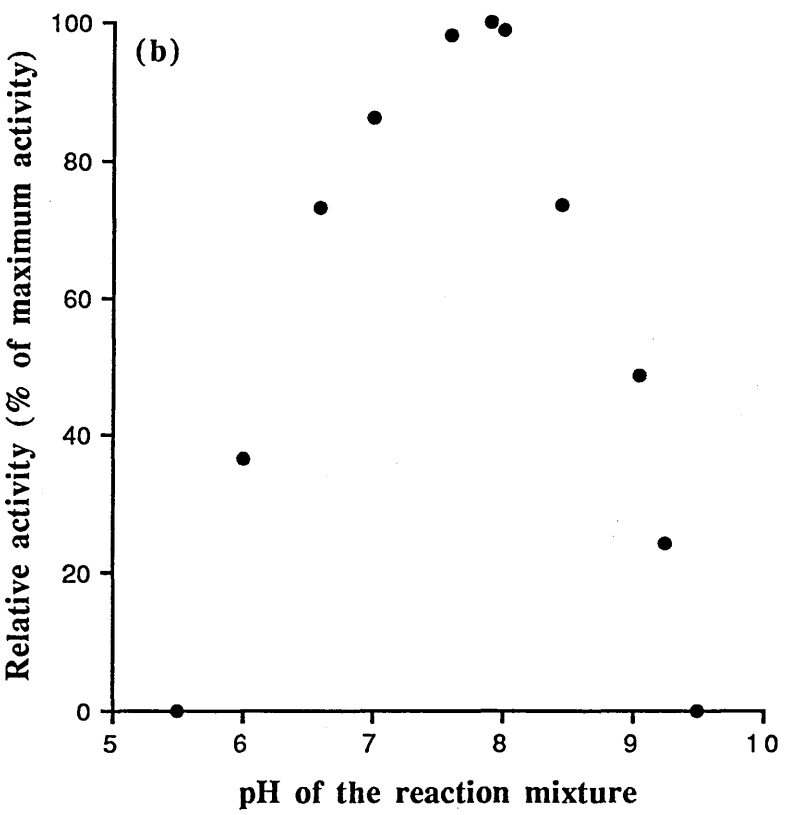
Figure 4.16 The effect of pH on the activity of L(+)-mandelate dehydrogenase in the presence of BSA or Triron X-100

The effect of pH on L(+)-mandelate dehydrogenase activity was estimated over the pH range 5.0 to 10.0. The molar absorption coefficients for DCIP were determined over this range (Fig. 2.1) and used to calculate rates at the respective pH values. The pH values of the reaction mixtures were determined as described in Methods 2.2.1, and these, rather than the pH values of the original buffer, are used in this Figure. The dependence of L(+)-mandelate dehydrogenase activity on the pH was measured using 0.1 M phosphate buffer in the presence of BSA or Triton X-100 with assay conditions described in Methods 2.4.2. The results shown in this Figure are means of two independent experiments.

(a) pH profile of purified L(+)-mandelate dehydrogenase in the presence of (•) 66.7 μg of BSA ml^{-1} or (○) 300 μg of Triton X-100 ml^{-1}

(b) pH profile of L(+)-mandelate dehydrogenase in high speed supernatant (Methods 2.3.9), measured in the presence of 66.7 μg of BSA ml^{-1}.


(a)
Relative activity (% of maximum activity)
100
80
60
40
20
0
5
6
7
8
9
10


(b)
Relative activity (% of maximum activity)
100
80
60
40
20
0
5
6
7
8
9
10
pH of the reaction mixture

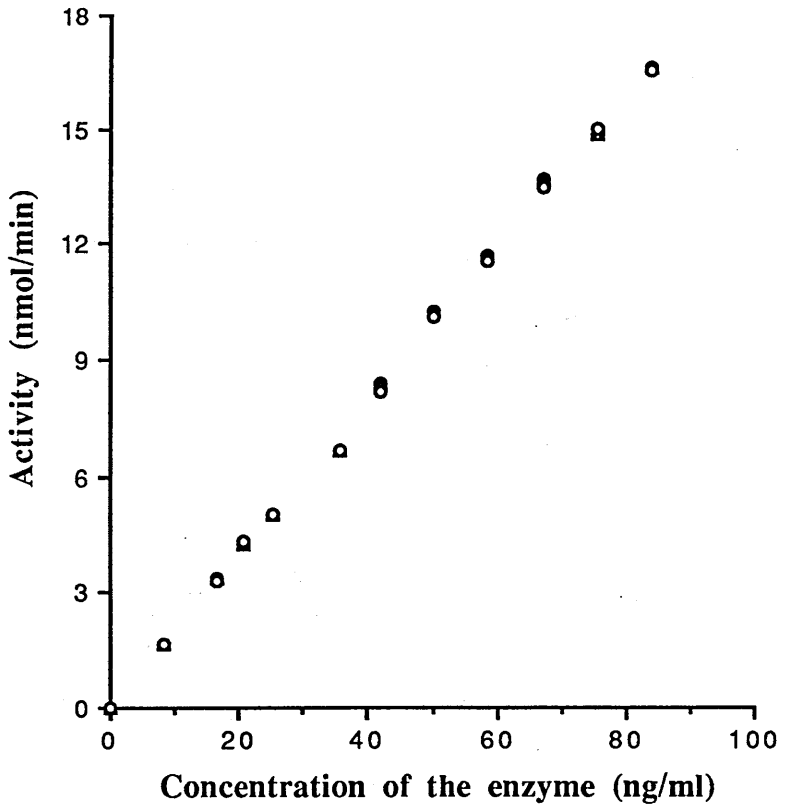


Figure 4.17 Dependence of the rate of L(+)-mandelate dehydrogenase upon its concentration in the reaction mixture.

Purified L(+)-mandelate dehydrogenase from *R. graminis* was diluted 12-fold in 20 mM Tris buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. Various amounts of enzyme were used using the standard assay conditions as described in Methods 2.4.2. Results are given as means of two independent experiments and triplicate assays were done in each. Identical values were obtained where only one point is shown in the graph.

4.4.2 Effects of BSA and Triton X-100 on the activity of L(+)-mandelate dehydrogenase.

The activity of L(+)-mandelate dehydrogenase was measured in the presence of BSA or Triton X-100 (Fig. 4.13-4.14 & Table 4.4-4.5). There was little or no activity in the absence of either BSA or Triton X-100, especially at low enzyme concentration i.e. 21 ng of L(+)-mandelate dehydrogenase per ml in the reaction mixture. Maximum activity of the enzyme was observed with either 50-200 μg of BSA per ml or 300-500 μg of Triton X-100 per ml in the reaction mixture. Optimum BSA and Triton X-100 concentration when added together to the reaction mixture did not give more activity than either of them alone, but the effects of sub-optimal concentrations were more or less additive (Table 4.4). In the presence of optimum concentrations of BSA or Triton X-100, rate of reduction of DCIP was linear upto a maximum of three min reaction time. At higher BSA (more than 300 μg ml^{-1}) or Triton (more than 1 mg ml^{-1}) concentrations the activity was depressed (Fig. 4.13b & 4.14b).

Activity of L(+)-mandelate dehydrogenase was increased by 10 to 100-fold by the addition of optimal BSA or Triton concentration for purified enzyme but only 30 to 50% increase in activity was observed for the crude extracts.

4.4.3 Effects of PMS on the activity of L(+)-mandelate dehydrogenase

The presence of PMS did not increase the activity of the purified enzyme in the absence of either BSA or Triton X-100. However, one and a half to three-fold increase in activity was observed when higher BSA and Triton concentrations were present (Fig. 4.15 & Table 4.5). Inhibition of activity due to high BSA and Triton concentrations was reversed in the presence of PMS (Fig. 4.15). A similar effect of PMS was observed on the activity of enzyme in crude extracts. Replacing PMS with PES gave identical increases in the activity of the enzyme.

4.4.4 The pH optimum of L(+)-mandelate dehydrogenase

Purified L(+)-mandelate dehydrogenase has a fairly broad pH profile in the presence of BSA or Triton X-100 (Fig. 4.16a) with half maximal activity at about pH 6.2 and 9. The

Table 4.6 Effect of salts on the activity of L(+)-mandelate dehydrogenase of *R.graminis*

L(+)-Mandelate dehydrogenase of *R. graminis* was diluted 12-fold in 20 mM Tris buffer, pH7.5, containing 0.5 mg Triton X-100 ml^{-1}. The mixtures were then incubated on ice for 80 min with the various salts and then samples were taken for assay as described in Methods 2.4.2. The concentrations of L(+)-mandelate dehydrogenase in the incubation and assay mixtures were 1.25 $\mu g\ ml^{-1}$ and 21 $ng\ ml^{-1}$ respectively. The initial velocity in the absence of salts was taken as 100%. Results are given as means of duplicate experiments. Ferric chloride was also tried but it gave precipitates with phosphate buffer in the reaction mixture.

Compound	Concentration in incubation mixture (mM)	Activity (%)
Sodium chloride	1	100
Potassium chloride	1	100
Calcium chloride	1	99
Magnesium chloride	1	100
Magnesium sulphate	1	99
Ammonium sulphate	1	101

Table 4.7 Possible effects of metal chelators on the activity of L(+)-mandelate dehydrogenase of *R.graminis*

L(+)-Mandelate dehydrogense of *R. graminis* was diluted 12-fold in 20 mM Tris buffer, pH7.5, containing 0.5 mg Triton X-100 ml^{-1} with various concentrations of metal chelators shown in the table. The mixtures were incubated on ice for 20 min and then samples from these mixtures were taken for assay as described in methods 2.4.2. The concentrations of L(+)-mandelate dehydrogense in the incubation and assay mixtures were 1.25 $\mu g\ ml^{-1}$ and 21 ng ml^{-1} respectively. The initial velocity in the absence of inhibitor was taken as 100%. Results are given as means of duplicate experiments .

Chelating agent	Concentration in the incubation mixture (mM)	Activity (%)
EDTA	1	100
	10	99
2,2-Bipyridyl	1	100
	10	99
Pyrazole	1	100
	10	99
8-Hydroxyquinoline	5	100
	10	96
Sodium azide	1	102
	10	99

pH optimum of the purified L(+)-mandelate dehydrogenase was 7.9 in phosphate buffer. An almost identical pH optimum was estimated for the enzyme in crude extract (Fig. 4.16b). Very similar results were obtained with 0.1 M Tris/HCl, glycylglycine, Mes, Tes, Mops, Bes, imidazole, Hepes, Tricine and citrate buffers. However, the enzyme was slightly less active in citrate buffer.

4.4.5 Dependence of the rate of L(+)-mandelate dehydrogenase upon its concentration and estimation of the reproducibility of the assay

The rate of DCIP reduction was directly proportional to the concentration of purified enzyme upto at least 84 ng of L(+)-mandelate dehydrogenase per ml in the reaction mixture (Fig. 4.17). Eighteen assays were done at the concentration of 21 ng of L(+)-mandelate dehydrogenase ml^{-1} in the reaction mixture and the mean rate was found to be 4.24 ± 0.12 (SD) nmol min^{-1}. The standard deviation was thus only 2.9% of the mean.

4.4.6 Stoichiometry of reaction

When 0.017 or 0.034 mM of L(+)-mandelate was added to a reaction mixture and the assay was allowed to go to completion, the ΔA_{600} (0.118 or 0.234) was equivalent to the reduction of 0.017 or 0.034 mM DCIP, indicating that equimolar concentration of the two substrates were used in the reaction mixture.

4.4.7 Effects of salts and metal chelators

L(+)-Mandelate dehydrogenase activity from *R. graminis* was not affected by $CaCl_2$, KCl, $MgCl_2$, $MgSO_4$, NaCl, or $(NH_4)_2 SO_4$ (Table 4.6).

The enzyme activity was apparently not affected by EDTA, 2,2'-bipyridyl, pyrazole, 8-hydroxyquinoline or sodium azide (Table 4.7).

Thus the behaviour of L(+)-mandelate dehydrogenase activity from *R. graminis* with respect to salts and metal chelators is like that of L(+)-lactate dehydrogenase from *S. cerevisiae* (Appleby & Morton, 1954; Morton *et al.*, 1961; Symons & Burgoyne, 1966; Morton & Sturtevant, 1964) and L(+)-mandelate and lactate dehydrogenase from *A. calcoaceticus* (Allison & Fewson, 1986; Hoey *et al.*, 1987).

Table 4.8 Possible effects of thiol-blocking reagents on the activity of L(+)-mandelate dehydrogenase of *R. graminis*

L(+)-Mandelate dehydrogenase of *R. graminis* was diluted 12-fold in 20-mM Tris buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} with various concentrations of thiol-blocking regents. The mixtures were incubated on ice for 10 min and then samples from these mixtures were taken for assays as described in Methods 2.4.2. The concentrations of L(+)-mandelate dehydrogenase from *R. graminis* in the incubation and assay mixtures were 1.25 μg ml^{-1} and 21 ng ml^{-1} respectively. The initial velocity in the absence of inhibitor was taken as 100% and remained unchanged throughout the experiment. Results are given as means of duplicate experiments.

Inhibitor	Concentration in the incubation mixture (mM)	Inhibition (%)
Iodoacetate	1	2
	10	5
Iodoacetamide	1	1
	10	2
N-Ethylmaleimide	1	16
	10	35
	25	55
Mercuric chloride	0.001	49
	1.0	100
P-Chloromercuribenzoate	0.001	49
	0.0125	76

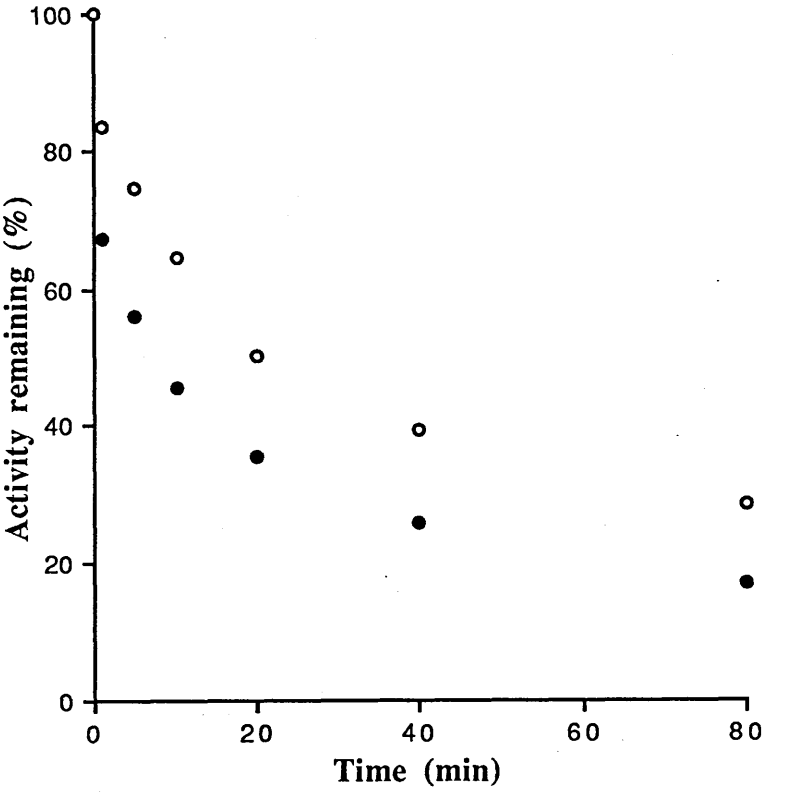


Figure 4.18 Effect of *N*-ethylmaleimide on the activity of L(+)-mandelate dehydrogenase of *R. graminis*

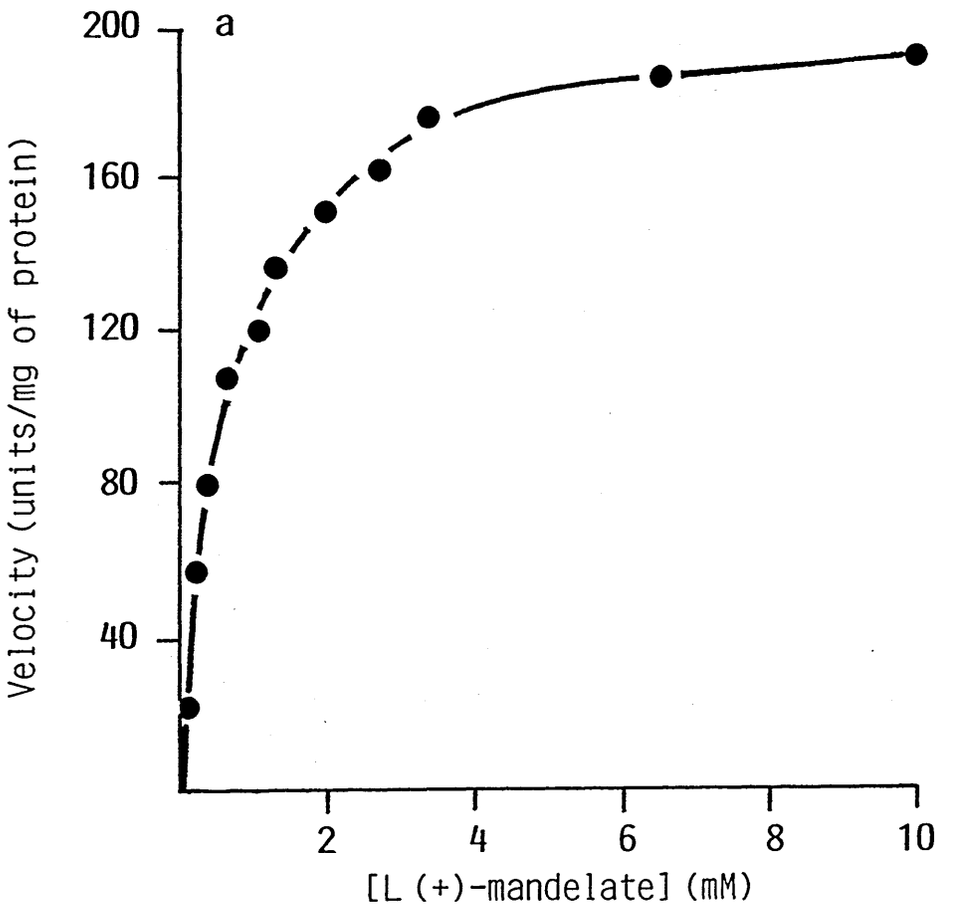
L(+)-Mandelate dehydrogenase of *R. graminis* was diluted 12-fold in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} and 25 mM N-ethylmaleimide in the presence (o) and absence (•) of 20 mM L(+)-mandelate. The mixtures were incubated on ice and samples were taken for assay at various times to measure enzyme activity described in Methods 2.4.2. The concentrations of L(+)-mandelate dehydrogenase in the incubation and assay mixtures were 1.25 μg ml^{-1} and 21 ng ml^{-1} respectively. The initial velocity in the absence of inhibitor was taken as 100% and it remained unchanged throughout the experiment. Results are given as averages of duplicate experiments.

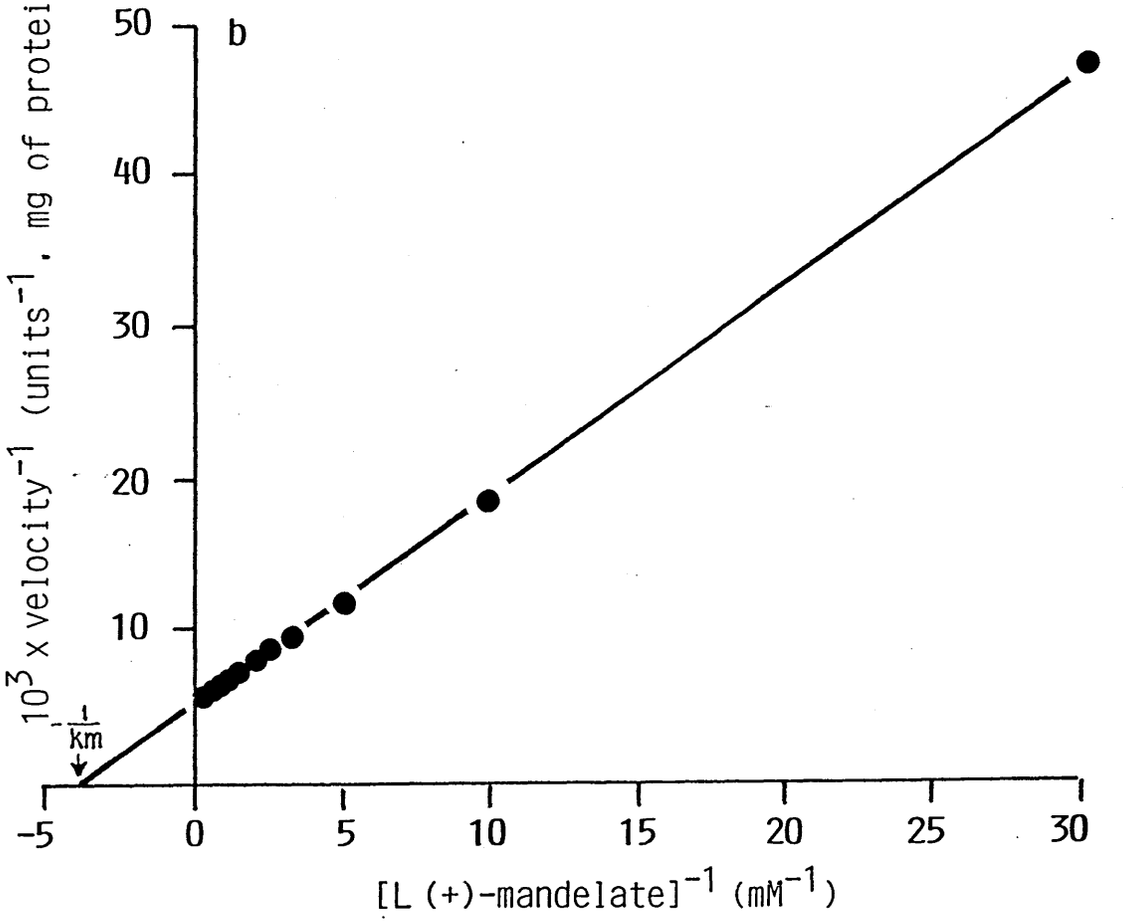
Figure 4.19 Typical plot of initial velocities of L(+)-mandelate dehydrogenase of *R. graminis* as a function of concentration of L(+)-mandelate

Results of a representative experiment are shown in this Figure. The enzyme was assayed as described in Methods 2.4.2.

(a) Direct plot of initial velocities against concentrations of L(+)-mandelate .

(b) Lineweaver-Burk double reciprocal plot of initial velocities against concentrations of L(+)-mandelate.


a
200
160
120
80
40
Velocity (units/mg of protein)
2
4
6
8
10
[L (+)-mandelate] (mM)


b
50
40
30
20
10
10^3 x velocity^{-1} (units^{-1}, mg of protein)
$\frac{1}{Km}$
-5
0
5
10
15
20
25
30
[L (+)-mandelate]$^{-1}$ (mM^{-1})

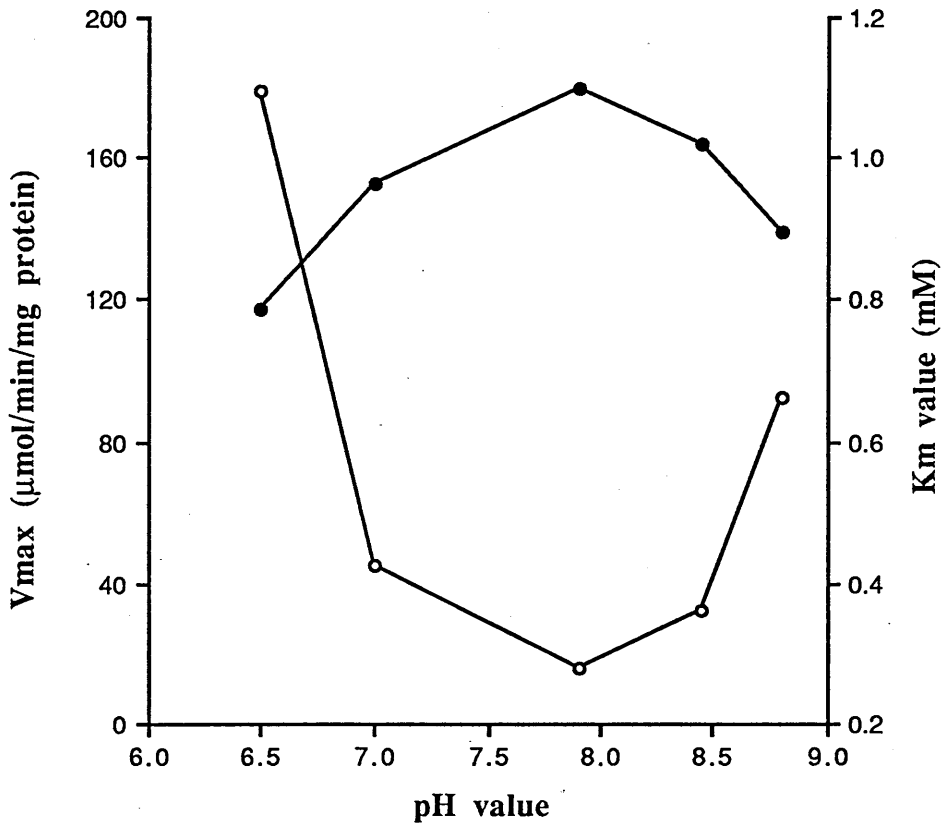


Figure 4.20 Effect of pH on the apparent K_m and V_{max} values of L(+)-mandelate dehydrogenase of *R. graminis*

Purified L(+)-mandelate dehydrogenase of *R. graminis* was diluted 12-fold in 20 mM Tris buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} and was assayed as described in Methods 2.4.2. The concentrations of L(+)-mandelate dehydrogenase in the diluted sample and assay mixtures were 1.25 µg ml^{-1} and 21 ng ml^{-1} respectively. (○) K_m and (●) V_{max} values were determined using the Enzpack computer program using the direct linear method (William, 1985). The pH of the reaction mixtures was that measured after completion of the reaction.

4.4.8 Effects of thiol-blocking reagents

Iodoacetate and iodoacetamide gave no or very little inhibition of enzyme activity (Table 4.8). The enzyme was slightly inhibited by *N*-ethylmaleimide (Table 4.8). However, mercuric chloride and *p*-chloromercuribenzoate strongly inhibited the enzyme (Table 4.8). The presence of substrate did not protect the enzyme from inactivation by mercuric chloride and *p*-chloromercuribenzoate but did slightly protect the enzyme from *R. graminis* against inhibition by *N*-ethylmaleimide (Fig. 4.18). This behaviour against thiol-blocking reagents is similar to that reported for of L(+)-lactate dehydrogenase from *S. cerevisiae* (Appleby & Morton, 1954; Morton *et al.*, 1961; Morton & Sturtevant, 1964; Symons & Burgoyne, 1966) and D(–)- and L(+)-mandelate and lactate dehydrogenases from *A. calcoaceticus* (Allison *et al.*, 1985a,b; Allison & Fewson, 1986; Hoey *et al.*, 1987).

4.5 Steady state kinetics of (+)-mandelate dehydrogenase from *R. graminis*

4.5.1 Determination of apparent K_m value for the substrate

The rate of L(+)-mandelate dehydrogenase showed a hyperbolic dependence on L(+)-mandelate concentration (Fig. 4.19). The K_m for L(+)-mandelate was 0.266 $\pm$ 017 mM (6) in the presence of saturating concentration of DCIP, as determined by the direct plot using the Enzpack computer program (Williams, 1985). The K_m determined by the direct linear method was in good agreement with K_m values determined by Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots. Enzyme in crude extract gave an apparent K_m value for L(+)-mandelate of 0.266 $\pm$ 0.02 mM (3) as determined by the direct linear plot .

4.5.2 Effect of pH on the apparent K_m and V_{max} values

The apparent K_m and V_{max} values of the enzyme were minimal and maximal, respectively at pH 7.9 (Fig. 4.20).

4.5.3 Determination of apparent K_m values for DCIP and cytochrome *c*

L(+)-Mandelate dehydrogenase was assayed in the presence of several non-saturating concentrations of DCIP or cytochrome *c* and a fixed (8.33 mM) concentation of

Table 4.9 Comparison of relative activity of purified L(+)-mandelate dehydrogenase of *R. graminis* and L(+)-lactate dehydrogenase from *S. cerevisiae* with DCIP or potassium ferricyanide or cytochrome *c* as electron acceptors

Values for L(+)-mandelate dehydrogenase of *R. graminis* are taken from section 4.6.3 and those for L(+)-lactate dehydrogenase from *S. serevisiae* are calculated from Morton *et al.* (1961).

Enzyme	Electron acceptor	pH	Relative activity as a function of activity with DCIP = 1	K_m (μM)
L(+)-Mandelate	Potassium ferricyanide	7.9	3.03	N.D.
dehydrogenase of	Cytochrome *c*	7.9	1.18	40
R. graminis	DCIP	7.9	1	27
L(+)-Lactate	Potassium ferricyanide	8.0	4	190
dehydrogenase of	Cytochrome *c*	8.0	1.81	44
S. serevisiae	DCIP	8.0	1	34

N.D. = Not determined.

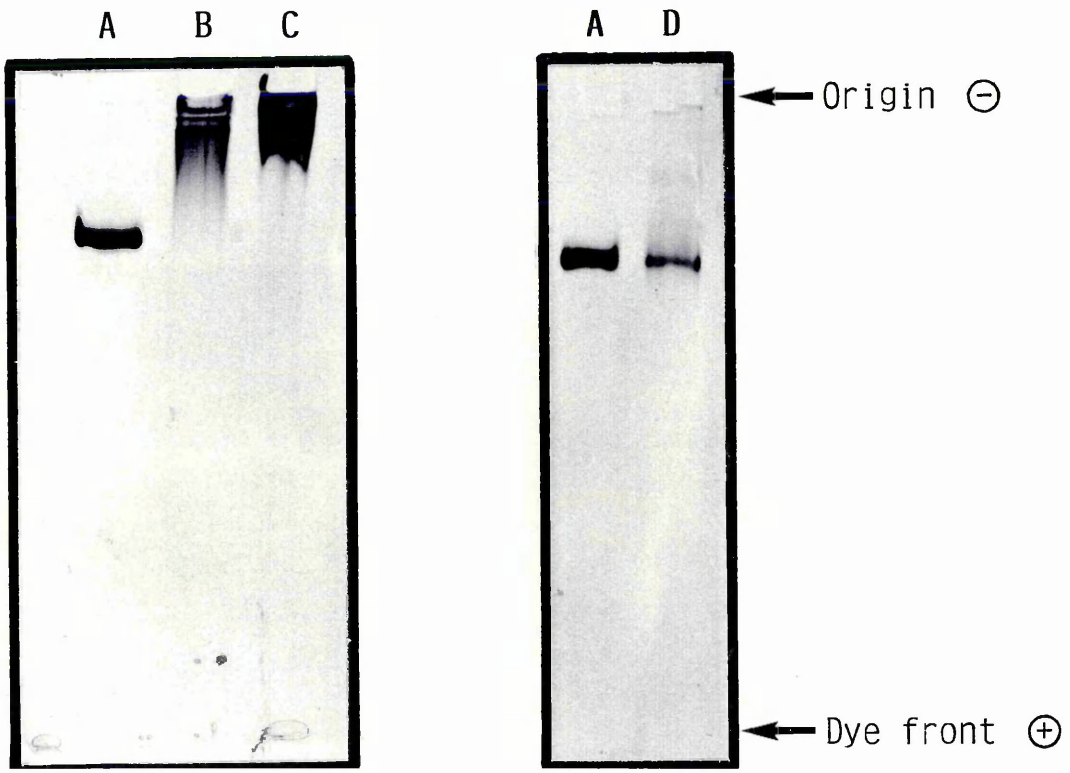


Figure 4.21 Non-denaturing polyacrylamide gel electrophoresis of crude extracts of *R. graminis*, *A. calcoaceticus*, *P. putida* and of purified L(+)-mandelate dehydrogenase of *R. graminis* followed by activity staining

The 6 % (w/v) non-denaturing-PAGE with a 5.6 % stacking gel was run as described in Methods 2.8.4 and was stained for activity as described in Methods 2.8.5.2.

A. 40 µg of crude extract of *R. graminis,*

B. 36 µg of crude extract of *A. calcoaceticus*

C. 36 µg of crude extract of *P. putida*

D. 1 µg of the concentrated purified L(+)-mandelate dehydrogenase from *R. graminis*.

L(+)-mandelate. The apparent K_m value for DCIP and cytochrome *c* as determined by the direct plot were 27 ± 3 μM (3) and 40.5 ± 4.6 μM (6) respectively (Table 4.9).

4.6 Comparative study of L(+)-mandelate dehydrogenases from different microorganisms

A few experiments were designed to study the comparative behaviour of L(+)-mandelate dehydrogenases from *R. graminis, A. calcoaceticus* and *P. putida.*

4.6.1 Effects of BSA and PMS

The effects of BSA and PMS on the activities of L(+)-mandelate dehydrogenases from *R. graminis, A. calcoaceticus* and *P. putida* are rather similar (Table 4.5). At higher BSA concentrations, enzyme activities from *R. graminis, A. calcoaceticus* and *P. putida* were strongly depressed but were restored to normal or even increased by approximately two-fold by the inclusion of PMS (Table 4.5). The presence of BSA and PMS had less effect on the activity of L(+)-mandelate dehydrogenases from extracts and washed cell membranes of *A. calcoaceticus* and *P. putida*, perhaps due to the presence of ethanediol and Triton X-100 in these enzyme preparations (Table 4.5).

4.6.2 Mobility on electrophoresis

A non-denaturing electrophoresis gel of L(+)-mandelate dehydrogenase from cell-free extracts of *R. graminis, A. calcoaceticus* and *P. putida* stained for enzyme activity is shown in Fig. 4.21. L(+)-Mandelate dehydrogenases from *A. calcoaceticus* and *P. putida* hardly entered the stacking gel and smeared badly, whereas L(+)-mandelate dehydrogenase in the crude extract of *R. graminis* or this enzyme after purification entered the main gel and ran as discrete bands (Table 4.21).

4.6.3 Electron acceptors

Purified L(+)-mandelate dehydrogenase from *R. graminis* was able to use DCIP, potassium ferricyanide or mammalian cytochrome *c* as electron acceptors and this is like L(+)-lactate dehydrogenase from *S. cerevisiae* and *H. anomala* (Appleby & Morton, 1954;

Table 4.10 Relative use of electron acceptors by L(+)-mandelate dehydrogenases of *R. graminis* , *A. calcoaceticus* or *P. putida* and L(+)-lactate dehydrogenases from *A. calcoaceticus* and *S. cerevisiae*

L(+)-Mandelate dehydrogenase of *R. graminis* was assayed in the presence DCIP or potassium ferricyanide or cytochrome *c* , used as electron acceptors (Methods 2.4.2, 2.4.3) and bacterial enzymes were assayed as described in Methods 2.4.6 using DCIP or cytochrome *c* as electron acceptors. Relative activities for L(+)-lactate dehydrogenase from *S. serevisiae* with DCIP or potassium ferricyanide or cytochrome *c* are calculated from Morton *et al.* (1961) as a function of activity with DCIP being one.

Enzyme	Activity (µmol/min/mg protein) with different electron acceptors		
	DCIP	Cytochrome *c*	Potassium ferricyanide
L(+)-Mandelate dehydrogenase from *R. graminis*	180	213	546
L(+)-Mandelate dehydrogenase from *A. calcoaceticus*	37	0	N.D.
L(+)-Mandelate dehydrogenase from *P. putida*	53	0	N.D.
L(+)-Lactate dehydrogenase from *A. calcoaceticus*	16	0	N.D.
L(+)-Lactate dehydrogenase from *S. serevisiae*	1	1.81	4

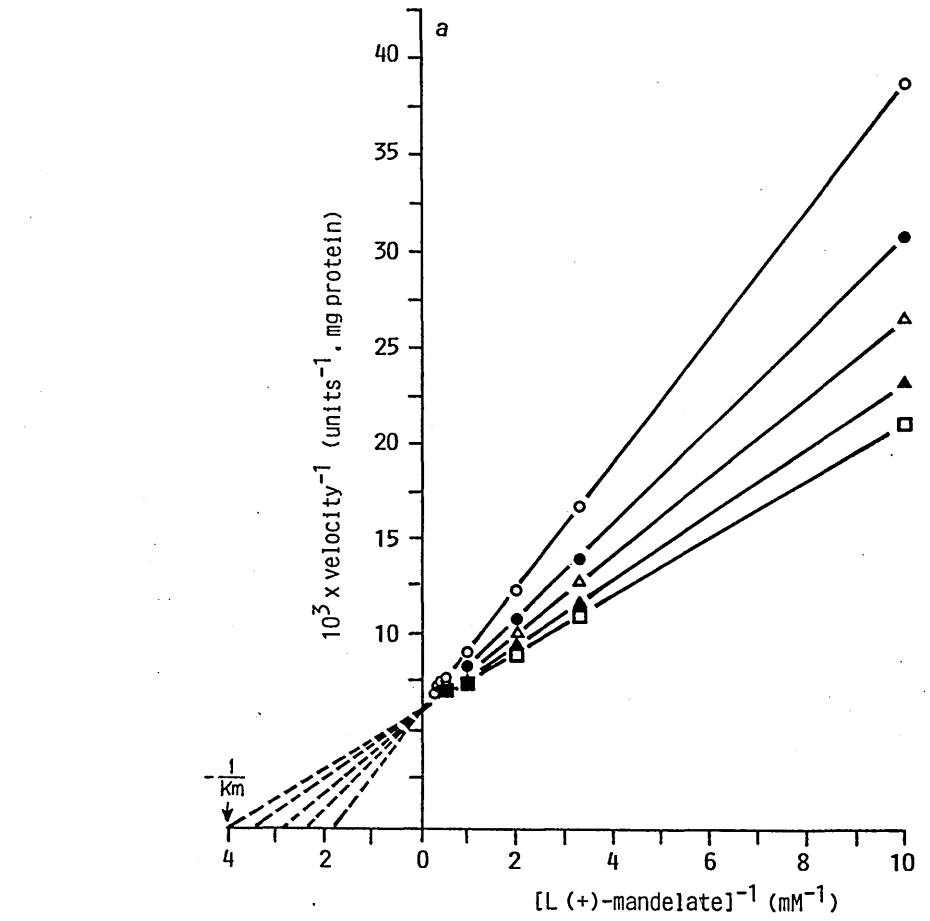
N.D. = Not determined.

Figure 4.22 Competitive inhibition of L(+)-mandelate dehydrogenase of *R. graminis* by D(-)-mandelate

The nature of the inhibition of L(+)-mandelate dehydrogenase was tested by measuring the activity of the enzyme (Methods 2.4.2) in the presence of various concentrations of L(+)-mandelate at four fixed concentrations of D(–)-mandelate. Assays were carried out in duplicate and means of the two results are given.

(a) Double reciprocal plot of initial velocities at different concentrations of L(+)-mandelate and (□) 0, mM (▲) 0.1 mM, (▲) 0.5 mM, (●) 1.0 mM, (○) 2.0 mM D(-)-mandelate.

(b) Secondary plot of slopes from Figure 4.19 (a) as a function of D(-)-mandelate concentration.


a
40
35
30
25
20
15
10
10^3 x velocity^{-1} (units^{-1}. mg protein)
$-\frac{1}{Km}$
4
2
0
2
4
6
8
10
[L (+)-mandelate]$^{-1}$ (mM^{-1})

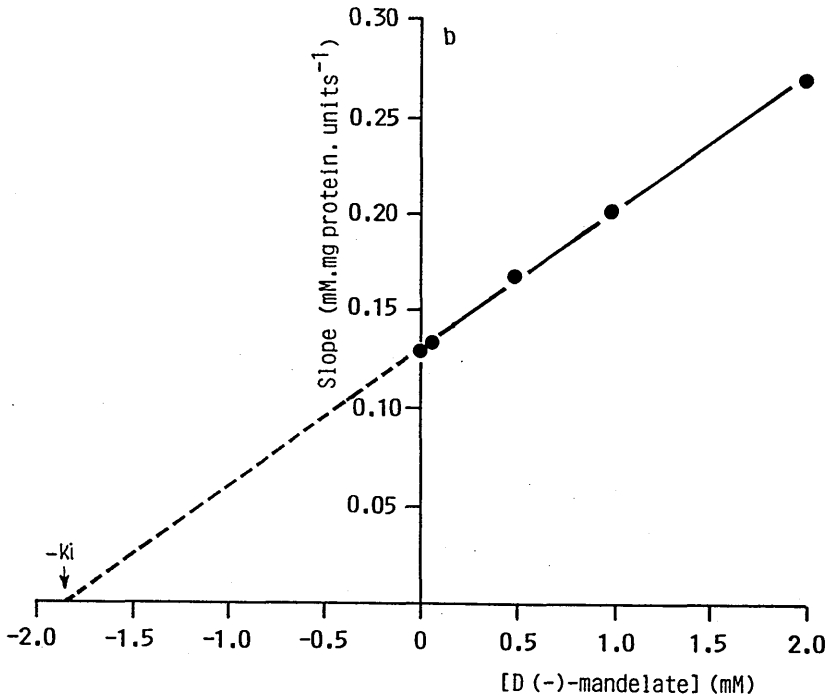

b
0.30
0.25
0.20
0.15
0.10
0.05
Slope (mM.mg protein. units^{-1})
-Ki
-2.0
-1.5
-1.0
-0.5
0
0.5
1.0
1.5
2.0
[D (-)-mandelate] (mM)

Table 4.11 Oxidation of ring-substituted mandelates by L(+)-mandelate dehydrogenases of *R. graminis* and *A. calcoaceticus*

Purified L(+)-mandelate dehydrogenases of *R. graminis* and *A. calcoaceticus* were diluted 12-fold and 30-fold respectively in 20 mM Tris buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. Substituted DL-mandelates were assayed at the concentrations shown in paretheses. The initial velocities with 0.3 mM (i.e. close to K_m) and 8.33 mM L(+)-mandelate (i.e. close to saturating concentration) for *R. graminis* enzyme and 0.2 mM (i.e. close to K_m) and 5 mM L(+)-mandelate (i.e. close to saturating concentration) for the *A. calcoaceticus* enzyme, with assay conditions, as described in Methods 2.4.2 and 2.4.6 respectively, were taken as 100% in each case. Activities for mandelonitrile and mandelate isoamyl ester in *R. gramunis* were calculated as a percent of the rate with 0.6 and 8.3 mM L(+)-mandelate in absolute alcohol. Values are given as means of duplicate experiments in which the individual results were well within 6% of the mean.

N.D. = Not determined

Compound	Activity as a percent of the rate with L(+)-mandelate at			
	0.3 mM	8.3 mM	0.2 mM	5.0 mM
	R. graminis		*A. calcoaceticus*	
DL-Mandelonitrile	11 (1.2)	23 (10)	N.D.	N.D.
DL-Mandelate, isoamyl ester	47 (1.2)	34 (10)	N.D.	N.D.
DL-Mandelate, methyl ester	40 (0.6)	76 (10)	N.D.	N.D.
DL-Mandelamide	0 (0.6)	0 (10)	0 (0.4)	0 (5)
4-Fluoro DL-mandelate	59 (0.6)	56 (10)	93 (0.4)	81 (5)
4-Chloro DL-mandelate	104 (0.6)	67 (10)	90 (0.4)	70 (5)
4-Bromo DL-mandelate	96 (0.6)	53 (10)	88 (0.4)	43 (5)
L(+)-Hexahydromandelate	0 (0.6)	0 (20)	0 (0.4)	0 (20)
D(-)-Hexahydromandelate	0 (0.6)	0 (20)	0 (0.4)	0 (20)
2-Methoxy DL-mandelate	43 (0.6)	35 (10)	2 (0.4)	3 (15)
3-Hydroxy DL-mandelate	40 (0.6)	52 (10)	79 (0.4)	76 (5)
3-Hydroxy 4-methoxy DL-mandelate	31 (0.6)	47 (10)	47 (0.4)	83 (5)
4-Hydroxy DL-mandelate	152 (0.1)	192 (1)	63 (0.4)	99 (5)
4-Hydroxy 3-methoxy DL-mandelate	173 (0.2)	169 (1)	19 (0.4)	53 (5)
3,4-Dihydroxy DL-mandelate	156 (0.6)	85 (5)	86 (0.4)	82 (5)

Jacq & Lederer, 1974; Gervais *et al.*, 1983; Haumont *et al.*, 1987; Xia *et al.*, 1987; Chapman *et al.*, 1991). However, L(+)-mandelate dehydrogenases from *A. calcoaceticus* and *P. putida* and and L(+)-lactate dehydrogenase from *A. calcoaceticus* could not use mammalian cytochrome *c* as an electron acceptor (Table 4.10). L(+)-Mandelate dehydrogenase from *R. graminis* gave maximum rate with ferricyanide and rates with DCIP and cytochrome *c* were 39% and 33% respectively, of the rate with ferricyanide (Table 4.10), again very similar to the literature values for L(+)-lactate dehydrogenase of *S. cerevisiae* (Table 4.9 & 10).

4.6.4 Substrate specificity

L(+)-Mandelate dehydrogenase from *R. graminis* was found to be stereospecific for its substrate and D(–)-mandelate was not oxidised to a detectable extent even at a concentration of 20mM. D(–)-Mandelate inhibited L(+)-mandelate dehydrogenase activity competitively (Fig. 4.22) with a K_i for D(–)-mandelate of 1.98 $\pm$ 0.18 mM (3) (Fig. 4.22b), which compares with an apparent K_m value for L(+)-mandelate of 0.25 mM calculated from Fig. 4.22a.

Purified L(+)-mandelate dehydrogenase from *R. graminis* can use a number of ring-substituted mandelates as substrates (Table 4.11). For comparative purposes, the substrate specificity of L(+)-mandelate dehydrogenase of *A. calcoaceticus* was also determined (Table 4.11). Most of the compounds tested were racemic mixtures and it is possible that the D(–)-enantiomers might be inhibitory. Purified L(+)-mandelate dehydrogenase from *R. graminis* gave maximum rates with 4-hydroxy D,L-mandelate and 4-hydroxy-3-methoxy D,L-mandelate whereas L(+)-mandelate dehydrogenase from *A. calcoaceticus* did not give higher rates with these compounds and in fact rather low rates were observed with 4-hydroxy 3-methoxy D,L-mandelate (Table 4.11).

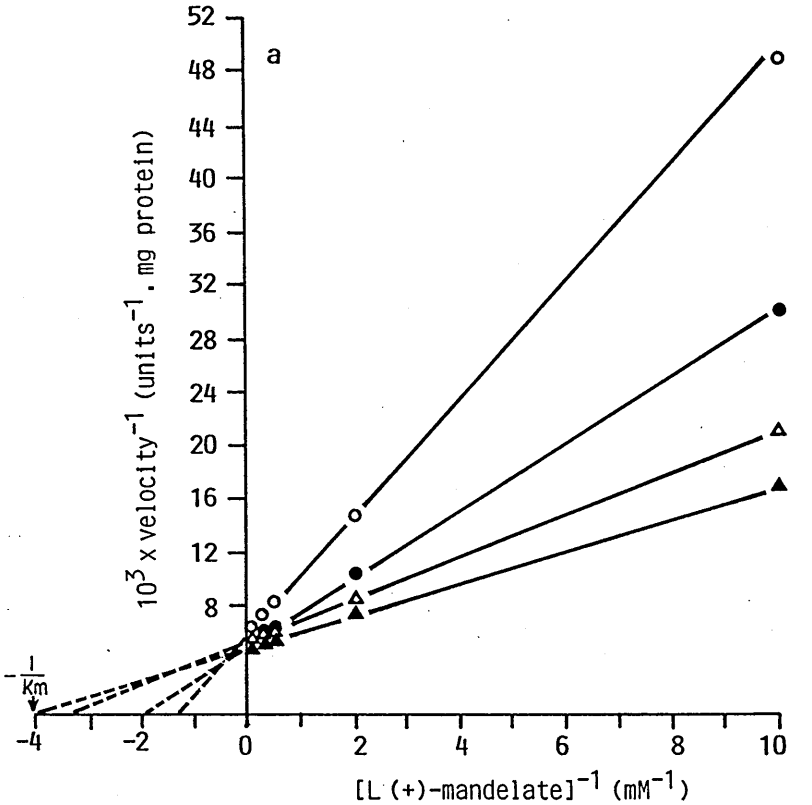
Purified L(+)-mandelate dehydrogenase from *R. graminis* and *A. calcoaceticus* could not use D(–)-hexahydromandelate, L(+)-hexahydromandelate or D,L-mandelamide as substrates. L(+)-Mandelate dehydrogenase from *R. graminis* gave low rates with some of the substituted mandelates at higher concentrations e.g. rate at 10 mM concentrations of

Figure 4.23 Competitive inhibition of L(+)-mandelate dehydrogenase of *R. graminis* by L(+)-hexahydromandelate

The nature of the inhibition of L(+)-mandelate dehydrogenase was tested by measuring the activity of the enzyme (Methods 2.4.2) in the presence of various concentrations of L(+)-mandelate at three fixed concentrations of L(+)-hexahydromandelate. Assays were carried out in duplicate and means of the two results are given.

(a) Double reciprocal plot of initial velocities at different concentrations of L(+)-mandelate and (▲) 0 mM, (▲) 0.1 mM, (●) 0.5 mM, (○) 1.0 mM L(+)-hexahydromandelate

(b) Secondary plot of slopes from Figure 4.16 (a) as a function of L(+)-hexahydromandelate concentration.


a
52
48
44
40
36
32
28
24
20
16
12
8
10^3 x velocity^{-1} (units^{-1}. mg protein)
$-\frac{1}{Km}$
-4
-2
0
2
4
6
8
10
[L (+)-mandelate]$^{-1}$ (mM^{-1})

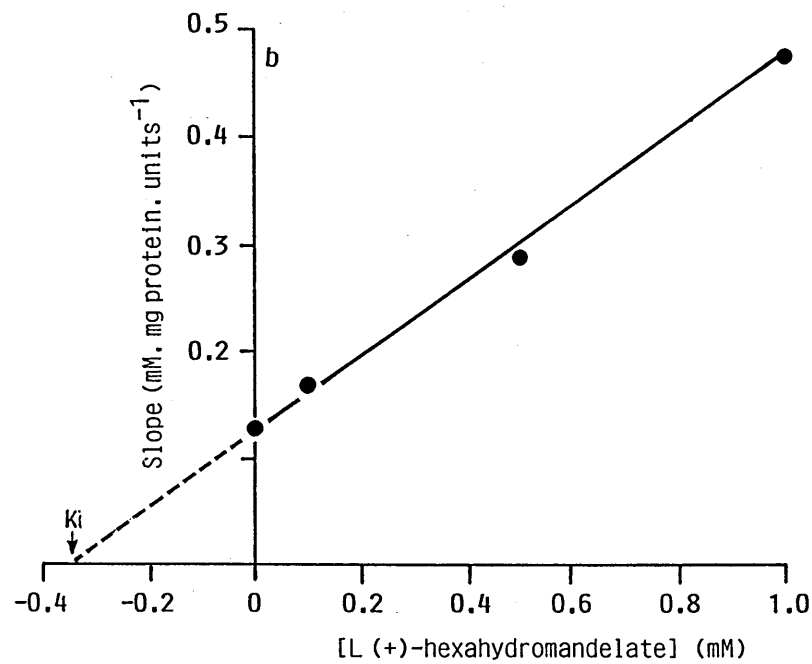

b
0.5
0.4
0.3
0.2
Slope (mM. mg protein. units^{-1})
Ki
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
[L (+)-hexahydromandelate] (mM)

Table 4.12 Inhibition of L(+)-mandelate dehydrogenases of *R. graminis* and *A. cacoaceticus* by compounds that are not substrates

Purified L(+)-mandelate dehydrogenase of *R. graminis* and *A. calcoaceticus* were diluted 12-fold and 30-fold respectively in 20 mM Tris buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. Samples were taken from these diluted samples for assay as described in Methods 2.4.2 and 2.4.6. The initial velocities in the presence of 0.3 mM (i.e. close to K_m) and 8.33 mM L(+)-mandelate (i.e. close to saturating concentration) for *R. graminis* enzyme and 0.15 mM (i.e. close to K_m) and 8.3 mM L(+)-mandelate (i.e. close to saturating concentration) for the *A. calcoaceticus* enzyme were taken as 100% in each case. The percentage inhibitions are means of duplicate experiments.

N.D. = Not determined

Compound	Concentration (mM)	Inhibition % in the presence of L(+)-mandelate at 0.3 mM	8.3 mM	0.2 mM	5.0 mM
		R. graminis		*A. calcoaceticus*	
L(+)-Lactate	2	N.D.	N.D.	3	2
	10	49	N.D.	N.D.	N.D.
	20	N.D.	N.D.	17	3
L(+)-β-Phenyllactate	1	91	48	18	N.D.
	5	N.D.	81	51	13
	10	100	94	N.D.	N.D.
4-Hydroxy	1	81	27	9	N.D.
DL-phenyllactate	5	N.D.	63	26	1
	10	97	78	N.D.	9
2-Hydroxy	1	37	8	N.D.	N.D.
DL-valerate	2	52	N.D.	N.D.	N.D.
	15	N.D.	50	N.D.	N.D.
2-Hydroxy	2	32	11	9	N.D.
DL-isovalerate	10	N.D.	32	34	7
	15	N.D.	44	N.D.	10
2-Hydroxy	1	47	12	N.D.	N.D.
DL-caproate	10	87	N.D.	40	9
	18	N.D.	59	N.D.	N.D.
2-Hydroxy	1	62	15	7	N.D.
DL-isocaproate	10	92	N.D.	50	18
	15	N.D.	70	N.D.	26
	18	N.D.	75	N.D.	N.D.
Oxalate	1	40	25	N.D.	N.D.
	2	56	N.D.	64	N.D.
	5	75	59	82	51
	10	85	75	N.D.	63

Table 4.13 Effects of various aromatic acids on the activity of L(+)-mandelate dehydrogenase of *R. graminis*

L(+)-Mandelate dehydrogenase of *R. graminis* was diluted 12-fold in 20 mM Tris buffer, containing 0.5 mg Triton X-100 ml^{-1}. The enzyme was assayed with various concentrations of aromatic acids using the standard assay procedures as described in Methods 2.4.2. The concentration of the enzyme in the assay mixture was 21 ng ml^{-1} and the concentrations of aromatic acids represent the final concentrations in the assay mixture. The initial velocity in the presence of mandelate and in the absence of other aromatic acids was taken as 100 percent. Values are given as means of duplicate experiment in which the individual results were well within 4% of each other. 3,5-Dihydroxybenzoate was also checked as an inhibitor but due to a high blank rate, the result is not included.

N.D. = Not determined

Compound	Concentration (mM)	Inhibition (%) in the presence of L(+)-mandelate at 0.3 mM	8.3 mM
Benzoate	1	0	N.D.
	10	18	N.D.
p-Hydroxybenzoate	1	1	N.D.
	10	30	N.D.
m-Hydroxybenzoate	1	0	N.D.
	10	30	N.D.
3,4-Dihydroxybenzoate	5	58	32
	10	73	46
2,4-Dihydroxybenzoate	5	32	9
	10	63	17
2,5-Dihydroxybenzoate	5	32	7
	10	62	18
2,6-Dihydroxybenzoate	5	32	7
	10	62	18
2,6-Dichlorobenzoate	5	35	15
	10	69	36

Table 4.14 Inhibition of L(+)-mandelate dehydrogenase of *R. graminis* and *A. calcoaceticus* by catechol, protocatechuic acid, glyoxylate and thiopheneglyoxylate

Purified L(+)-mandelate dehydrogenases of *R. graminis* and *A. calcoaceticus* were diluted 12-fold and 30-fold respectively in 20 mM Tris buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. Samples were taken from these diluted samples for assay as described in Methods 2.4.2 and 2.4.6. The initial velocities in the presence of 0.3 mM (i.e. close to K_m) and 8.33 mM L(+)-mandelate (i.e. close to saturating concentration) for *R. graminis* enzyme and 0.15 mM (i.e. close to K_m) and 3.3 mM L(+)-mandelate (i.e. close to saturating concentration) for the *A. calcoaceticus* enzyme, with standard assay conditions and in the absence of inhibitors were taken as 100% in each case. The percentage inhibitions are means of duplicate experiments.

Compound	Concentration (mM)	Inhibition % in the presence of L(+)-mandelate at			
		0.3 mM	8.3 mM	0.15 mM	3.3 mM
		R. graminis		*A. calcoaceticus*	
Catechol	1	39	25	N.D.	25
	5	82	55	N.D.	57
	10	100	81	N.D.	82
Protocatechuic acid	1	20	9	N.D.	8
	5	58	32	N.D.	30
	10	73	46	N.D.	46
Glyoxylate	3	73	77	47	49
	10	91	93	70	74
Thiopheneglyoxylate	10	62	N.D.	52	35

N.D. = Not determined

4-hydroxy D,L-mandelate is 73% of the maximum rate at 1mM concentration, rate at 10 mM concentrations of 4-hydroxy 3-methoxy D,L-mandelate is 63% of the maximum rate at 1mM concentration.

Both D(–)- and L(+)-hexahydromandelate were found to be inhibitors of the enzyme from *R. graminis* . L(+)-Hexahydromandelate inhibited L(+)-mandelate dehydrogenase competitively with a K_i of 0.33 $\pm$ 0.07 mM (Fig. 4.23b), which compares with an apparent K_m value for L(+)-mandelate of 0.25 mM calculated from Fig. 4.23a. Other compounds which were not substrates but which inhibited the enzyme activity are given in Table 4.12. Purified L(+)-mandelate dehydrogenase from *R. graminis* was particularly strongly inhibited by L(+)-β-phenylactate and 4-hydroxyphenyl D,L- lactate and was fairly strongly inhibited by 2-hydroxy D,L caproate, 2-hydroxy D,L-isocaproate or oxalate. However, except for oxalate, such strong inhibitions by these compounds were not observed for L(+)-mandelate dehydrogenase of *A. calcoaceticus* (Table 4.12).

Possible inhibitory effects of the mandelate pathway metabolites and related compounds were also tested (Table 4.13 & 4.14). Benzoate, *p*-hydroxybenzoate, *m*-hydroxybenzoate were not strong inhibitors whereas dihydroxybenzoates, 2,6-dichlorobenzoate, protocatechuate or thiopheneglyoxylate were fairly strong inhibitors of L(+)-mandelate dehydrogenase from *R. graminis* (Table 4.13 & 14). Possible inhibitory effects of benzoate or hexahydroxymandelate were not tested for L(+)-mandelate dehydrogenase from *A. calcoaceticus* but protocatechuate or thiopheneglyoxylate were also fairly strong inhibitors of this enzyme (Table 4.14). Catechol and glyoxylate were particularly strong inhibitors of L(+)-mandelate dehydrogenases from both *R. graminis* and *A. calcoaceticus* (Table 4.14).

The enzymes from *R. graminis* and *A. calcoaceticus* were neither inhibited nor stimulated at 1 mM concentrations of acetyl-CoA, ADP/Mg^{2+}, ATP/Mg^{2+}, benzene sulphonate, citrate, glucose, glyceraldehyde, glycolate, isocitrate, D,L-malate, oxaloacetate, 2-oxoglutarate, phenylacetate, phenylglyoxylate, phenylpyruvate, phenylsuccinate, pyruvate, succinate or tartrate in the presence of their K_m or optimum substrate concentrations. However, 67 mM citrate buffer slightly inhibited the *R. graminis* enzyme.

4.7 Discussion

The results described in this chapter show that L(+)-mandelate dehydrogenase from *R. graminis* is a flavohaem protein. As will be outlined in this section, its physical, chemical and kinetic properties along with its compositional and sequence homologies with other proteins suggest that it is very similar to L(+)-lactate dehydrogenase of *S. cerevisiae* (Morton *et al.*, 1961; Guiard, 1985; Chapman *et al.*, 1991) and *H. anomala* (Labeyrie & Baudras, 1972; Chapman *et al.*, 1991). Its physical, chemical and kinetic properties are also similar to those of L(+)-mandelate dehydrogenase of *A. calcoaceticus* (Hoey *et al.*, 1987; Fewson, 1992) and *P. putida* (Tsou *et al.*, 1990; Fewson, 1992), and L(+)-lactate dehydrogenase of *A. calcoaceticus* (Allison & Fewson, 1986) and *E. coli* (Futai & Kimura, 1977; Kimura & Futai, 1978). This all suggests evolutionary relationship amongst these proteins.

4.7.1 Structural studies

An understanding of the molecular mechanism of enzymic catalysis depends on a detailed knowledge of the structure of that enzyme. Therefore, those general features that lead to a proposed model for the structure of L(+)-mandelate dehydrogenase from *R. graminis* are discussed in the following pages.

4.7.2 Quaternary structure

L(+)-Mandelate dehydrogenase has a native M_r of around 240,000 and a subunit M_r of 59,000 (section 4.2.1), similar to those of L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala* (Monteihet & Risely, 1970; Pajot & Groundinsky, 1970; Labeyrie & Baudras, 1972; Mevel-Nino, 1972; Chapman *et al.*, 1991) suggesting that L(+)-mandelate dehydrogenase of *R. graminis* is a homotetrameric enzyme like L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala* (Chapman *et al.*, 1991; present project). By contrast, bacterial enzymes are of smaller size, L(+)-lactate dehydrogenase of *A. calcoaceticus* and *E. coli* , L(+)-mandelate dehydrogenase of *A. calcoaceticus* and *P. putida* are membrane proteins with subunit M_r values of about 40,000 (Hegeman *et al.*, 1970; Futai & Kimura, 1977; Kimura & Futai, 1978; Allison & Fewson, 1986; Hoey *et al.*, 1987; Tsou *et al.*,

1990; Fewson, 1992). Interestingly, the flavodehydrogenase moiety isolated by proteolytic cleavage of L(+)-lactate dehydrogenase of *H. anomala* has a M_r of 39,000 (Celerier *et al.*, 1989). A similar flavodehydrogenase moiety could not be isolated from the *S. cerevisiae* enzyme but a fragment of M_r 11,000 was isolated that contained haem but was devoid of flavin and had no lactate dehydrogenase activity (Labeyrie *et al.*, 1966, 1967; Guird, *et al.*, 1973; Pompon & Lederer, 1976; Gervais *et al.*, 1983). A proteolytic fragment of M_r of 44,000 derived from L(+)-mandelate dehydrogenase of *R. graminis* was found after long period of storage indicating that L(+)-mandelate dehydrogenase might have been cleaved into a flavodehydrogenase domain (subunit M_r 44,000) and a haem domain, although no attempt was made to look for FMN because of the small amount of peptide available (Fig. 3.7). However, no smaller M_r band corresponding to a haem moiety could be seen in the gel, perhaps because of its smaller size. The larger sizes of L(+)-mandelate dehydrogenase from *R. graminis,* L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala* are thus probably due to the presence of a haem domain, absent from the smaller L(+)-mandelate dehydrogenases of *A. calcoaceticus* and *P. putida* and L(+)-lactate dehydrogenase of *A. calcoaceticus* and *E. coli* . Therefore the possible flavodehydrogenase domain of L(+)-mandelate dehydrogenase from *R . graminis* , and flavodehydrogenase domains of lactate dehydrogenases of *S. cerevisiae* and *H. anomala* are comparable in size to the FMN-containing mandelate dehydrogenase of *A. calcoaceticus* (Hoey *et al.*, 1987), *P. putida* (Tsou *et al* ., 1990; Fewson, 1992) and L(+)-lactate dehydrogenase of *A. calcoaceticus* (Allison & Fewson, 1986) and *E. coli* , (Futai & Kimura, 1977). However these are all quite different from the non-haem but FAD-containing membrane-bound D(–)-lactate and D(–)-mandelate dehydrogenase of *A. calcoaceticus* with subunit M_r values of 63,000 and 60,000 respectively (Allison *et al.*, 1985b).

In contrast to the membrane-associated, flavohaem, tetrameric L(+)-mandelate dehydrogenase from *R. graminis*, D(–)-mandelate dehydrogenase from *R. graminis* is a soluble dimeric, NAD^+-dependent protein with a native M_r of 77,200 (2 x 38,000 subunits; Baker & Fewson, 1989, Baker, 1990). The M_r of D(–)-mandelate dehydrogenase from *R. graminis* (Baker & Fewson 1989) is similar to that of other NAD^+-dependent

Table 4.15 Amino acid compositions of related mandelate dehydrogenases, lactate dehydrogenases and glycolate oxidase of spinach

The amino acid composition of L(+)-mandelate dehydrogenase (LMDH) from *R. graminis* is taken from Table 4.1. The amino acid sequences of L(+)-mandelate dehydrogenase from *P. putida* (Tsou *et al.*, 1990) and L(+)-lactate dehydrogenase (LLDH) of *S. cerevisiae* (Guiard,1985; Lederer *et al.*, 1985) and *H. anomala* (Black *et al.*, 1989) and glycolate oxidase (GOX) from spinach (Volokita & Somerville, 1987; Cederlund *et al.*, 1988) were used to calculated amino acid compositions. The amino acid composition of L(+)-lactate dehydrogenase of *E. coli* is taken from results of Kimura and Futai (1978). The amino acid percent values are given parentheses.

	LMDH *P. putida*	GOX spinach	LLDH *E. coli*	LLDH *S. cerevisiae*	LLDH *H. anomala*	LMDH *R. graminis*
A* ala	38 (9.7)	48 (13)	49 (12.3)	36 (7.0)	37 (7.4)	59 (10.9)
C cys	05 (1.3)	01 (0.3)	02.9(0.7)	07 (1.4)	03 (0.6)	02 (0.4)
D asp	27 (6.9)	19 (5.1)	-	34 (6.7)	41 (8.2)	-
D+N asx	39 (10)	29 (7.8)	40.7(10.2)	56 (11)	54 (10.8)	48 (8.8)
E glu	17 (4.3)	20 (5.4)	-	37 (7.2)	42 (8.4)	-
E+Q glx	34 (8.6)	31 (8.4)	33.3 (8.4)	53 (10.3)	62 (12.4)	61 (11.3)
F phe	10 (2.5)	14 (3.8)	12.6 (3.2)	14 (2.7)	18 (3.6)	16 (2.9)
G* gly	32 (8.1)	27 (7.3)	34.4 (8.6)	35 (6.7)	35 (7)	47 (8.8)
H his	10 (2.5)	04 (1.1)	07.3 (1.8)	08 (1.6)	13 (2.6)	09 (1.7)
I* ile	15 (3.8)	26 (7)	23.9 (6)	29 (5.7)	39 (7.8)	35 (6.5)
K lys	19 (4.8)	18 (4.9)	16.3 (4.1)	47 (9.2)	33 (6.6)	37 (6.8)
L* leu	52 (13.2)	31 (8.4)	42.7 (10.7)	51 (10.0)	44 (8.8)	52 (9.6)
M met	11 (2.8)	13 (3.5)	09.7 (2.4)	09 (1.8)	11 (2.2)	02 (0.4)
N asn	12 (3.1)	10 (2.7)	-	22 (4.3)	13 (2.6)	-
P* pro	15 (3.8)	14 (3.8)	20.7 (5.2)	32 (6.3)	28 (5.6)	29 (5.3)
Q gln	17 (4.3)	11 (3)	-	16 (3.1)	20 (4.0)	-
R arg	26 (6.6)	26 (7)	23.9 (6)	20 (3.9)	28 (5.6)	40 (7.5)
S ser	24 (6.1)	23 (6.2)	20.1 (5.0)	27 (5.3)	25 (5)	25 (4.6)
T thr	18 (4.6)	21 (5.7)	15.7 (3.9)	24 (4.7)	19 (3.8)	24 (4.5)
V* val	28 (7.1)	29 (7.9)	30.1(7.6)	43 (8.4)	33 (6.6)	52 (9.7)
W try	07 (1.8)	05 (1.4)	05.4 (1.4)	04 (0.8)	04 (0.8)	-
Y tyr	10 (2.5)	09 (2.4)	09.5 (2.4)	16 (3.1)	14 (2.8)	09 (1.6)
Hydrophobic residues* (%)	46	47	50	44	43	50

Table 4.16 Amino acid compositional relationships among related mandelate dehydrogenases, lactate dehydrogenases and glycolate oxidase of spinach

The SΔQ values were calculated with the equation, $S\Delta Q = \Sigma (X_a - X_b)^2$ (Vanni *et al.*, 1990) where X is mol % of a particular amino acid and a and b are the proteins to be compared. The data in Table 4.15 were used for for this comparison. The SΔQ units for asx and glx of *R. graminis* and *E coli* were calculated using the sum of asparatic acid and asparagine; glutamic acid and glutamine respectively of other proteins compared as shown in Table 4.15. The SΔQ values for other proteins were calculated using individual amino acids.

(a)

Enzyme	SΔQ when compared with Glycolate oxidase from spinach	SΔQ when compared with L(+)Mandelate dehydrogenase from *P. putida*
L(+)Mandelate dehydrogenase from *P. putida*	58[1]	
L(+)Lactate dehydrogenase from *E. coli*	25	25

(b)

Enzyme	SΔQ when compared with L(+)-Mandelate dehydrogenase from *R. graminis*	SΔQ when compared with L(+)-Lactate dehydrogenase from *S. cerevisiae*
L(+)-Lactate dehydrogenase from *S. cerevisiae*	53[2]	
L(+)-Lactate dehydrogenase from *H. anomala*	43[3]	30

[1] 44 SΔQ units are contributed by difference due to alanine, leucine and isoleucine only. The sum of of alanine, leucine and isoleucine is 105 in both glycolate oxidase of spinach and L(+)-mandelate dehydrogenase of *P. putida*.

[2] 28 SΔQ units are contributed by difference due to alanine and arginine only.

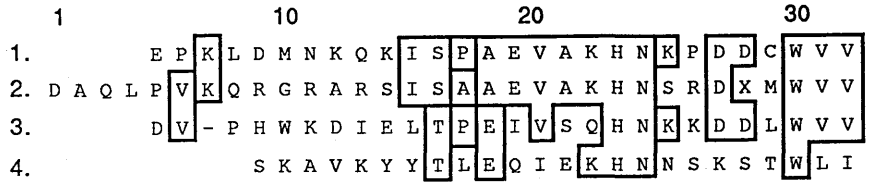
[3] 22 SΔQ units are contributed by difference due to alanine and valine only.

D(–)-enantiomer specific 2-hydroxyacid dehydrogenases from lactic acid bacteria, *Staphylococci*, *Streptococcus faecalis* and D(–)-mandelate dehydrogenase of *Lactobacillus curvatis* with native M_r of 70,000, 80,000, 72,000 and 60,000 respectively (Garvie, 1980; Yamazaki & Maeda, 1986a; Hummel *et al.*, 1988). The native M_r of D(–)-mandelate dehydrogenase from *R. graminis* (Baker & Fewson, 1989) is also similar to that of some other NAD-dependent, L(+)-specific 2-hydroxy acid dehydrogenase from *E. coli.*, *Bacillus subtillis* and *S. cerevisiae* (Murphy *et al.*, 1967; Holbrook *et al.*, 1975; Hagele *et al.*, 1978; Garvie, 1980; Fernley *et al.*, 1981; Turunen *et al.*, 1987).

4.7.3 Amino acid composition

Comparison of amino acid compositions often reveal evolutionary relatedness (Marchalonis & Weltman,1971; Cornish-Bowden, 1979; Yeh *et al.*, 1982; Vanni *et al.*, 1990). Comparison of the amino acid composition of purified proteins indicates the presence of overlapping evolutionary similarities amongst L(+)-mandelate dehydrogenase from *R. graminis*, L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala*, L(+)-mandelate dehydrogenase of *P. putida*, L(+)-lactate dehydrogenase of *E. coli*, and glycolate oxidase of spinach (Table 4.15, 4.16). All these proteins contain more than 43% hydrophobic residues (Table 4.15).

In a method developed by Marchalonis and Weltman (1971) for the pairwise comparison of protein amino acid compositions, the measure of relatedness, $S\Delta Q$, is the sum of the squares of the differences between percent of residues of two proteins being compared per subunit. Marchalonis and Weltman conducted 5,000 pairwise comparisons and found that a $S\Delta Q$ of about 50 reflects structural homology at the level of amino sequence (Marchalonis & Weltman, 1971; Yeh *et al.*, 1982). Therefore, a low $S\Delta Q$ value is indicative of a high degree or relatedness between two proteins whereas a high value (more than 100) indicates no relationship (Marchalonis & Weltman, 1971; Yeh *et al.*, 1982). This test has previously been used by many workers, for instance Yeh *et al*. (1982) compared transferase, hydrolase, decarboxylase and lactonizing enzyme of the β-ketoadipate pathway and found that $S\Delta Q$ values were less than 50 and similarly Vanni *et al*. (1990), obtained



Enzymes	Residues compared	Identity %	Similarity %
1-2	28	50	50
1-2	18	72	72
1-3	27	37	44
1-4	24	17	33
2-3	28	28	39
2-4	24	13	33
3-4	24	21	33

Figure 4.24 ***N*** **-Terminal amino acid sequence alignments of:**

1. L(+)-Lactate dehydrogenase of *Saccharomyces cerevisiae* (Guiard, 1985; Lederer *et al.*, 1985)
2. L(+)-mandelate dehydrogenase of *Rhodotorula graminis* (Present project)
3. L(+)-Lactate dehydrogenase of *H. anomala* (Black *et al* ., 1989)
4. Microsomal cytochrome b_5 of beef (Guiard *et al.*, 1974; Guiard & Lederer, 1976; Haumont *at al.*, 1987)

Identical residues are boxed and percent identities and percent similarities are given. Conservative replacements are based on the PAM 250 scoring matrix (Dayhoff *et al.*, 1978). The conservative replacements are I/L/V, D/E, K/R, T/S.

SΔQ values of less than 50 when comparing isocitrate lyases from nine different microorganisms.

In Table 4.16 SΔQ values are given for (a) FMN-containing enzymes of M_r of approximately 40,000 and (b) flavohaemproteins of M_r approximately 60,000. The SΔQ values of 25 to 58 are indicative of relatedness amongst these proteins (Table 4.16a and b).

4.7.4 Primary structure

The genes encoding L(+)-lactate dehydrogenase from *S. cerevisiae* (Guiard, 1985) and *H. anomala* (Black *et al.*, 1989; Risler *et al.*, 1989) have been sequenced and the corresponding amino acid sequences were deduced (Guiard, 1985; Black *et al.*, 1989). The DNA sequence of L(+)-lactate dehydrogenase from *S. cerevisiae* revealed 80-residues at the *N* -terminal are pre-sequence. The pre-sequence residues direct the enzyme into the mitochondrial intermembrane space where, after two successive proteolytic steps, the mature enzyme is formed (Daum *et al.*, 1982; Gasser *et al.*, 1982; Reid *et al.*, 1982; Hartle & Neuport, 1990). By analogy, the L(+)-mandelate dehydrogenase of *R. graminis* may have the same location and this could explain the experimental results which indicated it to be membrane-associated protein (see section 3.2.2).

The limited amino-terminal sequence of L(+)-mandelate dehydrogenase from *R. graminis* shows strong homologies with the amino-terminal sequence of the mature L(+)-lactate dehydrogenase from *S. cerevisiae* (Guiard, 1985, Lederer *et al.*, 1985) and *H. anomala* (Black *et al.*, 1989; Fig. 4.24). It has 50% identity with L(+)-lactate dehydrogenase of *S. cerevisiae* when the first 28 amino acids are compared. The percent identity increases to 72% when amino acid numbers from 14 to 32 (18 amino acids) are compared (Fig. 4.24). A similar increase in percent identity is seen when amino terminal sequence of L(+)-mandelate dehydrogenase of *R. graminis* is compared with L(+)-lactate dehydrogenase from *H. anomala* (Black *et al.*, 1989) and beef cytochrome b_5 (Ozols & Strittmatter, 1968, 1969; Fig. 4.24). The percent identities and similarities amongst L(+)-mandelate dehydrogenase from *R. graminis*, L(+)-lactate dehydrogenase from *S. cerevisiae* (Guiard, 1985, Lederer *et al.*, 1985) and *H. anomala* (Black *et al.*, 1989) (Fig. 4.24) strongly suggest that these proteins have close evolutionary relationship.

Figure 4.25 Sequence alignments of :

1. L(+)-Lactate dehydrogenase of *Saccharomyces cerevisiae* (Guiard, 1985; Lederer *et al.*, 1985)

2. L(+)-Lactate dehydrogenase of *Hansenula anomala* (Black *et al.*, 1989)

3. L(+)-Mandelate dehydrogenase of *Rhodotorula graminis* (present project)

4. Glycolate oxidase of spinach (Volokita & Somerville, 1987; Cederlund *et al.*, 1989)

5. L(+)-Mandelate dehydrogenase of *Pseudomonas putida* (Tsou *et al.*, 1990)

6. L(+)-Mandelate dehydrogenase of *Acinetobacter calcoaceticus* (C.A. Fewson, personal communication)

7. L(+)-Lactate dehydrogenase of *Acinetobacter calcoaceticus* (C.A. Fewson, personal communication)

The sequence alignments are shown with a identical residues in bold type. Possible amino acids near the active sites (based on knowledge of glycolate oxidase), conserved in all enzymes as shown by asterisks. The interdomain hinge region and protease sensitive loop in L(+)-Lactate dehydrogenase of *S. cerevisiae* and *H. anomala* are underlined.

```
1      EPKLDMNKQKISPAEVAKHNKPDDCWVVINGYVYDLTRFLPNHPGGQDVIKFN          53
2      DV-PHWKDIELTPEIVSQHNKKDDLWVVLNGQVYDLTDFLPNHPGGQKIIIRY          52
3  DAQLPVKQRGRARSISAAEVAKHNSRDDMWVV                                   32

1  AGKDVTAIFEPLHAPNVIDKYIAPEKKLGPLQGSMPPELVCPPYAPGETKEDIARKEQLKS     114
2  AGKDATKIFVPIHPPDTIEKFIPPEKHLGPLVGEFEQE------EEELSDEEIDRLERIER     107

1  LLPPLDNIINLYDFEYLASQTLTKQAWAYYSSGANDEVTHRENHNAYHRIFFKPKILVDVR     175
2  -KPPLSQMINLHDFETIARQILPPPALAYYCSAADDEVTLRENHNAYHRIFFNPKILIDVK     167
4       MEITNVNEYEAIAKQKLPKMVYDYYASGAEDQWTLAENRNAFSRILFRPRILIDVT      56
5     MSQNLFNVEDYRKLAQKRLPKMVYDYLEGGAEDEYGVKHNRDVFQQWRFKPKRLVDVS      58
6       PHKMINVEDYQKLAKSTLPKVIYDYLEGGADDEKGLHHNRQVFDQKWFKP            49
7       MIISSANDYREAARRRLPPFLFHYIDGGAYAEYTLKRNVEDLXWIALWQ             49
                              *

1  KVDISTDMLGSHVDVPFYVSATALCKLGNPLEGEKDVARGCGQGVTKVPQMISTLASCSPE     236
2  DVDISTEFFGEKTSAPFYISATALAKLGHP-EGEVAIAKGAGRE--DVVQMISTLASCSFD     225
4  NIDMTTTILGFKISMPIMIAPTAMQKMAHP-EGEYATARAASAAGT-----IMTLSSWATS     111
5  RRSLQAEVLGKRQSMPLLIGPTGLNGALWP-KGDLALARAATKAGI-----PFVLSTASNM     113
                         *

1  EIIEAAPSDKQIQWYQLYVNSDRKITDDLVKNVEKLGVKALFVTVDAPSLGQREKDMKLKF     297
2  EIADARIPGQQ-QWYQLYVNADRSITEKAVRHAEERGMKGLFITVDAPSLGRREKDMKMKF     285
4  SVEEVASTGPGIRFFQLYVYKDRNVVAQLVRRAERAGFKAIALTVDTPRLGRREADIKNRF     172
5  SIEDLARQCDGDLWFQLYV-IHREIAQGMVLKALHTGYTTLVLTTDVAVNGYRERDLHNRF     173
                  * *                          * *

1  ------------------___SNTKAGPKAMKKTNVEESQGAS-----RALSKFIDPSLTWK     333
2  ------------------EADS-DVQG----DDEDIDRSQGAS-----RALSSFIDPSLSWK     319
4  VLPPFL--------------TLKNFEGIDLGKMDKANDSGLS-----SYVAGQIDRSLSWK     214
5  KIPMSYSAKVVLDGCLHPRWSLDFVRHGMPQLANFVSSQTSSLEMQAALMSRQMDASFNWE     234

1  DIEELKKKTKLPIVIKGVQRTEDVIKAAEIGVSGVVLSNHGGRQLDFSRAPIEVLAETMPI     394
2  DIAFIKSITKMPIVIKGVQRKEDVLLAAEHGLQGVVLSNHGGRQLDYTRAPVEVLAEVMPI     380
4  DVAWLQTITSLPILVKGVITAEDARLAVQHGAAGIIVSNHGARQLDYVPATIMALEEVV--     273
5  ALRWLRDLWPHKLLVKGLLSAEDADRCIAEGADGVILSNHGGRQLDCAISPMEVLAQSV--     293
                  *                          *  *

1  LEQRNLKDKLEVFVDGGVRRGTDVLKALCLGAKGVGLGRPFLYANSCYGRNGVEKAIEILR     455
2  LKERGLDQKIDTFVDGGVRRGTDVLKALCLGAKGVGLGRPFLYAMSSYGDKGVTKAIQLLK     441
4  ---KAAQGRIPVFLDGGVRRGTDVFKALALGAAGVFIGRPVVFSLAAEGEAGVKKVLQMMR     331
5  -----AKTGKPVLIDSGFRRGSDIVKALALGAEAVLLGRATLYGLAARGETGVDEVLTLLK     349

1  DEIEMSMRLLGVTSIAELKPDLL--DLSTLKARTVGVPNDVLYNEVYEGPTLTEFEDA       511
2  DEIEMNMRLLGVNKIEELTPELL--DTRSIHNRAVPVAKDYLYEQNYQRMSGAEFRPGIED    500
4  DEFELTMALSGCRSLKEISRSHIAADWDGPSSRAVARL                           369
5  ADIDRTLAQIGCPDITSLSPDYLQ-NEGVTNTAPVDHLIGKGTHA                    393
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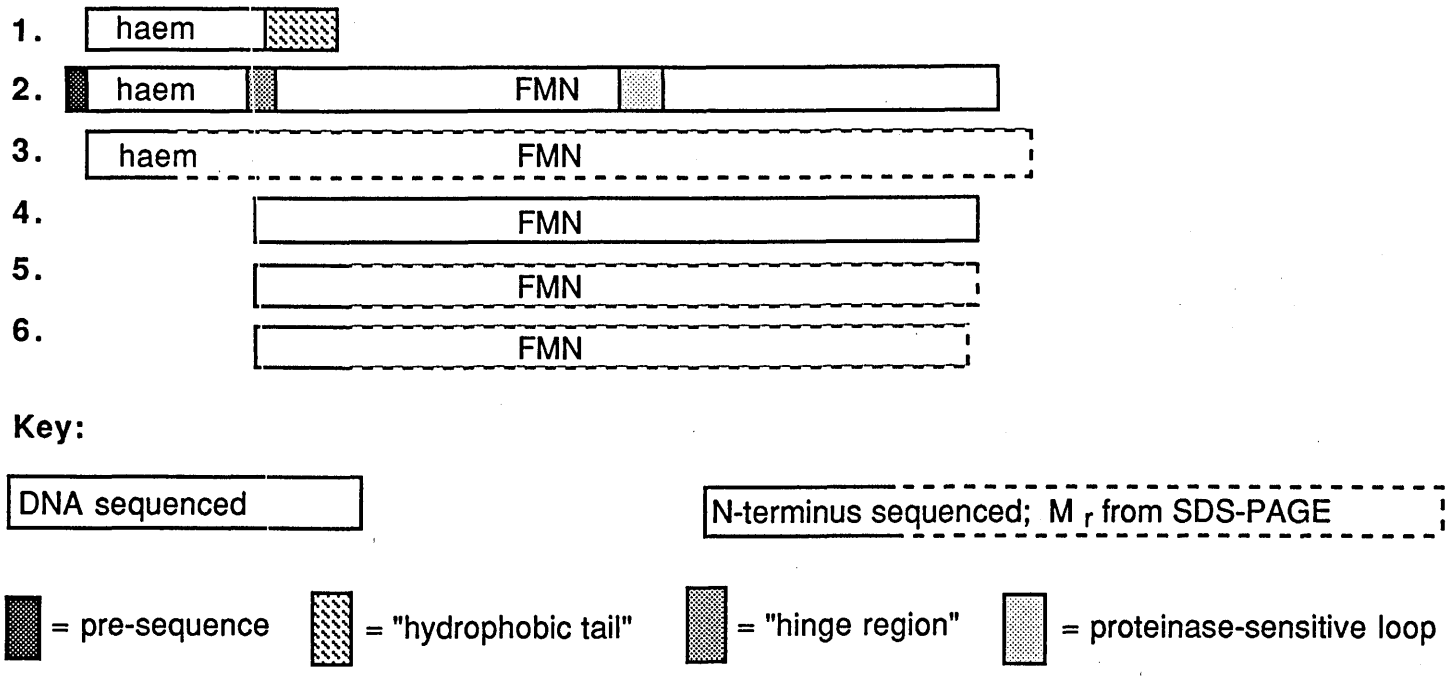


Figure 4.26 Diagramatic representation of domain structures based on cofactor studies, DNA sequences and amino-terminal sequences and M_r values determined by SDS-PAGE of:

1. Beef microsomal cytochrome b_5 (Guiard *et al.*, 1974; Guiard & Lederer, 1979)
2. L(+)-Lactate dehydrogenase from *Saccharomyces cerevisiae* (Guiard, 1985; Lederer *et al.*, 1985; Chapman *et al.*, 1991)
3. L(+)-Mandelate dehydrogenase from *Rhodotorula graminis* (Present project)
4. L(+)-Mandelate dehydrogenase from *Pseudomonas putida* (Tsou *et al.*, 1990)
5. L(+)-Mandelate dehydrogenase from *Acinetobacter calcoaceticus* (Hoey *et al.*, 1987; C.A. Fewson, personal communication)
6. L(+)-Lactate dehydrogenase from *Acinetobacter calcoaceticus* (Allison & Fewson, 1986; C.A. Fewson, personal communication)

4.7.5 Proposed domain structures

A diagrammatic representation of the domain structure for different dehydrogenases is proposed in Fig. 4.26.

(a) The haem domain

The spectral properties of native, purified L(+)-mandelate dehydrogenase (Fig. 4.3) were like that of microsomal cytochrome b_5 and L(+)-lactate dehydrogenase from *S. cerevisiae* (Labeyrie *et al.*, 1966; Pajot & Groudinskry, 1970; Chapman *et al.*, 1991). These similarities in the spectral properties can be extended to homology amongst amino-terminal amino acid sequences of L(+)-mandelate dehydrogenase of *R. graminis*, L(+)-lactate dehydrogenase of *S. cerevisiae* (Guiard, 1985, Lederer *et al.*, 1985) and *H. anomala* (Black *et al.*, 1989), haem domains of sulphite oxidase from rat and chicken and cytochrome b_5 of beef and other species (Guiard & Lederer, 1977 & 1979a,b) and assimilatory nitrate reductase (Le & Lederer, 1983). Guird and Lederer (1979a) suggested the existence of common "cytochrome b_5 fold" which is supported by similarity between three-dimensional structures of microsomal cytochrome b_5 (Mathews *et al.*, 1972) and the haem domain of L(+)-lactate dehydrogenase from *S. cerevisiae* (Mathews & Xia, 1987; Xia *et al.*, 1987; Xia & Mathews, 1990).

The haem domain of L(+)-mandelate dehydrogenase of *R. graminis* is located at the amino-terminus of the protein, as suggested by amino acid sequence comparison with L(+)-lactate dehydrogenase from *S. cerevisiae* (Guiard, 1985), *H. anomala* (Black *et al.*, 1989) and other haem containing proteins (Fig. 4.24 & 4.25). The haem containing domain of *S. cerevisiae* is much smaller than the FMN-containing domain, as shown in Fig. 4.27 and it consists of residues 1-100 whereas residues 101-488 constitute the flavodehydrogenase domain (Mathews & Xia, 1987; Xia *et al.*, 1987; Xia & Mathews, 1990). Residues at the C-terminal tail wraps around the centre of the enzyme (Xia & Mathews, 1990) and its contacts with subunit structures perhaps are important in stabilising the L(+)-lactate dehydrogenase of *S. cerevisiae*. However, such studies have not been conducted for L(+)-mandelate dehydrogenase of *R. graminis* but Fig. 3.7 suggests that perhaps haem domain is also smaller than the flavodehydrogenase domain and C-terminal

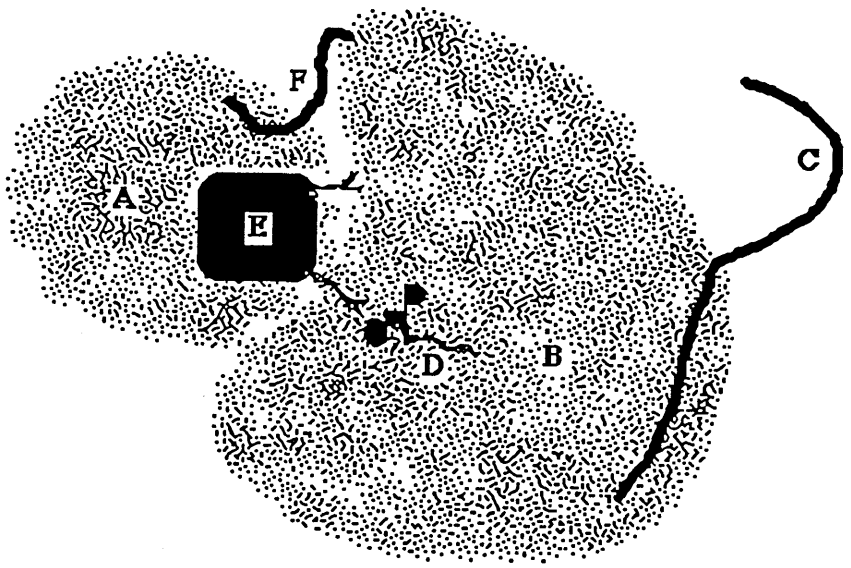


Figure 4.27 Schematic representation of the structure of a flavocytochrome b_2 A, Haem domain; B, Flavodehydrogenase domain; C, C-Terminal tail; D, FMN; E, Haem; F, Hinge region linking the flavin and haem domains. Adapted from Chapman *et al.*, 1991.

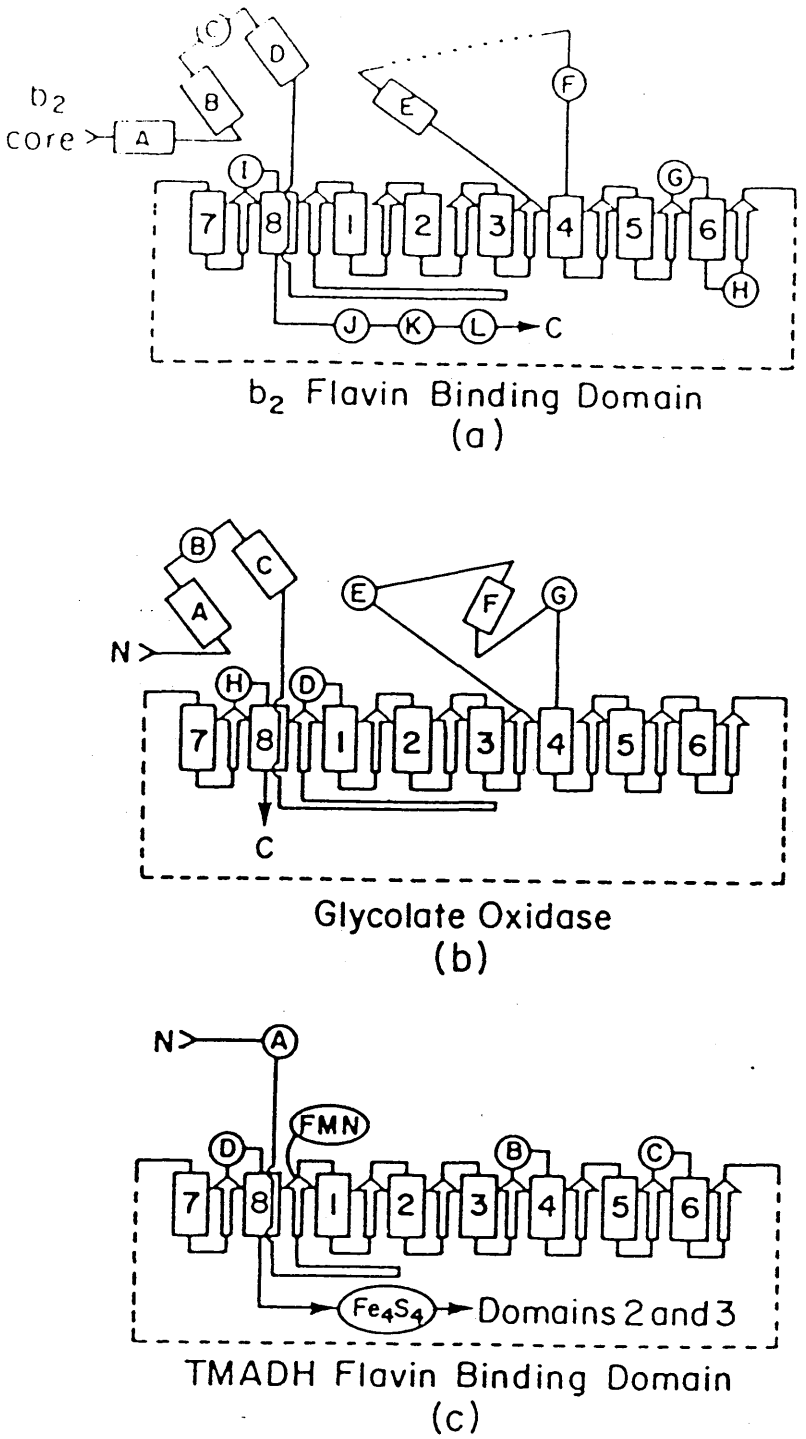


Figure 4.28 Folding topology of flavin binding domains of: (a) L(+)-lactate dehydrogenase of *S. cerevisiae* (b) glycolate oxidase of spinach (c) trimethylamine dehydrogenase (TMADH) of methylotrophic bacterium. From Xia *et al.*, 1987.

tail contacts explained for *S. cerevisiae* enzyme may also be present for L(+)-mandelate dehydrogenase of *R. graminis* .

(b) The flavodehydrogenase domain

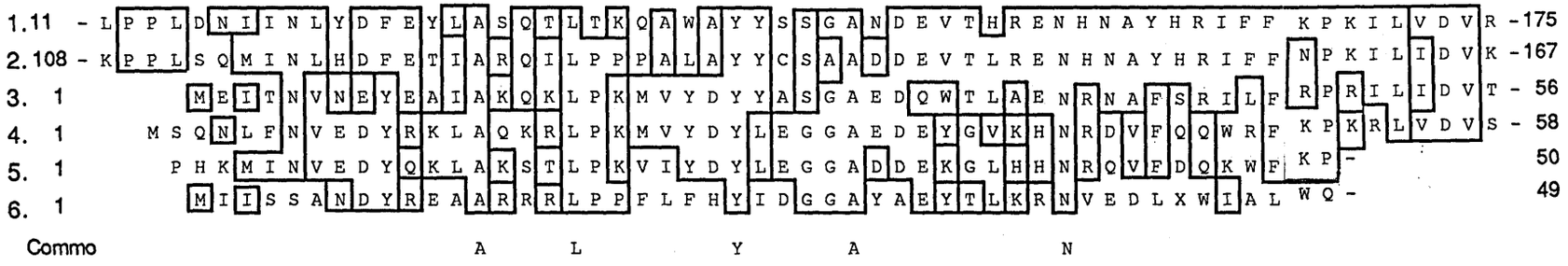
An apparent band of M_r of 44,000 separated from the native L(+)-mandelate dehydrogenase of *R. graminis* (Fig. 3.7) was tentatively identified as the flavodehydrogenase domain, as in L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala* (Gervais *et al.*, 1983; Celerier *et al.*, 1989). The separation of flavodehydrogenase domain and haem domain and recovery of ferricyanide reductase activity suggests an autonomous folding of two domains in L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala* (Gervais *et al.*, 1983; Celerier *et al.*, 1989). A similar autonomous flavodehydrogenase domain may be present in L(+)-mandelate dehydrogenase (Fig. 3.7 & section 3.8) of *R. graminis* but experiments were not done to confirm this.

Recently, the amino-terminal sequences of FMN-linked L(+)-lactate dehydrogenase and L(+)-mandelate dehydrogenase of *A. calcoaceticus* have been determined (C.A. Fewson, personal communication). The complete amino acid sequences of FMN-linked glycolate oxidase from spinach (Volokita & Somerville, 1987; Cederlund *et al.*, 1988) and L(+)-mandelate dehydrogenase of *P. putida* (Tsou *et al.*, 1990) are also available (Fig. 4.25). The amino acid sequence of L(+)-mandelate dehydrogenase from *P. putida* has an overall identity of 43% with either L(+)-lactate dehydrogenase from *S. cerevisiae* or glycolate oxidase of spinach (Tsou *et al.*, 1990). It also showed 62% identity with limited *N*-terminal sequences of L(+)-mandelate dehydrogenase of *A. calcoaceticus* (C.A. Fewson, personal communications & Fig. 4.25, 4.29). The limited amino-terminal sequences of L(+)-mandelate dehydrogenase of *A. calcoaceticus* and *P. putida* , L(+)-lactate dehydrogenase of *A. calcoaceticus*, glycolate oxidase of spinach, flavodehydrogenase domains of L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala* share significant percent identities (Fig. 4.25, 4.29). L(+)-Mandelate dehydrogenase from *R. graminis* has not been sequenced fully, therefore it is not possible to compare with enzymes in Fig. 4.29. However, a comparison of amino acid sequences of L(+)-mandelate dehydrogenase of

Figure 4.29 Sequence alignments of

1. L(+)-Lactate dehydrogenase of *Saccharomyces cerevisiae* (Guiard, 1985; Lederer *at al.*, 1985)
2. L(+)-Lactate dehydrogenase of *Hansenula anomala* (Black *at al.*, 1989)
3. Glycolate oxidase of spinach (Volokita & Somerville, 1987; Cederlund *et al.*, 1988)
4. L(+)-Mandelate dehydrogenase of *Pseudomonas putida* (Tsou *et al.*, 1990)
5. L(+)-Mandelate dehydrogenase of *Acinetobacter calcoaceticus* (C.A. Fewson, personal communication)
6. L(+)-Lactate dehydrogenase of *Acinetobacter calcoaceticus* (C.A. Fewson, personal communication)

Identical residues are boxed and percent identities and percent similarities are given. Conservative replacements are based on the PAM 250 scoring matrix (Dayhoff *et al.*, 1978). The conservative replacements are I/L/V, D/E, K/R, T/S.



Enzymes	Residues compared	Identity %	Similarity %
1-2	60	67	70
1-3	56	46	57
1-4	58	34	41
1-5	50	34	36
1-6	49	22	24
2-3	56	46	55
2-4	58	26	36
2-5	50	32	38
2-6	49	27	29

Enzymes	Residues compared	Identity %	Similarity %
3-4	56	41	55
3-5	50	40	48
3-6	49	29	39
4-5	50	62	70
4-6	49	29	43
5-6	49	24	33

R. graminis with L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala* (Guiard, 1985, Black *et al.,* 1989 & Fig. 4.24) and comparison of internal sequences of L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala* with amino-terminal sequences of L(+)-mandelate dehydrogenase of *A. calcoaceticus* (C.A. Fewson, personal communication) and *P. putida* (Tsou *et al.,* 1990), L(+)-lactate dehydrogenase of *A. calcoaceticus* (C.A. Fewson personal communication), glycolate oxidase of spinach (Volokita & Somerville, 1987) (Fig. 4.29) give indirect evidence that L(+)-mandelate dehydrogenase of *R. graminis* and the bacterial FMN enzymes may share sequence homoloies. Limited amino acid sequence information of lactate oxidase of *Mycobacterium smegmatis* (Giegel *et al.,* 1990) and long chain α-hydroxy acid oxidase from rat kidney (Urban *et al.,* 1988) also shows significant homologies with the FMN-containing L(+)-mandelate dehydrogenase of *A. calcoaceticus* (C.A. Fewson, personal communication) and *P. putida* , (Tsou *et al.,* 1990), L(+)-lactate dehydrogenase of *A. calcoaceticus* (C.A. Fewson, personal communication), glycolate oxidase of spinach (Volvokita & Somerville, 1987; Cederlund *et al.,* 1988) and L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala* (Guird, 1985; Black *et al.,* 1989). The flavodehydrogenase domain of L(+)-lactate dehydrogenase of *S. cerevisiae* (Xie *et al.,* 1987) is folded similarly to that of glycolate oxidase of spinach (Lindqvist & Branden, 1985) and trimethylamine dehydrogenase of methylotrophic bacterium W3A1 (Lim *et al.,* 1986) and all contain similar eight stranded α/β-barrel domains. The three flavodehydrogenase domains are schematically compared in Fig. 4.28 (Xia *et al.,* 1987). The similar folding patterns further suggest an evolutionary relationship amongst FMN containing enzymes.

The flavocytochrome b_2 enzymes from *S. cerevisiae*, (Guiard, 1985; Lederer *et al.,* 1985), *H. anomala* (Black *et al.,* 1989), *R. graminis* and FMN-containing L(+)-mandelate dehydrogenase of *A. calcoaceticus* (Hoey *et al.,* 1987; C.A. Fewson, personal communication) and *P. putida* (Tsou *et al.,* 1990; C.A. Fewson, personal communication), glycolate oxidase from spinach (Volokita & Somerville, 1987; Cederlund *et al.,* 1988) show no detectable sequence homologies with the lactate or mandelate dehydrogenases which use FAD and NAD as cofactor. However, FAD-linked

D(–)-mandelate dehydrogenase from *A. calcoaceticus* does show sequences homologies with D(–)-lactate dehydrogenase from the same organism and *E. coli* (C.A. Fewson, personal communications) and thus indicating their possible common evolutionary origin.

The amino terminal sequence of D(–)-mandelate dehydrogenase (Baker, 1990) shows no detectable sequence homologies with any of the proteins compared in Fig. 4.25. Baker (1990) could not find any homology with sequences in the University of Leeds OWL composite database (Release 1.1) which contained all the proteins sequences in the NBRF PIR and NEW 16.0, SWISSS-PROT 6.0, Gen-Bank 54, NEWAT 86, JIPID and Brockhaven (October, 1987) data bases (Akrigg *et al.,* 1988).

These amino acid sequence comparisons suggest different evolutionary origin for the NAD-dependent, FAD-linked and FMN-linked enzymes.

4.7.6 Active site

Because no detailed investigation of the active site of L(+)-mandelate dehydrogenase of *R. graminis* was undertaken, it is only possible to speculate about its nature. However, 13 amino acids have been described at the active site of the homologous enzyme, glycolate oxidase (Lindqvist & Branden, 1985 & 1989). Of these 13 amino acids, tyrosine 143 is conserved in all enzymes in Fig. 4.25. Similarly threonine 197, glutamine 252, tyrosine 254, threonine 280, aspartic acid 282, lysine 349, histidine 373, arginine 376 (all shown with asterisk in Fig. 4.25) are conserved in L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala* and L(+)-mandelate dehydrogenase of *P. putida* (using numbering system of *S. cerevisiae* enzyme; Fig. 4.25). Leucine 230, which is in van der Waals contacts with the methyl group of the substrate in L(+)-lactate dehydrogenase of *S. cerevisiae* (Chapman *et al.,* 1991), is also conserved in L(+)-lactate dehydrogenase of *H. anomala,* glycolate oxidase of spinach and L(+)-mandelate dehydrogenase of *P. putida* (Fig. 4.25). The 10 amino acids (out of 14, including leucine 230), presumably serve equivalent functions in the enzymes given in Fig. 4.25. Tyrosine 144, alanines 196 and 231 and cysteine 233 present in L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala* are replaced by leucine 144, proline 196, serine 231 and tryptophan 233 respectively in L(+)-mandelate dehydrogenase of *P. putida.* Perhaps these changes at the active site of L(+)-mandelate

dehydrogenase of *P. putida* may provide space at the active site to accommodate the phenyl group of mandelate and are probably responsible for the different substrate specificity. Indeed L(+)-lactate is a potent inhibitor of the mandelate dehydrogenase of *R. graminis* (Table 4.9) and L(+)-mandelate is a potent inhibitor of L(+)-lactate dehydrogenase of *S. cerevisiae* (Dikstein, 1959; Morton *et al.*, 1961). Although we cannot identify the active site amino acids of the L(+)-mandelate dehydrogenases of *R. graminis* , *A. calcoaceticus* , and the L(+)-lactate dehydrogenase of *A. calcoaceticus* because of limited sequence data, but on the basis of domain studies, amino acid sequence and compositional similarities (Fig. 4.24-4.26, 4.28-4.29 & Tables 4.15, 4.16) and other properties discussed in the next sections, it is suggested that to some degree all these enzymes may have conserved active site amino acids that are present in L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala* , L(+)-mandelate dehydrogenase *P. putida* and glycolate oxidase from spinach. To confirm this, full sequences would have to be determined. Perhaps the effect of pH on K_m and V_{max} values (Fig. 4.20) could be explained partly in terms of ionization of these active site amino acids. The proposed role of different active site amino acids is diagrammatically represented in Fig. 4.30 (for detail of mechanism see Reid *et al.*, 1988; Dubois *et al.*, 1990; Chapman *et al.*, 1991).

The pH optima of L(+)-mandelate dehydrogenases of *R. graminis* , *A. calcoaceticus* , *P. putida* , L(+)-lactate dehydrogenases from *S. cerevisiae* , *H. anomala* , *A. calcoaceticus* , *E. coli* and glycolate oxidase range from 7 - 8.5 (Futai & Kimura, 1977; Allison & Fewson, 1985a; Hoey *et al.*, 1987; Chapman *et al.*, 1991; present project). At this pH all substrates for these enzymes will be anions. At pH 7.5 arginine 376 (pKa ~12) and lysine 349 (pKa ~10.5) will be positively charged whereas histidine 373 will be neutral (pKa ~6.2) (Dikstein, 1959; Creighton, 1983; Dawson *et al.*, 1986). Therefore, the proposed role for side chains of positively charged amino acids in a carbanion mechanism, proposed for the oxidation of L(+)-lactate by L(+)-lactate dehydrogenase (Fig. 4.30) of *S. cerevisiae* (Reid *et al.*, 1988; Dubois *et al.*, 1990; Chapman *et al.*, 1991), may also apply for the oxidation of substrates by L(+)-mandelate dehydrogenase of *R. graminis* and *A. calcoaceticus* and L(+)-lactate dehydrogenases of *A. calcoaceticus* or *E. coli* or glycolate oxidase of spinach (Boeri & Rippa, 1961; Frigerio & Harbury, 1958; Futai & Kimura, 1977;

Allison & Fewson, 1985a,b; Hoey *et al.*, 1987; Chapman *et al.*, 1991, present project). Each of these enzymes has a single pH optimum which is different from two pH optima values for the soluble D(–)-mandelate dehydrogenase of *R. graminis* (Baker & Fewson, 1989, Baker, 1990) and *L. curvatus* (Hummel *et al.*, 1988) and D(–)-2-hydroxy isocaproate dehydrogenase of *S. faecalis* (Yamazaki & Maeda, 1986a). These NAD^+-dependent dehydrogenases have one pH optimum for the oxidation reaction and the other for the reduction reaction (Yamazaki & Maeda, 1986a,b; Hummel *et al.*, 1988; Baker & Fewson, 1989). Similarly, a number of other bacterial and fungal NAD^+-dependent 2-hydroxyacid dehydrogenases have two pH optimal values (Le John, 1971; Garvie, 1980).

L(+)-Mandelate dehydrogenase from *R. graminis* catalyses an essentially irreversible oxidation of mandelate (section 4.4.6), which is similar to that of mandelate and lactate dehydrogenases from *A. calcoaceticus* (Allison *et al.*, 1985a; Allison & Fewson, 1986; Hoey *et al.*, 1987), L(+)-lactate dehydrogenases from *E. coli* (Futai & Kimura, 1977) and *S. cerevisiae* (Chapman *et al.*, 1991). By contrast, the NAD^+-dependent D(–)-mandelate dehydrogenases from *R. graminis* (Baker & Fewson, 1989), *L. curvatus* (Hummel *et al.*, 1988) and D(–)-2-hydroxy isocaproate dehydrogenase or *S. faecalis* (Yamazaki & Maeda, 1986a,b) catalyse reversible reactions.

Some bacterial and yeast lactate dehydrogenases require divalent metal ions for activity (Boeri & Tosi, 1956; Hatefi & Stiggal, 1976; Futai & Kimura, 1977). However, the activity of L(+)-mandelate dehydrogenase of *R. graminis* , like the L(+)-lactate and mandelate dehydrogenases of *A. calcoaceticus* (Allison *et al.*, 1985 a, b; Allison & Fewson, 1986; Hoey *et al.*, 1987) is not affected by a range of salts, metal ions or metal chelating agents (Table 4.6 & 4.7). These results presumably indicate that L(+)-mandelate dehydrogenase of *R. graminis* has no metal ion requirement other than iron in the haem group. Similarly, addition of iron to L(+)-lactate dehydrogenase of *S. cerevisiae* gave no activation (Appleby & Morton 1959) and metal chelating agents caused no loss in enzyme activity (Boeri & Ripa, 1961; Morton *et al.*, 1961).

The presence of one or more cysteine residues can perhaps be inferred from the amino acid compositions given in Table 4.15. Effect of thiol blocking reagents were checked in order to study whether cysteine residues are important for the activity of these

enzymes. L(+)-Mandelate dehydrogenase activity is insensitive to iodoacetate and iodoacetamide (Table 4.8), which is similar to the position with L(+)-mandelate dehydrogenase of *A. calcoaceticus* (Hoey *et al.*, 1987), L(+)-lactate dehydrogenase of *A. calcoaceticus* (Allison & Fewson, 1986), *S. cerevisiae* and *E. coli* (Morton *et al.*, 1961; Morton & Sturterrant, 1964, Futai & Kimura, 1977). *N*-Ethylmaleimide is an effective inhibitor of L(+)-mandelate dehydrogenase activity of *R. graminis* (Table 4.8, *A. calcoaceticus* (Hoey *et al.*, 1987) and L(+)-lactate dehydrogenase activity of *S. cerevisiae* (Morton *et al.*, 1961). *p*-Chloromercuribenzoate inhibited the L(+)-lactate dehydrogenase activity of *S. cerevisiae* with the appearance of strong fluorescence due to the displacement of FMN (Morton *et al* . 1961; Symons & Burgoyne, 1966). The appearance of fluorescence was much reduced in the presence of substrate which protected enzyme activity Labeyrie *et al.*, 1978), perhaps by maintaining the FMN group in the reduced state and thus lowering its dissociation (Morton *et al.*, 1961). *p*-Chloromercuribenzoate also strongly inhibits the activity of L(+)-mandelate dehydrogenase of *R. graminis* (Table 4.8) and *A. calcoaceticus* (Hoey *et al.*, 1987) but the appearance of fluorescence was not determined. Presence of substrate apparently does not protect enzyme activity against *p*-chloromercuribenzoate but substrate slightly protects activity against *N*-ethylmaleimide inhibition in the *R. graminis* enzyme (Fig. 4.18). *p*-Chloromercuribenzoate and mercuric chloride also strongly inhibit L(+)-lactate dehydrogenase, D(–)-lactate dehydrogenase and D(–)-mandelate dehydrogenase activity of *A. calcoaceticus* (Allison *et al.*, 1985b; Allison & Fewson 1986, Hoey *et al.*, 1987). These results suggest that perhaps cysteine is located at or near the active site of these enzymes, however, more detailed experiments are needed to confirm this hypothesis.

4.7.7 Inter domain interaction and protease sensitive regions

The interdomain hinge region and protease-sensitive loop are shown in Fig. 4.25 and 4.27 for the L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala* (Chapman *et al.*, 1991). These inter-domain regions were sensitive to *S. aureus* V 8 protease activity and L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala* were thereby separated into cytochrome b_2 and flavodehydrogenase domains. However in *S. cerevisiae* another

protease sensitive loop was cleaved 20 times faster than the interdomain hinge region and therefore the isolated nicked flavodehydrogenase domain retained only about one percent of the original activity (Pompon & Lederer, 1976; Ghrir & Lederer, 1981; Gervais *et al.*, 1983). The interdomain hinge region L(+)-lactate dehydrogenase of *H. anomala* was cleaved highly specifically with *Staphylococcus aureus* V 8 proteinase 1 and the isolated dehydrogenase domain retained 70-80% of the original activity (Gervais *et al.*, 1983; Celerier *et al.*, 1989). This shows that the cytochrome b_2 domain is not needed for lactate dehydrogenase activity if DCIP or ferricyanide are used as electron acceptors. However, the cytochrome b_2 domain is necessary when cytochrome *c* is used as an electron acceptor in the reaction mixture (Gervais *et al.*, 1983; Chapman *et al.*, 1991).

A similar interdomain hinge region may perhaps be present in L(+)-mandelate dehydrogenase of *R. graminis* and Fig. 3.7 is the first evidence for the possible existence of such a region.

4.7.8 Physical and chemical properties

4.7.8.1 Effect of potential stabilizing agents and PMS on the activity of L(+)-mandelate dehydrogenases

Membrane bound or membrane associated enzymes are often activated in the presence of detergents (Kimura & Futai, 1978; Prat *et al.*, 1979; Kovatchev *et al.*, 1981). Detergents and BSA have similar effect on L(+)-mandelate dehydrogenase from *A. calcoaceticus*, *P. putida* and *R. graminis* (Table 4.5 & Hoey *et al.*, 1987) and L(+)-lactate dehydrogenase from *A. calcoaceticus* (Allison *et al.*, 1988b). Significant increase in activity (two to many-fold) was obtained for L(+)-mandelate dehydrogenases from *A. calcoaceticus*, *P. putida* and *R. graminis* in the presence of Triton X-100 or BSA (Table 4.4-4.5; Fig. 4.13-4.14 & Hoey *et al.*, 1987). Similarly mixture of phospholipids increase the activity of L(+)-lactate dehydrogenase and D(–)-lactate dehydrogenase from *E. coli* by three-fold and five-fold respectively (Kimura & Futai, 1978; Kovatchev *et al.*, 1981; Ho *et al.*, 1989). The exact reason for the increase in activities is not known but perhaps the enzyme conformation is changed to a more active form in the presence of detergent or BSA (Coleman, 1973; Gennis *et al.*, 1976; Kimura & Futai, 1978).

Increase in activity was also observed for L(+)-mandelate dehydrogenase from *A. calcoaceticus* , *P. putida* and *R. graminis* , if PMS was added to the reaction mixture (Table 4.4, 4.5; Fig. 4.15 & Hoey *et al.,* 1987). This increase was perhaps due to efficient flow of electrons from enzymes to electron acceptors.

4.7.8.2 Thermal stability and reactivation of L(+)-mandelate dehydrogenase from *R. graminis*

L(+)-Mandelate dehydrogenase from *R. graminis* is very unstable even at 0^0C (Table 4.3 & Fig. 4.8-4.11). The enzyme seems to be more thermostable when incubated with Triton X-100 or substrate (Table 4.3 & Fig. 4.8). Other detergents and BSA, have very similar effects on the stabilization (Table 4.3 & Fig. 4.8-4.11). Durham (1984) found that the time required for 50% inactivation of the enzyme activity in crude extract was 45 min at 52°C which is much longer time than that required for 50% inactivation of the purified enzyme (Table 4.3). Other proteins or phospholipids in the crude extracts may be protecting the enzyme and their effects can be replaced by Triton X-100 or BSA or Lubrol PX for the purified enzyme. L(+)-Mandelate dehydrogenase from *R. graminis* was remarkably stabilized by the presence of Triton X-100 and substrate together (Table 4.3 & Fig. 4.8). In the presence of Triton X-100 and substrate together, it retained almost 100% activity for more than 72 h at 27^0C. The heat stability was also pH dependent, with enzyme being relatively stable at neutral pH and less stable at acidic or alkaline pH values (Table 4.3 & Fig. 4.10). Rather similar detergent and pH dependent thermal stabilities have also been observed for L(+)- and D(–)-lactate dehydrogenases from *E. coli* (Kimura & Futai, 1978; Kovatchev *et al.,* 1981). In that case it was suggested that secondary structures of the enzymes when preincubated with detergents or detergent like substances were more thermostable than in detergent free environments. Purified L(+)-lactate dehydrogenase from *E. coli* was as stable in the presence of detergent as was memberane-bound enzyme (Kimura & Futai, 1978). At low protein concentration, detergent-free L(+)-lactate dehydrogenase lost half of its activity in 30 min at 0^0C and at pH 8.0, whereas, little loss was noticed when phosphatidylglycerol was present (Kimura & Futai, 1978). Similarly, in the presence of Triton X-100, even diluted L(+)-mandelate dehydrogenase from *R. graminis*

does not lose activity at 0^0C (Table 4.3 & Fig. 4.8-4.11). Kimura and Futai (1978) noticed that detergent-free L(+)-lactate dehydrogenase could not be reactivated. They suggested that this was possibly because of irreversible detachment of FMN and irreversible denaturation. They provided preliminary evidence that detergents protect the FMN binding sites of the enzymes. Prat (1978) has suggested that the tetramers of L(+)-lactate dehydrogenase from *H. anomala* dissociate at low ionic strength to give inactive monomers. These monomeric subunits can then reassociate with recovery of the enzyme activity (Prat, 1978; Chapman *et al.,* 1991). Perhaps both phenomena described by Kimura and Futai (1978) and Prat (1978), may have occurred in L(+)-mandelate dehydrogenase from *R. graminis* when enzyme samples with Triton X-100 or Triton X-100 and substrate were reactivated to more than 80% of it original activity whereas samples without any detergent suffered approximately 50% irreversible inactivation (Fig. 4.12). Perhaps Triton X-100 protects the FMN binding site in the enzyme and also facilitates reassociation of subunits dissociated at high temperature.

In the presence of substrate, proteins may become more rigid and so they are more stable at higher temperatures, e.g. L(+)-lactate dehydrogenase of *S. cerevisiae* is protected against denaturation in the presence of substrate (Labeyrie, *et al.,* 1978). Triton perhaps protects hydrophobic residues from direct exposure to an aqueous environment and so the proteins are protected from denaturation.

The folding of denatured protein is a thermodynamically controlled process (Jaenicke & Rudolph, 1989; Honeycut & Thirumalai, 1990) and that may be the reason why reactivation of L(+)-mandelate dehydrogenase is successful at 27^0C and not at 0^0C (Fig. 4.12). Similarly, Greiger and Gorisch (1989) reported that glucose dehydrogenase from *A. calcoaceticus* inactivated at 50^0C, was reactivated to about 95% of its original activity at 25^oC in the presence of prosthetic group and Ca^{2+} and pyruvate dehydrogenase from *Bacillus stearothermophillus* is not reactivated at 0^0C but was observed at 55^0C (Jaenicke & Rudolph, 1989).

In vivo studies have indicated that thermal inactivation is also associated with insolubilization of proteins (Evan & Handcock, 1985; Littlewood *et al.,* 1987; Luscher & Eisenman, 1988; Maridonneau-Parini, *et al.,* 1988; Dubois *et al.,* 1989; Fisher *et al.,* 1989;

Bensaude *et al.*, 1990). Pinto *et al*. (1991) suggested that β-galactosidase and luciferase were inactivated because of reversible insolubilization and not because of irreversible protein denaturation. The D_20 and glycerol which protect against insolubilization also protected β-galactosidase and luciferase against thermal inactivation. However, in the present work the extent of insolubilization of L(+)-mandelate dehydrogenase was not determined.

4.7.9 Spectroscopic properties

Absorption spectra of the L(+)-mandelate dehydrogenase from *A. calcoaceticus* (Hoey *et al.*, 1987) or L(+)-lactate dehydrogenase from *A. calcoaceticus* (Allison & Fewson, 1986) and *E. coli* (Futai & Kimura, 1977) indicated the presence of FMN and none of them gave absorption spectra characteristic of cytochrome b_2. However the visible spectra of L(+)-lactate dehydrogenase from *S. cerevisiae* and *H. anomala* was shown to be similar to that of microsomal cytochrome b_5 that indicated the presence of haem in these enzymes (Morton *et al.*, 1961; Watari *et al.*, 1967; Baudras, 1971; Groudinsky, 1971; Chapman *et al.*, 1991). The absorption spectrum of purified L(+)-mandelate dehydrogenase from *R. graminis* (Fig. 4.3) was similar to that of *S. cerevisiae* and *H. anomala* which indicated the presence of haem. L(+)-Mandelate dehydrogenase from *R. graminis* contain about one mole of haem per subunit. Similar values have been reported for L(+)-lactate dehydrogenase from *S. cerevisiae* and *H. anomala* (Appleby & Morton, 1959; Baudras, 1971; Jacq & Lederer, 1972, 1974; Chapman *et al.*, 1991).

Absorption spectra of TCA extracted flavin from *R. graminis* enzyme was similar to that of flavin absorption spectra of standard solutions of FMN and FAD (Fig. 4.4). The presence of flavin was also confirmed by fluorescence spectra of TCA extract of purified L(+)-mandelate dehydrogenase from *R. graminis*. TCA extracts of L(+)-mandelate dehydrogenase from *R. graminis* (present project), *A. calcoaceticus* (Hoey *et al.*, 1987) and L(+)-lactate dehydrogenase of *A. calcoaceticus* (Allison & Fewson, 1986) and *E. coli* (Futai & Kimura, 1977) all gave similar fluorescence spectra. The presence of FMN was confirmed when *Naja naja* snake venom did not change the fluorescence emission spectra for the enzymes in *R. graminis* (Fig. 4.5) and *A. calcoaceticus* (Allison & Fewson, 1986; Hoey *et al.*, 1987; present project). The presence FMN in the *E. coli* enzyme was

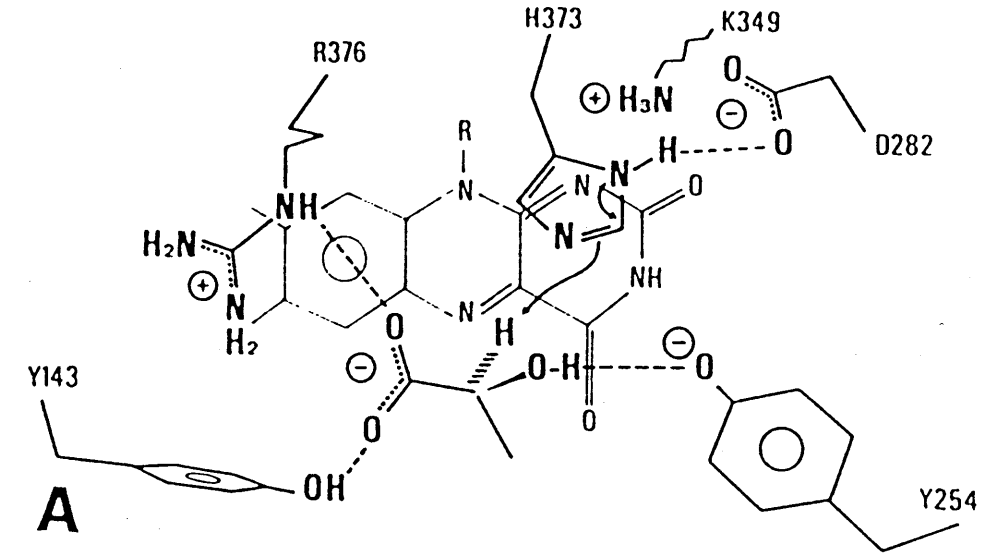
confirmed by comparing its R_f value with that of standard FMN (Futai & Kimura, 1977). The prosthetic group of *P. putida* is most likely to be FMN because its physical and chemical properties in general and especially amino acid composition are similar with that of glycolate oxidase from spinach and *E. coli* (Table 4.15 & 4.16). It also shares extensive amino acid sequence homologies with L(+)-mandelate dehydrogenase of *A. calcoaceticus* , glycolate oxidase from spinach, L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala* (Fig. 4.25, 4.29). Moreover, it shares similar α/β motifs that contain the FMN binding site (Lindqvist & Branden, 1985; Xia *et al.*, 1987, Tsou *et al.*, 1990). However the unrelated D(+)-mandelate dehydrogenases of *A. calcoaceticus* , D(–)-lactate dehydrogenase of *A. calcoaceticus* and *E. coli* (Futai, 1973; Allison *et al.*, 1985b) contain FAD.

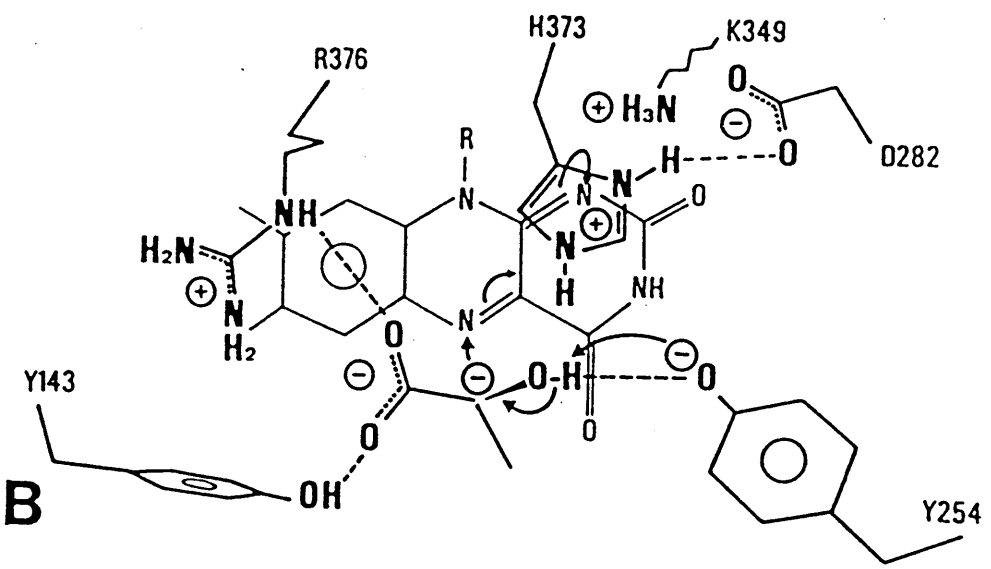
Approximately one mole of FMN per subunit M_r value was found in L(+)-mandelate dehydrogenase from *R. graminis* using absorption and fluorescence spectra of TCA extracts (Fig. 4.4, 4.5). Non-covalently bound haem and FMN are present in equimolar amounts i.e. each subunit of L(+)-mandelate dehydrogenase of *R. graminis* carries one flavin mononucleotide and one haem molecule as prosthetic groups. The presence of equimolar amounts of FMN and haem is similar to that of L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala* (Appleby & Morton, 1954 & 1959; Baudras, 1971; Jacq & Lederer, 1972 & 1974; Hatefi & Stiggall, 1976; Chapman *et al.*, 1991).

4.7.10 Substrate and inhibitor specificity

L(+)-Mandelate dehydrogenase of *R. graminis* is completely stereospecific for its substrate, but, is capable of oxidizing a broad range of substituted mandelates (Table 4.11). It is competitively inhibited by D(–)-mandelate (Fig. 4.22) like the L(+)-mandelate dehydrogenase of *A. calcoaceticus* (Table 4.17; Hoey *et al.*, 1987). The apparent K_m could not be determined for ring-substituted mandelates because most of these compounds were racemic and it is possible that D(–)-enantiomers might be inhibitory. Therefore, L(+)-mandelate dehydrogenase from *R. graminis* and *A. calcoaceticus* can oxidise a number of ring-substituted mandelate and can tolerate substitution at the 2-, 3-, and 4- positions of the benzene ring (Table 4.11). However, substitution at the carboxylic group e.g. presence of isoamyl or methyl esters and especially nitrile group gives very little

Figure 4.30 The proposed carbanion mechanism of of oxidation of L(+)-lactate by L(+)-lactate dehydrogenase. (A) Substrate is bound via electrostatic and hydrogen-bonding with Arg^{376} and by hydrogen-bonding to Tyr^{143}. His^{373} is poised to abstract C_α hydrogen as a proton. (B) The imidazolium ion is stabilised by Asp^{282} and removal of the hydroxyl proton by Tyr^{254} allows transfer of electron from carbanion to FMN. (C) The resultant reduced FMN is stabilised by Lys^{349}. However the structure does not explain the electron transfer from lactate to FMN. For details of mechanism see Reid *et al.*, 1988. From Reid *et al.*, 1988.


H373
K349
R376
D282
Y143
Y254
A


H373
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Y254
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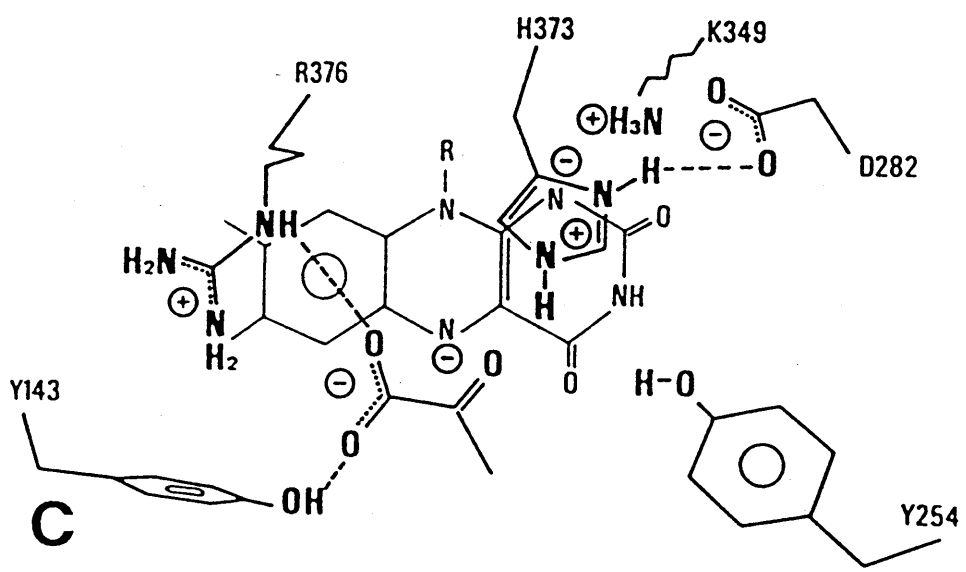

H373
K349
R376
D282
Y143
Y254
C

activity, perhaps because these substitutions interfere with proper bonding (may be hydrogen bonding) of the substrate at the active site as explained for L(+)-lactate dehydrogenase of *S. cerevisiae* in Fig. 4.30 (Chapman *et al.*, 1991). L(+)-Hexahydro-mandelate is not oxidised by either enzyme and it competitively inhibits L(+)-mandelate dehydrogenase of *R. graminis* (Fig. 4.23). Similarly, L(+)-phenyllactate is a very potent inhibitor of L(+)mandelate dehydrogenase of *R. graminis* and L(+)-mandelate dehydrogenase of *A. calcoaceticus* (Table 4.12) but the latter enzyme is inhibited to a lesser extent. Other compounds which were not substrates but inhibited the enzyme activity include aliphatic 2-hydroxyacids, oxalate, glycolate, thiopheneglyoxylate, substituted or non-substituted benzoates, catechol and protocatechuate (Tables 4.12 - 4.14). Comparison of the compounds that are substrates or inhibitors leads to the conclusion that the minimum requirements for the substrate is a carboxylic acid with an α-hydroxy, an α-hydrogen and an aromatic ring (Fig. 1.7a). The L(+)-lactate dehydrogenase from *S. cerevisiae* and *H. anomala* oxidise L(+)-lactate to pyruvate and their minimum requirement for the substrate is the presence of a carboxylate with α -hydrogen and α-hydroxyl group and alkyl group (Chapman *et al.*, 1991). Substrates of L(+)-mandelate dehydrogenase of *R. graminis*, *P. putida* and *A. calcoaceticus* are potent inhibitors of L(+)-lactate dehydrogenase of *S. cerevisiae* (Dikstein, 1959; Morton *et al.*, 1961). L(+)-Lactate dehydrogenase gives best activity with L(+)-lactate but it can use chlorolactate, fluorolactate, bromolactate, glycerate, phosphoglycerate, glycolate, α-hydroxybutyrate, α-hydroxy-isocaproate, isocitrate, malate and tartrate (Chapman *et al.*, 1991). Most of the substrates of L(+)-lactate dehydrogenase are inhibitors of the L(+)-mandelate dehydrogenases of *A. calcoaceticus* and *R. graminis* (Table 4.12). Therefore, the amino acid composition and structure of the active sites of L(+)-lactate dehydrogenase of *S. cerevisiae* , L(+)-mandelate dehydrogenase of *R. graminis* , *P. putida* and *A. calcoaceticus* are perhaps similar with extra hydrophobic residues at the active site of L(+)-mandelate dehydrogenases, that bind the phenyl group of mandelate. At the active site of mandelate racemase the hydrophobic side chains of tyrosine and phenylalanine residues interact with the phenyl group of mandelate (G. Petsko, personal communication to D. P. Baker). It is possible that the aromatic ring plays an important role to give correct orientation to the substrate at the active site. In the

Table 4.17 Comparison of the properties of purified L(+)-mandelate dehydrogenase of *R.graminis* and *A. calcoaceticus* with those of the enzymes in extracts

In the D(-)-mandelate inhibition studies, the initial velocity in the absence of D(–)-mandelate was taken as 100% and remained unchanged throughout the experiment. Some of the values are given as mean values with the number of determinations in the parentheses and others are given as means of two experiments.

+Present project; *From Hills (1979), Hills and Fewson (1983a) and Hoey *et al.* (1987); N.D., not determined

	R. graminis +		*A. calcoaceticus* *	
	Pure enzyme	Extract	Pure enzyme	Extract
Km value for L(+)-mandelate (μM)	266 ± 17 (6)	266 ± 19 (3)	186 ± 19 (3)	207 ± 12 (3)
pH value for maximum activity (with pH values for half maximum activity)	7.9 (6.1, 9.1)	7.5-8 (6.0, 9.1)	7.5 (5.2, 9.0)	8 (6.0, 8.9)
Inhibition (%) obtained with 20 mM D-mandelate in the presence of				
100 μM L(+)-mandelate	N.D.	N.D.	60	69
300 μM L(+)-mandelate	81	81	N.D.	N.D.
8.6 mM L(+)-mandelate	31	29	N.D.	N.D.

absence of phenyl group correct orientation is not acheived, therefore aliphatic 2-hydroxy acids are not substrates but inhibit the enzymes from *R. graminis* and *A. calcoaceticus* (Table 4.9). Inhibition of L(+)-mandelate dehydrogenase of *R. graminis* by L(+)-hexahydromandelate (Fig. 4.23) may indicate that the aromaticity of the benzene ring is important for the compounds to be recognised as substrates. Perhaps the aromaticity also stabilise the possible cabanion formed during oxidation of mandelate.

As shown in Table 4.11, L(+)-mandelate dehydrogenase of *R. graminis* and *A. calcoaceticus* are similarly active with different substituted mandelates. The use of substituted mandelates as substrates (Table 4.11), similar K_m for L(+)-mandelate and similar pH optimum values (Table 4.17) all suggest homology at the active sites of L(+)-mandelate dehydrogenases of *R. graminis* and *A. calcoaceticus* and possibly this homology can be extended to L(+)-lactate dehydrogenases of *S. cerevisiae*, *H. anomala*, *E. coli*, *A. calcoaceticus* which use L(+)-lactate as substrate as all these enzymes catalyse the removal of the same α-hydrogen and hydroxyl hydrogen as protons (Fig. 4.30).

Hexahydromandelate, a potent inhibitor of L(+)-mandelate dehydrogenase of *R. graminis*, was a substrate for D(–)-mandelate dehydrogenase of *R. graminis* (Baker & Fewson, 1989), indicating the difference in the mechanisms of action of two enzymes from the same microorganism. Similarly, D(–)-mandelate dehydrogenases from *L. curvatus* and D(–)-2-hydroxyisocarporate dehydrogenase from *S. faecalis* show broad substrate specificities as they can use phenyllactate and phenylpyruvate (Yamazaki & Maeda, 1986a; Hummel *et al.*, 1988) as compared to mandelate dehydrogenases from *R. graminis* (Baker & Fewson, 1989, present project). However *L. curvatus* and *S. faecalis* enzymes do not readily tolerate ring substitution as they give very low activity with 4-hydroxyphenylpyruvate or 4-hydroxyphenylglyoxylate respectively (Yamazaki & Maeda, 1986a; Hummel *et al.*, 1988). It seems as if the mechanism of substrate binding in L(+)-mandelate dehydrogenase from *R. graminis* is different from that of D(–)-mandelate dehydrogenase of *R. graminis.*, *L. curvatus* and *S. faecalis* (Yamazaki & Maeda, 1986a; Hummel *et al.*, 1988; Baker & Fewson, 1989). Moreover, L(+)-mandelate dehydrogenase of *R. graminis* is more specific for its substrates than is D(-)mandelate dehydrogenase of *L. cuvatus* (Hummel *et al.*, 1988)

and D(–)-2-hydroxyisocaproate dehydrogenase of *S. faecalis* (Yamazaki & Maeda, 1986a,b) which use a wide range of substrates.

L(+)-Mandelate dehydrogenase of *A. calcoaceticus* and *R. graminis* can use many substituted mandelates (Table 4.11) but can not use L(+)-lactate where D(–)-mandelate dehydrogenase of *A. calcoaceticus* can use D(–)-lactate (Allison *et al.*, 1985a,b; Hoey *et al.*, 1987). This indicates that not only FMN-dependent L(+)-mandelate dehydrogenases of *R. graminis* and *A. calcoaceticus* are different from the NAD^+-dependent dehydrogenases but they are also different from the FAD-linked enzymes in the mechanism of oxidation of their substrates.

4.7.11 Catalysis, electron acceptors and electron transfer

L(+)-Mandelate dehydrogenase from *R. graminis* and L(+)-lactate dehydrogenase of *S. cerevisiae* can use DCIP, cytochrome *c* and ferricyanide as electron acceptors (Morton *et al.*, 1961; Chapman *et al.*, 1991), whereas L(+)-mandelate dehydrogenase of *P. putida*, D(–)- and L(+)-mandelate dehydrogenases of *A. calcoaceticus* , D(–)- and L(+)-lactate dehydrogenase of *A. calcoaceticus* have no capacity to use cytochrome *c* but do use DCIP as electron acceptor (Table 4.10). Similarly flavodehydrogenase domains of *S. cerevisiae* and *H. anomala* obtained by tryptic cleavage have no cytochrome *c* reductase activity (Iwatsubo *et al.*, 1977; Gervais *et al.*, 1983; Chapman *et al.*, 1991). It has thus been suggested that DCIP, ferricyanide and cytochrome *c* are reduced by different mechanisms (Morton *et al.*, 1961; Chapman *et al.*, 1991; present project).

Cytochrome *c* forms a complex with L(+)-lactate dehydrogenase of *S. cerevisiae* (Baudras *et al.*, 1971; Yoshimura *et al.*, 1977; Prat, 1978; Tegoni *et al.*, 1983) and the stability of the complex depends on the ionic strength and pH of the solution (Baudras *et al.*, 1971 & 1972). A similar complex perhaps is formed with L(+)-mandelate dehydrogenase of *R. graminis*. It has been suggested that ferricyanide can accept electrons from FMN as well as from haem (Iwatsubo *et al.*, 1977). However, cytochrome *c* can accept electron from the haem prosthetic group only as indicated by flavodehydrogenase studies in *H. anomala* and *S. cerevisiae* (Iwatsubo *et al.*, 1977; Gervais *et al.*, 1983; Chapman *et al.*, 1991). The apparent K_m and relative rate values of L(+)-lactate dehydrogenase of *S. cerevisiae* for

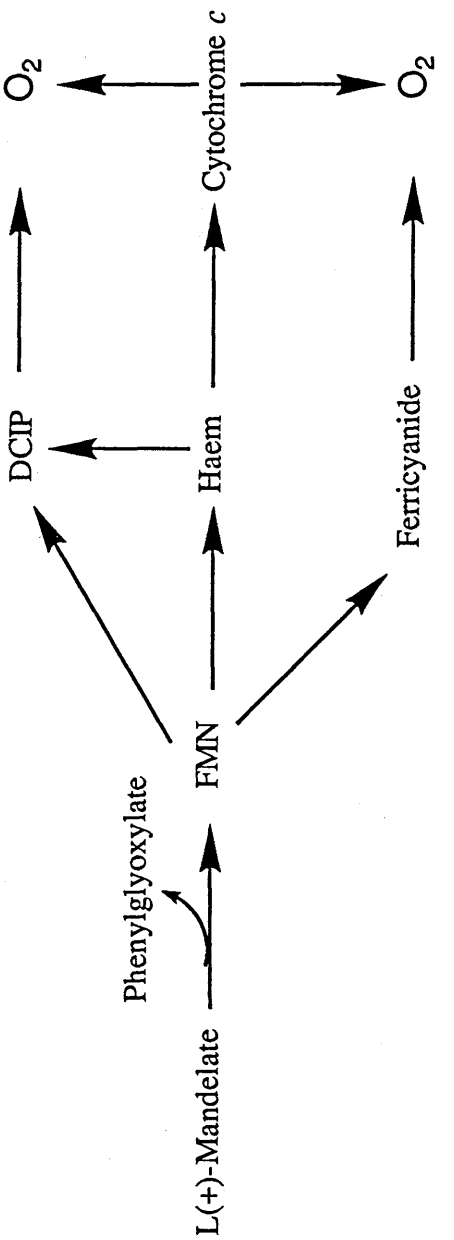


Figure 4.31 Schematic representation of electron transfer involving different electron acceptors.

DCIP, and cytochrome *c* are similar to the values obtained for L(+)-mandelate dehydrogenase of *R. graminis* (Table 4.9). Considering the electron acceptor specificities of L(+)-mandelate dehydrogenase of *R. graminis* for DCIP, cytochrome *c* and ferricyanide and flavodehydrogenase activity studies in L(+)-lactate dehydrogenase of *H. anomala* (Gervais *et al.,* 1983; Celerier *et al.,* 1989), the possible electron transfer from substrate to final electron acceptor, in L(+)-mandelate dehydrogenase of *R. graminis* , can be summarised as shown in Fig. 4.31.

4.7.12 Feedback regulation of mandelate enzymes

Fewson (1988b) suggested that feedback inhibition is not important in regulation of mandelate enzyme activities. However, L(+)-mandelate dehydrogenase of *R. graminis* and *A. calcoaceticus* were inhibited to the same extent by catechol and protocatechuate (Table 4.14) and catechol seems to be a more potent inhibitor of both enzymes. The inhibition by catechol and protocatechuate suggests that perhaps product inhibition mechanism operates for these two enzymes. Feed back inhibition is usually needed to regulate flux and to avoid the accumulation of intermediates which might have harmful effects (Fewson, 1988b). However, inhibition by catechol and protocatechuate may simply be due to similarities in binding sites with no physiological relevance.

End-product repression has been reported for mandelate pathway enzymes in different microorganisms e.g. the presence of benzoate and catechol in the growth medium repressed mandelate oxidation and benzoate oxidation in *Pseudomonas fluorescence* (Mandelstam & Jacoby, 1965). Similarly, mandelate pathway enzymes were repressed by the presence of succinate in the growth medium in *P. putida* (Ornston, 1971) and *A. calcoaceticus* (Livingstone & Fewson, 1972; Cook *et al.,* 1975). The presence of glucose in the growth medium repressed the synthesis of mandelate enzymes in *Byssochlamys fulva* and *R. graminis* (Durham, 1984; Iyayi & Dart 1986; Fewson, 1988b). The repression of mandelate enzymes by pathway products suggest that some sort of end-product repression mechanism exists for peripheral pathways but it is not clear whether these feed-back mechanisms are due to specific or non-specific repressions (Fewson, 1988b).

4.7.13 Overall relatedness of dehydrogenases

From the comparison of tertiary and primary structures, domain structure and other physical, chemical and kinetic properties it is reasonable to conclude that L(+)-mandelate dehydrogenases of *P. putida* , *A. calcoaceticus* and *R. graminis* , L(+)-lactate dehydrogenase of *E. coli* , *S. cerevisiae* and *H. anomala* and glycolate oxidase of spinach are evolutionarily related to each other and that these enzymes are different from FAD-linked mandelate dehydrogenase and even more different from NAD/NADH-dependent 2-hydroxy acid dehydrogenases and mandelate dehydrogenases.

CHAPTER 5

POSSIBLE IMMUNOLOGICAL RELATIONSHIPS AMONGST MANDELATE AND LACTATE DEHYDROGENASES

5.1 Introduction: Immunochemical comparisons of lactate and mandelate dehydrogenases

Complete deduced amino acid sequences of the L(+)-mandelate dehydrogenase of *Pseudomonas putida* and L(+)-lactate dehydrogenase of *Saccharomyces cerevisiae* have recently been reported (Guiard, 1985; Tsou *et al.*, 1990; Chapman *et al.*, 1991). In addition, the *N*-terminal sequences of L(+)-mandelate dehydrogenases of *Rhodotorula graminis* and *Acinetobacter calcoaceticus* and D(–)- and L(+)-lactate dehydrogenases of *A. calcoaceticus* and D(–)-mandelate dehydrogenase of *A. calcoaceticus* and *R. graminis* have also been determined (Baker, 1990; C.A. Fewson, personal communications; present project). However, in the absence of the complete amino acid sequence data for these enzymes, it was thought that immunological studies might reveal structural and amino acid sequence homologies among these enzymes. It was also of interest to determine their relationships with D(–)-mandelate dehydrogenase from *Lactobacillus curvatus* and D(–)-2-hydroxyisocaproate dehydrogenase from *Streptococcus faecalis* (see section 1.5.2).

Four types of immunological cross-reaction assays were conducted during these studies :

(a) *Immunoinhibition*: Antisera raised against native antigens were incubated with homologous or heterologous antigens (Methods 2.10.2) and the mixtures were assayed for enzyme activity.

(b) *Immunoprecipitation*: Antisera raised against native antigens were incubated with homologous or heterologous antigens and microfuged as described in Methods 2.10.3. The supernatants were assayed for enzyme activity.

(c) *Immunoprecipitation* with *Staphylococcus aureus* cells: Antisera raised against native antigens were first incubated with homologous or heterologous antigens for one hour and the mixture were then microfuged (Methods 2.10.4b). The supernatants were then incubated with *Staphylococcus aureus* cells for another hour and the mixtures were microfuged again (Methods 2.10.4b). The supernatants were assayed for enzyme activity. In this type of immunoprecipitation, the ability of *S. aureus* to bind to the antibody-antigen complex was relied on (Kessler, 1975).

Table 5.1 Activities of mandelate and lactate dehydrogenases

R. graminis KGX 39, *R. graminis* NCYC 980, DB 2 and DB 3 were grown on Sabouraud-dextrose agar plates (Methods 2.3.2). Five loopful of each yeast was inoculated into minimal medium containing D,L-mandelate (Methods 2.3.5a) and grown for 24-48 h (Methods 2.3.5a). *L.curvatus* and *S. faecalis* were grown in MRS broth (Methods 2.3.5b) and were then inoculated into 2 x 400 ml volumes of MRS broth and grown for 48 h (Methods 2.3.5b). *A. calcoaceticus* was grown in complex medium containing lactate and mandelate (Methods 2.3.5a). The cultures were harvested (Methods 2.3.7) and cell-free extracts were prepared (Methods 2.3.8). The purified L(+)-mandelate dehydrogenase of *R. graminis* ; partially purified L(+)-mandelate dehydrogenases from *A. calcoaceticus* and *P. putida* and purified L(+)-lactate dehydrogenase of *S. cerevisiae* (Sigma) were also used. In all cases the extraction/ dilution buffer (20 mM Tris/HCl buffer, pH 7.5) containing 0.5 mg of Triton X-100 ml^{-1} was used. The mandelate and lactate dehydrogenases were assayed for enzyme activity (Methods 2.4.1, 2.4.2, 2.4.4 & 2.4.6). The values represent the mean of duplicate assays.

Dye-linked L(+)-mandelate dehydrogenase activity

	Purified*/partially purified enzyme activity		Cell-free extract enzyme activity	
	µmol/min/ml	units (mg protein)$^{-1}$	µmol/min/ml	units (mg protein)$^{-1}$
R. graminis KGX 39	2.49*	208*	1.49	0.186
R. graminis NCYC 980	ND	ND	1.08	0.086
DB2	ND	ND	1.98	0.123
DB 3	ND	ND	1.97	0.127
A. calcoaceticus	37.2	16.9	9.46	0.473
P. putida	33.0	53.0	5.08	0.132

Dye-linked L(+)-lactate dehydrogenase activity

	Purified*/partially purified enzyme activity		Cell-free extract enzyme activity	
	µmol/min/ml	units (mg protein)$^{-1}$	µmol/min/ml	units (mg protein)$^{-1}$
A. calcoaceticus	3.10	15.74	1.52	0.076
S. cerevisiae	3.73*	0.60*	NA	NA

Dye-linked D(–)-mandelate dehydrogenase activity

	Purified*/partially purified enzyme activity		Cell-free extract enzyme activity	
	µmol/min/ml	units (mg protein)$^{-1}$	µmol/min/ml	units (mg protein)$^{-1}$
A. calcoaceticus	NA	NA	4.52	0.32

Dye-linked D(–)-lactate dehydrogenase activity

	Purified*/partially purified enzyme activity		Cell-free extract enzyme activity	
	µmol/min/ml	units (mg protein)$^{-1}$	µmol/min/ml	units (mg protein)$^{-1}$
A. calcoaceticus	ND	ND	4.52	0.226

NAD-dependent D(–)-mandelate dehydrogenase activity

	Purified*/partially purified enzyme activity		Cell-free extract enzyme activity	
	µmol/min/ml	units (mg protein)$^{-1}$	µmol/min/ml	units (mg protein)$^{-1}$
R. graminis KGX 39	9.50*	63.33*	3.10	0.388
R. graminis NCYC 980	N.D	ND	0.635	0.052
DB 2	N.D.	ND	3.15	0.185
DB 3	N.D.	ND	1.83	0.118
L. curvatus	N.D.	ND	0.21	0.208
S. faecalis	N.D.	ND	0.55	0.043

ND, Not determined; NA, not applicable; *, purified to homogeneity

(d) *Immunoblottting*: Homologous and heterologous antigens were first denatured and separated by SDS-PAGE and were then electroblotted to nitrocellulose. The transferred protein were then probed with antisera as described in Methods 2.10.5.

An immunological comparison of various dehydrogenases was carried out using polyclonal antisera raised against: (a) purified, presumably native L(+)-mandelate dehydrogenase of *R. graminis*. prepared in the present project, (b) native D(–)-mandelate dehydrogenase of *R. graminis* (Baker, 1990), (c) native D(–)-mandelate dehydrogenase of *A. calcoaceticus* (Dr. I.D. Hamilton , personal communication) and (d) SDS-denatured L(+)-mandelate dehydrogenase of *A. calcoaceticus* (Hoey *et al.*, 1987). The antiserum raised against SDS-denatured L(+)-mandelate dehydrogenase did not give a positive cross-reaction in immunoinhibition experiments but it did give a positive cross-reaction with its homologous antigen during immunoblot experiments (Hoey *et al.*, 1987), therefore it was used for immunoblotting experiments. The antisera raised against L(+)-mandelate dehydrogenase of *R. graminis* were used for immunological experiments of types a-d, whereas antisera raised against D(–)-mandelate dehydrogenase of *R. graminis* and *A. calcoaceticus* were used for immunological experiments of type a and b only.
In order to reiteration the following two terms are used in the text :

1. 'Homologous antigen' is used to describe the protein (enzyme) against which antiserum was raised.
2. 'Heterologous antigen' is used to describe the protein (enzyme) other than the homologous antigen.

5.2 Results

5.2.1 Mandelate and lactate dehydrogenase activities

In preparation for immunological studies, the yeasts *R. graminis* KGX 39, *R. graminis* NCYC 980, unidentified yeasts DB2, DB3 and the bacteria *Acinetobacter calcoaceticus* C1219, *Pseudomonas putida* 9494, *Lactobacillus curvatus* NCIMB 9710 and *S. faecalis* IFO 12964 were grown on selective media (Methods 2.3.5) and cell-free extracts were prepared and assayed for mandelate or lactate dehydrogenases activities (Table 5.1). Purified L(+)-mandelate dehydrogenase and D(–)-mandelate dehydrogenase of *R. graminis*

Table 5.2 Potency of antisera, raised against purified L(+)-mandelate dehydrogenase of *R. graminis* KGX 39, obtained from two rabbits

Antisera were raised in two New Zealand white rabbits (No.s 1153 & 1154) as described in Methods 2.10.1. The purified L(+)-mandelate dehydrogenase and antisera were diluted with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. The purified L(+)-mandelate dehydrogenase was diluted to give the same activity per ml as in the extract of *R. graminis* NCYC 980 (Table 5.1). Serial dilutions of antisera 1153:1, 1153:2, 1154:1, 1154:2 and normal rabbit serum (NRS) were prepared in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. Equal amounts of enzymes and antisera were mixed and incubated on ice for one hour and then microfuged (Methods 2.10.3). The supernatants were assayed for the remaining activity (Methods 2.4.2). Assays were carried out in duplicate and mean values were calculated as percent of the enzyme activity incubated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. The percentage inhibition was plotted against the dilution of antisera to calculate the dilution of antiserum required to give 50 % inactivation.

Code number of antiserum	Protein concentration in serum (mg ml^{-1})	Antiserum dilution needed to give 50 % inactivation	Activity remaining (%) when mixed in 1 : 1 ratio
1153:1	45	1 : 8 - 1 : 16	5
1153:2	44	1 : 4 - 1 : 8	19
1154:1	46	1 : 32 - 1 : 64	0
1154:2	50	1 : 32 - 1 : 64	0
NRS	51		101

NRS, Normal rabbit serum.

Table 5.3 Effect of antisera raised against L(+)-mandelate dehydrogenase of *R. graminis* KGX 39 on the mandelate dehydrogenase activities from *R. graminis* KGX 39, *R. graminis* NCYC 980, DB 2, DB 3, *A. calcoaceticus* and *P. putida*

Cell-free extracts (Table 5.1) were prepared as described in Methods 2.3.8. The extracts were diluted with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} to give the same enzyme activity per ml as in the extract of *R. graminis* NCYC 980 (Table 5.1). Two samples of antisera, raised in separate rabbits against the L(+)-mandelate dehydrogenase of *R. graminis* KGX 39 and normal rabbit serum (NRS) were used. Equal volumes of enzymes and antisera (300 µl each) were incubated on ice and assayed for enzyme activity (Methods 2.4.1, 2.4.2 & 2.4.6). The same sample was later microfuged (Methods 2.10.3) and the supernatants were again assayed for enzyme activity. Assays were carried out in duplicate and mean values were calculated as percentage of enzyme activity incubated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}.

NRS, Normal rabbit serum; LMDH, L(+)-mandelate dehydrogenase;
DMDH, D(–)-mandelate dehydrogenase.

Extract used	Enzyme assayed	Remaining enzyme activity (%)					
		Before centrifugation			After centrifugation		
		NRS	batch 1153:1	batch 1154:2	NRS	batch 1153:1	batch 1154:2
R. graminis KGX 39	LMDH	104	55	20	103	8	0
R. graminis NCYC 980	LMDH	99	69	25	99	60	8
DB 2	LMDH	98	81	29	102	63	9
DB 3	LMDH	96	80	31	100	61	7
A. calcoaceticus	LMDH	104	99	100	105	103	102
P. putida	LMDH	102	99	100	104	98	100
R. graminis KGX 39	DMDH	103	103	101	104	101	102
R. graminis NCYC 980	DMDH	98	100	106	97	99	100
DB 2	DMDH	106	104	105	109	102	104
DB 3	DMDH	96	105	107	97	101	104
A. calcoaceticus	DMDH	100	102	95	102	103	96

KGX 39, partially purified L(+)-mandelate dehydrogenases of *A. calcoaceticus* mutant C1219 and *P. putida* 9494, partially purified L(+)-lactate dehydrogenase of *A. calcoaceticus* C1219 and commercial purified lactate dehydrogenase of *S. cerevisiae* were also used. The specific activities of the enzymes in cell-free extracts and the specific activities of purified and partially purified samples from different microorganisms are shown in Table 5.1.

5.2.2 Production of antisera against L(+)-mandelate dehydrogenase *R. graminis* KGX 39 and their potency

Antisera against purified L(+)-mandelate dehydrogenase from *R. graminis* KGX 39 was raised by immunising two rabbits, 1153 and 1154 (Methods 2.10.1 & Table 2.1). The potency of antisera from rabbit 1154 was found to be greater than that from 1153 (see detail in the next section).

5.2.3 Immunoinhibition and immunoprecipitation of homologous and heterologous enzymes by anti-L(+)-mandelate dehydrogenase of *R. graminis*

Immunological cross-reactions were observed between anti-L(+)-mandelate dehydrogenase of *R. graminis* KGX 39 and the L(+)-mandelate dehydrogenases of *R. graminis* KGX 39, *R. graminis* NCYC 980 and yeasts DB2 and DB3 as well as L(+)-lactate dehydrogenase of *S. cerevisiae* (Table 5.3 - 5.5 & Fig. 5.1 - 5.3).

Anti-L(+)-mandelate dehydrogenase of *R. graminis* from rabbit 1154 strongly inhibited and precipitated purified L(+)-mandelate dehydrogenase of *R. graminis* KGX 39 and the enzymes in extracts of *R. graminis* KGX 39, *R. graminis* NCYC 980, DB2 and DB3 whereas anti-L(+)-mandelate dehydrogenase of *R. graminis* from rabbit 1153 inhibited and precipitated them to a somewhat lesser extent (Table 5.3 & 5.4). The presence of either 50 mM L(+)-mandelate or 50 mM D(–)-mandelate or 0.5 mg Triton X-100 ml^{-1} did not affect the immunological behaviour of anti-L(+)-mandelate dehydrogenase of *R. graminis* towards its homologous antigen (Table 5.5).

These immunological cross-reactions were quantified by immunotitration (Fig. 5.1 to 5.3). The strongest cross-reactions were observed between anti-L(+)-mandelate dehydrogenase of *R. graminis* and the homologous antigens, both in purified form

Table 5.4 Quantitative estimation of immunoprecipitation of L(+)-mandelate dehydrogenases of *R. graminis* KGX 39, *R. graminis* NCYC 980, DB 2, DB 3

Cell-free extracts and the purified L(+)-mandelate dehydrogenases (Table 5.1) were prepared as described in Methods 2.3.8. The extracts and purified enzymes were diluted with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} to give the same activity per ml as in the extract of *R. graminis* NCYC 980 (Table 5.1). Serial dilutions of antisera 1153:1 and 1154:2 were prepared in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. Equal volumes of enzymes and antisera were mixed and incubated on ice and then microfuged (Methods 2.10.3). The remaining activities were assayed in the supernatants (Methods 2.4.2). Assays were carried out in duplicate and the mean remaining activity values were calculated as percentages of enzyme activity incubated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. The results are means of two independent experiments.

	Dilution of antisera required to give 50 % inactivation of L(+)-mandelate dehydrogenase activity	
	Antiserum 1153 : 1	Antiserum 1154 : 2
R. graminis KGX 39		
purified enzyme	1 : 8 - 1 : 16	1 : 32 - 1 : 64
extract	1 : 16	1 : 64
R. graminis NCYC 980 extract	1 : 2 -1 : 4	1 : 16 - 1 : 32
DB 2 extract	<1 : 2	1 : 16 - 1 : 32
DB 3 extract	<1 : 2	1 : 16 - 1 : 32

Table 5.5 Immunoinhibition and immunoprecipitation of the purified L(+)-mandelate dehydrogenase of *R. graminis* and L(+)-lactate dehydrogenase of *S. cerevisiae* by anti-L(+)-mandelate dehydrogenase of *R. graminis*

Purified L(+)-mandelate dehydrogenase (Table 5.1) was diluted with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} to the same activity per ml as in the cell-free extract of *R. graminis* NCYC 980 (Table 5.1). The L(+)-lactate dehydrogenase of *S. cerevisiae* was also diluted in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} to the same activity per ml as found in the extract of *R. graminis* NCYC 980 (Table 5.1), but the purified enzyme had a very low specific activity (0.6 units/mg of protein). Equal volumes of enzymes and antisera (300 µl each) were incubated on ice and then microfuged (Methods 2.10.4b). The supernatants were assayed for remaining enzyme activity before and after centrifugation (Methods 2.4.2 & 2.4.4). The microfuged supernatants were then incubated with *S. aureus* cells on ice for a further 1 h and microfuged the mixture (Methods 2.10.4b). The supernatants were again assayed for the enzyme activity. Assays were carried out in duplicate and mean values are calculated as percentages of enzyme activity incubated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. The results of one experiment are shown, but this experiment was done twice independently and gave very similar results on both occasions.

ND, not determined; NRS, normal rabbit serum

(A) Immunoinhibition and immunoprecipitation of the purified L(+)-mandelate dehydrogenase of *R. graminis* KGX 39

Rabbit serum code No	Activity remaining (%) before centrifugation	Activity remaining (%) after centrifugation	Activity remaining (%) after treatment with *S. aureus* cells and centrifugation
NRS	104	102	100
1153:1	44	5	2
1153:2	37	19	9
1154:1	26	0	0
1154:2	14	0	0
1154:2 + 50 mM L(+)-mandelate	19	0	0
1154:2 + 50 mM D(–)-mandelate	18	0	0

(B) Immunoinhibition and immunoprecipitation of purified L(+)-lactate dehydrogenase of *S. cerevisiae*

Rabbit serum code No	Activity remaining (%) before centrifugation	Activity remaining (%) after centrifugation	Activity remaining (%) after treatment with *S. aureus* cells and centrifugation
NRS	100	101	100
1153 : 1	95	98	101
1153 : 2	95	99	ND
1154 : 1	43	47	ND
1154 : 2	29	31	30

Table 5.6 Effects of antisera raised against D(–)-mandelate dehydrogenases of *R. graminis* and *A. calcoaceticus* on the dehydrogenase activities of *R. graminis* KGX 39, *R. graminis* NCYC 980, DB 2, DB 3, *A. calcoaceticus* and *P. putida*

Cell-free extracts (Table 5.1) were prepared as described in Methods 2.3.8. The extracts were diluted with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} to give the same activity per ml as in the extract of *R. graminis* NCYC 980 (Table 5.1). Two samples of antisera, raised in separate rabbits, against the D(–)-mandelate dehydrogenase of *R. graminis* KGX 39 (A) and *A. calcoaceticus* (B) were used. Equal volumes of enzymes and antisera (300 µl each) were incubated on ice and then microfuged (Methods 2.10.2 & 2.10.3). The supernatants were assayed for remaining enzyme activity before and after centrifugation (Methods 2.4.1, 2.4.2, 2.4.4 & 2.4.6). Assays were carried out in triplicate and mean values were expressed as percentage of enzyme activity incubated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}.

NRS, Normal rabbit serum; LMDH, L(+)-mandelate dehydrogenase; LLDH, L(+)-lactate dehydrogenase; DMDH, D(–)-mandelate dehydrogenase; DLDH, D(–)-lactate dehydrogenase.

(A) Anti-D(–)-mandelate dehydrogenase of *R. graminis*

Extract used	Enzyme assayed	Remaining enzyme activity (%)					
		Before centrifugation			After centrifugation		
		NRS	batch 319	batch 253	NRS	batch 319	batch 253
R. graminis KGX 39	DMDH	103	9	≤ 4	104	0	≤ 0
R. graminis KGX 39	LMDH	100	106	101	100	102	101

(B) Anti-D(–)-mandelate dehydrogenase of *A. calcoaceticus*

Extract used	Enzyme assayed	Remaining enzyme activity (%)			
		Before centrifugation		After centrifugation	
		NRS	antiserum	NRS	antiserum
A. calcoaceticus	DMDH	105	51	106	49
R. graminis KGX 39	DMDH	103	102	104	102
R. graminis KGX 39	LMDH	106	104	105	105
A. calcoaceticus	LMDH	105	105	110	112
P. putida	LMDH	105	106	107	109
A. calcoaceticus	DLDH	106	106	102	103
A. calcoaceticus	LLDH	104	106	103	104

(Fig. 5.1) and in crude extract (Fig. 5.2). It also gave considerable cross-reactions with the L(+)-mandelate dehydrogenase of all the yeasts (Fig. 5.2 & 5.3). However none of the bacterial D(–) or L(+)- mandelate dehydrogenases or D(–) or L(+)- lactate dehydrogenases gave cross-reactions with antisera raised against L(+)-mandelate dehydrogenase of *R. graminis* (Table 5.3). Similarly, the activities of D(–)-mandelate dehydrogenases of yeasts *R. graminis* KGX 39, *R. graminis* NCYC 980, DB2 and DB3 were not inhibited by anti-L(+)-mandelate dehydrogenase of *R. graminis* (Table 5.3).

L(+)-Lactate dehydrogenase from *S. cerevisiae* was inhibited by antiserum from rabbit 1154, but not by antiserum from rabbit 1153 (Table 5.5).

5.2.4 Immunological inhibition of mandelate and lactate dehydrogenase activities by antisera raised against D(–)-mandelate dehydrogenase of *R. graminis* and *A. calcoaceticus*

Anti-D(–)-mandelate dehydrogenase of *A. calcoaceticus* and *R. graminis* were tested for immunoinhibition and immunoprecipitation of activities of their homologous and heterologous antigens. Anti-D(–)-mandelate dehydrogenase of *R. graminis* from rabbit 319 and 253 strongly inhibited the homologous antigen (Table 5.6 A) but the anti-D(–)-mandelate dehydrogenase of *A. calcoaceticus* inhibited its homologous antigen (enzyme) by about half (Table 5.6B). L(+)-Mandelate dehydrogenase from *R. graminis* was not inhibited by the anti-D(–)-mandelate dehydrogenase of *R. graminis* (Table 5.6 A) and neither L(+)-mandelate dehydrogenase nor D(–)-mandelate dehydrogenase from *R. graminis* was inhibited by anti-D(–)-mandelate dehydrogenase of *A. calcoaceticus* (Table 5.6 B). Similarly, anti-D(–)-mandelate dehydrogenase of *A. calcoaceticus* did not inhibit L(+)-mandelate dehydrogenases of *A. calcoaceticus*, *P. putida* or the D(–)- and L(+)-lactate dehydrogenases of *A. calcoaceticus* (Table 5.6B).

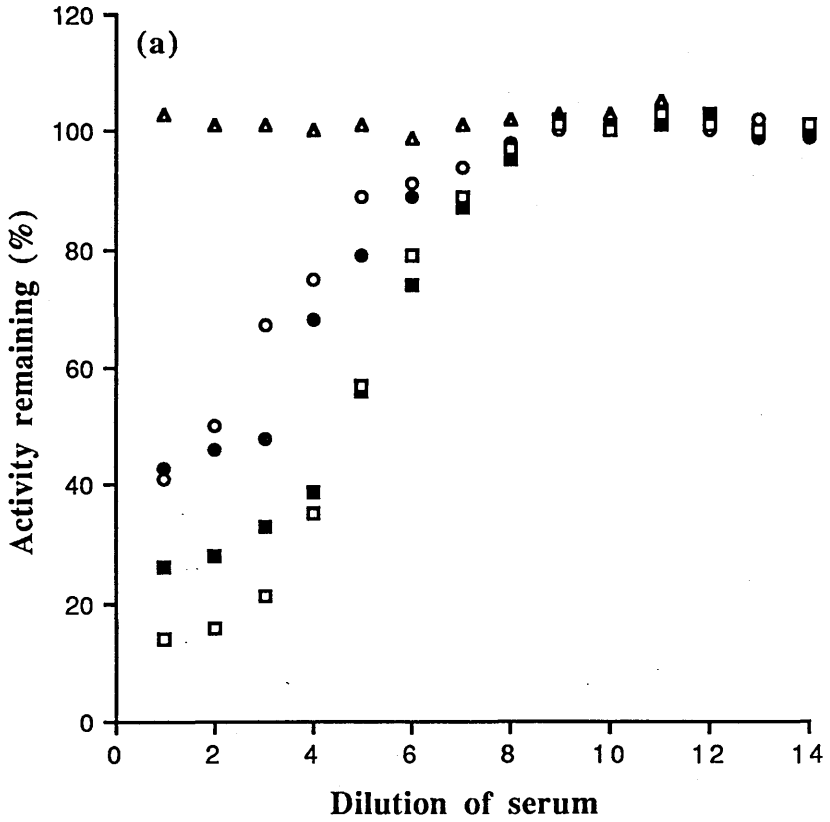
5.2.5 Immunoblotting (Western blotting)

Anti-L(+)-mandelate dehydrogenase of *R. graminis* (raised against the presumably native enzyme) and anti-L(+)-mandelate dehydrogenase of *A. calcoaceticus* [raised against L(+)-mandelate dehydrogenase isolated as a Coomassie Blue stained band from an

Figure 5.1 Immunotitration of the purified L(+)-mandelate dehydrogenase of *R. graminis* KGX 39

Purified L(+)-mandelate dehydrogenase, normal rabbit serum and antisera were diluted with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. The purified L(+)-mandelate dehydrogenase was diluted to give the same activity ml^{-1} as in the cell-free extract of *R. graminis* NCYC 980 (Table 5.1). Serial dilutions (1:2-1:16384) of antisera 1153:1, 1153:2, 1154:1, 1154:2 and normal rabbit serum were prepared. Equal volumes of enzymes and various dilutions of antisera were mixed and incubated on ice and then microfuged (Methods 2.10.2 & 2.10.3). The supernatants were assayed for remaining activity before and after centrifugation (Methods 2.4.2). Assays were carried out in triplicate and mean values were calculated as percent of enzyme activity incubated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}.

(a) Before centrifugation, (b) after centrifugation ; (▲) normal rabbit serum, (o) antiserum batch 1153:1, (●) antiserum batch 1153:2, (□) antiserum batch 1154:1, (■) antisera 1154:2.


(a)
Activity remaining (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Dilution of serum

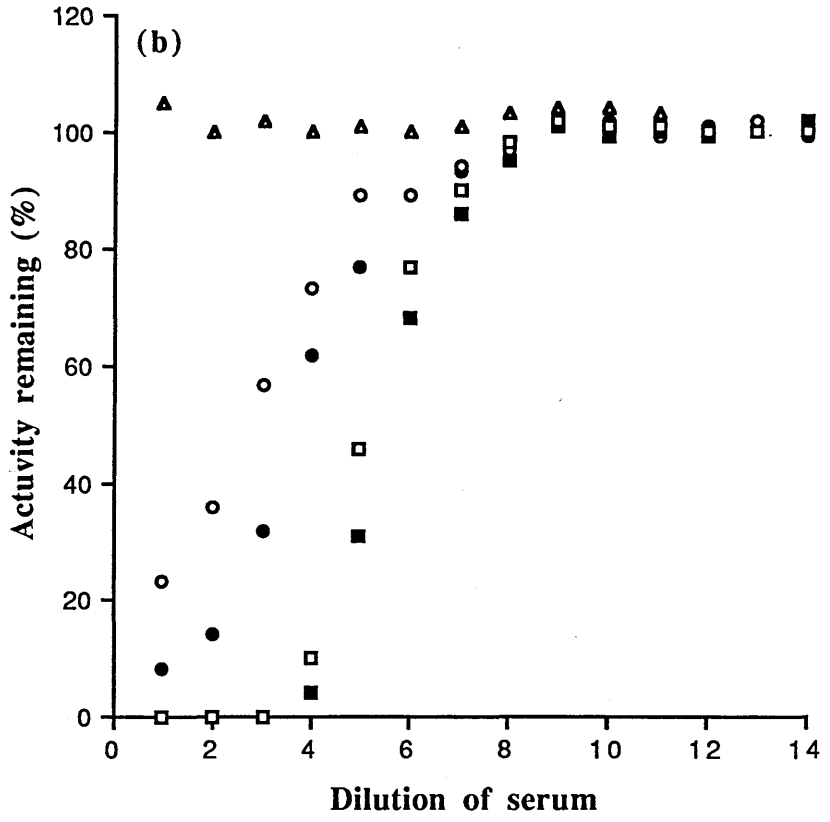
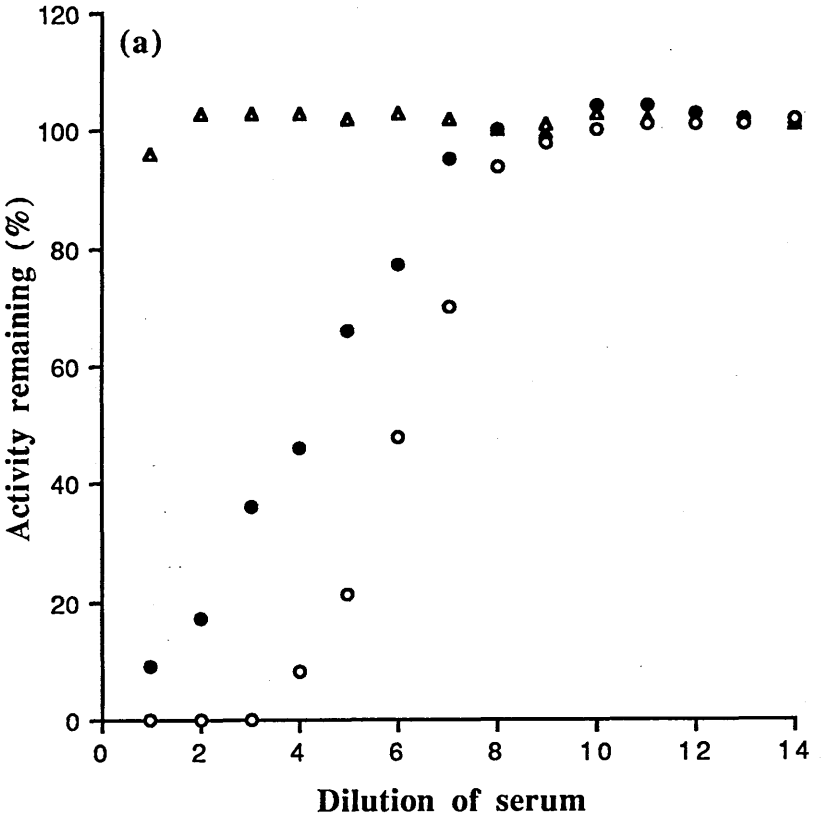

(b)
Actuvity remaining (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Dilution of serum

Figure 5.2 Immunotitration of the L(+)-mandelate dehydrogenases in extracts of *R. graminis* KGX 39 and *R. graminis* NCYC 980

Cell-free extracts of *R. graminis* KGX 39 and *R. graminis* NCYC 980, grown on mandelate, were prepared as described in Methods 2.3.8. The extract of *R. graminis* KGX 39 was diluted with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} to give the same activity per ml as in the extract of *R. graminis* NCYC 980 (Table 5.1). Serial dilutions (1:2-1:16384) of antisera 1153:1, 1154:2 and normal rabbit serum were prepared in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. Equal volumes of enzymes and various dilutions of antisera (100 µl each) were mixed and incubated on ice and then microfuged (Methods 2.10.3). The supernatants were assayed for remaining activity after centrifugation (Methods 2.4.2). Assays were carried out in triplicate and mean values were calculated as percent of enzyme activity incubated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}.

(a) L(+)-Mandelate dehydrogenase of *R. graminis* KGX 39, (b) L(+)-mandelate dehydrogenase of *R. graminis* NCYC 980; (▲) normal rabbit serum, (●) antiserum batch 1153:1, (o) antiserum batch 1154:2.


(a)
Activity remaining (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Dilution of serum

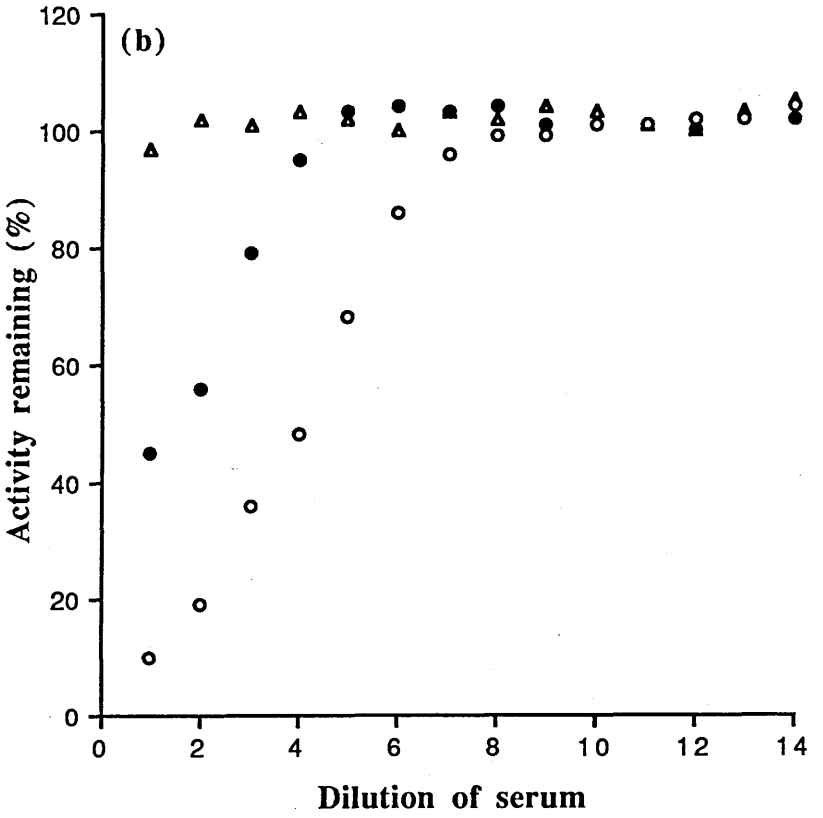
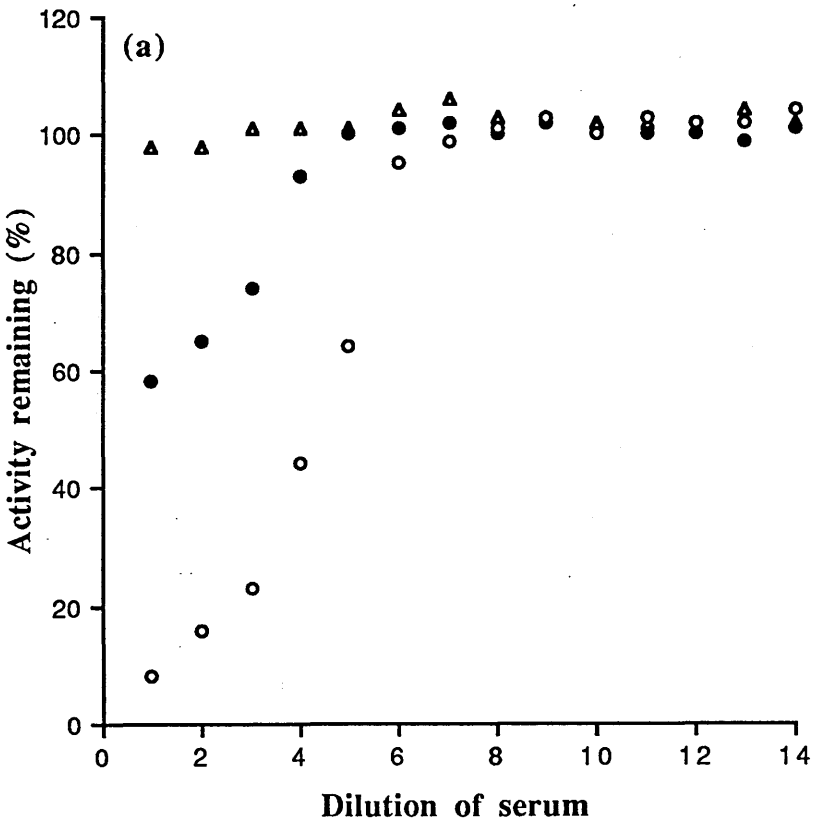

(b)
Activity remaining (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Dilution of serum

Figure 5.3 Immunotitration of the L(+)-mandelate dehydrogenases in extracts of yeasts DB 2 and DB 3

Cell-free extracts of yeasts DB 2 and DB 3, grown on mandelate, were prepared as described in Methods 2.3.8. The extracts of yeasts DB 2 and DB 3 were diluted with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1} to give the same enzyme activity per ml as in the extract of *R. graminis* NCYC 980 (Table 5.1). Serial dilutions (1:2-1:16384) of antisera 1153:1, 1154:2 and normal rabbit serum were prepared in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}. Equal volumes of enzymes and various dilutions of antisera (100 µl each) were mixed and incubated on ice and then microfuged (Methods 2.10.3). The supernatants were assayed for remaining activity after centrifugation (Methods 2.4.2). Assays were carried out in duplicate and mean values were calculated as percent of enzyme activity incubated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mg Triton X-100 ml^{-1}.

(a) L(+)-Mandelate dehydrogenase of yeast DB 2, (b) L(+)-mandelate dehydrogenase of yeast DB 3; (▲) normal rabbit serum, (●) antiserum batch 1153:1, (o) antiserum batch 1154:2.


(a)
Activity remaining (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Dilution of serum

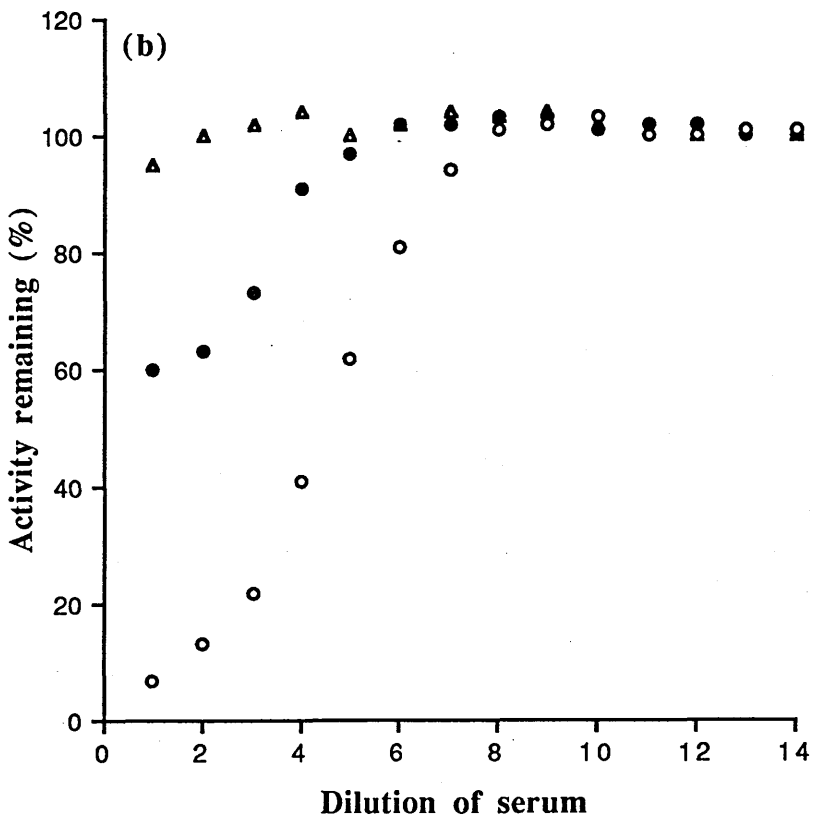

(b)
Activity remaining (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Dilution of serum

Figure 5.4 Specificity of immunoblotting: immunoblots of purified L(+)-mandelate dehydrogenase of *R. graminis* KGX 39 and cell-free extracts of *R. graminis* NCYC 980 and yeasts DB 2, DB 3, probed with anti-L(+)-mandelate dehydrogenase of *R. graminis* KGX 39

Cell-free extracts of *R. graminis* KGX 39, *R. graminis* NCYC 980 and yeasts DB 2 and DB 3, grown on mandelate and glucose, were prepared as described in Methods 2.3.8. Extracts of *R. graminis* KGX 39, *R. graminis* NCYC 980 and yeasts DB 2 and DB 3, grown on mandelate, with approximately the same enzyme activity and an equivalent amount of protein from extracts the same organisms grown on glucose were electrophoresed on SDS-polyacrylamide gel (Methods 2.10.5a). Proteins were then electroblotted onto nitrocellulose (Methods 2.10.5b) and later probed with anti-L(+)-mandelate dehydrogenase of *R. graminis* (Methods 2.10.5c).

Tracks :

A,K, 33 ng of purified L(+)-mandelate dehydrogenase of *R. graminis* KGX 39

B,J, 23 μg of extract of *R. graminis* KGX 39 grown on mandelate

I, 23 μg of extract of *R. graminis* KGX 39 grown on glucose

C, 50 μg of extract of *R. graminis* NCYC 980 grown on glucose

D, 50 μg of extract of *R. graminis* NCYC 980 grown on mandelate

E, 36 μg of extract of DB 2 grown on glucose

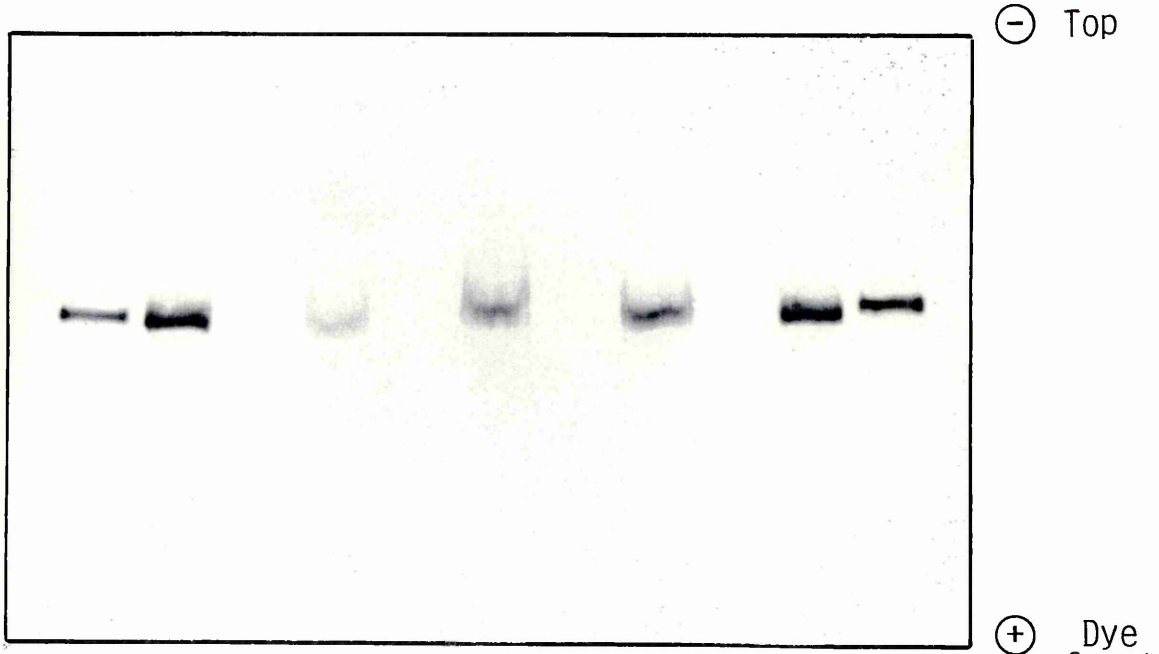
F, 36 μg of extract of DB 2 grown on mandelate

G, 34 μg of extract of DB 3 grown on glucose

H, 34 μg of extract of DB 3 grown on mandelate

(a) Probed with antisera batch 1153:1, (b) probed with antisera batch 1154:2

(a)



(b)

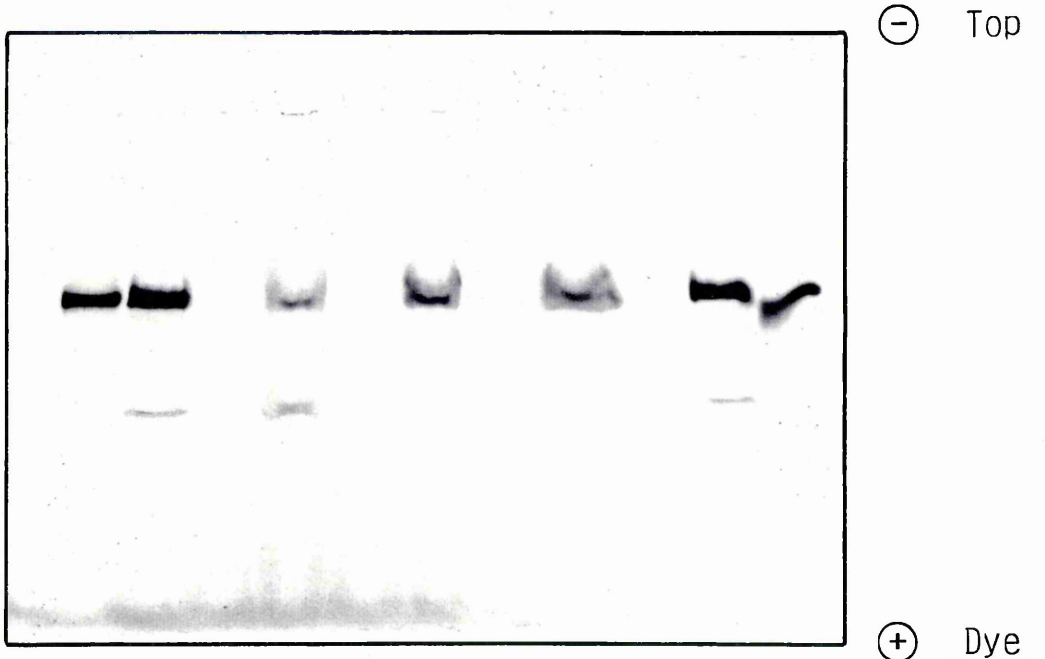


Figure 5.5 Specificity of immunoblotting : immunoblots of purified L(+)-mandelate dehydrogenase of *R. graminis* KGX 39 and purified L(+)-lactate dehydrogenase of *S. cerevisiae*, probed with anti-L(+)-mandelate dehydrogenase of *R. graminis* KGX 39

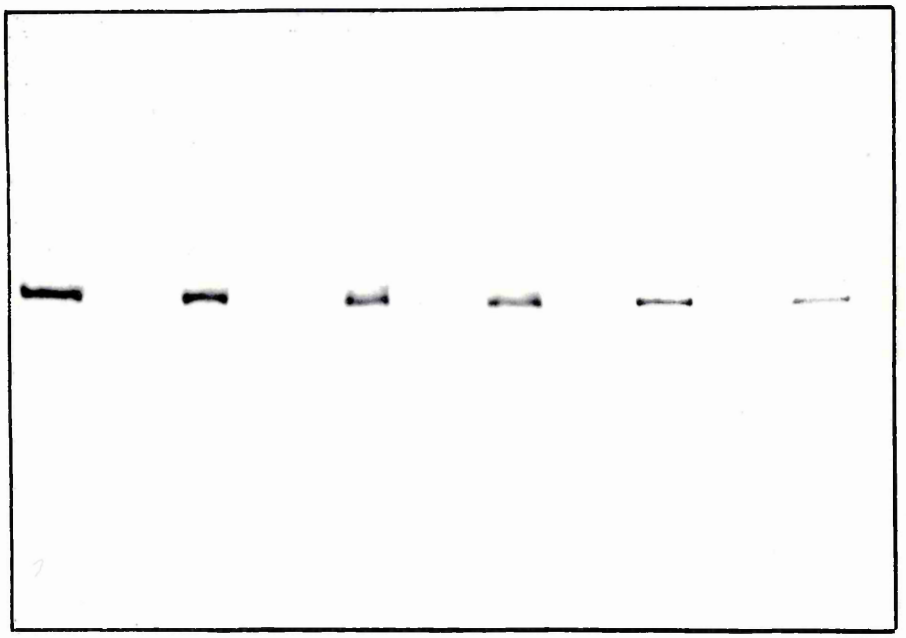
Purified dehydrogenases (Table 5.1) were electrophoresed on 10 % (w/v) SDS-polyacrylamide gel (Methods 2.10.5a). Proteins were then electroblotted onto nitrocellulose (Methods 2.10.5b) and later probed with anti-L(+)-mandelate dehydrogenase of *R. graminis* (Methods 2.10.5c).

Track :

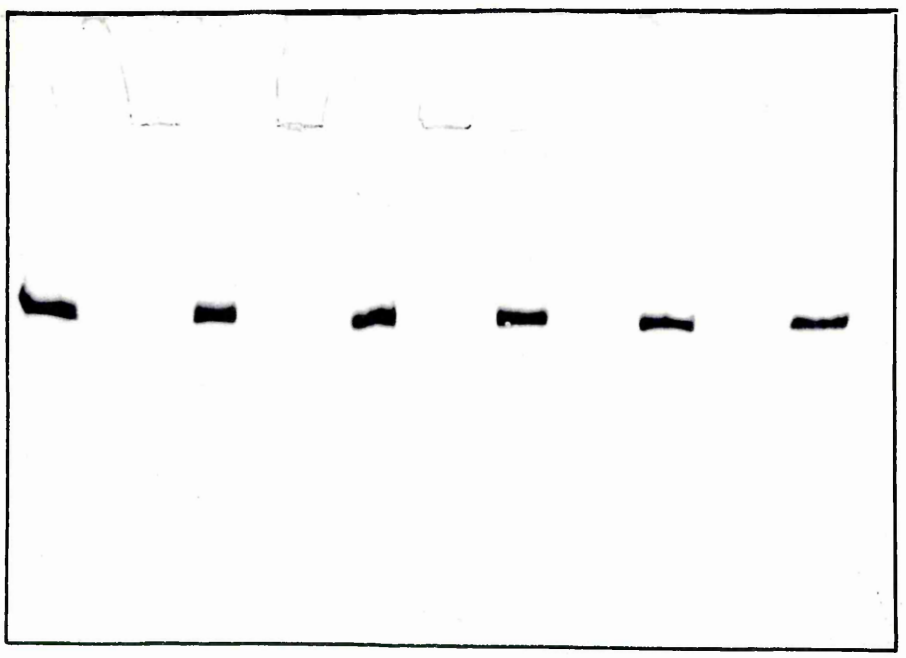
A,	40 ng of purified L(+)-mandelate dehydrogenase of *R. graminis*
C, E, G, I, K,	33 ng of purified L(+)-mandelate dehydrogenase of *R. graminis*
B,	1.0 μg of purified L(+)-lactate dehydrogenase of *S. cerevisiae*
D,	2.0 μg of purified L(+)-lactate dehydrogenase of *S. cerevisiae*
F,	4.0 μg of purified L(+)-lactate dehydrogenase of *S. cerevisiae*
H, J,	10 μg of purified L(+)-lactate dehydrogenase of *S. cerevisiae*

(a) Probed with antiserum batch 1153:1, (b) probed with antiserum batch 1154:2

(a)



(b)



SDS-polyacrylamide gel (Hoey, 1986)], were used to investigate the cross-reaction with SDS-treated mandelate and lactate dehydrogenases.

The positive cross-reactions indicated by immunoinhibition and immunoprecipitation between anti-L(+)-mandelate dehydrogenase of *R. graminis* and L(+)-mandelate dehydrogenases from *R. graminis* KGX 39, *R. graminis* NCYC 980, DB2, DB3 and purified L(+)-lactate dehydrogenase of *S. cerevisiae* were also analysed by immunoblotting. In addition to extracts of mandelate grown cells, equal loading of extracts from glucose grown cells of *R. graminis* KGX 39, *R. graminis* NCYC 980, DB2, DB3 were run as controls. Two batches of antisera 1153:1 and 1154:2 cross-reacted with homologous antigen in the purified and crude extract form and they also cross-reacted with extracts of *R. graminis* NCYC 980, DB2 and DB3, giving single bands (Fig. 5.4). Labelling of bands at molecular weight other than that expected for L(+)-mandelate dehydrogenase was not observed except with the extract of *R. graminis* KGX 39 (Fig. 5.4). The extra band obtained was perhaps a result of proteolytic cleavage of the enzyme. The immunoblot bands were more intense for L(+)-mandelate dehydrogenase of *R. graminis* KGX 39 than for L(+)-mandelate dehydrogenase of *R. graminis* NCYC 980, DB2 and DB3 (Fig. 5.4). The purified L(+)-mandelate dehydrogenase of *R. graminis* KGX 39 and extracts of *R. graminis* KGX 39, *R. graminis* NCYC 980, DB 2, DB 3 gave bands at the same distance, indicating a similar subunit size (Fig. 5.4).

However, none of the batches of antisera raised against L(+)-mandelate dehydrogenase of *R. graminis* cross-reacted with purified L(+)-lactate dehydrogenase of *S. cerevisiae* (Fig. 5.5) during immunoblot experiments, in contrast to immunoinhibition by the same antisera (See section 5.2.3). They also did not cross-react with any of the bacterial dehydrogenases or yeast D(–)-mandelate dehydrogenases during immunoblot experiments (Fig. 5.6) but this was in agreement with the immunoinhibition and immunoprecipitation experiments (section 5.2.3).

Anti-L(+)-mandelate dehydrogenase of *A. calcoaceticus* gave a positive blot with its homologous antigen (Fig. 5.7 & 5.8) but also gave some artifactual cross-reactions however, the L(+)-mandelate dehydrogenase band was positively identified by comparing it with the L(+)-mandelate dehydrogenases run on SDS-PAGE and stained for protein.

Figure 5.6 Specificity of immunoblotting: immunoblots of purified D(–)- and L(+)-mandelate dehydrogenases of *R. graminis* KGX 39, purified L(+)-mandelate and L(+)-lactate dehydrogenase of *A. calcoaceticus*, purified L(+)-mandelate dehydrogenase of *P. putida* and cell-free extracts of *R. graminis* KGX 39, *A. calcoaceticus*, *P. putida*, *L. curvatus* and *S. faecalis* probed with anti-L(+)-mandelate dehydrogenase of *R. graminis*.

Cell-free-extracts of *A. calcoaceticus*, *R. graminis*, *P. putida*, *L. curvatus*, *S. faecalis* and purified dehydrogenases of *R. graminis*, *P. putida* and *A. calcoaceticus* were prepared (Methods 2.3.8). Extracts and purified dehydrogenases were electrophoresed on a 10 % (w/v) SDS-polyacrylamide gel (Methods 2.10.5a). Proteins were then electroblotted onto nitrocellulose (Methods 2.10.5b) and later probed with anti-L(+)-mandelate dehydrogenase of *R. graminis* (Methods 2.10.5c). The immunoblot probed with antiserum batch 1153:1 is shown here but antiserum batch 1154:2 gave virtually identical results.

Tracks :

A,L, 33 ng of purified L(+)-mandelate dehydrogenase of *R. graminis* KGX 39

B,J, 23 μg of extract of *R. graminis* KGX 39 grown on mandelate

K, 0.5 μg of purified D(–)-mandelate dehydrogenase of *R. graminis* KGX 39

C, 2.0 μg of purified L(+)-mandelate dehydrogenase of *A. calcoaceticus*

D, 2.1 μg of purified L(+)-lactate dehydrogenase of *A. calcoaceticus*

E, 70 μg of extract of *A. calcoaceticus*

F, 50 μg of of extract of *P. putida*

G, 2.0 μg of purified L(+)-mandelate dehydrogenase of *P. putida*

H, 50 μg of extract of *L.curvatus*

I, 50 μg of extract of *S. faecalis*

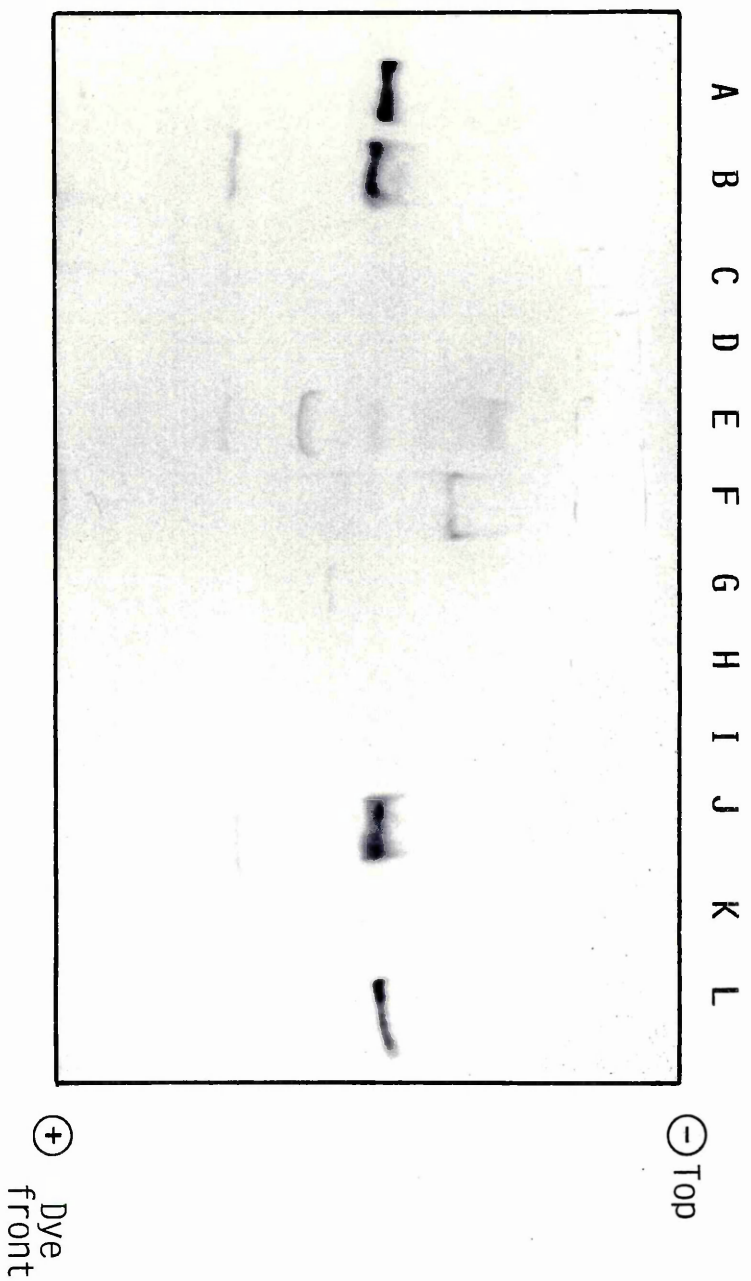

A B C D E F G H I J K L
⊖ Top
⊕ Dye front

Figure 5.7 Specificity of immunoblotting: immunoblots of purified L(+)-mandelate and L(+)-lactate dehydrogenases of *A. calcoaceticus*, purified L(+)-mandelate dehydrogenase of *P. putida*, purified D(–)- and L(+)-mandelate dehydrogenases of *R. graminis* and extracts of *A. calcoaceticus*, *P. putida*, *R. graminis*, *L. curvatus* and *S. faecalis* probed with anti-L(+)-mandelate dehydrogenase of *A. calcoaceticus*

Cell-free extracts of *A. calcoaceticus*, *R. graminis*, *P. putida*, *L. curvatus*, *S. faecalis* and purified dehydrogenases of *R. graminis*, *P. putida* and *A. calcoaceticus* were prepared (Methods 2.3.8). Cell free extracts and purified dehydrogenases were electrophoresed on a SDS-polyacrylamide gel (Methods 2.10.5a). Proteins were then electroblotted onto nitrocellulose (Methods 2.10.5b) and later probed with anti-L(+)-mandelate dehydrogenase of *A. calcoaceticus* (Methods 2.10.5c).

Tracks :

A,L, 2.0 μg of purified L(+)-mandelate dehydrogenase of *A. calcoaceticus*

B,J, 50 μg of extract of *A. calcoaceticus*

C, 2.1 μg of purified L(+)-lactate dehydrogenase of *A. calcoaceticus*

D, 2.0 μg of purified L(+)-mandelate dehydrogenase of *P. putida*

E, 50 μg of extract of *P. putida*

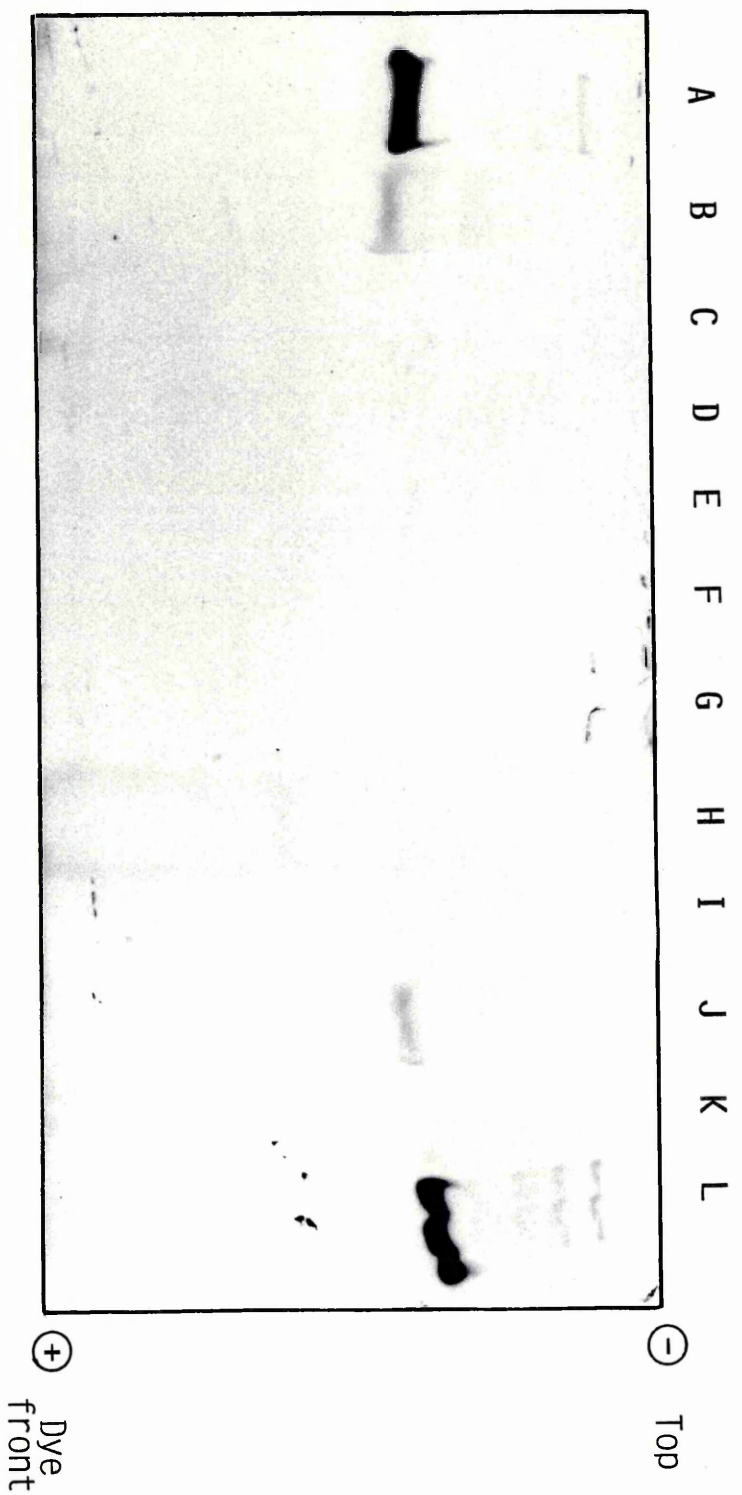
F, 2 μg of purified L(+)-mandelate dehydrogenase of *R. graminis* KGX 39

G, 2.0 μg of purified D(–)-mandelate dehydrogenase of *R. graminis* KGX 39

H, 50 μg of extract of *R. graminis* KGX 39 grown on mandelate

I, 50 μg of extract of *L.curvatus*

K, 50 μg of extract of *S.faecalis*


A B C D E F G H I J K L
⊖ Top
⊕ Dye front

Anti-L(+)-mandelate dehydrogenase of *A. calcoaceticus* did not cross-react with yeast proteins or bacterial D(–)-mandelate dehydrogenases (Fig. 5.7), however, it did give a weak cross-reaction with partially purified L(+)-mandelate dehydrogenase and the cell-free extract (Methods 2.3.8) from *P. putida*. The cross-reaction was positively identified when approximately 100 times more *P. putida* protein than that of *A. calcoaceticus* was loaded onto SDS-PAGE gels and afterwards the nitrocellulose membrane was developed for more than 20 minutes with two changes of developing mixture; the L(+)-mandelate dehydrogenases of *A. calcoaceticus* and *P. putida* co-migrated indicating a similar size for the two proteins (Fig. 5.8).

5.3 Discussion

Immunological cross-reactions are often used to investigate the degree of homology amongst proteins. For example, Durham and Ornston (1980) compared the immunological behaviour of protocatechuate-3, 4-dioxygenases from different bacteria to test for possible evolutionary relationships among them. They found that immunological studies provide reasonable estimates of amino acid homologies among these proteins. The antisera raised against protocatechuate-3, 4-dioxygenase of *Azotobacter vinelandii* gave immunological cross-reactions of various degrees with the same enzyme from different bacteria. On the basis of these immunological cross-reactions they concluded that protocatechuate-3, 4-dioxygenase of *A. vinelandii*, *Pseudomonas putida* and *Pseudomonas aeruginosa* had a common evolutionary origin whereas protocatechuate-3, 4-dioxygenase of *A. calcoaceticus* was only distantly related to these proteins. This conclusion was supported by comparison of amino acid compositions of these enzymes (Durham & Ornston, 1980). Systematic studies on avian lysozymes have demonstrated that a linear relationship exists between the sequence homology and immunological cross-reactivity (Prager & Wilson, 1971a). There seems to be only one known example where proteins with no significant amino acid sequence similarities have strong immunological similarity. Monellin and thaumatin are very sweet proteins but have quite different sequences and yet antibodies raised against either of them gave immunological cross-reaction with both (Ogata *et al.*, 1987). The two

proteins do not taste sweet when bound to the antibodies, indicating that the common antigenic sites lie within the sweet receptors of the two proteins (Ogata *et al.*, 1987).

5.3.1 Artifactual immunological cross-reactions

Artifactual labelling (i.e. non-specific labelling of bands not related to antigen) is often observed, depending on chance sequence similarities and such artifactual labelling of proteins is apparently a more common problem in yeasts than in other organisms (Lillie & Brown , 1987). During the the present immunoblotting work, in addition to cell free extracts of mandelate grown cells, equal loading of cell free extracts of glucose grown cells were run as controls to identify artifactual labelling. The results showed not only the absence of significant artifactual cross-reactions but also confirmed that glucose grown cells [which have no mandelate dehydrogenase activity (Durham, 1984 & present work)] do not contain an inactive but immunologically recogniseable mandelate dehydrogenase (Fig. 5.4). In experiments of this type, control proteins are usually treated in the same way as experimental proteins and are selected arbitrarily (Zakin *et al.*, 1978; Chaffotte *et al.*, 1980; Pekkala-Flagan & Ruoslahti, 1982). Such arbitrarily selected control proteins were not used in the present work but unrelated D(–)-mandelate dehydrogenase from *R. graminis* and *A. calcoaceticus,* D(–)-lactate dehydrogenase from *A. calcoaceticus* and cell free extracts from *L. curvatus* and *S. faecalis*, all enzymes with different physical, chemical properties and no obvious sequence homologies when compared to L(+)-mandelate dehydrogenases of *R. graminis* KGX 39 or *A. calcoaceticus* or *P. putida* or L(+)-lactate dehydrogenase of *A. calcoaceticus* and *S. cerevisiae*, may be considered as control proteins. Artifactual labelling was observed during immunoblot experiment when cell free extracts of *R. graminis* KGX 39, *R. graminis* NCYC 980, DB 2, DB 3 and *P. putida* were run with higher loading during preliminary immunoblotting experiments. Some of these immunological cross-reactions were avoided with proper loading of samples and careful incubation with antisera and extensive washing (Figure 5.4-5.8). During immunoinhibition and immunoprecipitation experiments, enzyme rates with with 20 mM Tris-HCl buffer containing 0.5 mg Triton X-100 ml^{-1} or normal rabbit serum were also used as controls. The lactate dehydrogenase from *S. cerevisiae* incubated with normal rabbit serum could not be taken as a control because an

Figure 5.8 Specificity of immunoblotting: immunoblots of purified L(+)-mandelate dehydrogenase of *A. calcoaceticus* and *P. putida*, probed with anti-L(+)-mandelate dehydrogenase of *A. calcoaceticus* C1219

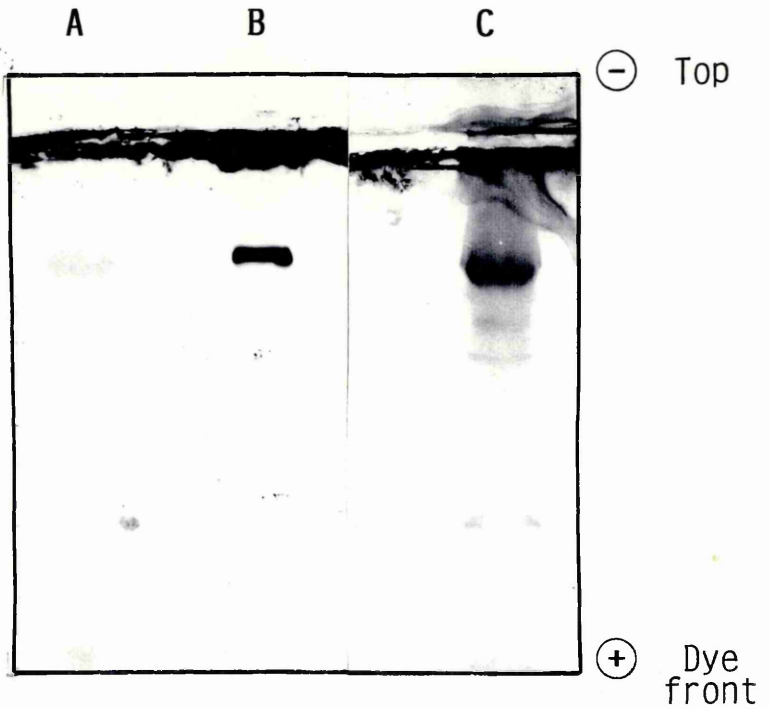
Purified dehydrogenases (Table 5.1) were electrophoresed on SDS-polyacrylamide gel (Methods 2.10.5a). Proteins were then electroblotted onto nitrocellulose (Methods 2.10.5b) and later probed with anti-L(+)-mandelate dehydrogenase of *A. calcoaceticus* (Methods 2.10.5c).

Tracks :

A, 10.0 μg of purified L(+)-mandelate dehydrogenase of *P. putida*

B, 2.0 μg of purified L(+)-mandelate dehydrogenase of *A. calcoaceticus*

C, approximately 100 times more of purified L(+)-mandelate dehydrogenase of *P. putida* than purified L(+)-mandelate dehydrogenase of *A. calcoaceticus*


A
B
C
⊖ Top
⊕ Dye front

increase in rate was observed by increasing the amount of normal rabbit serum in the reaction mixture even in the absence of L(+)-lactate. It was assumed that normal rabbit serum contained L(+)-lactate, substrate for the L(+)-lactate dehydrogenase. However, when antiserum from White New Zealand rabbits immunised with L(+)-mandelate dehydrogenases of *R. graminis* was added in the reaction mixture, no increase in the reaction rate of the control was observed, even if the amount of antisera was increased in the reaction mixture. The heterologous antigens giving no immunological cross-reaction with any of the antisera described in section 5.1, increased the activity of some of the enzymes and that was attributed to stabilisation or activation of the enzymes, which was consistent with the known effects of BSA or Triton X-100 (Yamazaki & Maeda 1986a; see section 4.4.2).

5.3.2 Possible structural and amino acid sequence homologies among L(+)-mandelate and lactate dehydrogenases from bacteria and yeasts based on immunological studies

There are two types of antigenic determinants :

(1) linear (sequential or segmental) antigenic determinants, represented by a continuous stretch of amino acid residues which are found together in a single short segment of the primary structure of protein,

(2) conformational (topographic) antigenic determinants, antigenic determinants formed by amino acid residues that may be far apart in the primary structure but are brought together by three-dimentional folding of the protein (Benjamin *et al.*, 1984; Berzofsky, 1985; van Regenmortel, 1987). The antibodies raised against native proteins are most probably directed against conformational antigenic determinants.

Anti-L(+)-mandelate dehydrogenase of *R. graminis* gave immunological cross-reactions with L(+)-mandelate dehydrogenase from *R. graminis* KGX 39, *R. graminis* NCYC 980 and yeasts DB 2 and DB 3 as shown by immunoinhibition, immunoprecipitation, and Western blotting methods (Table 5.2-5.5 & Fig. 5.1-5.4). The presence of strong immunological cross-reactions suggested the presence of more than 60% sequence homology among these proteins (Prager & Wilson, 1971 b), although this homology is less than 100% as the heterologous cross-reactions are weaker than the homologous one

(Fig. 5.5). Immunoinhibition experiments using the anti-L(+)-mandelate dehydrogenase of *R. graminis* revealed immunological relationships between L(+)-mandelate dehydrogenase of *R. graminis* and L(+)-lactate dehydrogenase of *S. cerevisiae* (Table 5.5) and this is consistent with the similarity between their physical, chemical and kinetic properties and high homology found at their *N* -terminal amino acid sequences (Table 4.6, 4.15, 4.16, 5.5 & Fig. 4.24, 4.25, 4.27). Although, anti-L(+)-mandelate dehydrogenase of *R. graminis* immunoinhibited L(+)-lactate dehydrogenase from *S. cerevisiae* (Table 5.5), it did not cross-react in Western blotting (Fig. 5.5). It is most likely that anti-L(+)-mandelate dehydrogenase of *R. graminis* has been raised against both conformational and linear antigenic determinants and these determinants have been conserved in L(+)-mandelate dehydrogenase from *R. graminis* NCYC 980, DB 2 and DB 3. Although L(+)-lactate dehydrogenase from *S. cerevisiae* has similar conformational antigenic determinants, its primary structure is sufficiently different that it does not give a detectable immunological cross-reaction in the denatured form.

Anti-L(+)-mandelate dehydrogenase of *R. graminis* did not give immunological cross-reactions with L(+)-mandelate dehydrogenases of *A. calcoaceticus* and *P. putida* and L(+)-lactate dehydrogenase of *A. calcoaceticus* (Fig. 5.6) despite indirect evidence of amino acid sequence homologies (Fig. 4.25), similar physical and chemical properties (Table 4.4, 4.8, 4.11, 4.15-4.17). The absence of any detectable immunological cross-reaction (Table 5.3 & Fig. 5.6) is presumably due to absence of the haem domain in L(+)-mandelate dehydrogenases of *A. calcoaceticus* and *P. putida* and L(+)-lactate dehydrogenase of *A. calcoaceticus* (see section 4.7). However, immunological dissimilarity between two antigens using antisera directed against native structure does not necessarily mean dissimilarity amongst homologous proteins, e.g. antisera raised against human leukaemia and hen egg-white lysozymes do not cross-react with reciprocal antigens although they have 52% identity of the surface amino acids, 74% identity of the internal residues and also they have very similar three-dimensional structure (Arnon & Maron, 1971; Arnheim *et al.*, 1971; Blake & Swan, 1971). Arnheim *et al.* (1971) have observed an immunological cross-reaction between antisera raised against denatured lysozyme and denatured antigens which did not cross-react in their native conformation (Arnon & Maron, 1971; Arnheim *et al.*,

1971; Blake & Swan, 1971). Many other workers also have demonstrated that proteins do not show immunological relationships when using antisera raised against native protein but considerable immunological resemblance is observed using antibodies raised against the unfolded structure of a protein (Prager & Wilson, 1971a, b; Zakin *et al.*, 1980; Ragione *et al.*,1990).

It is a common observation that surface residues are subjected to more substitutions than the residues in the interior of the native molecule and these residues are subjected to less environmental constraints (Prager & Wilson, 1971a, b). These conserved amino acids become accessible upon denaturation and can initiate antibody formation in the organism. The presence of immunological cross-reaction between antisera raised against denatured proteins and denatured proteins indicates the presence of homology of their sequences (Arnon & Maron, 1971; Arnheim *et al.*, 1971; Zakin *et al.*, 1978), but these immunological cross-reactions may not reveal conformational similarities.

It follows that lack of immunological cross-reaction among L(+)-specific dehydrogenases mentioned above does not necessarily mean insignificant amino acid sequence homologies or total dissimilarity of three-dimensional structure (Arnheim *et al.*, 1971; Black & Swan, 1971; Prager & Wilson, 1971a, b). Antisera raised against the native conformation of the protein may not expose the more antigenic hydrophobic residues which may be buried in the molecule (Miller *et al.*, 1990). A similar explanation can be given for the absence of cross-reaction during immunoblotting among L(+)-mandelate dehydrogenases of *R. graminis*, *A. calcoaceticus*, *P. putida* and L(+)-lactate dehydrogenase of *S. cerevisiae* or *A. calcoaceticus* (all these proteins contain more than 43% hydrophobic residues, (Table 4.15) against anti-L(+)-mandelate dehydrogenase of *R. graminis* or *A. calcoaceticus*.

The other possible reason for the absence of cross-reaction is that there may be be less than 40% overall amino acid sequence homology among these proteins. Perhaps it would be worth raising antibodies against SDS-denatured L(+)-mandelate dehydrogenase of *R. graminis* in order to test for possible additional cross-reactivity.

Tricot *et al.* (1989) observed that antiserum raised against anabolic ornithine carbamoyltransferase of *Aeromonas formicans* was more reactive towards its homologous antigen in the presence of KCl and less reactive towards anabolic ornithine carbamoyl-

transferase in the presence of substrate. Similarly, to acheive 50% inhibition about 4-fold more antibody was required with purified L(+)-lactate dehydrogenase from *E. coli* than with the membrane-bound enzyme (Futai & Kimura, 1977). Perhaps the antibodies are directed against antigenic determinants which undergo conformational changes when exposed to either KCl or the substrate, ornithine, in *A. formicans* or membranes in *E. coli* (Futai & Kimura, 1977; Tricot *et al.*, 1989). It contrast,the immunological behaviour of L(+)-mandelate dehydrogenase of *R. graminis* was not affected by the presence of substrate or Triton X-100 towards anti-L(+)-mandelate dehydrogenase (Table 5.5).

Anti-L(+)-mandelate dehydrogenase of *A. calcoaceticus* did not cross-react with L(+)-mandelate dehydrogenase of *R. graminis* or L(+)-lactate dehydrogenase of *A. calcoaceticus* (Fig. 5.7), it did give cross-reactions with L(+)-mandelate dehydrogenase of *P. putida* (Fig. 5.8) and this is consistent with the 63% amino acid identity at their amino – terminal sequence (Fig. 4.29).

Anti-D(–)-mandelate dehydrogenase of *A. calcoaceticus* did not give immunological cross-reactions with any of the mandelate or lactate dehydrogenases except its homologous antigen. The *N* -terminal amino acid sequence of D(–)-mandelate dehydrogenase from *A. calcoaceticus* shows no significant sequence homologies with other mandelate and lactate dehydrogenases except that it has 30% amino acid sequence homology with D(–)-lactate dehydrogenase from *A. calcoaceticus* (C.A. Fewson, personal communications) and this is less than the sensitivity limit generally needed to have immunological cross-reactions (Prager & Wilson, 1971b)

Anti-D(–)-mandelate dehydrogenase of *R. graminis* gave cross-reactions with purified D(–)-mandelate dehydrogenase from *R. graminis* KGX 39 and enzymes in extracts of *R. graminis* KGX 39, *R. graminis* NCYC 980 and the yeasts DB 2 and DB 3 indicating that perhaps these proteins might have more than 60% amino acid sequence homology (Baker, 1990) and these proteins are apparently of similar size (Baker, 1990). However, no immunological cross-reaction between the anti-D(–)-mandelate dehydrogenase of *R. graminis* and D(–)-mandelate dehydrogenases of *L. curvatus* and D(–)-2-hydroxy isocaproate dehydrogenase of *S. faecalis*, was demonstrated (Baker, 1990) and this is similar to the immunological specificity of anti-L(+)-mandelate dehydrogenases of

R. graminis (Fig. 5.6). However, neither of the two antisera, anti-D(–)-mandelate dehydrogenase or anti-L(+)-mandelate dehydrogenase of *R. graminis* gave cross-reaction with bacterial FMN or FAD or NAD-dependent mandelate or lactate dehydrogenases (Baker, 1990; this thesis). The results in this thesis show that the L(+)-mandelate dehydrogenases are very similar in all the yeasts examined, and they are clearly immunologically distinguishable from the equivalent bacterial enzymes.

Antibodies raised against denatured enzymes would be useful in future work as there is evidence that such antibodies give cross-reaction even at lower levels of sequence homology (Arnheim *et al.*, 1971; Arnon & Maron, 1971; Arnon, 1973; Zakin *et al.*, 1978; Lompre *et al.*, 1979; Schwartz *et al.*, 1980; Pekkaala-Flagan & Rouslathi, 1982).

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

The partial characterization of L(+)-mandelate dehydrogenase of *R. graminis* has allowed a comparison with other mandelate and lactate dehydrogenases from both prokaryotes and eukaryotes. L(+)-Mandelate dehydrogenase from *R. graminis* appears to be a loosely membrane-associated enzyme as most of it is easily removed from the membrane using French pressing while the rest is solubilised by detergent treatment (Table 3.2). However, the nature of this association is not at all clear. It may be loosely anchored in the membrane *in vivo*, perhaps allowing transfer of electrons from the haem to the next component of the electron transport chain. It may, like the cytochrome b_2 L(+)-lactate dehydrogenase of *S. cerevisiae*, be located between the inner and outer mitochondrial membranes (Daum *et al.*, 1982) which gives the appearance of it being membrane associated when distribution studies are performed. Or, it may non-functionally bind to membranes during extraction, i.e. the appearance of membrane association may be artifactual.

The L(+)-mandelate dehydrogenase of *R. graminis*, a haem containing protein, has similar native and subunit molecular weights to those of cytochrome b_2 L(+)-lactate dehydrogenase of *S. cerevisiae* and *H. anomala*. Pairwise alignment of amino acid sequences and comparison of compositions (Table 6.1) revealed that L(+)-mandelate dehydrogenase of *R. graminis* has significant degree of homologies with L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala* (Fig. 4.24 & Tables 4.15, 4.16 & 6.1). Furthermore the quaternary structure of this enzyme together with the presence of FMN and haem as prosthetic groups, the ability to dehydrogenate 2-hydroxyacids and the use of DCIP, ferricyanide and cytochrome *c* as electron acceptors are the properties it shares with the L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala* (Table 6.1 & see section 4.7). Moreover, immunological cross-reactions between anti-L(+)-mandelate dehydrogenase of *R. graminis* and L(+)-lactate dehydrogenase of *S. cerevisiae* (Summarised in Table 6.1 and chapter 5) suggest the possibile existence of conformational homologies between L(+)-mandelate dehydrogenase of *R. graminis* and L(+)-lactate dehydrogenase of *S. cerevisiae*. In conclusion, L(+)-mandelate dehydrogenase from *R. graminis* is a cytochrome b_2 mandelate dehydrogenase and it is evolutionarily related to cytochrome b_2 L(+)-lactate dehydrogenase of *S. cerevisiae*.

Table 6.1 Evidence for evolutionary relatedness among L(+)-mandelate dehydrogenases, L(+)-lactate dehydrogenases and glycolate oxidase

These pairs of enzymes also share similarities among their physical, chemical and kinetic properties

LMDH, L(+)-mandelate dehydrogenase; LLDH, L(+)-lactatate dehydrogenase; NA, not available

Enzymes	Approximate subunit M_r/cofactors	Amino acid composition (SΔQ)	Amino acid sequence identities (%)*	Additional evidence for relatedness
LMDH of *P.putida* and LLDH of *E. coli*	44,000/ FMN	25	NA	
LMDH of *P.putida* and LMDH of *A. calcoaceticus*	44,000/ FMN	NA	62 (50)	Immunologcal cross-reaction
LMDH of *P.putida* and glycolate oxidase of spinach	44,000/ FMN	58	33 (393)	
LLDH of *E. coli* and glycolate oxidase of spinach	43,000/ FMN	25	NA	
LMDH of *R. graminis* and LLDH of *S. cerevisiae*	58,000/ FMN and haem	53	50 (28)	Immunological cross-reaction
LMDH of *R. graminis* and LLDH of *H. anomala*	58,000/ FMN and haem	43	28 (28)	
LLDH of *S. cerevisiae* and LLDH of *H. anomala*	58,000/ FMN and haem	30	60 (500)	

*Number of amino acids compared are given in the parentheses.

Information from Futai & Kimura (1977), Lindqvist (1989), Tsou *et al.* (1990), Chapman *et al.* (1991), Fewson (1992) and this thesis.

Because of the limited amino-terminal sequence data that is available for L(+)-mandelate dehydrogenase of *R. graminis,* it cannot be compared at the sequence level with any relevant bacterial enzyme. However, L(+)-mandelate dehydrogenase of *R. graminis* is very similar to L(+)-mandelate dehydrogenase of *A. calcoaceticus* and *P. putida* and L(+)-lactate dehydrogenase of *A. calcoaceticus* with respect to substrate specificity, the presence of FMN as prosthetic group, the effects of thiol-blocking reagents, the reversibility of the reaction and the conditions required to give maximum activity (Hoey *et al.,* 1987, Tsou *et al.,* 1990, Fewson, 1992; this thesis, Table 6.2). In addition, indirect amino acid sequence homologies are found when the amino-terminal sequences of the FMN-containing L(+)-lactate dehydrogenase of *A. calcoaceticus*, L(+)-mandelate dehydrogenases of *A calcoaceticus* and *P. putida* , and glycolate oxidase of spinach (Fig. 4.22, 4.25 and Table 6.1) are compared to the FMN-domain sequences of cytochrome b_2 L(+)-lactate dehydrogenase *S. cerevisiae* and *H. anomala* which have amino-terminal sequence homologies with the amino-terminus sequence of L(+)-mandelate dehydrogenase of *R. graminis.* Also the amino acid composition of L(+)-mandelate dehydrogenase of *R. graminis* possesses significant similarities with that of L(+)-lactate dehydrogenases of *S. cerevisiae* and *H. anomala* (Table 4.15, 4.16 & 6.1). Finally, L(+)-mandelate dehydrogenase of *P. putida,* L(+)-lactate dehydrogenase of *E. coli* and glycolate oxidase of spinach have significant similarities in their amino acid compositions (Table 4.15, 4.16 & 6.1). The amino acid composition of L(+)-mandelate dehydrogenase of *A. calcoaceticus* has not been determined but the immunological cross-reaction between anti-L(+)-mandelate dehydrogenase of *A. calcoaceticus* and L(+)-mandelate dehydrogenase of *P. putida* strengthens the view that the two proteins are very homologous to each other (Table 6.1). From these comparisons of primary structures, physical, chemical and kinetic properties and from immunological studies, it can be concluded that L(+)-mandelate dehydrogenase of *P. putida, A. calcoaceticus, R. graminis* and L(+)-lactate dehydrogenase of *A. calcoaceticus, E. coli, S. cerevisiae* and *H. anomala* belong to the same evolutionarily related group of proteins. Glycolate oxidase of spinach with respect to its amino acid sequence homologies with these proteins (Fig. 4.25 & 4.29) can be considered as one of the member of this group

Table 6.2 Comparison of the NAD(P)-independent lactate and mandelate dehydrogenases

	A. calcoaceticus		*P. putida*	*R. graminis*	*S. cerevisiae*
	L(+)-Mandelate dehydrogenase	L(+)-Lactate dehydrogenase	L(+)-Mandelate dehydrogenase	L(+)-Mandelate dehydrogenase	L(+)-Lactate dehydrogenase
Location	Membrane bound	Membrane bound	Membrane bound	Membrane associated	Soluble
Subunit M_r	44000	40000	44000	59100	57500
Subunit composition	Monomer	Monomer	Monomer	Tetramer	Tetramer
Cofactor	FMN	FMN	FMN	FMN + haem	FMN + haem
Reversibility of reaction	All the enzymes catalyse essentially irreversible reactions				
pH optimum	7.5	7.5	7.5	7.9	7.5-8.0
Electron acceptor	DCIP	DCIP	DCIP	DCIP or cyt. *c*	DCIP or cyt. *c*
Stereospecifity	All the enzymes are stereospecific for their substrates				
Inhibitors	Not affected by a wide range of chelating agents, but susceptible to several thiol-blocking reagents specially to *p*-chloromercuribenzoate; inhibited by oxalate				

Information from Allison and Fewson (1986), Hoey *et al.* (1987), Tsou *et al.* (1990), Chapman *et al.* (1991) and this thesis.

Table 6.3 Comparison of the NAD-dependent D(–)-mandelate dehydrogenases from *Rhodotorula graminis, Lactobacillus curvatus* and *Streptococcus faecalis*

	R. graminis	*L. curvatus*	*S. faecalis*
Location	All the enzymes are soluble		
Subunit M_r	38000	30000	34000
Subunit composition	All the enzymes are dimers		
Reversibility of reaction	All the enzymes catalyse the reversible oxidation of mandelate to phenylglyoxylate		
Stereospecificity	All the enzymes are stereospecific for their substrate		
pH optimum (mandelate oxidation)	9.5	8.5	9.2
pI value	5.9	ND	4.9
Km value (μM) for D(–)-mandelate	325	500	ND
Km value (μM) for NADH	36	36	35
Heat lability	Labile	Stable	Stable
Inhibitors	Not affected by a wide range of chelating agents, but susceptible to some thiol-blocking reagents		
Inducer	Probably mandelate	Probably constitutive	Probably constitutive

Based on (Fewson, 1992) with information from Yamazaki and Maeda (1986), Hummel *et al.* (1988) and Baker and Fewson (1989); ND, not determined.

(Volokita & Somerville, 1987; Cederlund *et al.*, 1988). Representatives of this group of enzymes are compared in Tables 6.1 and 6.2.

In sharp contrast to these FMN-linked enzymes, D(–)-mandelate dehydrogenase from *R. graminis* is an NAD-dependent, soluble, dimeric enzyme with subunit M_r of 38,000 (Baker & Fewson, 1989). This enzyme is analogous to the D(–)-mandelate dehydrogenase of *L. curvatus* and the D(–)2-hydroxyisocaproate dehydrogenase of *S. faecalis* (Yamazaki & Maeda, 1986a,b; Hummel *et al.*, 1988) with similar quaternary structure, cofactor and substrate specificity, reversibility of reaction and pH dependence of activity (Table 6.3; Baker & Fewson, 1989; Fewson, 1992).

On the basis of these comparisons, mandelate and lactate dehydrogenases seem to fall into two quite different classes (Table 6.4):

(1) NAD-dependent, soluble enzymes catalysing reversible reactions, and

(2) NAD-independent flavo or flavohaem enzymes catalysing essentially irreversible reactions.

On the basis of these criteria, the D(–)-mandelate dehydrogenase and the D(–)-lactate dehydrogenases of *A. calcoaceticus* (Allison *et al.*, 1985a) and *E. coli* (Futai, 1973; Campbell *et al.*, 1984; Ho *et al.*, 1989) apparently belong to the second class of enzymes, as they contain FAD as prosthetic group and catalyse essentially irreversible reactions. D(–)-Mandelate dehydrogenase and D(–)-lactate dehydrogenase of *A. calcoaceticus* and *E. coli* have quite similar amino-terminal sequences but these are quite different from those of the FMN-linked enzymes (C.A. Fewson, personal communication). Based on differences of prosthetic groups, sequences and substrate specificities, the NAD-independent enzymes are subdivided (Table 6.4) into:

(a) FMN-dependent enzymes, and

(b) FAD-dependent enzymes .

FMN-dependent enzyme can further be subdivided (Fig. 6.4) into:

(i) FMN-containing non-haem enzymes with subunit Mr of about 44,000, and

(ii) FMN and haem-containing enzymes with subunit Mr of about 59,000.

It has been suggested that cytochrome b_2 lactate dehydrogenase could have evolved as a result of fusion between the structural genes encoding cytochrome reductase and lactate

Table 6.4 Types of mandelate and lactate dehydrogenases

1. NAD-dependent

D(–)-mandelate dehydrogenase of *Rhodotorula graminis*
D(–)-mandelate (?) dehydrogenase of *Lactobacillus curvatus*
D(–)-hydroxyisocaproate dehydrogenase of *Streptococcus faecalis*

2. NAD(P)-independent

(a) FMN-dependent

(i) FMN, non-haem, approx. M_r = 44,000

L(+)-mandelate dehydrogenase of *Acinetobacter calcoaceticus*
L(+)-mandelate dehydrogenase of *Pseudomonas putida*
L(+)-lactate dehydrogenase of *Acinetobacter calcoaceticus*
L(+)-lactate dehydrogenase of *Escherichia coli*

(ii) FMN, haem, approx. M_r = 59,000

L(+)-mandelate dehydrogenase of *Rhodotorula graminis*
L(+)-lactate dehydrogenase of *Saccharomyces cerevisiae*
L(+)-lactate dehydrogenase of *Hansenula anomala*

[2-hydroxyacid oxidase from rat kidney (Urban *et al.*,1988) and probably mandelate dehydrogenases of *Pseudomonas aeruginosa* (Kemp, 1972) and *Rhizobium leguminosarum* (Chen *et al.*, 1989) are also related to these FMN-dependent enzymes].

(b) FAD-dependent

D(–)-mandelate dehydrogenase of *Acinetobacter calcoaceticus*
D(–)-lactate dehydrogenase of *Acinetobacter calcoaceticus*
D(–)-lactate dehydrogenase of *Escherichia coli*

dehydrogenase enzymes (Leberie & Baudras, 1972; Guiard *et al.*, 1974; Guiard & Lederer, 1979b) . This is supported by the evidence that trypsin rapidly cleaves the link between the two domains in *S. cerevisiae* and *H. anomala* haem and FMN domain with the flavodehydrogenase domain still retaining the reducing capacity (Gervais *et al.*, 1983; Celerier *et al.*, 1989). The covalent association of the flavoreduction-cytochrome system in yeasts can be compared with the system present in liver microsomes where flavoreductase and cytochrome b_5 are separately anchored in the membrane (Gervais *et al.*, 1977). Amino acid sequence homologies, together with the common folding patterns of cytochrome b_2 and cytochrome b_5 core, support the hypothesis that both proteins may have been derived from a common ancestor (Gervais *et al.*, 1977).

From the above comparisons, it is concluded that the mandelate dehydrogenases have almost certainly evolved by recruitment of aliphatic 2-hydroxyacid dehydrogenases of other established pathways. Both NAD-dependent and NAD-independent mandelate dehydrogenases are found in both prokaryotes and eukaryotes (Tables 6.4) and it seems as though the ability to metabolise different enantiomers of mandelate has been acquired after gene recruitment independently. The close similarity between L(+)-mandelate dehydrogenases and L(+)-lactate dehydrogenases invites the immediate speculation that they might have evolved from a common ancestor or that the mandelate dehydrogenases may have arisen from lactate dehydrogenases. Similarly, NAD-dependent or FAD-dependent mandelate dehydrogenases might have arisen from homologous NAD-dependent or FAD-dependent lactate dehydrogenases respectively following gene duplication and recruitment to the new function. Some of the bacterial and fungal aliphatic 2-hydroxyacid dehydrogenases show broad substrate specificity and can dehydrogenate aliphatic as well as aromatic 2-hydroxy acids such as phenyllactate or 4-hydroxyphenyllactate or even mandelate itself (e.g. Bode *et al.*, 1986; Yamazaki & Maeda; 1986a; Bhatnager *et al.*, 1989; Hummel & Kula; 1989; Hendry *et al.*, 1990). It is not difficult to imagine that the recruitment of such an enzyme to a new function could be achieved by only a few mutations . Clark *et al* . (1987) showed that the introduction of three amino acid substitutions, changing glutamate-102, aspartate-197 and threonine-246 to arginine, asparagine and glycine respectively, in the lactate dehydrogenase of *Bacillus stearothrermophillus* , changed the substrate specificity of the

enzyme from pyruvate reduction/lactate oxidation to oxoloacetate reduction/malate oxidation. It is therefore postulated that mandelate dehydrogenase activity might have resulted from the recruitment of a duplicated gene coding for an aliphatic 2-hydroxy acid dehydrogenase which had undergone a few mutations, leading to replacement of some of the active site amino acids with hydrophobic residues to bind the aromatic ring of mandelate.

6.2 Future work

Purification, primary characterisation and comparison of L(+)-mandelate dehydrogenase from *R. graminis* with other mandelate and lactate dehydrogenases has allowed us to speculate about their evolutionary origin. To further investigate its evolutionary relationship with other mandelate and lactate dehydrogenase, the following experiments would be especially useful.

6.2.1 Cloning and sequencing of the gene encoding L(+)-mandelate dehydrogenase

The immediate priority would be to clone and sequence the structural gene coding for L(+)-mandelate dehydrogenase of *R. graminis* . From the first 32 residues, Aspartate-27 to Valine-32 could best be used to construct an oligonucleotide probe for use as a 5' complement primer.

Position	27	28	29	30	31	32
Amino acid	Aspartate	Aspartate	Methionine	Tryptophan	Valine	Valine
codon	-GA[T/C]	GA[T/C]	ATG	TGG	GT[C/A/G]	GT-
Degeneracy	2	2	1	1	4	1

For the reverse complement primer, Serine-370 to Glycine-374 of L(+)-lactate dehydrogenase of *S. cerevisiae* could be used because the sequence is highly conserved in

evolutionarily related L(+)-lactate dehydrogenases of *S. cerevisiae* , *H. anomala* , glycolate oxidase of spinach and L(+)-mandelate dehydrogenase of *P. putida* .

Position	370	371	372	733	374
Amino acid	Serine	Asparagine	Histidine	Glycine	Alanine/Glycine
codon	GT T/C/A/G	AA T/C	CA T/C	GG T/C/A/G	G C/G
Degeneracy	4	2	2	4	2

If the gene is sequenced it should be possible to modify the enzyme by site-directed mutagenesis and construct enzymes with different properties, e.g. altered substrate specificity, perhaps turning it into a lactate dehydrogenase. It would also make it possible to make a thorough comparison with nucleotide sequences of cytochrome b_2 dehydrogenases of *S. cerevisiae* (Guiard, 1985) *H. anomala* (Risler *et al.*, 1989) and flavoproteins L(+)-mandelate dehydrogenase of *P. putida* (Tsou *et al.*, 1990) and glycolate oxidase of spinach (Volokita & Somerville, 1987). The comparison of nucleotide sequences may be more useful than amino acid sequence because of the degeneracy of the genetic code (Yokoyama *et al.*, 1990). The amino acid sequence would also be helpful to identify active site residues conserved in cytochrome b_2 dehydrogenases of *S. cerevisiae, H. anomala* , L(+)-mandelate dehydrogenase of *P. putida* and glycolate oxidase of spinach (see section 4.7.6).

6.2.2 Determination of three-dimensional structure

Immunological studies indicated that the three-dimensional structure of L(+)-mandelate dehydrogenase of *R. graminis* and L(+)-lactate dehydrogenase of *S. cerevisiae* may be similar. At present, three-dimensional structures of L(+)-lactate dehydrogenase of *S. cerevisiae* (Mathew & Xia 1987; Xia *et al.*, 1987) and glycolate oxidase of spinach (Lindqvist & Branden, 1985) are available. Therefore it would be worthwhile to determine the crystal structure of L(+)-mandelate dehydrogenase to allow a comparison with known

structures of related proteins. For crystallographic studies, it would be useful to have an overexpressing strain containing the cloned gene coding for this enzyme so that enough protein could be purified for these studies. If the three-dimensional structures are found to be similar, it will be even more reasonable to hypothesise the recruitment of L(+)-mandelate dehydrogenase activity from aliphatic 2-hydroxyacid dehydrogenases of other pathways by gene duplication followed by mutations. In addition, the three-dimensional structure of L(+)-mandelate dehydrogenase could also be compared with the crystal structure of the mandelate racemase of *P. putida* (Neidhart *et al.*, 1988) which is evolutionarily related to muconolactonizing enzyme of the same organism (Tsou *et al.*, 1990). The crystallographic studies would also reveal information about domain structures and residues associated with substrate binding at the active site and thus provide further ideas as to how the enzymes could have evolved both in terms of domain structure and substrate specificity.

6.2.3 Immunological studies

Immunolocalisation of L(+)-mandelate dehydrogenase of *R. graminis* with gold-labelling could be done using anti-L(+)-mandelate dehydrogenase of *R. graminis* which has shown positive cross-reactions with its homologous antigen and L(+)-mandelate dehydrogenase from DB2, DB3 and *R. graminis* NCYC 980 and L(+)-lactate dehydrogenase of *S. cerevisiae* (see references in section 3.8). This would help to identify its *in vivo* location and perhaps its physiological role in the electron transport chain.

6.2.4 Survey of other organisms

It would also be worth examining a range of other prokaryotes and eukaryotes to see if there are further examples of enzymes that fit into the categories in Table 6.4 and perhaps to discover new groups of mandelate dehydrogenases which might have quite different properties.

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